THE ENDOCRINE SYSTEM IN SPORTS AND EXERCISE

VOLUME XI OF THE ENCYCLOPAEDIA OF SPORTS MEDICINE

AN IOC MEDICAL COMMISSION PUBLICATION

IN COLLABORATION WITH

THE INTERNATIONAL FEDERATION OF SPORTS MEDICINE

EDITED BY

WILLIAM J. KRAEMER AND ALAN D. ROGOL

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Development Editor: Nick Morgan
Production Controller: Kate Charman

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Contents

List of Contributors

OSCAR ALCAZAR PhD, *Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, MA 02215, USA*

LAWRENCE E. ARMSTRONG PhD, *Departments of Kinesiology and Physiology-Neurobiology, University of Connecticut, Storrs, CT 06269, USA*

GERHARD BAUMANN MD, *Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Feinberg School of Medicine and Veterans Administration Chicago Health Care System, 303 East Chicago Avenue, Chicago, IL 60611, USA*

BETH A. BEIDLEMAN DSc, *Biophysics and Biomedical Modeling Division, US Army Research Institute for Environmental Medicine, Natick, MA 01760, USA*

SHALENDER BHASIN MD, *UCLA School of Medicine, Drew-UCLA Reproductive Science Research Center, Division of Endocinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, CA 90059, USA*

MARTIN BIDLINGMAIER MD, *Neuroendocrine Unit, Medizinische Klinik—Innenstadt, Klinikum der LMU, Ziemssenstr. 1, 80336 Munich, Germany*

ROBERT H. BONNEAU PhD, *Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, PA 17033, USA*

JACK A. BOULANT PhD, *Department of Physiology and Cell Biology, Ohio State University College of Medicine, Columbus, OH 43210, USA*

PIERRE BOULOUX MD, *Department of Medicine, Royal Free and University College Medical School, University of London, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK*

JILL A. BUSH PhD, *Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Houston, TX 77204, USA*

JOHN W. CASTELLANI PhD, *Thermal and Mountain Medicine Division, US Army Research Institute for Environmental Medicine, 42 Kansas Street, Natick, MA 01760-5007, USA*

DAN M. COOPER MD, *Center for the Study of Health Effects of Exercise in Children, Department of Pediatrics, Irvine College of Medicine, University of California, Irvine, CA 92868, USA*

ROSS C. CUNEO PhD, *Department of Diabetes and Endocrinology, University of Queensland, Princess Alexandra Hospital, 4102 Brisbane, Queensland, Australia*

DAVID W. DEGROOT MS, *Thermal and Mountain Medicine Division, US Army Research Institute for Environmental Medicine, 42 Kansas Street, Natick, MA 01760-5007, USA*

MICHAEL R. DESCHENES PhD, *Department of Kinesiology, The College of William and Mary, Williamsburg, VA 23187-8795, USA*

MARY JANE DE SOUZA PhD, *Women's Exercise and Bone Health Laboratory, Faculty of Physical Education and Health, 52 Harbord Street, University of Toronto, Toronto, Ontario, M5S 2W6, Canada*

KEIICHIRO DOHI PhD, *Osaka University of Health and Sport Sciences, Asashirodai, Kumatori-cho, Sennan-gun, Osaka, 590-0496, Japan*

ALON ELIAKIM MD, *Sackler School of Medicine, Tel-Aviv University and Child Health and Sports Center, Pediatric Department, Meir General Hospital, Kfar-Saba 44281, Israel*

KARL E. FRIEDL PhD, *US Army Research Institute of Environmental Medicine, Natick, MA 01760-7007, USA*

ANDREW C. FRY PhD, *Exercise Biochemistry Laboratory, 135 Roane Field House, The University of Memphis, Memphis, TN 38152, USA*

ELLEN L. GLICKMAN PhD, *School of Exercise, Leisure and Sport, Kent State University, Kent, OH 44513, USA*

ALLAN H. GOLDFARB PhD, *Exercise and Sport Science Department, University of North Carolina-Greensboro, Greensboro, NC 27402-6170, USA*

GEOFFREY GOLDSPINK PhD, ScD, *Department of Surgery, Royal Free and University College Medical School, University of London, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK*

LAURIE J. GOODYEAR PhD, *Joslin Diabetes Center, One Joslin Place, Boston, MA 02215, USA*

SCOTT E. GORDON PhD, *Human Performance Laboratory, East Carolina University, Greenville, NC 27858, USA*

RICHARD E. GRINDELAND PhD, *Life Science Division, NASA-Ames Research Center, Moffett Field, CA 94035, USA*

MAHJABEEN HAMEED PhD, *Department of Surgery, Royal Free and University College Medical School, University of London, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK*

HEINZ W. HARBACH MD, *Department of Anesthesiology, Intensive Care Medicine, Pain Therapy, University Hospital, Giessen, Rudolf-Buchheim-Str. 7, D-35385 Giessen, Germany*

STEPHEN HARRIDGE PhD, *Department of Physiology, Royal Free and University College Medical School, University of London, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK*

GUNTER HEMPELMANN MD, *Department of Anesthesiology, Intensive Care Medicine, Pain Therapy, University Hospital, Giessen, Rudolf-Buchheim-Str. 7, D-35385 Giessen, Germany*

RICHARD C. HO PhD, *Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, MA 02215, USA*

JAY R. HOFFMAN PhD, *Department of Health and Exercise Science, College of New Jersey, Ewing, NJ 08628, USA*

WESLEY C. HYMER PhD, *Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA*

WARRICK J. INDER MD, *Department of Medicine, St. Vincent's Hospital, University of Melbourne, Fitzroy, VIC 3065, Australia*

DANIEL A. JUDELSON MA, *Human Performance Laboratory, Department of Kinesiology, University of Connecticut, Storrs, CT 06269-1110, USA*

FAWZI KADI PhD, *Department of Physical Education and Health, Örebro University, 70182 Örebro, Sweden*

MICHAEL KJÆR MD, PhD, *University of Copenhagen, Sports Medicine Research Unit, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark*

WILLIAM J. KRAEMER PhD, *Human Performance Laboratory, Department of Kinesiology, University of Connecticut, Storrs, CT 06269-1110, USA*

ANNE B. LOUCKS PhD, *Department of Biological Sciences, Ohio University, Irvine Hall 053, Athens, OH 45701, USA*

CARRIE E. MAHONEY BS, *Department of Kinesiology, University of Connecticut, Storrs, CT 06269-1110, USA*

CARL M. MARESH PhD, *Human Performance Laboratory, Department of Kinesiology, University of Connecticut, Storrs, CT 06269-1110, USA*

ANDREA M. MASTRO PhD, *Department of Biochemistry and Molecular Biology, 431 South Frear Building, Pennsylvania State University, University Park, PA 16802, USA*

ROMAIN MEEUSEN PhD, *Faculty of Physical Education and Physiotherapy, Vrije Universiteit Brussel, Brussels, 1050, Belguim*

MARY P. MILES PhD, *Department of Health and Human Development, Montana State University, Bozeman, MT 59717, USA*

DAN NEMET MD, *Sackler School of Medicine, Tel-Aviv University and Child Health and Sports Center, Pediatric Department, Meir General Hospital, Kfar-Saba 44281, Israel*

BRADLEY C. NINDL PhD, *Military Performance Division, US Army Research Institute of Environmental Medicine, Natick, MA 01760, USA*

CHARLES T. ROBERTS, JR PhD, *Department of Pediatrics (NRC5), Oregon Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, USA*

CAROL D. RODGERS PhD, *Department of Physical Education and Health, University of Toronto, Toronto, Ontario, Canada, and Department of Physiology, Faculty of Medicine, University of Toronto, Ontario, M5S 2W6, Canada*

JAMES N. ROEMMICH PhD, *Department of Pediatrics, Division of Behavioral Medicine, State University of New York at Buffalo, 3435 Main Street, Buffalo, NY 14214-3000, USA*

ALAN D. ROGOL MD, PhD, *Clinical Pediatrics, University of Virginia, ODR Consulting, 685 Explorers Road, Charlottesville, VA 22911-8441, USA*

CLIFFORD J. ROSEN MD, *Maine Center for Osteoporosis Research and Education, St. Joseph Hospital, 900 Broadway, Bangor, ME 04401, USA*

WILHELM SCHÄNZER PhD, *Institute of Biochemistry, German Sport University Cologne, Carl-Diem Weg 6, 50933 Cologne, Germany*

MATTHEW J. SHARMAN MA, *Human Performance Laboratory, Department of Kinesiology, 2095 Hillside Road, Unit 110, University of Connecticut, Storrs, CT 06269-1110, USA*

JANET E. STAAB BS, *Thermal and Mountain Medicine Division, US Army Research Institute for Environmental Medicine, 42 Kansas Street, Natick, MA 01760-5007, USA*

CHRISTIAN J. STRASBURGER MD, *Division of Endocrinology, Department of Internal Medicine, Charité—Campus Mitte, Schumannstrasse 20/21, 10117 Berlin, Germany*

JUERGEN M. STEINACKER MD, PhD, *Section of Sports and Rehabilitation Medicine, University of Ulm, 89070 Ulm, Germany*

MARIO THEVIS PhD, *Institute of Biochemistry, German Sport University Cologne, Carl-Diem Weg 6, 50933 Cologne, Germany*

N. TRAVIS TRIPLETT PhD, *Department of Health, Leisure and Exercise Science, Appalachian State University, Boone, NC 28608, USA*

JACI L. VANHEEST PhD, *Department of Kinesiology, University of Connecticut, Storrs, CT 06269, USA, and Adjunct in Department of Physical Education and Health, University of Toronto, Toronto, Ontario, M5S 2W6, Canada*

JOHANNES D. VELDHUIS MD, *Division of Endocrinology and Metabolism, Department of Internal Medicine, Mayo Medical and Graduate Schools of Medicine, General Clinical Research Center, Mayo Clinic, Rochester, MN 55905, USA*

ATKO VIRU DSc, PhD, *Institute of Sports Biology, University of Tartu, 18 Ylikooli, Tartu 51014, Estonia*

MEHIS VIRU PhD, *Institute of Sports Biology, University of Tartu, 18 Ylikooli, Tartu 51014, Estonia*

JEFF S. VOLEK PhD, *Department of Kinesiology, University of Connecticut, Storrs, CT 06269-1110, USA*

JENNIFER D. WALLACE PhD (Med) pending, *Metabolic Research Unit, Department of Medicine, University of Queensland, Princess Alexandra Hospital, 4102 Brisbane, Queensland, Australia*

ARTHUR L. WELTMAN PhD, *Department of Human Services, Department of Medicine, General Clinical Research Center and Exercise Physiology Laboratory, Memorial Gymnasium, University of Virginia, Charlottesville, VA 22908, USA*

JUDY Y. WELTMAN MS, *General Clinical Research Center, University of Virginia, Charlottesville, VA 22908, USA*

LAURIE WIDEMAN PhD, *Department of Exercise and Sport Science, University of North Carolina-Greensboro, Greensboro, NC 27402-6170, USA*

NANCY I. WILLIAMS ScD, *Noll Physiological Research Center and Department of Kinesiology, 108 Noll Laboratory, Penn State University, University Park, PA 16802, USA*

GARY A. WITTERT MD, *Department of Medicine, Royal Adelaide Hospital, University of Adelaide, Adelaide, SA 5000, Australia*

ZIDA WU MD, *Division of Endocrinology, Department of Internal Medicine, Charité—Campus Mitte, Schumannstrasse 20/21, 10117 Berlin, Germany*

SHI YU YANG PhD, MVet Sci, *Department of Surgery, Royal Free and University College Medical School, University of London, Royal Free Campus, Rowland Hill Street, London, NW3 2PF, UK*

Foreword

The Encyclopaedia of Sports Medicine is a highly valuable series that has been produced during the last 17 years by the IOC Medical Commission. The present edition becomes the eleventh volume in the series.

A volume that is focused on the physiology of sport and exercise could be expected to concentrate on skeletal muscle metabolism and the supporting functions of the cardiovascular and respiratory systems. Indeed, these aspects of the biology of human performance have been featured in certain of the earlier editions of The Encyclopaedia of Sports Medicine.

It is now entirely appropriate that attention be given to the ductless glands that produce the hormones that modify and control so many of the important body functions. A great number of the chronic adaptations of the human organism to sport conditioning result in major changes in endocrine function. The co-editors and contributing authors of Encyclopaedia Vol. XI, *The Endocrine System in Sports and Exercise*, have provided a comprehensive and authoritative coverage of this highly complex system. This volume will serve as a leading reference for physicians, scientists and graduate students for many years to come.

I am pleased to congratulate the co-editors and the contributing authors on the quality of their work and to welcome the newest volume into the prestigious Encyclopaedia of Sports Medicine series.

Dr Jacques Rogge
IOC President

Preface

It is an honor for each of us to serve as Co-Editors for this important contribution in the field of endocrinology, more specifically sport and exercise endocrinology. We were fortunate to have an exceptional group of scientists to participate in this seminal work in our field. Thus, each chapter is written by one or more of the world's leading scientists in their specific area of expertise. Their enthusiasm and excitement for the project and its importantance is reflected in the very dynamic of each chapter. We acknowledge many of our other distinguished colleagues who have also made influential contributions to this field but were unable to participate in this project, as this field of study has undergone dramatic growth over the past 20 years.

Each author was asked to develop a working paradigm that would not only provide a cutting edge overview of the field as it currently exists but would also provide a template for research over the coming years. This encyclopedia provides one of the few resources for such a comprehensive view of the many aspects of endocrinology in sport and exercise. It is important to understand that each chapter was not meant to be a comprehensive review of the existing literature, but rather a state-of-the-art conceptual framework for the area covered. Thus, it was not the purpose to provide exhaustive references but rather a "cutting edge" perspective for use by both clinical and basic scientists. It is our hope that this encyclopedia serves to educate as well as inspire future research in the area of endocrinology in sport and exercise.

For so many years the field of exercise endocrinology has been folded into the many other areas of physiology, lacking direct acknowledgment of its own value as a discipline. The study of endocrinology as a specialty in medicine has been in existence for decades, while its applications in the field of exercise and sport has been a more recent phenomenon with typical focus on one or, at most, a few hormones. This encyclopedia provides the reader with a more complete view of the many targeted areas of study that have propelled this field forward over the past several decades. The number of publications listed on PubMed with hormones and exercise has increased by more than 30-fold over the past 20 years. Advances in technology as well as greater interest in the endocrine system and its integration with the nervous and immune systems have fueled the exponential rise in publications in endocrinology and exercise.

The encyclopedia begins with a basic overview of the principles and concepts in the field of endocrinology. It then takes the reader through the different endocrine glands allowing multiple perspectives on function. The interactive effects of hormonal influences on the immune system, muscle and bone are then examined. The interactions with nutrition are then covered, providing a unique view of one of the most important (and controversial) aspects of sport and exercise performance. The text then provides a unique examination of the hormonal interfaces with environmental stresses. The study of the underlying hormonal aspects of sport itself remains a much less developed area of study due to the inability to access athletes under competitive stress in game/match/meet conditions. In this encyclopedia, chapters examining overtraining,

the young athlete's growth and development, and the endocrinology of sport competition are covered, providing some unique perspectives for future work. The problem of anabolic drug use is then examined in detail. Ending with the study of endocrine mechanisms involved with competitive stress, this volume of The Encyclopaedia of Sports Medicine series permits the student, clinician and practicing scientist the chance to view this field more comprehensively. We are particularly indebted to the International Olympic Committee's Medical Commission and Sub-Commission on Publications in the Sport Sciences for providing us this opportunity to present to the world our field of study in its full array.

William J. Kraemer
Storrs, Connecticut
Alan D. Rogol
Charlottesville, Virginia

Chapter 1

Introduction

ALAN. D. ROGOL AND WILLIAM J. KRAEMER

What is endocrinology?

Endocrinology is the science of intercellular and intracellular communication. Classically, as defined by Bayliss and Starling more than 100 years ago, it was the secretion of a chemical substance (hormone, from the Greek word *hormaein*, to stimulate, to spur into action) into the general circulation to cause an effect at a distal site (target organ). Hormone receptors were unknown at that time. The hypothesis as stated was used to describe the secretion and action of secretin, a chemical substance secreted by the small intestine into the blood stream to stimulate pancreatic exocrine secretion (Bayliss & Starling 1902).

Initially all hormones were considered produced in specialized glands—for example, the thyroid, the adrenals or the pituitary—to act on single or multiple organs as a means of homeostatic regulation of metabolism (biochemical control mechanisms)—gene expression; biosynthetic pathways and their enzymatic catalysis; the modification, transformation and degradation of biological substances; the biochemical mediation of the actions and interactions of such substances; and the means for obtaining, storing and mobilizing stored energy (Table 1.1). This fundamental concept has been subsequently (and greatly) expanded to account for many forms of intercellular (and even intracellular) transfer of information in plants and animals (Table 1.2). Hormones represent many classes of biological molecules (Table 1.3). It now includes almost every tissue and cell for they either produce or respond to hormones. These hormonal systems integrate information in conjunction with the neural and immune systems to transmit it to define ambient conditions and to maintain metabolic homeostasis. These fundamental concepts have also been greatly expanded by advances in cell biology, molecular biology and genetics to help explain hormonal synthesis, action and perhaps integration, but the backbone of the endocrine system remains unchanged—integration of multiple systems and the maintenance of cellular and metabolic homeostasis.

Classically, hormones were defined by an ablation–replacement paradigm. Endocrine glands were removed from normal animals, the gland ground-up and extracted for the active substance. Restoration, by the extract of the function lost by removal of the gland, closed the loop and led to the discovery of many active hormonal compounds. Over 150 years ago, Berthold showed the 'proof-of-concept' of hormones by castrating roosters and noting regrowth of the wattles and comb by replacement of the testis into a body cavity (Berthold 1849). The transplant was both ectopic and lacked innervation, permitting him to conclude that the testes released a product that controlled the development (and maintenance) of the secondary sexual characteristics. The concept of homeostasis was stated by Claude Bernard, who showed that the liver could release glucose into the blood:

> The constancy of the internal environment is the condition that life should be free and independent. . . . So far from the higher animal being indifferent to the external world, it is on the contrary in a precise and informed relation

Table 1.1 Selected hormones of the endocrine system and their actions.

Endocrine organ	Hormone	Major actions
Testes	Testosterone	Stimulates development and maintenance of male sex characteristics, growth and protein anabolism
Ovaries	Estrogens	Develops female secondary sex characteristics; matures the epiphyses of the long bones
	Progesterone	Develops female sex characteristics; maintains pregnancy; develops mammary glands
Anterior pituitary	Growth hormone	Stimulates IGF-I and -II synthesis; stimulates protein synthesis, growth and intermediary metabolism
	Adrenocorticotropic hormone (ACTH)	Stimulates glucocorticoid release in adrenal cortex
	Thyroid-stimulating hormone (TSH)	Stimulates thyroid hormone synthesis and secretion
	Follicle-stimulating hormone (FSH)	Stimulates growth of follicles in ovary, seminiferous tubules in testes and sperm production
	Luteinizing hormone (LH)	Stimulates ovulation and production and secretion of sex hormones in ovaries and testes
	Prolactin (Prl)	Stimulates milk production in mammary glands
Posterior pituitary	Antidiuretic hormone (ADH)	Increases reabsorption of water by kidneys and stimulates contraction of smooth muscle
	Oxytocin	Stimulates uterine contractions and release of milk by mammary glands
Adrenal cortex	Glucocorticoids	Inhibits or retards amino acid incorporation into proteins (cortisol); stimulates conversion of proteins into carbohydrates (gluconeogenesis); maintains normal blood sugar level; conserves glucose; promotes metabolism of fat
	Mineralcorticoids (aldosterone, deoxycorticosterone, etc.)	Increases or decreases sodium–potassium metabolism; increases body water
Adrenal medulla	Epinephrine	Increases cardiac output; increases blood sugar, glycogen breakdown and fat mobilization
	Norepinephrine (some)	Similar to epinephrine plus constriction of blood vessels
	Proenkephalins (e.g. peptide F, E)	Analgesia; enhances immune function
Thyroid	Thyroxine	Stimulates oxidative metabolism in mitochondria and cell growth
	Calcitonin	Reduces blood calcium levels; inhibits osteoclast function
Heart (cardiocytes)	Atrial natriuretic hormone	Facilitates the excretion of sodium and water; regulates blood pressure and volume homeostasis and opposes the actions of the renin–angiotensin system
Pancreas	Insulin	Stimulates absorption of glucose and storage as glycogen
	Glucagon	Increases blood glucose levels
Parathyroids	Parathyroid hormone	Increases blood calcium; decreases blood phosphate
Skin	Vitamin D	Produces vitamin D from 7-dehydrocholesterol and sunlight
Adipose tissue	Leptin	Regulates appetite and energy expenditure

IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II.

Table 1.2 Cellular communication.

Endocrine	Chemical messenger (hormone) formed in a specialized tissue or organ (gland) and released into a circulation to affect another organ at a distance
Paracrine	Hormone synthesized in specialized cells to act on nearby cells
Juxtacrine	Hormone synthesized in one cell and acts on a contiguous cell
Autocrine	Hormone synthesized in one cell and acts on the surface of the same cell
Intracrine	Hormone synthesized in one cell and acts on the internal machinery of that cell without the necessity of cell surface receptors

Table 1.3 Chemical classes of hormonal molecules.

Amino acid derivative	Epinephrine, thyroid hormones
Steroid	Testosterone, cortisol, vitamin D
Peptide	Gonadotropin-releasing hormone, secretin
Fatty acid derivatives	Prostaglandins, leukotrienes

> with it, in such a way that its equilibrium results from a continuous and delicate compensation, established as by the most sensitive of balances (Bernard 1957).

Both of these results were known to Bayliss and Starling when they stated the secretin hypothesis and coined the word *hormone.* Later, Walter Cannon emphasized that the constancy of the internal environment could only be achieved through the operation and integration of exquisitely co-ordinated physiologic processes which he named *homeostatic* (Cannon 1939).

Hormones may be fully active when secreted, for example cortisol; however, some may require modification to become fully active, for example the conversion of tetraiodothyronine (thyroxine, T_4) to triiodothyronine (T_3) by a specific deiodinase, the conversion of testosterone to dihydrotestosterone (DHT) by 5-α reductase and the conversion of vitamin D_3 to 1,25 dihydroxyvitamin D by two distinct hydroxylases. Post-translational modification by phosphorylation, sulfation, or the addition of a lipid chain may be required to permit solubility in the particular environment that the hormone finds itself.

Because hormones may work at a distance, novel transport systems have evolved. Some steroid hormones have specific binding proteins to permit solubility in aqueous environments (sex steroids bind to sex hormone binding globulin [SHBG] and to albumin). There is a large series of insulin-like growth factor binding proteins (IGFBPs) that carry IGF-I and II to their sites of action, prolong their circulating half-life and dampen their effects as hypoglycemic agents (LeRoith 2003).

Most hormones are not secreted at a constant rate. Thus, the circulating concentrations undergo fluctuations that may be due to intermittent secretion or alterations in metabolism (clearance). For example, the circulating concentrations of insulin are not uniform, but vary from minute-to-minute according to the timing and composition of meals; from a baseline that is often determined by the relative mass of lean and fat tissue and the regional distribution of the fat tissue (visceral versus subcutaneous). The concentrations of growth hormone vary in 'pulses' throughout the day in response to meals and to sleep. Those of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and the sex steroids, estradiol and progesterone, vary over the approximately 28 days of the menstrual cycle in addition to the 1- to 2-hourly pulses of LH during the day. Adrenocorticotropic hormone (ACTH) and cortisol usually maintain a daily rhythm (high early in the morning and low at night) in those who sleep nocturnally. Disruption of these rhythms can lead to infertility or jet-lag.

What do hormones do?

For signal recognition the target cell produces a highly specific 'receptor' that recognizes the chemical

message (primary messenger) and begins a cascade of chemical reactions that lead to an alteration in the output of that cell, for example protein synthesis. Receptor proteins may be expressed at the cell surface, cytoplasm and the nucleus. The receptors themselves may be part of the overall regulating mechanism, for the signals from the hormones may be modified by their number and affinity for their ligand. Receptor endocytosis leads to the internalization of cell surface receptors; the hormone–receptor complex is subsequently dissociated and leads to a diminution of hormonal effect. Receptor desensitization (attenuated receptor signaling in the presence of ligand) may alter the receptor's affinity for the ligand, for example by phosphorylation (for epinephrine). The major hormonal signaling pathways use G-protein coupled receptors, tyrosine kinase receptors, serine/threonine kinase receptors, ion channels, cytokine receptors and nuclear receptors to activate the intracellular machinery.

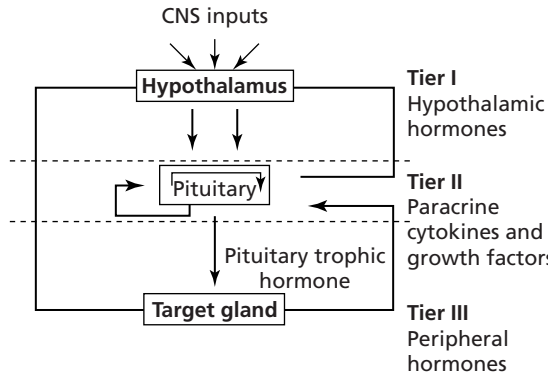

Fig. 1.1 Model for regulation of anterior pituitary hormones secretion by three tiers of control. Hypothalamic hormones impinge directly on their respective target cells. Intrapituitary cytokines and growth factors regulate tropic cell function by paracrine (and autocrine) control. Peripheral hormones exert negative feedback inhibition of respective pituitary trophic hormone synthesis and secretion. (Reproduced with permission from Ray & Melmed 1997.)

Control of hormone secretion

The endocrine system is organized in a hierarchal manner. As an example, the hypothalamus releases thyrotropin-releasing hormone (TRH) which stimulates the anterior pituitary to produce thyroid-stimulating hormone (TSH) which, in turn, acts on the thyroid to increase the synthesis and release of the thyroid hormones, T_4 and T_3. The thyroid hormones circulate bound to a specific binding protein, thyroxine binding protein (TBG), to affect virtually all cells in the body through intracellular thyroid hormone receptors and usually regulate the rate of metabolism of their targets (Fig. 1.1).

All of these systems are regulated by a series of negative (and some positive) feedback loops to maintain the system within a narrow range of operation (homeostasis). However, this complex, hierarchal system does not operate in a vacuum. There are multiple points of contact of the many endocrine axes with the neural and immune systems, as exemplified by the organism's response to stress (Fig. 1.2) (Selye 1950), which is served by a complex interaction of the central nervous system (CNS) and peripheral organs. This system must receive (and integrate) multiple neurosensory signals, for example visual and visceral inputs, blood borne and limbic signals. The concerted activation of this system leads to multiple outputs including physical and behavioral changes. When these are adaptive, one usually remains within the homeostatic range (see above). The hierarchy of this response is instructive for many of the other interactions of the neural, endocrine and immune systems.

The central components include the parvocellular corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) neurons within the paraventricular nucleus of the hypothalamus, the CRH neurons of the paragigantocellular and parabranchial nuclei of the medulla and the locus ceruleus (LC), and other mostly noradrenergic (norepinephrine [NE]) cell groups of the medulla and pons (the LC/NE sympathetic system, see Fig. 1.2). The peripheral limbs are the hypothalamic–pituitary–adrenal (HPA) axis, the efferent sympathetic–adrenomedullary system and some components of the parasympathetic nervous system. AVP and CRH activate the pituitary to produce proopiomelanocortin (POMC), which is eventually cleaved to produce ACTH (and many other centrally active peptides, such as α-melanocyte stimulating hormone, α-MSH). ACTH activates

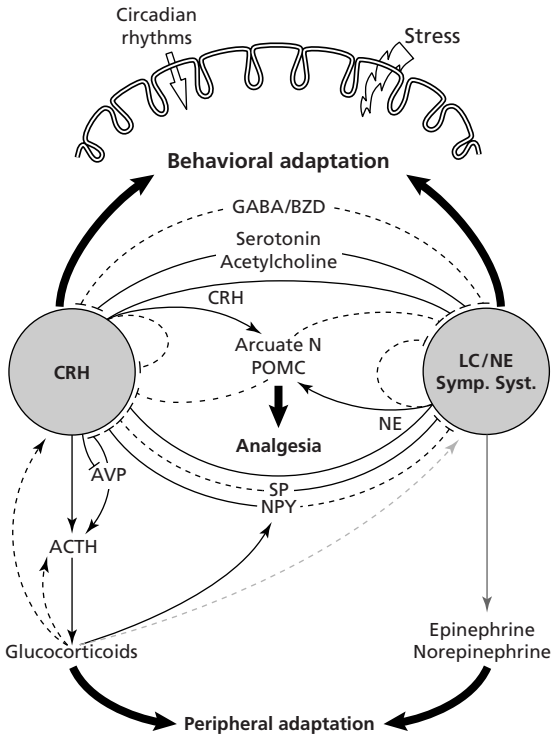

Fig. 1.2 Simplified representation of the central and peripheral components of the stress system, their functional interrelations and their relationships to other central nervous systems involved in the stress response. Activation is represented by solid lines and inhibition by dashed lines. ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; BZD, benzodiazepine; CRH, corticotropin-releasing hormone; GABA, γ-aminobutyric acid; LC/NE Sym. Syst., locus ceruleus/norepinephrine–sympathetic system; NE, norepinephrine; NPY, neuropeptide Y; POMC, proopiomelanocortin; SP, substance P. (Adapted with permission from Chrousos & Gold 1992. Copyright 1992, American Medical Association.)

the adrenal glands to produce cortisol and other steroids that affect metabolic homeostasis and the immune system. There are further interactions with the gonad axis (inhibition at multiple levels), the hypothalamic–pituitary–growth hormone/IGF-I axis (inhibition at multiple levels) and the thyroid axis (decreased production of TSH and inhibition of the conversion of T_4 to the more metabolically active T_3—in fact it has been called the 'euthyroid sick' syndrome in which there are diminished amounts of

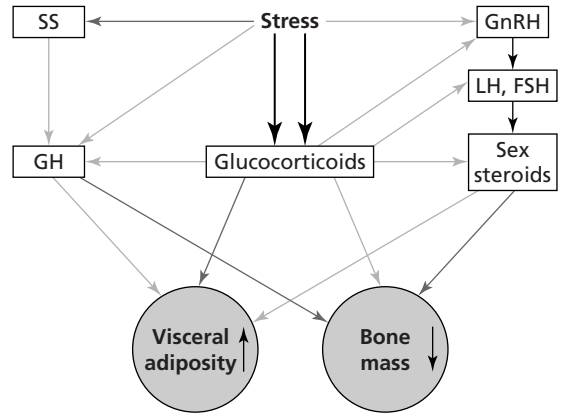

Fig. 1.3 Schematic representation of the detrimental effects of chronic stress on adipose tissue metabolism and bone mass. Stimulation is represented by solid lines and inhibition by light lines. FSH, follicle-stimulating hormone; GH, growth hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; SS, somatostatin. (Adapted with permission from Tsigos & Chrousos 1995.)

TSH and the peripheral thyroid hormones are less active). Further multiple metabolic axes are affected by antagonizing the effects of growth hormone and sex steroids on fat tissue catabolism and muscle and bone anabolism (Fig. 1.3).

Exercise and endocrinology

Exercise produces dramatic challenges to the homeostatic mechanisms. The acute exercise responses can see metabolic increases of 10-fold or more. Force production can be repetitively high and maximal limits reached in typical training sessions. The challenges to the body under conditions of athletic competition are also dramatic—ranging from performance of a marathon race in under 2 h and 10 min to an Olympic weightlifter lifting a weight four times his body mass. The mechanisms by which exercise is tolerated and adapted to are intimately related to hormonal regulation of physiological systems, but with both acute and chronic changes.

Over the past 50 years or more, exercise and sport physiology has continued to increase its study of hormonal mechanisms that mediate the exercise-induced adaptations. For example, in resistance

training of primary importance to acute exercise performance and subsequent tissue remodeling is the role(s) played by many members of the endocrine system (Kraemer & Ratamess 2003). Hormonal elevations in response to resistance exercise take place in a unique physiological environment. Acute elevations in circulating blood hormone concentrations (i.e. resulting from either increased secretion, reduced hepatic clearance, plasma volume reductions, reduced degradation rates) observed both during and immediately following a resistance exercise protocol present a greater likelihood of interaction with receptors on either the target tissue cell membrane (e.g. peptides) or with nuclear/cytoplasmic receptors located within the target tissue (e.g. steroid receptors) (Kraemer 2000). Coinciding with blood hormonal concentrations is the number of available receptors for binding and subsequent cellular changes. Interaction with the receptor initiates a myriad of events culminating in a specific response, such as an increase in muscle protein synthesis. Therefore, from the role of anabolic hormones (e.g. growth hormone, testosterone, IGFs) in protein synthesis in response to resistance training to insulin's role in glycogen metabolism with endurance training, hormonal mechanisms have become prominent in the study of exercise and sport. Due to the ubiquitous nature of hormones, no physiological system can adequately function without one or more involved with its response and adaptation to one form or another of exercise. Such dramatic hormonal influences have increased the interest in endocrinology for those who investigate exercise and sport.

With exercise creating such a unique physiological environment, one cannot merely extrapolate our understanding of resting homeostatic physiology (endocrinology). The conditions with exercise are such that the stimulus is highly specific in its nature. Different from the general nature of stress proposed by Selye (1950) over 50 years ago, we now know that stress is very specific in its 'fingerprint' and mediating mechanisms. Thus, the magnitude of hormonal responses as well as the biocompartments in which they occur can differ dramatically. For example, with a resistance exercise that just stimulates an arm muscle, one notes only small or no increases in circulating anabolic hormones, but growth factor concentrations (for example, IGF-I) may dramatically increase, specifically at the site of their action. Differences in hormonal responses also occur with the magnitude of the exercise intensity—low intensity exercise produces a smaller magnitude of hormonal response than higher intensity exercise. Thus, the influence of work, intensity, volume and frequency all help create the exercise stimulus that occurs acutely with a single exercise session or repetitively as one continues with training.

Understanding the role of different hormones within and among the various physiological communication systems is a challenge as few, if any, hormones act in isolation. Furthermore, with multiple-level communication being so important to optimal homeostatic regulation, complex integration of hormonal signals is required to respond to the multiple energy demands of exercise.

Finally, the study of hormones and their roles in exercise and sport has permitted a better understanding of the stresses of competition, overtraining and identified key factors in exercise prescription (e.g. intensities, frequencies and durations) that can be optimized to create improved training programs and, ultimately, performance. Ultimately, the underlying physiological basis of any exercise or sport stress has its underpinnings in endocrinological science.

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Chapter 2

Basic Principles and Mechanisms of Endocrinology

MICHAEL R. DESCHENES AND KEIICHIRO DOHI

Introduction and general principles

The overarching theme of physiology is the persistent effort to maintain homeostasis within the organism. This drive to sustain a constant internal milieu is challenged by regular perturbations of the organism's internal and external environment. In the face of these changes, the capacity to achieve homoeostasis is determined by effective communication among the cells of the organism. Two systems have evolved to provide such communication. The nervous system generally involves immediate and brief responses to stimuli. In contrast, the endocrine system typically is slower to respond to alterations in the environment, but its responses persist longer than those of the nervous system. The effects of the endocrine system are pervasive, and regulate the activity of virtually all cells within the body. Each of these cells is perfused with blood; the endocrine system capitalizes on this physiological arrangement to communicate effectively throughout the body.

The word 'hormone' derives from the Greek word *hormaein* which means 'to spur into action' or 'to stimulate'. In 1902, Bayliss and Starling (1902) described a substance secreted into the blood by one organ (small intestine) that gave rise to a response in another (pancreas). Thus, secretin became the first hormone identified. Today, a hormone is commonly defined as 'a chemical substance that is released into the blood in small amounts and that, after delivery by the circulation, elicits a typical physiological response in other cells' (Goodman 1994). It has been discovered, however, that in addition to this example of classical endocrine function, mediating agents can be released into the interstitial fluid to diffuse to and affect neighboring cells (paracrine effect), or even to interact with the same cell that secreted them (autocrine effect). Indeed, some substances, for instance insulin-like growth factor I (IGF-I), may exert their biological responses via endocrine, paracrine and autocrine routes (Yakar *et al*. 2002). More recently it has been proposed (Re 2003) that some growth factors and peptide hormones may directly regulate activity within the cell of synthesis without ever exiting that cell (intracrine effect). For the purpose of this chapter, only the features of the endocrine system will be addressed.

Although scores of hormones have been identified, and their biological activities regulate a plethora of physiological processes, several fundamental principles of endocrine function have been established. *First*, hormones are synthesized and secreted by specialized ductless endocrine glands into the bloodstream to be carried to 'target' cells that bind the hormone and respond to it by altering their biological activity in a specific, preprogrammed fashion. *Second*, although some endocrine glands are the main components of organs specialized for endocrine function (e.g. pituitary, thyroid), others are located in organs that have distinct, primary physiological functions (e.g. heart, gut, kidney). *Third*, a single endocrine gland may produce more than one hormone. *Fourth*, with rare exception, however, a single endocrine cell will synthesize and release only one hormone. *Fifth*, a particular hormone may be secreted by more than one endocrine gland. *Sixth*, a single hormone may elicit several

different physiological responses by binding with several different types of target cells. *Seventh*, any one hormone, however, elicits only a single response in any one type of target cell. *Eighth*, any one target cell may respond to numerous hormones, each inducing its own biological response within that cell. *Ninth*, a particular intracellular activity, glycolysis for example, can be regulated by more than one hormone. *Tenth*, a target cell's responsiveness to a specific hormone may vary according to its own state of differentiation, presence of other hormone, environmental conditions, etc.

While the endocrine system regulates a host of biological activities within the target cells of the organism, the physiological effects of hormones can be broadly assigned to four areas. These are: (i) the digestion and metabolism (anabolic and catabolic mechanisms) of food nutrients; (ii) salt and water balance; (iii) growth and development; and (iv) reproductive function.

Hormone categorization and synthesis

All of the known hormones can be classified according to their chemical composition and manner of synthesis as either: (i) steroids; (ii) peptides/proteins; or (iii) amines. *Steroid* hormones are derived from cholesterol and include the sex steroids (androgens, estrogens, progestins), which are produced in the gonads, as well as the glucocorticoids and mineralocoids, which are synthesized by the adrenal glands. In humans, the main androgen, or male sex steroid, circulating in the blood is testosterone. Similarly, estrogens are a family of female sex steroids, but in humans estradiol is the primary estrogen, and among progestins, progesterone dominates. Cortisol is the principal glucocorticoid in humans, and aldosterone is the major mineralocorticoid.

Since the same precursor molecule—cholesterol—is used in the formation of all steroid hormones, it is the biosynthetic enzyme pathways present in the endocrine gland that determines the specific steroid that is mainly produced by that gland. Typically, however, small amounts of a secondary hormone are concurrently synthesized as some diversion among the enzyme pathways occurs. For example, the bulk of steroids synthesized by the testes is in the form of testosterone, yet trace amounts of cortisol are also manufactured due to a minor presence of the enzymes that comprise that steroid's biosynthetic pathway. In viewing Fig. 2.1, it is easy to appreciate how this diversion among enzymatic pathways can occur.

The rate at which steroid hormones are produced is dictated—as in all enzymatic pathways—by the activity of the rate limiting enzyme of the pathway, i.e. the enzyme that catalyzes the slowest reaction within the cascade. In all steroid hormone production the rate limiting reaction is the conversion of cholesterol to pregnenolone. Thus, factors that increase the rate of steroid production do so primarily by accelerating the formation of pregnenolone, and secondarily by amplifying the uptake of cholesterol from the blood into the endocrine gland (Rhoades & Pflanzer 2003).

In the endocrine glands that manufacture steroids, there is no capacity for storage of the newly synthesized hormone. Accordingly, as the steroid is synthesized, it is secreted into the blood stream, and therefore the rate of the newly formed steroid's release into the blood equals the rate of its production within the endocrine cell.

Peptide and protein hormones are comprised of chains of amino acids. Should a small number of amino acids be involved (< 20), the hormone is typically referred to as a peptide hormone, but if 20 or more amino acids comprise its structure, the chain is considered a protein hormone (Goodman 1994). Examples of peptide hormones include oxytocin, vasopressin, and somatostatin. Among the many (~ 100) protein hormones identified to date are insulin, growth hormone, calcitonin and glucagon. Some of these proteins exist as relatively simple, single chains of amino acids, while others feature disulfide bonds to connect different regions of the polypeptide sequence to convey complex tertiary structure. Some protein hormones are even composed of multiple subunits that are bonded together to form a single structure.

Regardless of their final structure, all peptide/protein hormones are synthesized within the endocrine cell in a manner that is consistent with protein production in all cells. That is, peptide/protein hormone precursors are synthesized by ribosomes

Fig. 2.1 Biosynthetic pathways of steroid hormones. Involved enzymes are found in parentheses.

—in conjunction with tRNA and mRNA—as much longer products than they ultimately appear in their final form. These *preprohormones* contain signal sequences indicating that the protein is intended for release from the cell. Initial modifications of these molecules occur in the endoplasmic reticulum, where the ribosomes are attached, and include proteolytic events that remove amino acid sequences —including the signal sequence—resulting in shorter chains. The newly formed *prohormones* then journey to the Golgi complex where they undergo additional modifications that include further proteolytic cleavage and perhaps the addition of carbohydrate (glycosylation) or phosphate (phosphorylation) groups. Upon completion of these modifications, the Golgi complex pinches off a segment of its membrane to encapsulate the finished hormone in a vesicle. This secretory vesicle remains stored in the endocrine cell's cytoplasm until the cell receives an appropriate signal resulting in an influx of calcium. The increased intracellular concentration of calcium causes the membrane of the secretory vesicle to fuse with the cell's plasma membrane, thus releasing the hormone via exocytosis. Typically the amount of peptide/protein hormone stored by the endocrine cell is limited and as a result the signal that stimulates the secretion of the cell's hormone also triggers the synthesis of additional quantities of that hormone (Rhoades & Pflanzer 2003).

The proteolytic cleavage of the prohormone during protein hormone synthesis conveys great diversity in the number of hormones produced by the endocrine system. The same precursor molecule can undergo differential processing resulting in the assembly of numerous end products. Perhaps the best example of this is the precursor proopiomelanocortin (POMC) which contains the amino acid sequences of several peptide/protein hormones including adrenocorticotropic hormone (ACTH), β-endorphin and β-lipotropic hormone, among others

(Krieger *et al*. 1980; Chretian & Seidah 1981). The specific cleavage enzymes expressed in the particular endocrine gland synthesizing POMC determines the principal hormone manufactured by that gland. For example, the anterior pituitary contains a specific set of proteolytic enzymes that will yield ACTH as its primary end product from the POMC prohormone. In contrast, neurons within the brain that produce POMC possess enzymes that cleave the molecule in such a way that β-endorphin is mainly secreted. This differential processing of POMC also occurs in the placenta, reproductive organs, gastrointestinal tract and lung (Liotta *et al*. 1982; Margioris *et al*. 1982). This gland specific expression of different hormones from a common precursor molecule—based upon the enzyme profile present—is not unlike the biosynthesis of steroids.

Amine hormones—also referred to as amino acid derivatives—are those that use the amino acid tyrosine as their initial precursor. Included in this category are the thyroid hormones (thyroxine [T_4] and triiodothyronine [T_3]) and the catecholamines (epinephrine and norepinephrine). Despite sharing the same precursor molecule, the thyroid hormones and catecholamines differ in many respects including their synthesis, transport through the blood stream and mechanism of action at target cells. Here the amine hormones will be addressed separately in describing their synthesis.

Thyroid hormone formation is dependent upon the uptake of both tyrosine and the mineral iodide from the blood into the follicular cells of the thyroid gland. Tyrosine is used as a backbone for the manufacture of thyroglobulin which is a large glycoprotein that is stored in large amounts within the follicular cells. With the uptake of iodide from the blood, the tyrosine residues of thyroglobulin become iodinated through a multistep reaction, ultimately forming either T_4 or T_3, depending on the number of iodide ions that bind with thyroglobulin. Initially, thyroglobulin reacts with either one or two iodide ions resulting in the production of either monoiodotyrosine (MIT), or diiodotyrosine (DIT), respectively. In the next step of the enzymatic pathway of thyroid hormone synthesis, two iodide atoms are added to the thyroglobulin molecule converting MIT to T_3 and DIT to T_4. At this point the thyroid hormones are part of the larger thyroglobulin structure that is stored within the glandular cell. Upon stimulation to release thyroid hormone, proteolytic enzymes within the follicular cell break down the stored thyroglobulin liberating the T_3 and T_4 hormones intact, which are then released into the circulation.

The catecholamines—epinephrine and norepinephrine—also use tyrosine as their initial precursor, but are produced in the medulla region of the adrenal glands. This adrenomedullary tissue is actually a modified component of the sympathetic, or excitatory, branch of the autocrine nervous system. In fact, the adrenal medulla directly receives input from nerve terminals of the sympathetic nervous system serving as an example of neuroendocrine function.

Catecholamine synthesis occurs in the chromaffin cells of the adrenal medulla via a multistep biosynthetic pathway. First, tyrosine is converted to 3,4-dihydroxyphenylalanine (dopa) by tyrosine hydroxylase which acts as the rate limiting step in the production of catecholamines. Dopa is then converted to dopamine, which is then transformed into norepinephrine, most of which is methylated by the enzyme phenylethanolamine–*N*–methyl transferase (PNMT) resulting in the formation of epinephrine. Both norepinephrine and epinephrine are considered catecholamines, but the stoichiometry of their synthesis and release from the adrenal gland is 1 : 4. Although circulating levels of norepinephrine exceed those of epinephrine, the bulk of this norepinephrine originates from the sympathetic nervous system where it functions as a neurotransmitter and 'spills over' into the blood. Epinephrine, however, is the primary catecholamine hormone circulating in the bloodstream (Hedge *et al*. 1987).

Upon their production, the same glandular cells that synthesize the catecholamines store them as chromaffin granules. Stimulation by the sympathetic nervous system results both in the secretion of catecholamines through typical exocytotic actions, and increased activation of tyrosine hydroxylase affecting a greater rate of catecholamine synthesis by the chromaffin cells, so that intracellular catecholamine depots can be replaced (Rhoades & Pflanzer 2003).

Regulation of hormone secretion

The effect of a hormone on its target tissue is proportional to its concentration within the bloodstream. Several factors combine to determine the level of any biologically active hormone in the circulation. These variables include: (i) rate of secretion from the endocrine gland into the circulatory system; (ii) for some hormones, i.e. thyroid hormones, rate of activation—the conversion of T_4 to T_3—within the blood; (iii) for lipophilic hormones, i.e. steroid and thyroid hormones, degree of binding to plasma proteins; and (iv) rate of inactivation and clearance from the blood. Of these factors it is the first-rate of release into the blood—that is the principal determinant of circulating hormone levels, particularly under non-exercising conditions (Sherwood 2004).

In general, there are two modes of hormonal release into the bloodstream (Kelly 1985). *Constitutive* release refers to a continual, moderate discharge of endocrine agent into the circulation. In this release mechanism, hormone exits as it is synthesized; there is no storage capacity within the endocrine gland. Consequently, upon receipt of stimulatory signal, synthetic pathways increase their activity and newly formed hormone is directly released into the circulation via passive diffusion through the cell's plasma membrane. This release pattern governs circulating levels of steroid and thyroid hormones, which like the cell's plasma membrane, are lipophilic in nature. Constitutive secretion is mediated by alterations in the phosphorylation status of proteins acting as enzymes in endocrine biosynthetic pathways.

Regulated release is the second mode of delivery of hormone from the endocrine gland to the bloodstream. In this case, the rates of protein synthesis and release are not directly coupled, as they are in constitutive release. Rather, endocrine glands that employ regulated release possess the ability to store newly synthesized hormone. It should be noted, however, that even in this type of endocrine gland storage potential is limited. Indeed, for any particular hormone, it is rare for even a day's supply to be stored and available for release (Baulieu 1990).

In regulated release, a stimulus causes the exocytotic release of prepackaged vesicles containing hormone. In most cases, to assure a ready supply of hormone, the same stimulus that evokes the release of stored hormone also activates enzymatic pathways that synthesize that hormone. An influx of calcium into the endocrine cell's cytosol usually precedes the release of stored hormone and activation of biosynthetic pathways. This regulated form of release is evident in the secretion of peptide/protein hormones, as well as catecholamines.

With both constitutive and regulated hormonal release, the stimuli that govern secretion are typically: (i) changes in plasma nutrient or ion concentrations; (ii) neurotransmitters released from neurons onto endocrine cells; or (iii) the binding of hormones released by other endocrine glands. Generally, these stimuli do not operate independently of each other. To the contrary, alterations in endocrine secretion usually are a function of receiving input from more than one type of stimulus.

The responsiveness of endocrine glands to these stimuli is contingent upon a sensitive and effective feedback system that conveys information from target tissues back to the hormone releasing organ. The most prevalent form of communication regulating secretory rates of endocrine glands is *negative feedback*. This type of feedback occurs when the activity of one system (endocrine gland) adjusts to negate, or offset, a change in another system (target tissue), thereby re-establishing homeostasis. For example, an elevation in blood glucose triggers the secretion of insulin by the pancreas. The increased blood-borne insulin level expedites the uptake of glucose into fat and muscle cells—target cells of insulin—resulting in a normalization of glucose concentration in the blood.

There are even multiple forms of negative feedback regulation controlling hormone secretion. The release of several important hormones is directed by the hypothalamic–pituitary axis. A small region at the base of the brain, the hypothalamus produces several releasing hormones that are transported by a portal blood supply to the anterior segment of the pituitary gland. In this example of *neuroendocrine* function, the hypothalamus delivers releasing hormones to the pituitary which, in turn, alters its rate of secretion of a number of hormones into the systemic circulation where they can be transported

throughout the organism. These pituitary hormones may directly bring about effects on target tissues, or they may react with a third gland in the axis, thereby influencing this gland's secretion of hormone into the bloodstream.

Regarding negative feedback, this neuroendocrine axis can display both 'short-loop' and 'long-loop' regulation (Vander *et al*. 2001). In an example of short-loop negative feedback, high levels of prolactin in the bloodstream are detected by the hypothalamus causing it to increase its own secretion of dopamine to the pituitary, thus curtailing that gland's release of prolactin into the circulation. In the three organ neuroendocrine axis, long-loop negative feedback is also evident. To illustrate this feedback mechanism, the regulation of cortisol secretion will be discussed. The axis involved features the hypothalamus, pituitary, and adrenal cortex. The hypothalamus delivers corticotropin-releasing hormone (CRH) into the portal blood system where it is carried to the anterior pituitary. Upon binding of CRH, the pituitary releases ACTH into the systemic bloodstream so that it can be delivered to the adrenal glands. In the cortex region of the adrenal—recall that the adrenal medulla produces catecholamines—the binding of ACTH triggers the release of cortisol into the systemic circulation so that it can elicit glucocorticoid effects on target tissues such as the liver, skeletal muscle and adipose. In this case, negative feedback occurs when the elevation in blood-borne cortisol blunts the release of ACTH from the pituitary and/or the secretion of CRH from the hypothalamus. As an example of the exquisite integration of signals delivered and received throughout the endocrine system, the hormone secretion of a multiorgan endocrine axis can be regulated both by short-loop and long-loop feedback (Vander *et al*. 2001). Illustrations of short- and long-loop negative feedback are displayed in Fig. 2.2.

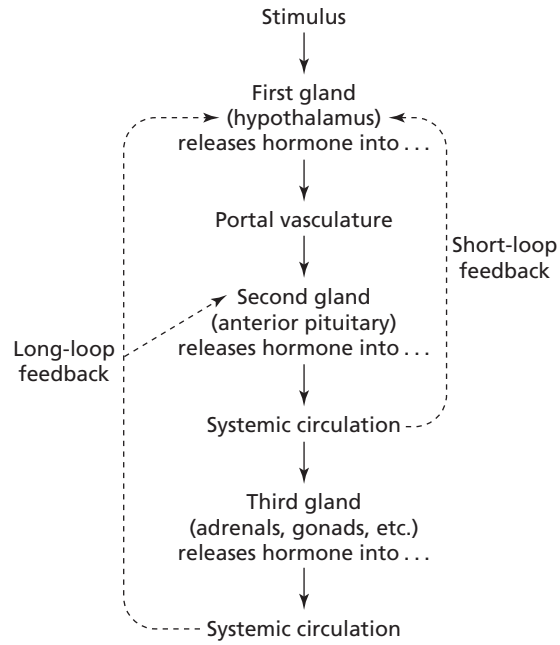

Fig. 2.2 Illustration of short-loop and long-loop negative feedback in the regulation of hormone release.

Although negative feedback is much more prevalent, *positive feedback* is also used to adjust hormone secretion. In positive feedback, the hormone induced change in the biological activity of the target tissue is monitored by the endocrine gland that initially released the hormone. Should the target tissue's response be insufficient in magnitude, the endocrine gland secretes additional hormone until the biological process it governs is adequate. An example of positive feedback is the regulation of endocrine function during childbirth. Oxytocin released by the posterior aspect of the pituitary gland stimulates muscle contraction of the uterus. As the birthing process continues and stronger uterine contractions are required, the activity of the uterus signals the pituitary to increase its secretion of oxytocin, thus amplifying the strength and frequency of uterine contractions enabling the completion of parturition.

Although the primary function of the endocrine system is to maintain homeostasis, changes in the body's internal and external environments are not the only regulators of hormone secretion. Indeed, circulating concentrations of most hormones display predictable fluctuations, or rhythms, over a given period of time. The best studied endocrine rhythm is the *circadian*, or *diurnal*, rhythm. 'Circadian' refers to the pattern of peaks and troughs throughout the roughly 24-h solar day, while 'diurnal' alludes to the day/night oscillations of hormone

secretion. Often these two terms are used interchangeably. These natural, preprogrammed rhythms of endocrine release originate in the suprachiasmatic nucleus region within the hypothalamus. This pacemaker regulates the secretion of hormones based upon its own internal clock, and elicits release patterns that are specific to each hormone. For example, cortisol levels in the blood are highest in the morning, while growth hormone peaks during the night-time hours (Illnerova *et al*. 2000).

In addition to variation over the 24-h day, hormone secretion often displays a regular pulsatile pattern sometimes referred to as an 'ultradian' rhythm that is superimposed on the underlying circadian rhythm. These episodic bursts of hormone release also appear to be directed by the hypothalamus and may have important physiological consequences. For example, it has been demonstrated that even when total dosage is the same, glucose uptake into target tissue is enhanced when insulin is delivered in pulses compared to a steady, non-pulsatile pattern (Porksen 2002).

Although not as well studied in humans as in animals, hormone secretion is also known to vary seasonally. These 'circannual' rhythms correspond to changes in the number of daylight hours, which are sensed by the pineal gland within the central nervous system (Short 1985). This gland, often alluded to as the 'third eye' due to its photosensitivity, adjusts the amount of melatonin it secretes in response to alterations in total daylight hours. In animals demonstrating seasonal breeding behavior, the production of gonadotropins is modulated by these circannual variations in melatonin production (Tamarkin *et al*. 1985). Other seasonal dependent behaviors, such as hibernation, migration and even changes in the color of fur, are guided by the predictable oscillations in circulating melatonin levels. In humans, increased melatonin production, which occurs as the number of daylight hours decreases, has been associated with altered mood states and even depression (Lewy *et al*. 1987). More recently it has been established that in all mammals—including humans—melatonin plays a major role in ensuring proper circadian rhythmicity by interacting with and influencing the suprachiasmatic nucleus (Pevet *et al*. 2002).

Hormone transport in the blood

Each hormone is released from the endocrine gland that produced it into the venous capillaries surrounding that gland. After passing through the lungs and heart, the hormone enters the general systemic circulation so that it may travel throughout the body. While traveling in the blood, a very small amount of hormone may be found adsorbed onto the plasma membrane of red blood cells, but mostly they are dissolved in the plasma (peptide/protein hormones), or bound to plasma proteins (steroids, thyroid hormones). Due to their chemical structure the peptide/protein hormones are hydrophilic in nature and readily dissolve in the blood's plasma. But once in the bloodstream these hormones are exposed to numerous proteolytic enzymes that break them down, thus preventing them from interacting with their target tissues. Recall, however, that endocrine glands that synthesize peptide/protein hormones are able to store them and secrete them upon receiving a stimulatory signal indicating a need for that particular hormone.

In contrast to the peptide/protein hormones, steroid hormones are hydrophobic in structure, and as a result, are not soluble in the plasma. Consequently, the vast majority of these hormones (> 95%) travel through the bloodstream bound to various plasma proteins. The amine hormones also are bound to proteins as they travel through the blood, albeit to various degrees. Only about 50% of blood-borne catecholamines are bound to carriers, while a much higher proportion (~ 99%) of thyroid hormones, which are hydrophobic in structure, are in the bound state.

Steroids may either bind to a plasma protein displaying high specificity for a particular hormone (Table 2.1), or with lower specificity and affinity to albumin and transthyretin, which are abundant circulating proteins. All carrier proteins, the high specificity transport proteins and the ubiquitous albumin and transthyretin, are synthesized by the liver before being released into the blood (Rhoades & Plfanzer 2003). The low affinity albumin and transthyretin molecules contain several binding sites which can be bound not only by hormones but also by other low molecular weight substances

Table 2.1 Examples of high specificity and affinity blood-borne hormone binding proteins.

Name	Abbreviation/alternate name	Hormone(s) bound
Sex hormone binding globulin	SHBG/sex steroid binding protein	Testosterone, estradiol
Corticosteroid binding globulin	CBG/transcortin	Glucocorticoids, progesterone, aldosterone (minor amounts)
Thyroxine binding globulin	TBG	Triiodothyronine (T_3), tetraiodothyronine (T_4)
Growth hormone binding protein	GHBP	Growth hormone
Insulin-like growth factor binding proteins	IGFBPs	IGF-I, IGF-II

found in the blood. In contrast, the high specificity transport proteins typically possess a single binding site which is configured to interact with a specific hormone, and displays greater affinity at its ligand binding site than does albumin or transthyretin (Baulieu 1990). The percentage of circulating hormone bound to albumin versus a specific transport protein differs among hydrophobic hormones. For example, equal amounts of bound aldosterone are complexed with specific binding proteins and albumin or transthyretin. But only 10% of circulating cortisol is bound to albumin or transthyretin, while the vast majority (> 85%) is bound to corticosteroid binding globulin.

Whether bound to albumin, transthyretin, or a specific transport protein, only the small amount of steroid or thyroid hormone that is unbound or 'free' is capable of binding with receptors sites expressed by target tissue. Thus, only the free portion of these hormones is able to stimulate biological activity within target cells. As this free steroid or thyroid hormone interacts with target tissue, some of the bound fraction dissociates from binding proteins, thereby forming a dynamic equilibrium between the bound and unbound fractions that is influenced by the need to maintain the organism's homeostasis. In effect, the bound fraction serves as a buffer to supply hormone to the target tissue as needed. This is presumed to compensate for the fact that unlike peptide/protein hormones, the glands that synthesize steroids and thyroid hormones are unable to store newly formed hormone to be released upon demand. Indeed, it is no longer considered that the primary function of transport proteins is to overcome the problem of insolubility in the plasma. It has been demonstrated that due to their small size, most steroid and thyroid hormones readily dissolve in the blood despite their hydrophobic chemical structure (Kronenberg *et al*. 2003). This suggests that the binding proteins mainly act to provide a buffering or storage capacity, and to prevent the small, intact hormones from passing from the blood into the renal tubules of the kidneys where they can be prematurely excreted from the body. In the case of insulin-like growth factors, which are hydrophilic in structure, specific binding proteins act to directly mediate the biological effect imparted by the hormone/receptor complex at the target cell (Firth & Baxter 2002).

Metabolic clearance of hormones

After entering the circulation most hormones are rapidly degraded and feature half-lives of no more than 30 min, although this can vary greatly. Catecholamines, for instance, have half-lives of only seconds, while thyroid hormones exhibit half-lives of several days. The length of any hormone's half-life in the bloodstream is dependent upon the metabolism and/or clearance from the circulation. As with transport, the chemical composition of the hormone will determine the specific method of its removal from the circulation, but in general, all hormones are primarily broken down within the liver and eliminated via the renal system. Peptide/protein hormones and catecholamines are typically degraded by proteolytic enzymes in the blood and the resultant amino acids are excreted through

the urinary tract. In contrast, steroid and thyroid hormones are resilient to circulating proteolytic enzymes, and they are mainly degraded in the liver through a series of reducing reactions which increase the solubility of these hydrophobic molecules. Since steroids are small in structure, the improved solubility then allows the majority of them to be eliminated via the urinary tract, although a small portion is secreted directly from the liver into the bile (Rhoades & Pflanzer 2003). Another, but less prevalent, mode of hormonal clearance is termed 'receptor-mediated endocytosis'. In this process some larger protein hormones such as insulin are internalized by the target cells after they have bound with receptors and elicited a response. After being engulfed by the target cell, the hormone is separated from its receptor and degraded by proteolytic enzymes located within the cell's cytoplasm.

Mechanism of action at target cells

General principles

The biological action(s) elicited by a hormone at its target cell is initiated by the binding of the hormone to its receptor produced by the cell. Indeed, this reaction between hormone and receptor lies at the foundation of the endocrine system. In this manner an endocrine gland may release a hormone into the general circulation where it will perfuse all tissues within the body, but will exert its influence only on those cells that express receptors specific for that hormone. Typically, a target cell will express 2000–100 000 receptors for any one hormone (Guyton & Hall 1996).

A receptor is a protein—sometimes a glycoprotein—that possesses one or more binding sites for ligand (hormone) and an effector, or active, site that triggers a biological response in the cell expressing that receptor. Each type of receptor displays a unique three-dimensional structure that is complimentary to the structure of a specific hormone, thus conferring the property of *specificity* to the receptor. The complimentary chemical structures apparent in the hormone and its receptor allow them to recognize each other and interact in what is sometimes referred to as a 'lock and key' formation. It is possible, however, that two hormones with very similar chemical structures, for example insulin and insulin-like growth factor, will cross-react with each other's receptors. Under normal conditions and hormone concentrations, such cross-reactivity is minimal and the resultant cellular activity is negligible.

The strength of the bond formed between the hormone and its receptor is described as the *affinity* of the ligand for its binding site. The affinity of the hormone–receptor complex determines how easily the bond can be broken. In high affinity reactions, much disturbance to environmental conditions, for example pH, temperature, etc., is necessary to disrupt the hormone–receptor complex. In contrast, low affinity bonds are easily broken and often require no change at all in the surrounding milieu. The cross-reactivity described above displays this low affinity binding, which is why those hormone–receptor complexes typically result in very little biological response in the cells where they occur.

A dynamic equilibrium exists between unbound hormone in the blood perfusing the target tissue and the hormone bound to the tissue's receptors. This equilibrium is essential to the endocrine system's regulatory function. Consistent with the laws of mass action, the greater the amount of hormone available to the target—circulating concentration—the greater the probability that receptor sites will be occupied by hormone, and thus the greater the biological response evident at the target. More directly stated, the target cell's change in biological activity is proportional to the number of hormone–receptor complexes formed, which is dictated by the hormone's concentration in the bloodstream. *Saturation* refers to the fraction of the target cell's receptors that is bound to hormone, and in physiological terms, this is critical in determining the response of the cell to the hormone. Two factors primarily account for the degree of saturation of a cell's binding sites: the concentration of unbound circulating hormone and the affinity of receptors for that hormone.

In considering the importance of saturation in regulating a target cell's response to a hormone, it is worth mentioning that, even at maximal biological response, not all of the cell's receptors for that hormone are bound. This observation has lead to the 'spare receptors' concept of endocrine function.

That is, 100% occupancy of receptors is not necessary to elicit maximal biological response, suggesting that the cell expresses more receptors than necessary. This 'overexpression' of receptors is, in fact, appropriate when one recalls that the formation of hormone–receptor complexes is governed by the rules of statistical probability. There are no forces of attraction that bring complimentary hormones and receptors together, although affinity sustains the bond once formed. There is simply a chance that circulating hormone and matching binding site will come into contact with each other, and then form a complex. Thus to enhance the probability that such random contacts will occur, target cells express more receptors than are needed to stimulate even maximal response. The expression of these 'spare receptors' is to be considered especially efficient and sensible given the very low concentrations at which hormones travel in the blood (10^{-8}–10^{-12} mol·L^{-1}).

Competition describes the process whereby different ligands—displaying similar chemical structure—may compete for binding at the same receptor. Under normal physiological conditions, little competition exists between hormones traveling through the bloodstream. The potential for such competition, however, enables the pharmacological management of some endocrine disorders, as drugs acting as antagonists may compete with endogenous hormones that are over-secreted for binding sites, thereby preventing an inappropriately amplified endocrine response. Also, the measurement of serum or plasma concentrations of hormones is predicated upon competition as endogenously produced hormones and labeled antigens vie for a given number of binding sites—antibodies—provided in the assay.

Although, in general, the concentration of any hormone present in the bloodstream dictates the degree of the biological response elicited by that hormone, the target cell is capable of fine tuning its response by adjusting the number—and perhaps affinity—of the receptors it expresses for that hormone. That is to ensure its proper function, target tissue may 'up-regulate' or 'down-regulate' its hormonal receptors to maintain its delicate homeostatic balance. The advantages of controlling the number of available binding sites is obvious when one considers that several types of cells, each with its own needs and homeostatic challenges, are sensitive to the same hormone that is secreted into the systemic circulation. For example, a disturbance in the environment of one cell type may trigger increased release of a particular hormone to counter it. But another cell that is also responsive to that hormone may be at risk of homeostatic imbalance as a result of the greater levels of that hormone. To prevent this, the cell may down-regulate, or temporarily decrease the number of receptors it makes available to bind the hormone. This would decrease the probability of hormone–receptor complex formation and dampen the cell's sensitivity to the hormone, thus maintaining its own homeostasis. Such adjustment in receptor number and/or affinity allows each type of target tissue to respond in a manner appropriate to its own needs when exposed to a given amount of hormone traveling throughout the entire organism. More long-term up- or down-regulation of receptor expression is evident with dramatic alterations in the production of hormone through surgical removal of endocrine glands (Dahlberg *et al*. 1981), disease (Potier *et al*. 2002; Pedersen & Vedickis 2003), or even as an adaptation to chronic exercise training (Tchaikovsky *et al*. 1986; Deschenes *et al*. 1994).

Not only do target cells express receptors for a host of different hormones so that various cellular functions can be regulated by the endocrine system, the same cell may respond to more than one hormone which affects the same biological process within the cell. This phenomenon is known as *redundancy*, but should not be considered a superfluous or inefficient means of controlling cellular physiology. For example, hepatocytes are sensitive to various hormones—including glucagon and epinephrine—that stimulate glycogenolysis and the release of glucose into the blood. However, different conditions may account for the need for additional circulating glucose. Missing a meal may result in depressed glucose levels in the bloodstream, which would be detected by the pancreas which would respond by secreting glucagon to break down glycogen stores in the liver. Epinephrine, too, stimulates glycogenolysis and the release of glucose from the

liver into the circulation. However, this is part of an overall 'fight or flight' response elicited by the sympathetic nervous system and the adrenal secretion of epinephrine that includes increased heart rate, blood pressure and sweat rate. Obviously, these responses would be inappropriate to address a simple fasting induced state of hypoglycemia. Seen in this context, redundancy in the expression of receptors for the glycogenolytic hormones does not appear wasteful, but rather a sophisticated approach to managing cellular homeostasis in the face of many different challenges.

Binding of hormone to receptor

The biological response attributed to a hormone must be preceded by the binding of that hormone with its receptors located at its target cells. In this scenario, it is best to view the receptor as a mediator that transducts the extracellular message carried by the hormone to an intracellular signal that ultimately leads to a specific cellular response.

At each hormonal receptor characterized to date, only a single binding site exists so that at any time, only a single molecule of hormone can occupy a binding site to form a hormone–receptor complex. Unlike enzymatic reactions where the substrate is altered by binding with enzyme, the hormone is unaffected by the receptor to which it is bound. It is also noteworthy that the hormone–receptor complex is formed by a non-covalent bond that is reversible, and thus transient.

Upon binding with its hormone, the receptor undergoes a modification to its three-dimensional structure. It is this conformational shift that activates the effector site of the receptor, setting forth a cascade of events which result in the cellular response that is ascribed to the involved hormone. It is important to recognize that while the binding of hormone to receptor is reversible, the biological events initiated by the formation of hormone–receptor complex continue for some time after the dissolution of that complex. On the other hand, this hormone induced cellular response is limited in the absence of additional hormone–receptor complex formation. Consequently, if the target cell is to remain activated for an extended period of time in its attempt to achieve homeostasis, the availability of hormone molecules must be sustained so that new molecules may bind with freshly unoccupied receptors.

Types of receptors and post-receptor actions

At all target cells and with all hormones, the biological response evoked by a hormone must be preceded by the binding of the ligand (hormone molecule) with its specific receptor expressed by the hormone sensitive cell. Despite the plethora of hormones produced by the endocrine system and the many cell types that respond to these hormones, all receptors can be broadly categorized as either membrane bound or intracellular receptors. Membrane bound receptors are constituents of the target cell's plasma membrane and bind to peptide/protein hormones, as well as catecholamines. As indicated by their name, intracellular receptors are located within the cell, and bind to steroid and thyroid hormones which are small lipophilic molecules that easily diffuse across the plasma membrane to enter the cell.

INTRACELLULAR RECEPTORS

In their unbound state there is some disparity in the precise localization of intracellular receptors. Initially, it was believed that unbound intracellular receptors were found in the cell's cytosol and that, upon hormone binding, the hormone–receptor complex was translocated into the nucleus. However, it is now understood that even in the unbound state, intracellular receptors for most steroid hormones are located within the nucleus. An exception is the glucocorticoid receptor, which when unoccupied is anchored to the cytoplasmic exterior of the nucleus (Lazar 2003). One feature that receptors of lipophilic hormones have in common is that they are considered inactive when they are not bound with ligand. Characteristic of this inactive state is that these receptors are found in association with heat shock proteins (HSPs), particularly HSP90 (Joab *et al*. 1984; Catelli *et al*. 1985). Hormone binding to the receptor results in a dissociation of the receptor from these chaperone proteins and dimerization of

newly liberated receptors. Upon this 'activation', both cytoplasmic and nuclear receptors are translocated to nuclear DNA, in order to interact with hormone response elements (HREs). Once adhered to these HREs, the hormone–receptor complex regulates the transcription of specific genes on the DNA. Accordingly, intracellular receptors are considered members of the larger family of transcription regulatory factors.

Determining the amino acid sequence of these intracellular receptors has provided much insight into the mechanisms of their interactions with hormones and DNA. At the C-terminal segment of the protein structure is a sequence of about 250 amino acids that are lipophilic in nature and form a structural pocket capable of binding steroid and thyroid hormones. The unique sequence of amino acids comprising this pocket determines the receptor's specificity for a particular hormone.

Binding of the hormone–receptor complex to specific HREs linked to hormone sensitive genes is directed by a DNA binding domain consisting of approximately 70 amino acids that are also located at the C-terminus of the receptor protein. Common to all DNA binding domains are two highly conserved regions referred to as 'zinc fingers' which directly interact with DNA (Scheidereit *et al*. 1986). The ensuing genetic transcription is carried out by a transcription complex that, in addition to the hormone–receptor complex, also includes 'general transcription factors' and 'positively acting cofactors' (McKenna *et al*. 1999). These coactivators enhance the rate of transcription, at least in part, by unwinding the DNA double helix and stimulating RNA polymerase activity (Kuo & Allis 1998).

Thus the cascade of events that result in the actions of steroid or thyroid hormones at target cells includes:

1 entrance of hormone via simple diffusion;
2 formation of hormone–receptor complex;
3 dissociation from HSPs and receptor dimerization;
4 translocation to DNA;
5 binding to HREs;
6 assembly of transcription complexes;
7 synthesis of gene specific mRNA;
8 within cytosol, translation of proteins coded by mRNA.

As an illustration of the efficiency and integration of the endocrine system, it appears that the actions of a single lipohilic hormone bound to its intracellular receptors can regulate the transcriptional activity of several genes, altering the synthesis of several proteins. Yet this occurs in a co-ordinated manner in that each of the newly synthesized proteins participates in a single, overall biological response within the cell. For example, aldosterone functions to maintain proper sodium levels within the body. It does this by stimulating the reabsorption of sodium from the renal tubules when blood concentrations of that mineral are low. To achieve this end, aldosterone increases the synthesis not only of sodium channels and sodium pumps within the membranes of the tubules, but also the enzymes that manufacture the adenosine triphosphate (ATP) used by those pumps. This synchronized production of several proteins subserves the reabsorption of sodium from the renal filtrate in order to maintain proper osmolality and water balance within the body.

Since the mechanism(s) of action of intracellular endocrine receptors involves the management of protein production, the biological activities stimulated by steroid and thyroid hormones are typically slow to occur and are long lasting. Recent evidence, however, suggests that in some cases lipophilic hormones induce fast acting responses of short duration in target cells, without any change in protein expression (Oichinik *et al*. 1991). Although the exact machinations of these rapid responses remain undefined, it appears that they are triggered by the interaction of steroid hormone with receptors located on the plasma membrane of the target cell, rather than with intracellular binding sites.

MEMBRANE BOUND RECEPTORS

As opposed to receptors that bind with lipophilic hormones which easily diffuse across the target cell's membrane to enter the cell, receptors for lipophobic hormones (peptides/proteins) are located on the target's plasmalemma since these hormones are not able to enter the cell. And because formation of the hormone–receptor complex occurs at the membrane's extracellular surface, the resultant intracellular activity is triggered by a pre-existing

signal transduction mechanism that provides a rapid response. In short, because membrane bound receptors are linked to second messenger (the hormone acts as the first message) systems that are already in place—simply needing to be activated by formation of hormone–receptor complex—the cellular response elicited is quickly turned 'on' and can be just as quickly turned 'off'.

It is the chemical structure of membrane bound receptors that enables the forwarding of an extracellular message presented by a hormone molecule to pre-existing signal transduction pathways within the cell. All membrane receptors are comprised of three distinct sections, even though the receptor itself is generally a single polypeptide chain (Spiegel *et al*. 2003). The *extracellular* region is located at the N-terminal end of the chain, and consistently expresses glycosylation sites. The carbohydrate residues found at those sites appear to be involved in hormone binding, which occurs specifically in cysteine rich pockets. Each of the receptor's *transmembrane* segments—membrane receptors typically feature several membrane spanning regions—consists of about 25 amino acids that are lipophilic in nature and form a helical structure. The *intracellular* region is at the C-terminal end of the chain and is responsible for the effector function of the receptor. Generally, the intracellular region—which is composed of lipophobic amino acids—contains regulatory elements including phosphorylation sites.

Although the actions of membrane bound receptors are carried out by numerous second messenger pathways, these post-receptor mechanisms can be categorized as: (i) ligand gated channels; (ii) receptor bound kinases; (iii) receptor bound guanylate cyclase; (iv) cytokine receptors; or (v) G protein coupled receptors. In the first three categories, when hormone binding occurs, the receptor itself directly stimulates cellular responses, but in cytokine receptors and G protein coupled receptors, the receptor activates another molecule within the target cell to carry out the final response attributed to the involved hormone.

In the case of *ligand gated channels*, the receptor expresses not only an extracellular hormone binding site, but also a channel within the membrane spanning regions that allows specific ions to transport when opened. The opening of the channel occurs upon binding of ligand, resulting in a conformational shift within the membrane spanning helices enabling specific ions to cross the plasmalemma to enter, or exit, the cell. This type of receptor is found on excitable cells, i.e. neurons and myocytes, and the movement of ions electrically stimulates a response in the target cell.

The best studied and most common receptor that is directly linked with *kinase activity* involves tyrosine kinase. Within the human genome, approximately 100 receptor tyrosine kinases have been identified (Spiegel *et al*. 2003). Although characteristics of the membrane spanning region are common to all of them, a large degree of variability exists at the extracellular region accounting for the diversity of ligands that interact with these receptors. Unlike most membrane bound receptors, members of the tyrosine kinase family of receptors span the membrane but once. Yet similar to many other ligand receptors, these proteins dimerize upon binding of hormone, a key step in activating the receptors. Upon this activation, a specific region of the receptor's intracellular domain phosphorylates tyrosine residues of enzymes within the cell's interior, thus eliciting the desired response of the cell. Moreover, the activated receptor is capable of phosphorylating its own tyrosine residues located on its cytoplasmic region. This 'autophosphorylation' allows the activation state of the receptor to persist, therefore amplifying the signal carried by the hormone. A combination of the dissolution of the hormone–receptor complex at the surface of the cell's exterior and the activity of intracellular phosphatases, which de-phosphorylate enzymes, terminate the hormone induced biological response within the cell.

Other receptor bound kinases, less prevalent than tyrosine kinase receptors, demonstrate similar mechanisms of activity in that they phosphorylate pre-existing enzymes found within the target's cytoplasm to alter cellular function. Both serine and threonine residues of enzymes residing within the cell can be phosphorylated by membrane receptors occupied by hormone. Again, cellular responses cease when hormone–receptor complexes are no longer formed, and when phosphatase activity de-phosphorylates activated enzymes. This

demonstrates a common theme to the mechanism of membrane receptor activity; cellular response is turned 'off' both at the extracellular and intracellular levels.

Receptors that mediate their responses via *guanylate cyclase* compose the final category of membrane receptors where a component of the receptor protein itself stimulates cellular response. This category is less ubiquitous than the other types of membrane receptors; it appears that only *atrial natriuretic factor* utilizes this type of receptor to regulate the activity of its target cells. Here, again, the transduction of the first messenger (hormone) to intracellular activity is restricted to the membrane molecule. After binding of ligand to the extracellular domain, a conformational shift occurs throughout the structure resulting in the activation of guanylate cyclase; an enzyme that is a constituent of the receptor's intracellular region. Upon activation, this enzyme converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). The newly formed cGMP is linked to a nucleotide dependent protein kinase which, in turn, phosphorylates enzymes within the cell's cytoplasm.

Cytokine receptors are quite similar to tyrosine kinase receptors in their mechanism of action: these receptors also employ tyrosine kinases to evoke cellular responses. However, a major difference exists in that cytokine receptors do not express the kinase enzyme itself in their intracellular tails. Rather, the enzyme is structurally uncoupled from the cytokine receptor molecule. Members of the cytokine receptor family—which include growth hormone and prolactin receptors—are comprised of multiple subunits. Upon binding of ligand, these receptors form oligomers and activate the *janus tyrosine kinases*, or JAKs, that are located in close proximity to the receptor along the cytoplasmic lining of the cell's plasmalemma (Heim 1999). Cytokine receptors themselves display no enzymatic activity (Argetsinger *et al*. 1993). It appears that the activation of JAKs occurs as a result of the drawing together of neighboring JAKs when oligomers of hormone–receptor complexes form, allowing the JAKs to transphosphorylate each other's tyrosine residues. The activated kinases then phosphorylate cytosolic enzymes which, ultimately, carry out the hormone's message. As previously described, hormone induced cellular responses are halted by the unbinding of hormone from receptor, as well as the activity of intracellular phosphatases which inactivate enzymes within the target.

The most prevalent category of membrane bound receptors is the family of *G protein coupled* receptors. Over a thousand different ligands execute their biological activities via these receptors (Spiegel *et al*. 2003). In all cases, however, the G protein and its coupled receptor are two distinct proteins that are functionally, but not structurally, linked together. Although these receptors contain seven membrane spanning regions, they cannot directly alter intracellular activity upon binding of hormone. Instead, located at the cytoplasmic lining of the plasmalemma and neighboring the receptor is a G protein that stimulates cellular responses upon the formation of hormone–receptor complexes. These G proteins—so termed because they require GTP to function—can be either stimulatory (G_S) or inhibitory (G_I) in their actions, with G_S being far more commonplace.

All G proteins, whether G_S or G_I, consist of three subunits termed α, β and γ. In the quiescent state, the α subunit is bound to guanosine diphosphate, but upon binding of hormone to its receptor, this nucleotide is replaced by GTP, thus activating the G protein. Once activated, G proteins can stimulate a host of different intracellular second messenger systems, therefore regulating—even simultaneously—numerous intracellular activities. The major signal transduction mechanisms linked with G protein activation will be addressed individually.

1 *Cyclic adenosine monophosphate (cAMP)*. In this second messenger system, activated G proteins stimulate the enzyme adenylate cyclase—located on the cytoplasmic lining of the plasmalemma—to convert ATP into cAMP. This reaction is similar to the one stimulated by the interaction of hormone with guanylate cyclase receptors where the binding of hormone results in the conversion of GTP to cGMP. In addition to the nucleotide substrate used, the systems differ in that, unlike guanosine cyclase, the enzyme adenylate cyclase is not a constituent of the receptor molecule.

Newly formed cAMP is capable of triggering numerous intracellular processes, primarily by activating

various cAMP dependent protein kinases, also referred to as PKAs. As with all kinases, PKAs stimulate cellular responses by phosphorylating enzymes of specific biochemical pathways. Because each type of target cell expresses its own set of PKAs and kinase activated pathways, the same second messenger system may be used to control biochemical responses that are cell specific. And consistent with hormone action in general, at each step along the way the message of the hormone is amplified.

The production of cAMP occurs in a matter of seconds, consequently the cellular response is quickly evident; unless hormone–receptor complexes are continually renewed the cellular action will be brief. Two enzymes terminate the actions induced by cAMP; *phosphodiesterases* cleave the bonds of cAMP therefore inactivating it, and phosphatase activity de-phosphorylates the enzymes stimulated by PKAs. As a result, responses typically evoked by cAMP are fast acting, and brief in duration.

However, not all biological responses associated with cAMP are of short duration. It is now known that cAMP can alter the transcription of certain proteins—mimicking the actions of steroids and thyroid hormones—and therefore bring about long-term modifications in cellular activity. Genes that are sensitive to cAMP regulation contain sequences referred to as *cAMP response elements* that act as enhancers of transcriptional activity when stimulated. This stimulation occurs when a specific type of cAMP dependent protein kinase phosphorylates the *cAMP responsive element binding protein* (CREB). Once phosphorylated, these CREBs act as transcription factors and bind with the cAMP response elements located on the DNA. The genes responsive to these CREBs vary in accordance with target cell type. This, again, is an illustration of how different target cells respond to the same signal transduction mechanism in a specific manner that is dictated by pre-existing 'hard wiring' of the cell.

2 *Phosphatidylinositol*—in another G protein related second messenger system, a phospholipid constituent of the plasmalemma—phosphatidylinositol—is degraded by the activity of *phospholipase C*, a membrane bound enzyme that is stimulated by G protein activity. Upon binding of hormone to its membrane receptor, phospholipase C cleaves phosphatidylinositol into diacylglycerol (DAG) and inositol triphosphate (IP_3), each of which triggers a cellular response. Inositol triphosphate leaves the plasmalemma to enter the cell's cytoplasm where it reacts with the endoplasmic reticulum to release its stored calcium into the cytosol. Increasing cytosolic calcium levels is a common method of stimulating various cellular activities via calcium sensitive enzymes.

Unlike IP_3, the newly produced DAG remains bound to the cytoplasmic lining of the plasmalemma where it can activate the membrane bound *protein kinase C* (PKC) enzyme. As its name suggests, PKC can only be turned 'on' by DAG in the presence of elevated cytosolic calcium levels, thus the actions of IP_3 are synergistic to those of DAG. As with all kinases, PKC functions by phosphorylating, and thus activating, enzymes within the cell.

The activities stimulated by the phosphatidylinositol signal transduction mechanism cease when IP_3 is eliminated by its conversion to inositol through a process of de-phosphorylation, and DAG is inactivated by the addition of a phosphate group. The activities of intracellular enzymes stimulated by PKC are suppressed when cytosolic calcium levels are returned to resting values.

3 In addition to the direct coupling of ion channel opening with hormone binding as described earlier, receptor binding at the membrane may regulate channel opening through a G protein intermediary. In this process, the membrane bound hormone receptor and the channel that spans across the membrane are separate proteins. Upon formation of the ligand-receptor complex, a nearby G protein located in the plasmalemma interacts with a neighboring channel protein. This interaction causes a conformational shift in the channel, resulting in its opening and movement of ions across the membrane. Typically, there is a large influx of ions into the cell resulting in a substantial increase in the cytosolic concentration of that ion (Finn *et al*. 1996). Perhaps the best example of this occurs in smooth muscle cells where a sharp increase in cytosolic calcium levels occurs via G protein opening of ion specific channels in the plasmalemma. At rest, cytosolic

calcium concentrations of these cells are in the range of 0.1–0.2 $\mu mol \cdot L^{-1}$, but upon hormone induced stimulation of calcium channel linked G proteins these levels quickly rise to more than 1 $mmol \cdot L^{-1}$. This is a much greater increase than that observed following the opening of ligand gated ion channels.

In some cases, newly available calcium does not by itself stimulate a biological response within the affected cell. Instead, the greatly increased concentration of calcium dramatically augments the probability of its interaction with calcium specific intracellular binding proteins. *Calmodulin* is most prominent among these binding proteins and it exists in almost all cells. This high affinity protein possesses four binding sites. When these sites are occupied by newly available calcium—which can also originate from intracellular stores—the calcium-calmodulin complex activates enzymes, most commonly kinases. In turn, these activate enzymes that directly catalyze the cellular activities attributed to the actions of the hormone bound to the extracellular surface of the target. This cellular response subsides with the dissolution of hormone–receptor complexes, therefore closing ion channels. Adenosine triphosphate-driven calcium pumps then deliver cytosolic ions back to their intracellular (endoplasmic reticulum) or extracellular sources of origin.

Integration of target cell responses to hormones

The integrative processes that characterize the endocrine system are not only evident during the secretion of hormones but also in the responses of target tissue to those hormones. More specifically, the biological activity stimulated by one hormone in a target tissue can be modified by the action of another hormone. Such integrated responsiveness of target cells is best exemplified by the phenomena of permissiveness, synergism, and antagonism. *Synergism*, also referred to as 'potentiation', occurs when two different hormones stimulate the same biological activity in the target cell. However, rather than the effects of the two hormones being additive in magnitude, the response is greater than would be evident by simply summing the responses demonstrated when the cell responds to each hormone individually. To illustrate this, both growth hormone and cortisol elicit lipolysis in adipocytes but, when administered simultaneously, the rate of triglyceride break down is actually much greater than it would be if those hormones were given separately and their individual effects were added together.

In *permissiveness* the binding of one hormone at the target cell must precede the binding of another hormone, which only then stimulates a biological response in the cell. In this case, it is said that the initial hormone confers a permissive effect, allowing the target cell to respond to the second hormone. As an example of this, it has been found that in many target cells, the initial binding of thyroid hormone enables epinephrine to exert its biological effects on those cells. Lastly, *antagonism* occurs when the influence of one hormone opposes, and effectively minimizes or even prevents, the action(s) of another hormone at the target cell. To exemplify this, growth hormone is known to antagonize the effects of insulin at their shared target tissues. As a result, the binding of growth hormone interferes with the ability of insulin to promote glucose uptake and glycogen synthesis in the liver and skeletal muscle.

Concluding comments

Even in the brief description presented here, it is obvious that the mechanisms utilized by the endocrine system to regulate target tissue responses are characterized by a high degree of complexity and integration. Even within a single cell, physiological activities are governed both by steroid and protein hormones, while various intracellular signal transduction mechanisms are employed in an effort to maintain homeostasis in the presence of continuous and diverse environmental perturbations. However, it is now understood that many pathological conditions, for example type 2 diabetes, can be directly attributed to dysfunction of these signal transduction mechanisms. Accordingly, much research is currently being conducted that will enhance our understanding of hormone regulated signal transduction in particular, as well as the function of the endocrine system in general.

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Chapter 3

Exercise Testing: a Bridge Between the High-Tech and the Human—the Need for Innovative Technologies

DAN M. COOPER

Scope of exercise testing in clinical medicine

An increasing number of investigators are using measurements of human performance to probe pathophysiology and disease mechanisms in a variety of conditions. Figure 3.1 shows the exponential increase in the number of published, peer-reviewed randomized trials that utilized clinical exercise testing. The widespread appeal of exercise testing is that it allows clinicians to quantify physiological responses in a controlled setting that better reflects the natural environment of their patients. Measurements of almost any physiological variable, ranging from cardiovascular to hormonal, made only at rest, rarely predict the impact of disease on exercise responses.

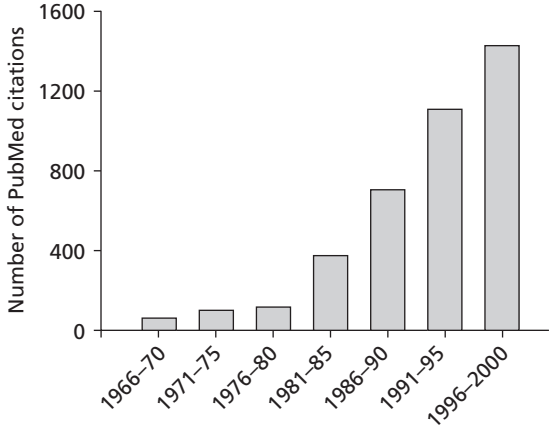

Fig. 3.1 Increasing number of clinical trials using exercise testing. Using the PubMed search engine, it is clear that the utilization of exercise testing in clinical trials is increasing rapidly.

Although tests like the 6-min walk (i.e. measuring the distance walked by a subject in 6 min) continues to be a useful assessment of integrated responses, such tools provide only crude insight into specific physiological mechanisms of disease. How much more information we could have from these simple protocols if, for example, in addition to the distance walked, we could measure body heat dynamics, mechanical work performed and shifts in intramuscular water with minimally invasive and intrusive devices.

Advances in the technology of exercise testing

Since the remarkable 'growth spurt' in human performance knowledge generated in the first half of the 20th century at such notable centers as the Harvard Fatigue Laboratory (Tipton 1998), the development of new, clinically useful, technologies to assess human performance in response to physiological stresses like exercise has not kept pace with the progress of many other areas of biomedical investigation. Treadmills and cycle ergometers have changed little since the first measurements of maximal oxygen uptake were made in the 1920s. These devices have proved to be very useful for testing the *upper limits* of gas exchange and metabolism, and are, therefore, suitable for studies of athletic performance in which near maximal efforts are critical. But the emphasis on maximal efforts does not readily reflect the level and type of physical activity that

Fig. 3.2 'Found art' in the General Clinical Research Center (GCRC) Human Performance Laboratory. A 7-year-old girl creatively expresses her thoughts on the $\dot{V}o_{2max}$ test. 'I'm very sorry Dr. Cooper. I'm very tierd [sic] and I don't want to do this again. I'm so sorry.'

determines quality of life in the clinical setting. Moreover, typical exercise protocols are exhausting and uncomfortable, and often inappropriate for young children, the elderly and persons with most diseases and disabilities (Cooper 1995; Metra *et al*. 1998) (Fig. 3.2).

Gas exchange during exercise can be measured precisely, but only with cumbersome and sometimes painful mouthpieces which, in and of themselves, alter normal respiratory patterns (Lowhagen *et al*. 1999). Beyond the measurement of oxygen and carbon dioxide, little progress has been made to exploit the potentially rich insight into disease mechanisms that could be gained from online, continuous measurement of nitric oxide (NO) and volatile organic compounds in the exhaled breath. Precise *non-invasive* quantification of physical activity and energy expenditure under field conditions in free-living human beings remains an elusive goal; such tools, like the use of stable-isotopically labeled bicarbonate (Zanconato *et al*. 1992; Coggan *et al*. 1993), could revolutionize research focused on health outcomes in many areas of clinical and basic science biomedical research.

Over the past 35 years, technological advances have facilitated an explosion of biomedical knowledge, particularly in molecular biology and neuroscience. Lagging behind this knowledge has been the development of tools for minimally invasive measures of neurological, intramuscular, cell signaling and vascular adjustments to exercise and other stresses that could be used easily and safely in human beings. Most troubling is that this lack of progress has occurred despite an increasing use of traditional exercise testing to probe mechanisms of human disease and to develop new therapies. Moreover, there is growing recognition that health impairment directly related to physical inactivity is increasing at an alarming rate (Booth *et al*. 2000; Cooper *et al*. 2004).

The *clinical* exercise testing technology gap has occurred despite a number of key technological and conceptual breakthroughs where basic engineering research is pointing the way toward novel clinical applications. In the 1970s and 1980s, the noted Harvard biomedical engineer T. A. McMahon (McMahon 1984) reconfigured our understanding of the biomechanical principals that govern human locomotion. His theories were tested on a running track built in his laboratory constructed so that mechanical forces generated during human running were matched by forces generated within the track. Equally remarkable was that McMahon developed a paradigm in which the biomechanical mechanisms

of human locomotion translated into theoretical constructs that involved biochemical, cardiovascular and neurological control mechanisms. Such novel uses of integrative physiology toward solving basic problems of human disease have proved to be a successful as a clinical research paradigm.

Imaging and spectroscopy approaches

A number of additional advances in non-invasive imaging approaches towards clinical human exercise testing also need to be mentioned in this context. One involves attempts to link the tools of remote imaging with physiological function during exercise. Anatomic imaging, such as can occur with amazing precision using magnetic resonance imaging, has been used to determine the profound changes in muscle water content and other physiological mechanisms that occur even with brief exercise. But Patten and colleagues in a recent comprehensive review of T_2 mapping of muscle noted:

> Despite demonstration of the capacity for imaging phenomena relevant to both exercise physiology and clinical diagnosis, to date there have been surprisingly few clinical applications of T_2 muscle mapping. Because MRI is noninvasive, it affords several advantages over traditional diagnostic modalities such as muscle biopsy or EMG for diagnosis of metabolic and neuromuscular disorders in sports medicine, occupational medicine, and neurorehabilitation. MRI studies provide results rapidly for purposes of diagnostic decision-making and also offer outcome assessment for clinical or exercise interventions without the patient suffering the risks or discomforts associated with repeated study (Patten *et al*. 2003).

In their review, Patten and coworkers went on to point out that the alterations in T_2 magnetic resonance imaging responses to exercise likely result from two mechanisms: osmotically driven shifts of muscle water that increase the volume of the intracellular space; and from intracellular acidification resulting from the end products of metabolism. An example of the use of these techniques in a paretic muscle and in a control subject are shown in Fig. 3.3.

Remote biochemical imaging (e.g. ^{31}P magnetic resonance spectroscopy), pioneered by Britton Chance and his colleagues (Chance 1994), also holds the possibility of providing clinical investigators with real time measurements of intramuscular high-energy phosphate during human performance (Zanconato *et al*. 1993). For example, Scheuermann-Freestone *et al*. (2003) recently examined both cardiac and skeletal muscle energetics during exercise in patients with type 2 diabetes (Fig. 3.4). Remarkably, although their cardiac morphology, mass and function appeared to be normal, the patients with diabetes had significantly lower phosphocreatine/adenosine triphosphate (PCr/ATP) ratios than the healthy volunteers. The cardiac PCr/ATP ratios correlated negatively with the fasting plasma free fatty acid concentrations. Although skeletal muscle energetics and pH were normal at rest, PCr loss and pH decrease were significantly faster during exercise in the patients with diabetes, who had lower exercise tolerance, and PCr recovery was slower in the patients. These investigators concluded that type 2 diabetic patients with apparently normal cardiac function have impaired myocardial and skeletal muscle energy metabolism related to changes in circulating metabolic substrates.

A major breakthrough in the understanding of exercise responses and their alteration in disease states resulted from yet another interaction between engineers and physicians. The development of breath-by-breath measurement of gas exchange was pioneered in large measure in the Harbor–University of California, Los Angeles (UCLA) Laboratory of Drs. Brian Whipp and Karlman Wasserman (Wasserman *et al*. 1973). Wasserman, an MD with a PhD in physiology, was a fellow in the noted Cardiovascular Research Institute (CVRI) at the University of California, San Francisco in the 1960s. Whipp and Wasserman collaborated primarily with two engineers, Dr. William Beaver and, subsequently, Dr. Norman Lamarra. Lamarra received his PhD at UCLA in aerospace engineering for a doctoral thesis devoted, oddly enough, entirely to analyzing the ontransient kinetics of oxygen uptake during exercise in human beings (Lamarra 1982)! Investigations into how disease influences these kinetics, and how these measurements prove to be

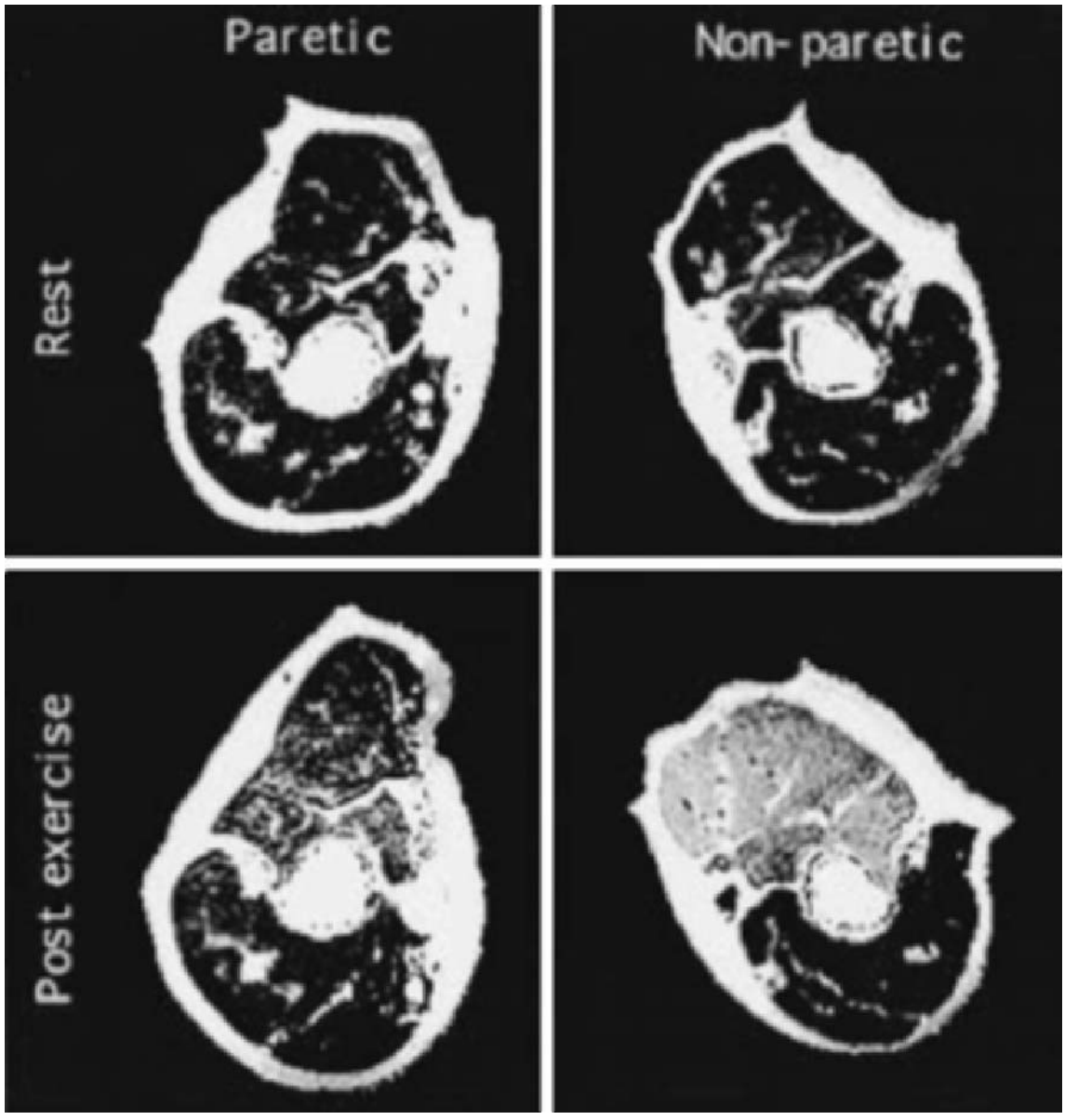

Fig. 3.3 T_2-weighted axial magnetic resonance images of the arm obtained using a multiecho sequence (TE 20, 40, 60, 80 ms, TR = 2000, I NEX, 18 cm FOV) with in-plane resolution of 0.6–0.8 mm. The upper panel images illustrate the paretic (left) and non-paretic (right) arms at rest in an adult with post-stroke hemiparesis of 24 months duration. The lower panels illustrate the same arms following 40 dynamic elbow flexions performed at 80% of maximal effort. Following exercise, increased signal intensity (T_2) is demonstrated in the flexor compartment bilaterally. The paretic side demonstrates markedly less activity-dependent increase in T_2 and documents impaired muscle activation associated with post-stroke hemiplegia. (Data from Patten *et al*. 2003.)

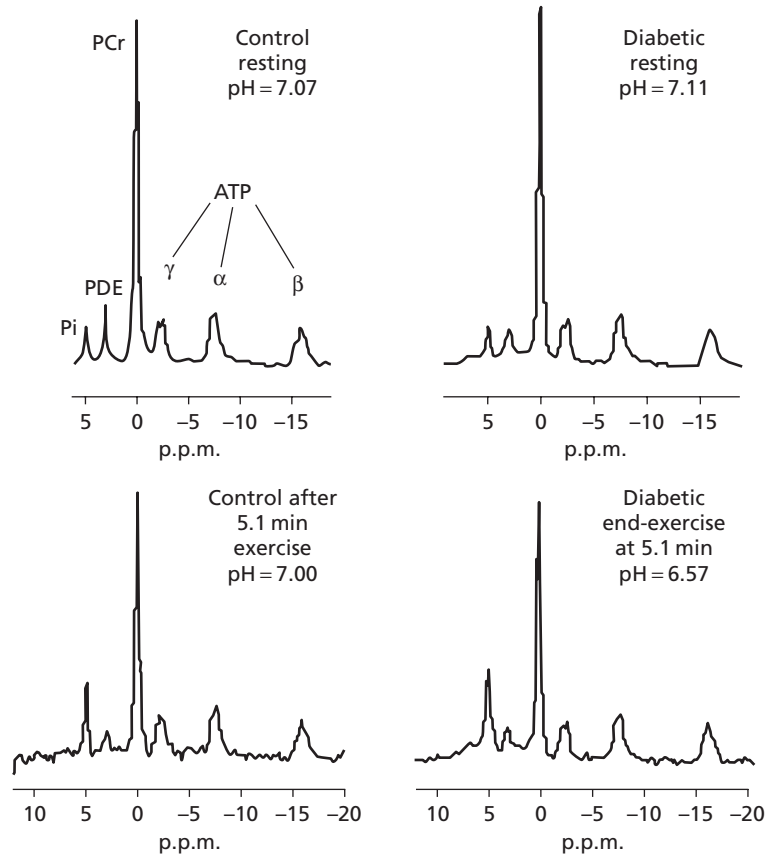

Fig. 3.4 Typical calf muscle ^{31}P magnetic resonance spectra from a control subject and a patient with type 2 diabetes at rest (upper panel, number of scans = 64), from the same patient at the end of exercise, and the same matched control at the equivalent time (5.1 min) of exercise (lower panel, number of scans = 16). PCr, phosphocreatine; PDE, phosphodiesters; Pi, inorganic phosphate; α, β and γ indicate the three phosphate groups of adenosine triphosphate (ATP). Cytosolic pH was calculated from the chemical shift of Pi relative to PCr. Abscissa shows chemical shift in parts per million (p.p.m.). (Data from Sheuermann-Freestone *et al*. 2003.)

useful indicators of disease and its therapy, have proceeded at a relatively slow pace.

The pioneers in the field of exercise physiology clearly recognized that the biological responses to the stress of exercise could ultimately be used to gain a greater understanding of fundamental processes at the cellular and subcellular level. Wasserman's 1975 cartoon of 'gears' linking cellular function to gas exchange measured at the mouth aptly illustrates this concept (Fig. 3.5). But the primary use of exercise testing in the clinical setting has remained almost invariably focused on cardiovascular measurements. As noted by Wasserman and colleagues:

> The authors would like to dispel a concept that has developed in medicine, i.e., that there is a cardiac stress testing and pulmonary stress testing. It is impossible to stress only the heart or only the lungs. Exercise requires the co-ordinated function of the heart, the lungs, and the peripheral and the pulmonary circulation to match the increased cellular respiration required to live and work (Wasserman *et al*. 1987).

We would add only that the 'co-ordinated function' includes the neuromuscular and cellular signal transduction adaptive mechanisms as well.

Development of new technologies—robots, strokes and maturation of motor control

In some cases, existing technologies are simply not sufficient to test exercise and motor control in certain individuals and new devices need to be developed. For example, in dealing with stroke victims, traditional treadmills or cycle ergometers are often not feasible as either diagnostic or therapeutic

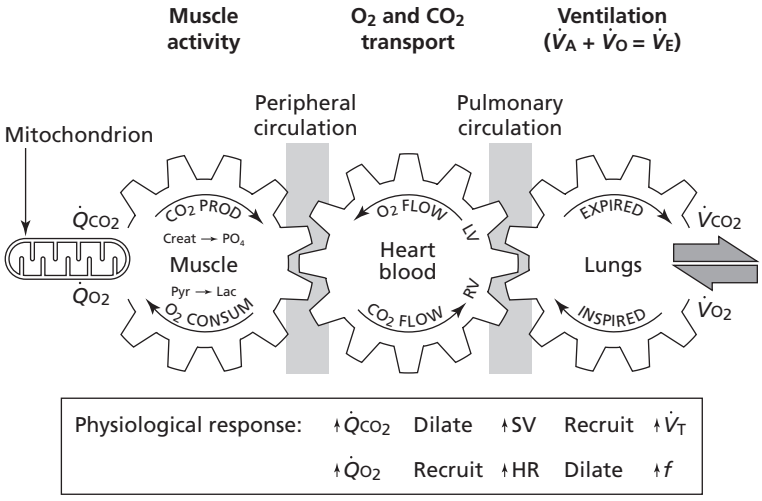

Fig. 3.5 The metabolic 'gears' that link ventilation during exercise to the cellular and subcellular level. (From Wasserman *et al*. 1987.)

tools because of the constraints and limitations to motor control caused by the brain injury. To get around these limitations, mechatronic devices and haptic robots have been developed by a number of investigators. Using these tools, new insight into the mechanisms that govern motor activity in stroke victims (Lum *et al*. 2002).

For example, recent evidence suggests that brain injury can impair the ability to independently activate shoulder and elbow muscles. Reinkensmeyer *et al*. (2002) hypothesized that if muscle activation patterns are constrained, then brain-injured subjects should not be able to accurately grade initial hand movement direction during reaching toward a broad range of target directions. To test this hypothesis, Reinkensmeyer *et al*. used the mechatronic haptic robot to measure hand trajectories during reaching in three-space by 16 subjects with hemiparetic stroke to an array of 75 targets distributed throughout the workspace (Fig. 3.6).

Results of these innovative studies clearly suggest that there are two identifiable classes of directional control following stroke: largely preserved and severely constrained. Since it has been hypothesized that lower pathways substitute for corticospinal ones following stroke, a possible explanation for

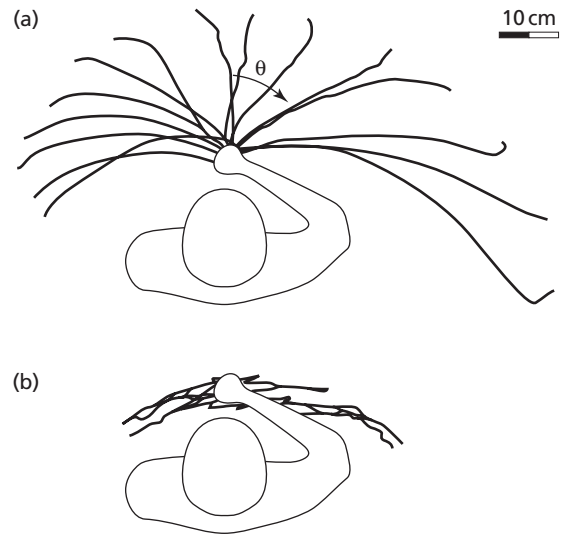

Fig. 3.6 Example hand trajectories for the ipsilesional (a) and contralesional arms (b) of a subject with severe stroke. The contralesional (left) arm trajectories are flipped about the sagittal plane. Reaching direction (θ) is defined positive for movements to the right of straight ahead. Note that movement was constrained to essentially two directions (medial and lateral) for the severely impaired subject, even though substantial active range of motion was preserved in these directions. (Data from Reinkensmeyer *et al*. 2002.)

these two classes is that directional control is largely preserved if some threshold fraction of corticospinal pathways is spared. This hypothesis should be testable in the future via detailed functional imaging of key neural tracts.

The use of robots in measuring motor control is not limited to brain-injured patients. Interestingly, a chance interaction between Reinkensmeyer's group and the author of this chapter led to the idea that mechatronic haptic robots could be used to measure motor control during development in normal children (Fig. 3.7). Children do not typically appear to move with the same skill and dexterity as adults, although they can still improve their motor performance in specific tasks with practice.

One possible explanation is that their motor performance is limited by an inherently higher level of movement variability, but that their motor adaptive ability is robust to this variability. To test this hypothesis, Takahashi *et al*. (2003) examined motor adaptation of 43 children (aged 6–17 years) and 12 adults as they reached while holding the tip of a lightweight robot. The robot applied either a predictable, velocity-dependent field (the 'mean field') or a similar field that incorporated stochastic variation (the 'noise field'), thereby further enhancing the variability of the subjects' movements. Children exhibited greater initial trial-to-trial variability in their unperturbed movements, but were still able to adapt comparably to adults in both the mean and noise fields. Furthermore, the youngest children (aged 6–8 years) were able to reduce their variability with practice to levels comparable to the remaining children groups, though not as low as adults. These results indicate that children as young as 6 years possess adult-like neural systems for motor adaptation and internal model formation that allow them to adapt to novel dynamic environments as well as adults on average despite increased neuromotor or environmental noise.

Performance following adaptation is still more variable than adults, however, indicating that movement inconsistency, not motor adaptation inability, ultimately limits motor performance by children and may thus account for their appearance of incoordination and more frequent motor accidents (e.g. spilling, tripping). The results of this study also suggest that movement variability in young children may arise from two sources—a relatively constant, intrinsic source related to fundamental physiological constraints of the developing motor system, and a more rapidly modifiable source that is modulated depending on the current motor context.

Exhaled nitric oxide and exercise

Clinicians are increasingly questioning the value of traditional measures of pulmonary function such as the forced expiratory volume in 1 s (FEV_1) so commonly used to assess childhood asthma (Spahn *et al*. 2004). A growing body of research has focused on arguably more direct measurements of lung inflammation, such as exhaled NO (Paredi *et al*. 2002). NO performs many important functions in the lungs and can be detected in the exhaled breath of humans. Inflammatory diseases such as asthma and cystic fibrosis alter exhaled NO levels. This has generated interest in utilizing exhaled NO as a non-invasive marker of lung inflammation.

However, the exchange dynamics of NO are markedly different from the respiratory gases (oxygen and carbon dioxide) whose exchange occurs predominantly in the alveolar region. In contrast, NO exchange occurs in both alveolar and airway compartments, and is thus highly dependent on the exhalation flow rate. This feature of NO exchange has confounded interpretation of exhaled NO in a variety of clinical and physiological settings. Given the nature of NO exchange dynamics, and the multisystem physiological responses to exercise, it is not surprising that there are inconsistencies in the reports of the impact of exercise on exhaled NO.

Recently, a number of investigators have developed paradigms to distinguish alveolar and airway contributions to exhaled NO (George 2004) (Fig. 3.8). This approach provides greater specificity than exhaled concentration alone, and thus may be able to address several unresolved questions regarding the impact of exercise on NO exchange. Given the nature of NO exchange dynamics, and the multisystem physiological responses to exercise, it is not surprising that there are inconsistencies in the reports of the impact of exercise on exhaled NO. After exercise, exhaled NO concentration has been reported to be increased (Bauer *et al*. 1994),

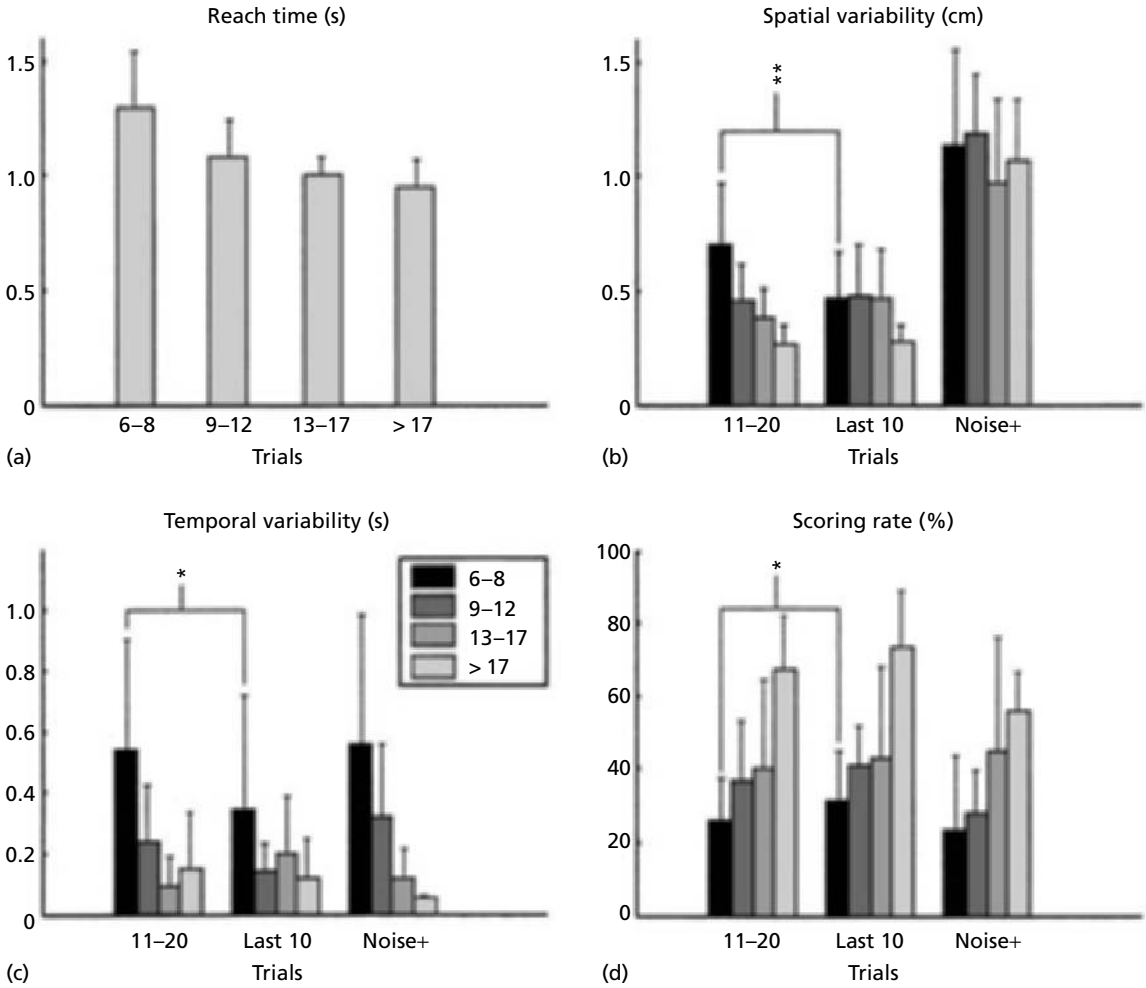

Fig. 3.7 Motor performance measures. (a) Reach time depended on age grouping (analysis of variance [ANOVA] linear contrast, $p < 0.001$). (b) Spatial variability depended on age grouping early in the experiment (mean over trials 11–20, ANOVA linear contrast over children groups, $p < 0.001$) but became independent of age grouping ($p = 0.99$) by the end of the experiment (i.e. last 10 trials). Children aged 6–8 years significantly reduced their spatial variability (paired one-sided *t*-test; ** $p < 0.01$) by the end of the experiment, but adults still maintained significantly lower levels compared to all other children groups (ANOVA planned comparison, $p = 0.002$). Spatial variability was significantly increased by the end of the noise field ('noise+' indicates last 10 trials of noise field) for all age groups (paired one-sided *t*-test, $p < 0.001$ all age groups) to levels that did not depend on age grouping (ANOVA linear contrast, $p = 0.39$). (c) Temporal variability also depended on age grouping over trials 11–20 (ANOVA linear contrast over children groups, $p < 0.001$) but became independent of age grouping by the last 10 trials of experiment ($p = 0.38$). Children aged 6–8 years significantly reduced their temporal variability (paired one-sided *t*-test, * $p \leq 0.05$) by the end of the experiment. (d) Children aged 6–8 years improved their timing score rate (percent 'just right', *t*-test, *$p \leq 0.05$) by the end of the experiment. Error bars show standard deviation across subjects. (Data from Takahashi *et al*. 2003.)

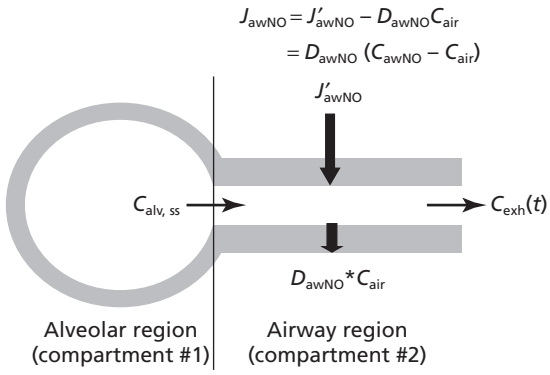

Fig. 3.8 Schematic of two-compartment model used to describe nitric oxide (NO) exchange dynamics. Exhaled NO concentration (C_ENO) is the sum of two contributions, the alveolar region and the airway region, which depends on three flow-independent parameters: maximum total volumetric flux of NO from the airway wall (J_{awNO}, pl_s), diffusing capacity of NO in the airways (D_{awNO}, pl_s^1, p.p.b.^{1}), and steady-state alveolar concentration (C_ANO, p.p.b.). J_{awNO}, total flux (pl_s) of NO between the tissue and gas phase in the airway and is an inverse function of the exhalation flow rate ($\dot{V}_E$); CNO, concentration of NO in the gas phase within the airway compartment. (From George *et al*. 2004.)

unchanged (Iwamoto *et al*. 1994), or decreased (Maroun *et al*. 1995). By distinguishing alveolar and airway contributions to exhaled NO, Shin *et al*. (2003) recently used a two-compartmental model of NO exchange during exercise in an attempt to provide greater specificity than exhaled concentration alone. They hoped to able to address several unresolved questions regarding the impact of exercise on NO exchange.

Significant changes were observed in J'_{awNO}, D_{awNO} and C_{awNO} 3-min post-exercise challenge, despite no significant changes in exhaled concentration (C_{NOplat}). D_{awNO} (mean ± SD) increased (37.1 ± 44.4%), whereas J'_{awNO} and C_{awNO} decreased (–7.27 ± 11.1%, –26.1 ± 24.6%, respectively) 3-min post-exercise. Shin *et al*. (2003) concluded that the flow-independent NO parameters provide greater specificity in characterizing NO exchange. It seems that exercise acutely enhances elimination of NO from airway tissue stores. This effect may be due to enhanced ventilation or an enhanced ability of NO to diffuse from the airway tissue to the gas phase. The latter suggests endogenously produced NO may be useful to probe metabolic and structural features of the airways during exercise.

Linking exercise testing to the new biology

The new technologies of genomic profiling, proteomics and flow cytometry have opened novel venues of research for exercise physiologists. The recent discoveries of the impact of physical activity on stress, inflammatory and immune function (Fleshner *et al*. 2003; Pedersen *et al*. 2003; Shephard 2003) have created a paradigm shift in our ability to understand the link between physical activity and health. For example, Fehrenbach *et al*. (2000) recently examined the role of exercise and fitness on leukocyte expression of key immune modulators, the heat shock proteins (HSPs) (Fig. 3.9). They found large increases in certain HSPs within the leukocytes of subjects following exercise. HSPs inhibit nuclear factor-κB, and this may explain the HSP cardioprotective effect that has been noted previously (Joyeux *et al*. 1999; Powers *et al*. 2002).

Summary

Investigators are increasingly requesting human performance/exercise studies on populations in whom the technology of 'traditional' exercise testing is inadequate; namely, the elderly, children and the disabled. The challenge we now face is to build on the past century of progress in exercise physiology by investing in new technologies and approach that can help us understand how exercise is linked to fundamental disease processes. Moreover, the new approaches and technologies must be used for integrating exercise testing with innovative, multidisciplinary, research tools in biology that can provide new insights into mechanisms of disease at the systemic and cellular level.

Acknowledgement

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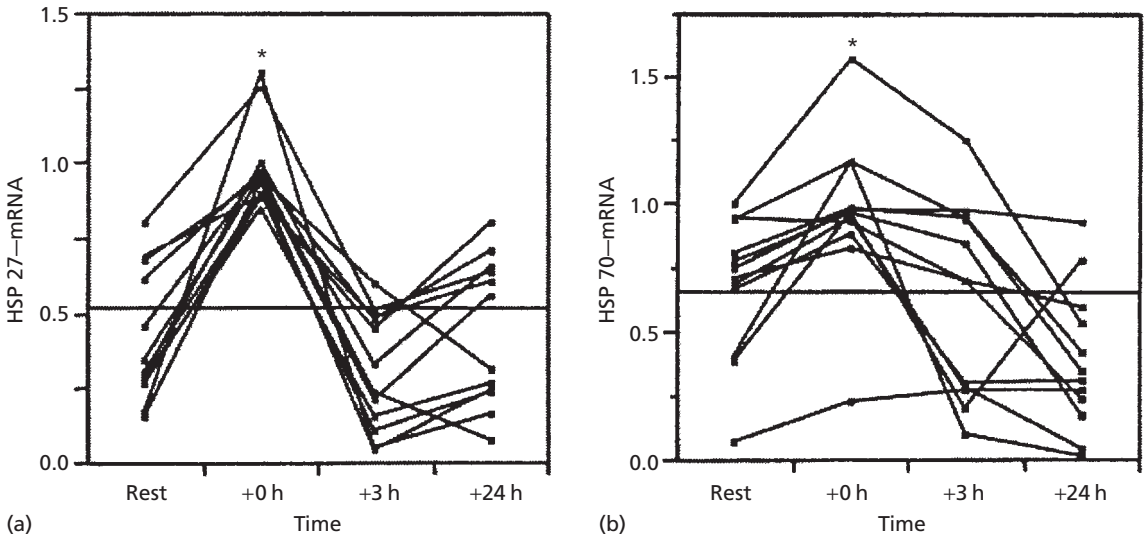

Fig. 3.9 Descriptional presentation of mRNA expression of heat shock proteins HSP27 (a) and HSP70 (b) in leukocytes of athletes at rest and immediately, 3 h and 24 h after the half-marathon (n = 12). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess mRNA expression. HSP27, HSP70 and β-actin were amplified under conditions to allow relative comparisons for a given mRNA. The specific mRNA values are described in relative units normalized to transcript levels of β-actin. Each curve represents a single subject. *Significant changes compared with resting values ($P < 0.05$). (Data from Fehrenbach *et al*. 2000.)

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Chapter 4

Measurement of Peptide Hormones

MARTIN BIDLINGMAIER, ZIDA WU AND CHRISTIAN J. STRASBURGER

Introduction

Methods to quantify peptide hormone concentrations in biological fluids are a basic requirement for medical practice and biomedical research in the field of endocrinology. Such methods ideally should be very specific, identifying precisely and quantifying accurately only the hormone of interest without interference from closely related peptides or unrelated matrix components. They must be extremely sensitive, allowing the detection of peptide hormones at the low concentrations observed in physiological and pathophysiological situations. Usually, peptide hormone concentrations in circulation are in the nano- or picomolar range, making the sensitivity of the methods a crucial issue. Finally, measurement methods should be easy and rapid, allowing application to large series of samples in an appropriate time. These methodological aspects of hormone measurement become even more important when the analyses are done in the framework of the fight against doping in sports. Because of the potentially severe consequences for the athlete, the commercial and political interests involved and the complicated ethical and legal background, the level of precision, specificity and reliability for hormone measurements must be extremely high in this field. For example, the cross-reactivity from specific molecular isoforms of peptide hormones in immunoassays is rarely known for commercial assays frequently used in clinical practice, whereas this is a crucial point for several doping tests described below (chorinonic gonadotropin [hCG], erythropoietin [EPO], human growth hormone [hGH]). Finally, large-scale analysis of samples in an extremely short time period is often required during competitive events, enforcing the need for rapid analytical methods.

During the last 30 years, enormous progress has been made in the attempt to develop analytical methods for peptide hormone quantification fulfilling the above mentioned criteria. However, from the early days of radioimmunoassays to the latest developments in mass spectrometry (MS) techniques, it remains crucial to be aware of the pitfalls each method includes to avoid misinterpretation of the data generated. In some cases, it might be impossible to combine different goals by one method: For example, there is no doubt that MS is a unique tool to identify a molecule with an extremely high degree of certainty (Binz *et al*. 2003; Gam *et al*. 2003; Kast *et al*. 2003). However, today's MS methods still are complex, time consuming and in many cases require a very sophisticated sample preparation. The progress made in the applicability of MS technologies to analyze purified peptide hormone preparations is not paralleled by the same progress in analyzing hormone mixtures in more complex biological matrices like serum (Liu & Bowers 1997; Black & Bowers 2000; Wu, S.L. *et al*. 2002), and the high degree of certainty achieved under optimal conditions by no means indicates that MS would not be susceptible to interferences (Annesley 2003). Finally, the costs of the equipment necessary are still much higher than those for immunological hormone measurement methods. The rapid development of new techniques in the area of MS together with the development of instruments designed for high throughput analyses, the introduction of

automated systems for sample preparation and the increase in sensitivity recently achieved by research groups might change the picture in the future (for review see Binz *et al*. 2003). However, until today the vast majority of hormone analyses in clinical practice and research is still done by the 'traditional' immunoassay techniques. Thus, this article is focused on the pitfalls and potentials of peptide hormone measurements by immunoassay techniques.

General aspects of immunoassay methods

Immunoassays employ the unique capability of specific immunoglobulins (antibodies) to recognize and bind a certain three-dimensional structure on the surface of a molecule (the so-called 'epitope'). This antigen–antibody reaction provides a high degree of specificity. In combination with the use of powerful labeling substances for antibodies or hormones, the dynamic range of immunoassays could be greatly improved. Traditionally, radioactivity had been used as a detection system in hormone assays. Driven by concerns regarding environmental, economical and health aspects of radiation, different non-isotopic methods have been developed for signal detection: These newer systems employ either an enzyme-catalyzed colorimetric or chemiluminescence reaction or—alternatively—a fluorescence dye for signal detection. Among the advantages of these non-isotopic methods is the stability of the label—radioactive labels undergo a decay, making lot-to-lot differences a common problem. In some cases, the sensitivity of modern non-isotopic detection systems exceeds that of radioactive labels, making their use even more popular. However, in general the sensitivity of an immunoassay method primarily depends on the affinity of the specific antibodies used, and often the detection label is chosen simply because a certain measurement device is present in a laboratory.

Technical aspects of immunoassay technology and its implications

From a methodological point of view, immunoassays can be divided in two major subtypes: the classical 'competitive' immunoassay and the 'sandwich type' immunoassay (Figs 4.1 and 4.2). In a competitive immunoassay, the hormone present in a sample 'competes' for antibody binding with a labeled form of the same hormone added to the sample. The higher the endogenous hormone concentration in a sample is, the lower the probability is that a labeled hormone molecule (called 'tracer') binds to the antibody. Thus, the hormone concentration in the sample is inversely correlated to the signal obtained in the assay. Depending on the label used, competitive assays have been named radioimmunoassay (RIA), enzyme-immunoassay (EIA), luminescence-immunoassay (LIA) or fluorescence-immunoassay (FIA). In contrast, 'sandwich type' immunoassays consist of a 'capture antibody', which binds the hormone from the sample, and a labeled 'detection antibody', which is directed against another epitope on the hormone surface and thus allows 'staining' of the hormone molecules bound by the capture antibody. The more the hormone is present in a sample, the more the hormone is bound by the capture antibody, which in turn is translated into a signal by the 'detection antibody'. Thus, the signal obtained is directly proportional to the amount of hormone present in the sample. Similar to the above mentioned nomenclature for the competitive assays, the names of sandwich type immunoassays are related to the detection system used: radioimmunometric assay (IRMA), enzyme linked immunosorbent assay (ELISA), luminometric assay (ILMA) or fluorometric assay (IFMA).

The difference in assay design between competitive and sandwich type immunoassays has major implications on their applications and on the interpretation of the results: whereas competitive assays require only one antibody and—correspondingly—one epitope, sandwich type immunoassays require two different antibodies and thus two different epitopes. These epitopes must be in spatial distance, because otherwise binding of the capture antibody would interfere with the binding of the detection antibody. Accordingly, sandwich type immunoassays require a 'larger molecule' and therefore are used to measure larger peptide hormones like insulin, growth hormone (GH) or EPO, whereas the smaller peptides (adrenocorticotropic hormone

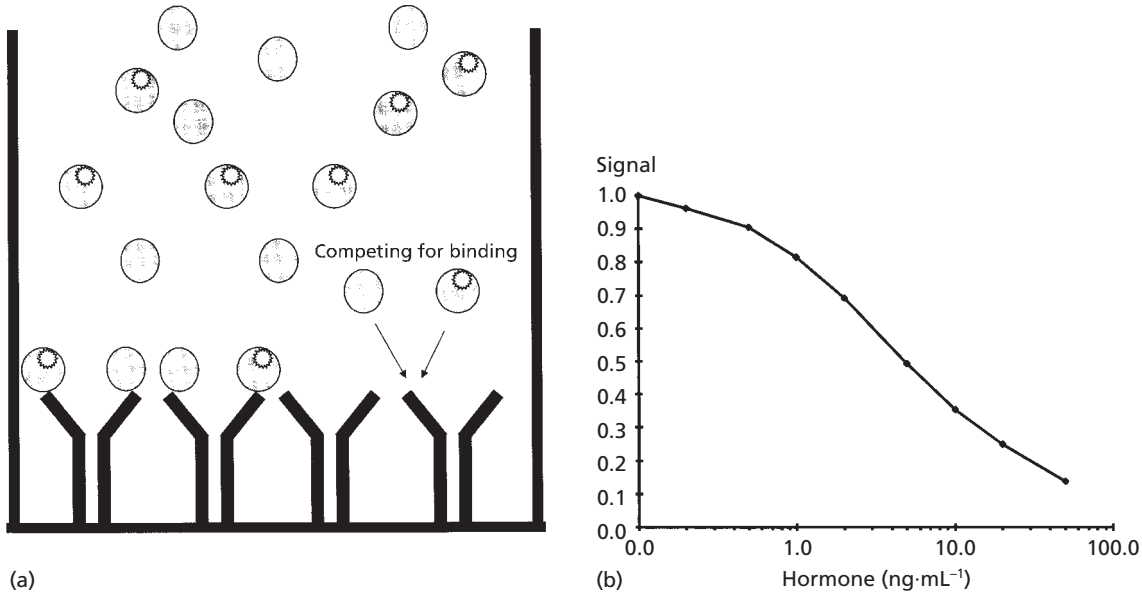

Fig. 4.1 (a) Competitive assay—principle. Labeled hormone ('tracer') and hormone from the sample compete for binding to antibody. (b) Competitive assay—calibration curve. The higher the hormone concentration, the lower the probability that a labeled tracer molecule is bound. Consequence: the more hormone, the lower the signal.

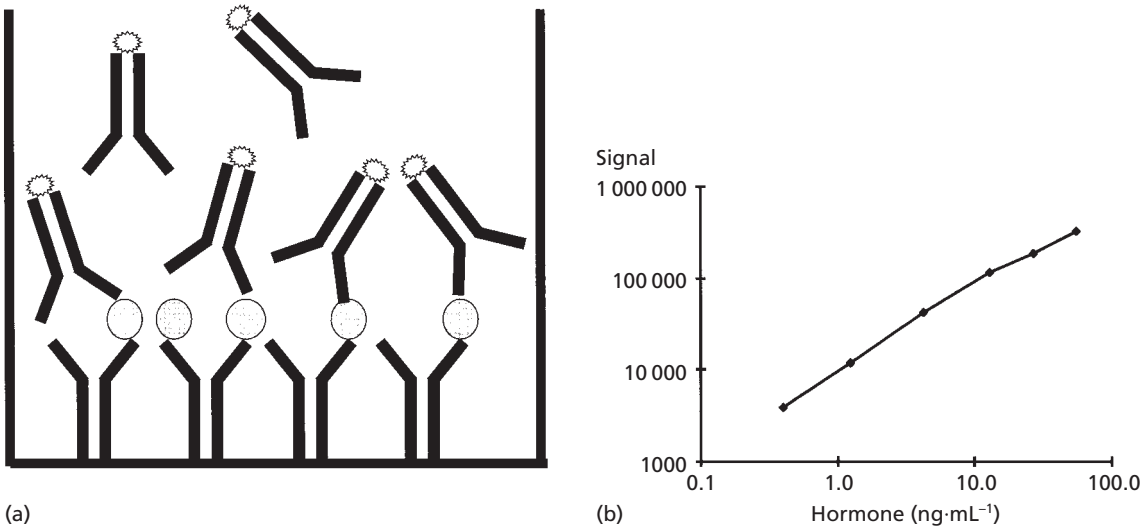

Fig. 4.2 (a) Sandwich assay—principle. (i) Hormone from the sample binds to capture antibody. (ii) Bound hormone is detected by labeled antibody. (b) Sandwich assay—calibration curve. The higher the hormone concentration, the higher the probability that a labeled detection antibody is bound. Consequence: the more hormone, the more signal.

[ACTH], corticotropin-releasing hormone [CRH], growth hormone releasing hormone [GHRH]) and steroid hormones frequently are measured by competitive assays. The advantage of the sandwich type assay format is that the recognition of a hormone molecule requires the presence of two independent epitopes, making the method—at least theoretically—more specific and less prone to cross-reactivity of partially related peptides. In addition, the analyte (the hormone to be measured) and the calibrator (the standard preparation used) are chemically identical, whereas in the case of competitive assays, the calibrator is a modified, labeled hormone. These modifications can lead to differences in the affinity of the antibody to the naturally occurring hormone in a sample and to the modified hormone used as the calibrator of the assay.

Standardization of immunoassays

It is important to mention that all immunoassay methods are 'relative' in nature. This means, that the concentration of the substance of interest in a sample is determined in comparison to the concentration contained in the calibrator. More precisely, the concentration is determined by comparing the ability of the antibodies used to bind the hormone in a biological sample (e.g. a patient's serum) and in the calibrator sample, respectively. Theoretically, this measurement technique relies on three fundamental assumptions: (i) the calibrator is identical to the substance of interest in its physicochemical properties and in its three-dimensional structure; (ii) the epitopes on the surface of the molecule of interest are freely accessible for the antibody in both the biological sample and in the calibrator; (iii) the matrix of the calibrator is identical to the matrix of the sample. Obviously it is very difficult to realize these assumptions in an assay for peptide hormones: In many cases, no international reference preparation (IRP) is available, whereas in other cases more than one reference preparation exists. In the case of hGH, the IRP 80/505 is of pituitary extraction, whereas the IRP 88/624 and IRP 98/574 are of recombinant origin. In addition, some of the reference preparations are of very poor quality and impure (e.g. the IRP for insulin-like growth factor I [IGF-I] contains only about 40% IGF-I [Quarmby *et al*. 1998]). To make the situation more complicated, many peptide hormones naturally occurring in the human body are a mixture of molecular isoforms rather than a homogenous substance (Nagy *et al*. 1994). This has been documented in very much detail, especially for hCG (Birken *et al*. 2003; Lottersberger *et al*. 2003), but also for hGH (Baumann 1999; Boguszewski 2003). Depending on the specific question an investigator or clinician attempts to answer by measuring a hormone's concentration, the answer which of these isoforms or which mixture of isoforms would be the suitable 'reference' preparation varies significantly. In 1991, Roger Ekins, one of the inventors of immunoassay technology wrote that standardization of immunoassays for heterogeneous antigens is impossible (Ekins 1991), and the ongoing discussions on assay standardization clearly demonstrate that we are far from having reached a consensus (Roger & Lalhou 1994; Quarmby *et al*. 1998; Ranke *et al*. 2001). As to the second assumption, one has to be aware that for many peptide hormones high- or low-affinity 'binding proteins' naturally occur in circulation (Baumann *et al*. 1988). Present in a sample, these binding proteins can interfere with the hormone measurement by either 'capturing' the tracer molecule in a competitive assay or by preventing antibody binding (Fisker & Orskov 1996). Depending on assay type, the interference from binding proteins in the sample will lead to under or overestimation of the hormone concentration. A source of interference with no relation to the hormone of interest is the presence of heterophilic antibodies in the patient's sample. These antibodies can 'link' the capture to the detection antibody directly, leading to falsely increased assay results (Kricka 1999). It has been described that such antibodies can be induced by pets housed by the patient (Park *et al*. 2003), but in many cases the origin remains unclear. Methods have been developed to avoid such interference, but the issue remains a problem (Emerson *et al*. 2003; Preissner *et al*. 2003). Finally, the matrix used to dissolve the calibrator rarely is identical to human serum—in many cases, animal sera or buffers supplemented with albumin are used. The behavior of antibodies sometimes can be influenced by

matrix components, leading to differences in the assay results depending on the calibrator's matrix.

Because all these factors can have profound effects on the hormone concentration measured, a careful interpretation of immunoassay results is required. Every single method must be evaluated in terms of cross-reacting substances, and ideally the exact nature of the epitope recognized by the antibodies involved is known. Obviously, 'references ranges' for hormone concentrations are method dependent (Strasburger *et al*. 1996, 2001; Stenman *et al*. 1997; Quarmby *et al*. 1998; Wood 2001; Sharpe *et al*. 2002), and the simple reference to 'general' normative data from textbooks should be avoided. Finally, there is still a need for the development of IRPs for many peptide hormones. As demonstrated most recently for hCG (Birken *et al*. 2003), the development of suitable reference reagents is a sophisticated task, but helps to eliminate some factors of uncertainty in immunoassay measurements.

Peptide hormone measurements in doping tests

The Olympic Movement Anti-Doping Code as of January 1st, 2003 contains the statement that 'the presence of an abnormal concentration of an endogenous hormone in class (E) or its diagnostic marker(s) in the urine of a competitor constitutes an offence unless it has been proven to be due to a physiological or pathological condition' (see www.wada-ama.org/docs). Unfortunately, in the case of peptide hormones the definition of an 'abnormal concentration' is extremely difficult or even impossible in many cases. Several peptide hormones are secreted by the human body in a pulsatil rather than a continuous manner, or the secretion exhibits a circadian profile. Furthermore, their concentration is usually influenced not only by age and gender but also by environmental factors (temperature, altitude), stress (psychological or physiological), sleep, nutrition state or training status. Many peptide hormones show an extremely short half-life time, leading to highly floatable concentrations in circulation. Therefore, a simple measurement of the hormone concentration in only a few cases (like hCG in men) is sufficient to demonstrate the misuse of the hormone. In contrast to some reports occurring in the laymen's press, a high concentration of hGH, for example, by no means is sufficient to prove that an athlete has been using recombinant hGH (Armanini *et al*. 2002).

Another problem with the detection of doping with peptide hormones is their recombinant origin. Derived from the expression of the protein encoded by the human gene sequence transferred to an *in vitro* system, the artificially produced hormones are identical to the naturally occurring hormone in their amino acid sequence and thus in their physicochemical properties. Once a peptide hormone was injected, for many years it was impossible to judge about the origin of a single hormone molecule.

Detecting peptide hormone doping is further complicated by the fact that—compared to the rather simple, small and stable steroid hormone molecules—peptide hormones are larger molecules exhibiting a very sensitive three-dimensional structure. In many cases, peptide hormones are rapidly degraded, metabolized and cleaved. The renal excretion involves complex processes, many of them being still poorly understood. Furthermore, the peptide hormone concentrations found in urine are often even much lower than those in blood. Therefore, the material traditionally used in doping analytics—urine—is of limited value for many peptide hormone tests. Blood sampling is required, bearing all the ethical and legal problems discussed elsewhere (Birkeland & Hemmersbach 1999).

Finally, the problem to establish doping tests for peptide hormones is related to the methodological difference in the measurement of peptide versus steroid hormones outlined above. For several years, steroid hormones have been identified by gas chromatography/mass spectrometry (GC/MS), and cutting edge experience together with the appropriate equipment is present in the International Olympic Committee (IOC) accredited laboratories. In many cases, GC/MS determination is referred to as a 'reference technology' or 'gold standard' for the quantitation of hormones. Unfortunately, this technology has not been available for peptide hormone analysis for many years, and there still is no established procedure shown to be ready to use in doping detection (Bowers 1997; Hilderbrand *et al*. 2003). All methods currently available or at least expected to

be available in the near future rely on immunoassay techniques, in the case of EPO complemented by an electrophoretic confirmation based on isoelectric focusing (IEF).

Specific aspects in doping analytics

Chorinonic gonadotropin

Chorinonic gonadotropin (hCG) is an important pregnancy-associated hormone that stimulates endogenous steroid production. The abuse of hCG in male athletes has been described, aiming to enhance endogenous anabolic steroids without changing the testosterone/epitestosterone ratio (Stenman *et al*. 1997). Normally, hCG concentrations in non-pregnant women and especially in men are extremely low. Only in a few pathological situations such as testicular cancer are know to be accompanied by elevated concentrations in men (Lottersberger *et al*. 2003). Therefore, the case of hCG is a comparably simple situation in peptide hormone doping analytics: The presence of high hCG levels in a male athlete are highly suspicious for doping. However, even frequently used in clinical practice, the quantification of hCG remains problematic. As a member of the glycoprotein hormone family, hCG is comprised of two distinct subunits (α and β). The α subunit is shared with all other members of this hormone family, whereas the β subunit is specific and responsible for receptor binding and biological activity. The carboxyl terminal of the hCG molecule contains a highly glycosylated region. Several molecular isoforms of hCG have been identified, and their heterogeneity contributes substantially to the large between-method differences in existing hCG immunoassays (Cole & Kardana 1992; Cole 1997; Cole *et al*. 2001). The situation is even worse in urine, where the spectrum of isoforms and degradation products is more complicated than in serum (Birken *et al*. 1996; O'Connor *et al*. 1999).

In addition to the heterogeneity of the analyte in a sample, for many years the standard preparations used to calibrate hCG assays contained substantial amounts of contaminating variants of hCG, which reacted to a variable degree with the antibodies in different immunoassays. It has been only recently that an international study group was able to establish six different IRPs for the most important hCG isoforms (Birken *et al*. 2003), and their introduction is expected to make assay results more comparable.

Most recently, a tandem mass spectrometric analysis technique has been described for confirmation of positive hCG tests (Gam *et al*. 2003). Following tryptic digestion, it was possible to identify a marker peptide (βT5) providing a specific fingerprint for hCG. Combined with a specific immunoextraction procedure, the proposed method currently is able to quantify hCG concentrations as low as 5 $IU \cdot mL^{-1}$ Whether this MS approach in the field of peptide hormone analysis will be reliable and practicable in doping analytics remains to demonstrated in large-scale studies.

Erythropoietin

The introduction of a doping test for recombinant human erythropoietin (rhEPO) at the 2000 Sydney Olympic games represents a major step forward in the fight against doping (Kazlauskas *et al*. 2002). For the first time, a detection method for a peptide hormone able to discriminate between the recombinant and the endogenous form of the hormone has been implemented into the official doping test program. To the present, detection of doping with rhEPO relies on two different methods. The first is the urine-based test as described by Lasne and de Ceaurriz (Lasne & de Ceaurriz 2000; Lasne *et al*. 2002), which utilizes an IEF method in combination with a technique called 'double-blotting' enabling the visualization of protein bands with a greatly reduced background staining (Lasne 2001, 2003). The rationale of the test is to detect the pattern of isoforms of EPO in a sample, which is different between recombinant and endogenous EPO (Lasne *et al*. 2002; De Frutos *et al*. 2003). The reason seems to be that especially glycosylation are sensitive to the cellular environment where a protein is produced. Because rhEPO is produced mainly in Chinese hamster ovary cells, the pattern of glycosylation is different from that in the human kidney (Sasaki *et al*. 1987; Rice *et al*. 1992). Meanwhile, this test procedure has been implemented in several IOC accredited laboratories and is used in doping analyses from many

sports associations (Catlin *et al*. 2002; Breidbach *et al*. 2003).

A major problem with this technique is that it is very expensive and time-consuming. In addition, the likelihood of catching a cheating athlete is given only for the first 3–4 days after injection (Wide *et al*. 1995; Souillard *et al*. 1996). The attempts to circumvent these problems led to the development of methods based on detecting changes in hematologic parameters after injection of rhEPO. These changes have been demonstrated to be more pronounced than can be expected from the normally observed variability, and some of them are detectable for as long as 4 weeks after rhEPO administration (Parisotto *et al*. 2001; Gore *et al*. 2003). The so-called 'second generation blood test' involves the measurement of EPO and of the soluble transferin receptor, both done by immunoassay techniques. Being much easier, cheaper and providing a much greater window of opportunity to detect rhEPO misuse, this test procedure unfortunately relies on blood samples and gives a comparably high rate of false positives when the cut off is set to a level where not too many abusers are missed. Thus, a current approach is to use the blood-based tests as a screening test to identify those samples which are more likely to contain rEPO, followed by the urine-based IEF test as a confirmatory test applied only to suspicious samples. Gore *et al*. (2003) have clearly shown that using the second-generation blood test for screening can drastically reduce the costs per positive sample as judged by the urine test. In addition, a lot of work has been done to establish normative data and to identify factors potentially influencing the test outcome (Sharpe *et al*. 2002; Ashenden *et al*. 2003; Parisotto *et al*. 2003). However, it is important to remember that the EPO molecules found in a urine sample can only be proven to be of recombinant origin by the IEF urinary approach visualizing the typical glycosylation pattern.

Growth hormone and growth hormone-dependent hormones

The detection of doping with recombinant human growth hormone (rhGH) has been thought to be impossible for many years. However, recently it has been shown that at least two different approaches are able to discriminate whether or not an athlete has taken rhGH. The test methods developed are still in the validation process and not yet implemented as an official doping test, but this is expected to happen soon.

A major problem remains that in contrast to the situation with EPO, none of the proposed methods for detecting hGH doping today can be applied in urine samples. This is primarily due to the extremely low hGH concentrations found in urine making the analysis not possible by currently available techniques. Furthermore, hGH secretion to urine is a complex process which seems to be highly variable and is still poorly understood (Saugy *et al*. 1996). The IEF method used for EPO in urine samples is not useful in the case of hGH, because hGH does not contain any glycosylation sites. According to our knowledge today, the hGH molecules of recombinant origin are almost identical to the main fraction of hGH molecules secreted by the pituitary gland, and no distinct physico-chemical properties of rhGH have been described.

However, even no glycosylation sites are present; hGH in circulation consists of a mixture of molecular isoforms (Baumann 1999). The investigation of these isoforms is not as advanced as in case of hCG, but during the last years it was possible to identify at least some of the major components. In addition to the 22 kDa major isoform, consisting of 191 amino acids, a shorter 20 kDa hGH isoform lacking amino acids 32–46 is the second most abundant form of hGH in circulation (Hashimoto *et al*. 1998; Tsushima *et al*. 1999; Leung *et al*. 2002). There are other even shorter isoforms described, but they are observed less constantly and are not yet fully analyzed. Some of them have been shown to be cleaved or degraded hGH molecules. The isoforms of hGH seem to exist in monomeric, dimeric and multimeric complexes formed by either identical (homodimers) or different isoforms (heterodimers).

Many of the hGH effects in the body are mediated through a factor called insulin-like growth factor I (IGF-I). Produced mainly in the liver, but even locally in cartilage, bone and many other tissues, IGF-I is secreted to the blood, where it is bound by specific binding proteins (Le Roith *et al*. 2001).

The most important ones are IGF binding protein-3 (IGFBP-3) and the acid labile subunit (ALS), both produced under hGH control as well. At least for IGFBP-3 it has been shown that this protein exerts effects independently from IGF-I binding and therefore can be a seen as a 'peptide hormone' itself. Together, IGF-I, IGFBP-3 and ALS form a 150 kDa ternary complex, possessing an increased half-life in comparison to each molecule alone.

It was one strategy in the search for a suitable test method to detect hGH doping to evaluate whether or not the increase in the pharmacodynamic endpoints of hGH action, especially the increase in the components of the ternary complex might exceed the normally observed variability (Dall *et al*. 2000). Such a test would include the advantage that the half-life of the pharmacodynamic endpoints of hGH action exceeds that of hGH, making possible a longer window of opportunity for detecting hGH abuse. An international consortium of researchers conducted a series of large-scale studies to investigate the behavior of such pharmacodynamic endpoints of hGH action in relation to various conditions like acute and chronic exercise, age, gender, ethnic background or injury (Wallace *et al*. 1999, 2000; Longobardi *et al*. 2000; Ehrnborg *et al*. 2003). The main outcome of these studies was that repeated administration of exogenous rhGH indeed induces changes in pharmacodynamic endpoints, which can be discriminated from changes induced by exercise or other stimulators. In more detail, the researchers developed a statistical model including more than one of the pharmacodynamic endpoints and tailored this model specifically for each gender. However, a crucial point is that the immunoassays used for determinations of the factors are sufficiently evaluated and that method specific reference ranges are known. Not all commercially available methods can fulfill these criteria, and therefore a careful selection of the immunoassays to be used is mandatory. Furthermore, because a highly sophisticated statistical model is the backbone of this test method, the variability of the assays must be known, and a continuous supply with an identical batch of antibodies must be ensured. This could provide problems because many of the immunoassays used until today rely on polyclonal rather than monoclonal antibodies. Polyclonal antisera, however, are inherently limited in their quantity and—once a batch is finished—cannot be reproduced identically. Therefore, one can understand that the international antidoping agencies put the development of suitable monoclonal antibodies onto the agenda. Once the methodological details have been clarified, this test method could be very useful, comparably to the blood test already in use for EPO doping detection.

Another approach more directly relies on the measurement of hGH: In contrast to the hGH isoform spectrum secreted by the pituitary gland, rhGH as produced in bacteria usually consist of 22 kDa hGH only. The recombinant production of 20 kDa hGH has been described, but until today this preparation has been used only in a few clinical trials. Recombinant hGH used to treat GH deficient children, adolescents and adults is 22 kDa hGH, and the same seems to apply for the hGH preparations abused for doping purposes in sports. This 'uniformity' or 'lack of heterogeneity' in the recombinant hGH preparations in comparison to the many isoforms secreted by the human pituitary gland and normally present in circulation forms the basis for the so-called 'differential immunoassay approach' to detect doping with recombinant hGH (Wu, Z. *et al*. 1999): the injection of recombinant monomeric 22 kDa hGH increases the relative abundance of this hGH isoform in circulation. This change in the isoform composition is further increased by the reduction of pituitary hGH secretion due to the negative feedback mechanism observed under chronic rhGH treatment (Wallace *et al*. 2001a). Screening of monoclonal antibodies raised against different hGH preparations led to the development of two different immunoassays for hGH. The capture antibody of the first assay preferentially binds the 22 kDa isoform of human GH, whereas the capture antibody of the second assay preferentially binds 'pituitary derived hGH', consisting of multiple isoforms (Bidlingmaier *et al*. 2000). Measuring on serum sample by both assays allows to calculate the relative abundance of 22 kDa hGH versus the other isoforms ('total hGH'), and thus identifying samples containing an inappropriately high content of 22 kDa hGH. It could be demonstrated that the change in the molecular isoform composition is uniquely related

to rhGH application, whereas the increase in circulating hGH after exercise is accompanied by an increase in the non-22 kDa hGH isoforms as well (Wallace *et al*. 2001b). Meanwhile, the original approach has been dramatically improved in terms of sensitivity (Bidlingmaier *et al*. 2003). This was made possible by the identification of new monoclonal antibodies. In addition, an independent confirmatory set of assays was developed, based on new monoclonal capture antibodies, targeting independent epitopes. The latter is a prerequisite for the acceptability of immunoassays in a doping test setting: each assay has to be confirmed by another assay, targeting independent structures of the molecule of interest and thus providing further evidence for the identity of the molecule.

Inherently, the differential immunoassay approach is limited to the detection of rhGH abuse—cadaveric hGH preparations, derived from extraction of pituitary glands, have an isoform pattern which cannot be discriminated from that naturally occurring in the human body. Furthermore, due to the very short half-life time of hGH in circulation (about 15 min), the window of opportunity for the detection remains limited to 24–36 h. Obviously, even the development of more sensitive assays will not overcome this limitation, because after disappearance of the recombinant substance and cessation of the negative feedback, the normal pituitary isoform secretion has been shown to start again. On the other hand the fact that rhGH has to be taken by daily subcutaneous injections enhances the probability to catch a cheating athlete in unannounced out-of-competition tests.

The differential immunoassay approach also makes it necessary to rigorously validate the immunoassays selected. Furthermore, because a ratio is calculated, the within- and between-method variability must be determined exactly with respect to the potential impact of this variability on the ratio result. A reduction of the variability could be achieved by using the same capture antibody for both, the 22 kDa preferential and the total hGH assay: on one microtiterplate, the upper half is covered by the 22 kDa assay capture monoclonal antibody (mAb), whereas the lower half of the plate is covered by the total hGH capture mAb. After adding calibrators, controls and samples to each half of the plate, the whole plate is covered by the same detection mAb. This reduces significantly the variability always included in distributing samples on two different plates (Bidlingmaier *et al*. 2000).

Conclusions

The immunoassay techniques used for peptide hormone measurements represent very sensitive tools to quantify the concentrations of the hormones in various biological fluids specifically. Appropriately selected antibodies, which must be characterized regarding their epitopes, affinities and possibly cross-reacting peptides, confer a high degree of specificity in hormone measurements, making these methods suitable even in the setting of doping test procedures. Being much easier, cheaper and less time consuming than other methods for peptide hormone analysis, immunoassays have a high potential, especially in large-scale screening. However, for each assay used, method specific normative data have to be established. Furthermore, potentially confounding factors must be identified and appropriate methods for their elimination must be developed. If these precautions are taken into consideration, the accuracy of immunoassay analyses can be extremely high and very suitable for use in a forensic environment.

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Chapter 5

Analysis of Low Molecular Weight Substances in Doping Control

MARIO THEVIS AND WILHELM SCHÄNZER

Historical aspects of doping control analysis of low molecular weight drugs

Ever since sport competition has been known, the wish to artificially enhance power, skill and strength was also present. Philostratos and Galen already reported attempts of athletes of the ancient Olympic Games to improve performance and endurance by means of concoctions of mushrooms and plant seeds as well as special diets, for example with bovine testicles (Burstin 1963; Prokop 1970, 2002). Moreover, the athletes' animals were targets of manipulation as described for the ancient Romans, who fed their horses *hydromel*, a mixture of honey and water, in order to increase their strength (Morgan 1957; Ariëns 1965). Major reasons for the phenomenon of athletes trying to artificially obtain the cutting edge advantage in competition are supposed to be fame, glory and honor as well as financial aspects, which became even more important with the introduction of professional sports and horse and greyhound racing. At the beginning of the 20th century, first scientific procedures for the determination of doping agents in horse saliva were developed by Russian and Austrian scientists, and in 1910–1911 about 220 samples were investigated regarding administration of alkaloids. Between 1938 and 1954, principal procedures for the detection of stimulants such as amphetamines were presented, which demonstrated limited sensitivity and susceptibility to interferences by biological matrices (Richter 1938; Keller & Ellenbogen 1952; Axelrod 1954), but in 1956, a commonly accepted assay based on liquid extraction, paper chromatography and visualization was introduced (Vidic 1956). The fatality of doping became obvious in 1886, when the cyclist Linton died during a race Paris–Bordeaux caused by an overdose of caffeine (Prokop 2002). In the following 70 years, numerous cases of doping-related deaths were documented, primarily involving cyclists, such as the death of a cyclist caused by amphetamine poisoning (1949); the hospitalization of a cyclist for confusional toxical state from excessive use of amphetamines (1956); a cyclist in state of shock from excessive use of sympathomimetics (1958); and hospitalization of a cyclist for intoxication with amphetamines and analeptics (1962). All these cases were uncovered in Italy (Venerando 1963). With the formation of doping commissions in France (1959), Austria (1962) and Italy (1963) followed by the Medical Sub-Commission of the International Olympic Committee (IOC) in 1967, a comprehensive fight against doping was started. First official doping controls were conducted during the Olympic Games in Grenoble (1968) based on a first screening assay for a selection of stimulants (Beckett *et al*. 1967), and the list of prohibited substances and methods of doping has been modified and extended many-fold during the last decades. A huge variety of methods have been established in order to determine doping-relevant compounds, their metabolites and their influence on endogenous parameters.

Classification of drugs

Prohibited compounds

Owing to the wide variety of banned compounds, there is no list mentioning all restricted substances by name. Besides examples for each category, the

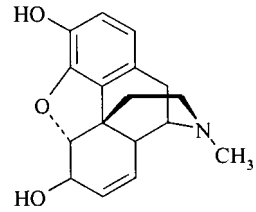
Table 5.1 Classification of prohibited compounds according to the International Olympic Committee (IOC) and World Anti-Doping Agency (WADA).

I. Prohibited classes of substances
A. Stimulants
B. Narcotics
C. Anabolic agents
D. Diuretics
E. Peptide hormones, mimetics and analogs
F. Agents with anti-estrogenic activity
G. Masking agents
II. Prohibited methods
A. Enhancement of oxygen transfer
B. Pharmacological, chemical and physical manipulation
C. Gene doping
III. Classes of prohibited substances in certain sports
A. Alcohol
B. Cannabinoids
C. Local anesthetics
D. Glucocorticosteroids
E. Beta-blockers

extension '. . . and related substances . . .' is added, resulting in prohibition of all compounds with correlated pharmacological or physicochemical properties. In Table 5.1, the classification of drugs according to the IOC and World Anti-Doping Agency (WADA) (International Olympic Committee 2003) is shown. For several remedies and substances such as beta-blockers, corticosteroids, local anesthetics, marihuana and alcohol, restriction is limited to selected sport sections or their medical indication.

STIMULANTS

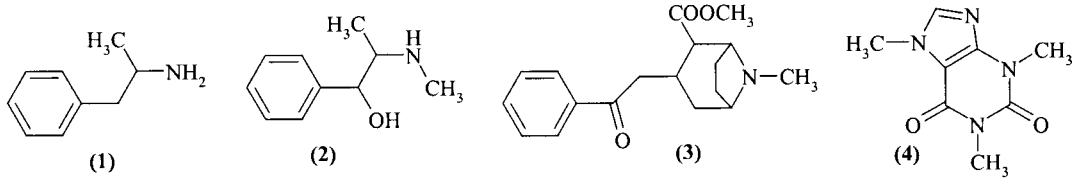
Stimulants are most likely the oldest doping substances known to human beings. They include compounds such as amphetamines, ephedrines, caffeine and also cocaine (Scheme 5.1). The latter was already used by Mexican Incas in the 16th century when they utilized coca leaves to cope with distances of more than 1000 miles (1609 km) between Cuzco and Quito within 5 days (www.g-o.de 2003), and the very first identified doping substances found in athletes' urine samples in the 20th century were related to amphetamines. Due to the presence of ephedrines in a series of cold medicines, and furthermore the caffeine content of several cold and hot beverages, urinary concentration limits for these selected compounds were established, requiring quantitation of these drugs in urine specimens, whereas new regulations valid from January 2004 do not prohibit caffeine anymore.



Scheme 5.2 Chemical structure of morphine, a representative of the class of opioid-like narcotics.

NARCOTICS

The class of narcotics includes compounds referred to as opioid-like analgesics such as morphine (Scheme 5.2) and related substances with a few exemptions, for example ethylmorphine and codeine, owing to their weak analgesic effect. Other non-opioid-like drugs, for instance acetylsalicylic acid or diclofenac, are allowed.



Scheme 5.1 Structure formulae of amphetamine (1), ephedrine (2), cocaine (3) and caffeine (4).

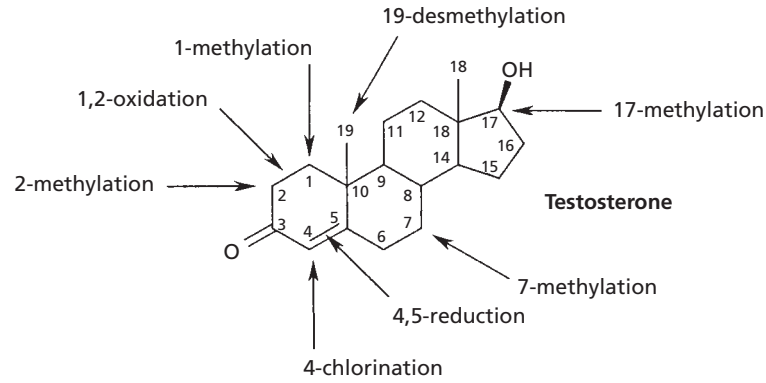


Fig. 5.1 Chemical structure of testosterone and modifications resulting in various synthetic anabolic steroids.

ANABOLIC AGENTS

While stimulants and narcotics are of interest primarily in competition, anabolic agents are effective during periods of exercise. Increasing muscle growth and strength gaining, the drugs belonging to this category were added to the list of prohibited compounds of the IOC in 1976, and, since 1993, not only are steroids related to testosterone (Fig. 5.1) included in the class of anabolic agents but also drugs such as clenbuterol, as β_2-agonists cause anabolic effects if administered in doses significantly higher than for therapeutic use (Reeds *et al*. 1988; Wagner 1989; Stallion *et al*. 1991; Höher & Troidl 1995).

DIURETICS

Diuretics are considered as doping agents based on two facts: (i) athletes competing in sports categorized by weight can decrease their body weight by artificially induced diuresis, i.e. increased urine flow; (ii) the increased loss of water dilutes the urine resulting in decreased concentration of excreted compounds. Regarding those drugs that are banned with respect to cutoff limits, diuretics can mask the abuse of prohibited substances. The class of diuretics in particular demonstrates the possible physicochemical heterogeneity of one category of drugs. Diuretic agents can be divided into at least six groups owing to their structures, place and mechanism of effect. There are so-called thiazides (e.g. hydrochlorothiazide), sulfamoylbenzoic acid derivatives (e.g. furosemide), osmotic diuretics (e.g. mannitol), carbonic anhydrase inhibitors (e.g. acetazolamide), phenoxyacetic acid derivatives (e.g. ethacrynic acid), and potassium-retaining diuretics (e.g. spironolactone), some of which are shown in Scheme 5.3 (Wilhelm & deStevens 1976; Lang & Hropot 1995; Möhrke & Ullrich 1995).

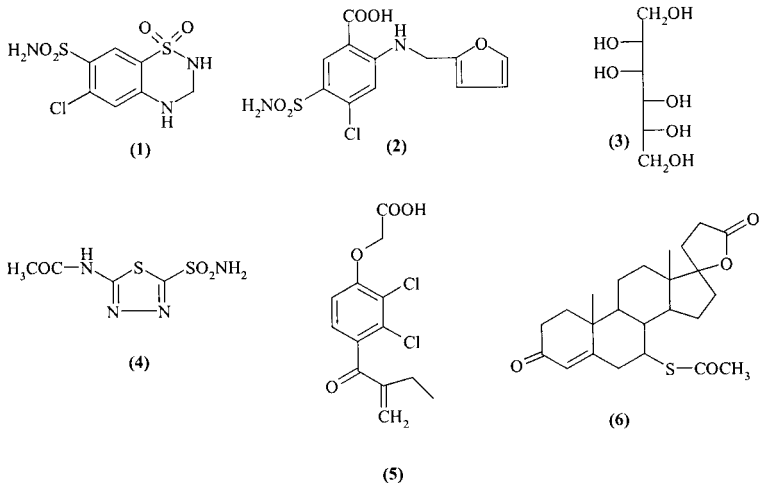
BETA-BLOCKERS

Beta-receptor blocking agents (also referred to as beta-blockers) are banned for sports such as shooting or ski jumping owing to their sedative effects. In general, beta-blockers consist either of a phenyl ring structure bearing an oxypropanolamine side chain that terminates in an isopropyl or *tert*. Butyl residue (e.g. propranolol, levobunolol), or a phenylethanolamine nucleus comprising substitutes such as nitrite functions (e.g. nifenalol). Modern β-receptor blocking agents partially diverge from this principal structure, for instance the remedy nebivolol. Since 1988 the class of beta-blockers belongs to the IOC list of prohibited compounds, and some examples are presented in Scheme 5.4.

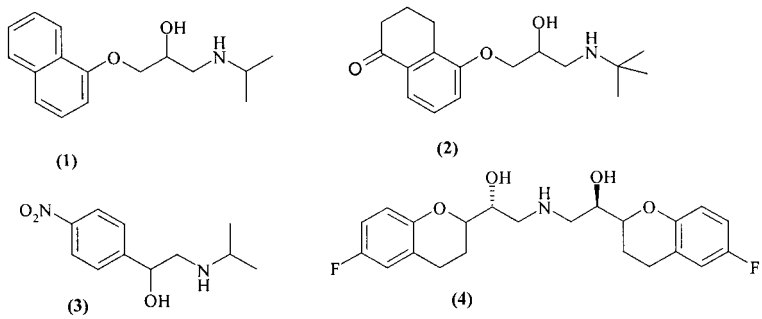
As shown in Table 5.1, additional classes of prohibited compounds, such as peptide hormones, belong to the list of banned substances, but in this overview we will focus only on a selection of low molecular weight drugs.

Analytical techniques

The first commonly accepted screening and confirmation methods for doping control analysis included sample preparations based on liquid–liquid



Scheme 5.3 Structure formulae of selected diuretics: (1) hydrochlorothiazide, (2) furosemide, (3) mannitol, (4) acetazolamide, (5) ethacrynic acid and (6) spironolactone.



Scheme 5.4 Structure formulae of selected beta-blockers: (1) propranolol, (2) levobunolol, (3) nifenalol and (4) nebivolol.

extraction of urine, concentration of the extracts and separation of analytes by means of gas–liquid chromatography (GLC) and thin-layer chromatography (TLC). A flame ionization detector (FID) was interfaced to GLC indicating the presence of stimulants of the amphetamine type by signals at comparable retention times as observed with reference compounds. Thin-layer chromatography was used for the identification of strychnine and also ring-hydroxylated stimulants such as *p*-hydroxyamphetamine and phenylephrine (Beckett *et al*. 1967). In case of 'positive' test results, additional information on the analytes was obtained by derivatization, micro infrared spectroscopy and also mass spectrometry (MS). With improvements and innovations in analytical techniques, commercially available instruments and sophisticated sample preparation procedures, numerous assays have been developed enabling a comprehensive analysis of elite athlete's urine samples for doping control purposes. Depending on the nature of compounds to be analyzed, different analytical tools have been employed, the principal techniques of some of which will be described in the following.

In general, chromatographic separation of extracts obtained from biological material such as blood, urine or hair, is more or less obligatorily preceding the analytical detection. Thus, more detailed information on analytes is provided; for example, by retention times that support for instance the distinction between stereoisomers, or by separating lowly concentrated from highly abundant compounds.

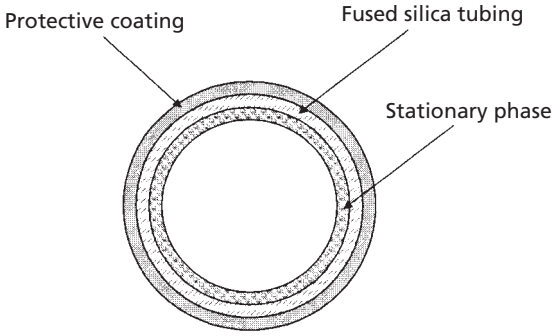


Fig. 5.2 Principal composition of capillary gas chromatography columns.

Modern chromatographic systems are based either on capillary gas chromatography or high performance liquid chromatography, both of which have become extremely sophisticated and elaborated sciences.

Gas chromatography

Nowadays, capillary columns are the first choice for gas chromatography (GC) in doping control analysis. Their excellent performance in terms of compound separation, robustness, and the enormous variety of different stationary phases provides numerous options for method development and possibilities to cope with tasks such as comprehensive screening assays or compound-specific analyses. The principal composition of a modern capillary GC column is presented as cross section in Fig. 5.2, and the basic scaffold is represented by three distinct parts: (i) outer protective coating; (ii) fused silica tubing; and (iii) stationary phase.

Fused silica tubing. Fused silica is a synthetic, quartz-like glass of very high purity regarding metal oxide contaminants. In general, fused silica bears a very active surface owing to its silanol functions, which can interact with polar groups of analytes, such as hydroxyl, carboxyl and thiol residues as well as primary and secondary amines, causing peak tailing and a decreased peak intensity. Hence, a deactivation of silanol groups by appropriate chemical treatment is performed, for instance by trimethylsilylation, and the derivatized fused silica creates a suitable surface for being coated by the stationary phase.

Stationary phase. The stationary phase is the most crucial component in terms of retention, separation and peak shape of analytes in GC. This particular part of capillary columns consists of either polysiloxanes/silicones, polyethylene glycols (PEGs) or so-called porous layer phases. The predominantly used stationary phases are the substituted polysiloxanes owing to their robustness and superior lifetime. In Fig. 5.3, the general chemical structure of polysiloxane-based stationary phases is presented, determined by the alternating silicon-oxygen backbone and two substitutes at each silicon atom. The structure and amount of the substitutes characterizes each stationary phase, and primarily four groups are utilized, mixed in different ratios: (i) methyl-; (ii) phenyl-; (iii) cyanopropyl-; and (iv) trifluoropropyl residues. With the choice of substitutes and their relative presence in the stationary phase, the polarity of the GC column is defined as listed in Fig. 5.4 for 14 common mixtures. Another stationary phase employed in GC is PEG that is characterized mainly by the molecular weight or the chain length of the polymer. A remarkable difference of this type of material to polysiloxane phases is the possibility to designate a certain pH range, i.e. acidic or alkaline columns are available demonstrating an improved capability to separate acidic or alkaline compounds, respectively. A main disadvantage is the high sensitivity to oxygen, in particular at elevated temperatures, resulting in destruction of the stationary phase and a correlated short lifetime. Porous layer stationary phases consist of small porous particles (e.g. polymers, aluminium oxide, molecular sieve) that are bound to the fused silica tubing by chemical linkers. Owing to their high affinity of gas adsorption, these phases are utilized primarily for the chromatography of very volatile compounds or gases, which usually demonstrate poor retention on stationary phases such as polysiloxane or PEG, and thus require temperatures below 35°C. With so-called PLOT columns (*p*orous *l*ayer *o*pen *t*ubular), gas–solid adsorption is the primary separation mechanism enabling efficient

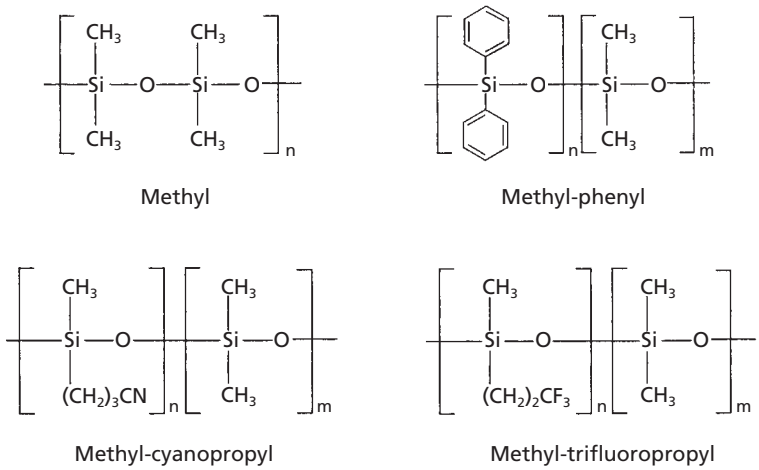


Fig. 5.3 General chemical structure of polysiloxane-based stationary phases with selected substitutes (methyl-, phenyl-, cyanopropyl- and trifluoropropyl residues).

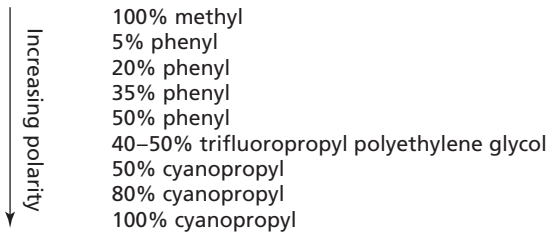


Fig. 5.4 Stationary phase polarities depending on the choice of substitutes and their relative amounts.

retention of very volatile substances, but the strong interaction between stationary phase and analyte excludes their use for less volatile substrates.

Outer coating. As fused silica tubing is fairly fragile, it requires a protective outer coating. For that purpose, polyimide is commonly employed and provides two advantages: first, polyimide-coated columns are robust, stable and do not require delicate handling, and second, it covers and fills flaws of the fused silica tubing, preventing the growth of defects.

After considering the composition of capillary GC column, two additional parameters impact the chromatographic performance remarkably: column dimensions, i.e. length, diameter and film thickness, and carrier gases.

While length and diameter mainly affect peak resolution, film thickness primarily influences the capacity of columns and the retention of analytes. The length of a column is directly proportional to the number of theoretical plates, and as the resolution is proportional to the square root of the number of theoretical plates, resolution is also proportional to the square root of column length. In other words, doubling the column length (and thus the number of theoretical plates) does not improve resolution by 100%, but in practise only by about 25–35%, and the reduction of column length by 50% causes a loss of resolution of approximately 15–25%. In addition, the number of theoretical plates is inversely proportional to the column diameter. Hence, decreasing column diameter increases efficiency of GC. Taking again into account that resolution is proportional to the square root of the number of theoretical plates, halving of the inner diameter also improves resolution by 25–35%; for instance, by exchanging a column with an inner diameter of 0.25 mm by 0.11 mm. The capacity of capillary columns is directly depending on the film thickness of the stationary phase. While phases of 0.1–0.2 µm are appropriate for 20–50 ng of analyte, 0.5 µm are necessary for amounts greater than 125 ng. Thus, film thickness must be adjusted to the expected load of compounds injected onto the GC column.

Also, carrier gas impacts the performance of GC, as commonly described by the Van Deemter curve. Frequently employed carrier gases are helium and

hydrogen, the latter one provides a superior optimal linear velocity resulting in an improved performance regarding resolution. The disadvantage of hydrogen is definitely its highly flammable character.

Liquid chromatography

Comparable to GC, liquid chromatographic accessories provide a wide variety of dimensions, packing materials and, as compounds are carried through the analytical columns by liquids, a series of organic solvents and buffers. In addition, several principles of liquid chromatography (LC) (normal phase, reversed phase, ion exchange, size exclusion) can be applied, depending on the nature of target analytes. In this brief overview, we will focus only on reversed phase chromatography (RPC) which is the most frequently used system in doping control analysis.

Cartridges. In LC in particular, numerous diameters of column cartridges are available. With the improvements in packing materials (i.e. stationary phases, see below), the most commonly employed dimensions for small molecule analysis in doping control laboratories have been reduced to column lengths of 30–120 mm and inner diameters of 1.0–4.6 mm compared to earlier days of high performance liquid chromatography (HPLC) where longer and broader columns were utilized. The shortening has become an option with the highly selective and specific mass analyzers, which enable separation of compounds by means of their mass spectra, and thus the need for optimal chromatographic distinction between analytes has become minor important. In addition, performance of LC columns has been incredibly ameliorated, facilitating peak separation in reduced time frames.

Stationary phase. As mentioned in the Gas chromatography section above, the stationary phase is the utmost crucial point in LC as well. Here, different parameters have to be considered such as particle size (3–50 µm), carrier (spherical or irregular silica), chemically bonded phases (e.g. C_4, C_8, C_{18}), and pore size (50–4000 Å).

For characterization of column performance, the number of plates n (or the plate height h) is frequently used as a measure. The Van Deemter equation in its simplest form describes the reciprocal relation of particle size to plate height, which means that a decrease in particle size entails an improvement in chromatography performance, namely resolution (Yamashita & Fenn 1984a; Engelhardt *et al*. 1985). Hence, there is a bias to reduce particle sizes with the use of very short columns in high speed chromatography in order to maintain required peak resolutions.

The most commonly employed carrier material for the preparation of stationary phases in RPC is silica. Commercially available silicas differ in physical properties such as specific surface area, average pore diameter, specific pore volume and shape. Assuming open cylindrical pores, the three first mentioned variables are related by the equation

$$\phi = 10^3 \times 4V_p/O_{sp}$$

with ϕ = average pore diameter (nm), V_p = specific pore volume ($mL \cdot g^{-1}$) and O_{sp} = specific surface area ($m^2 \cdot g^{-1}$) (Scott 1982). Average surface areas are approximately 300 $m^2 \cdot g^{-1}$ and pore diameters 10 nm, giving rise to an approximate specific pore volume of 1 $mL \cdot g^{-1}$. Nowadays, almost exclusively spherical silica is utilized as carrier for reversed phase LC stationary phases, owing to the higher density compared to silica prepared by polymerization of silicic acid yielding irregular particle shapes. On the surface, the silica carrier bears hydroxyl groups (silanol functions), which are utilized to chemically bind phases and thus define the principal nature of the HPLC column. An enormous variety of phases has been introduced to the market in order to provide suitable material for each chromatographic challenge, for instance the frequently used phenyl-, C_{18}-, C_8- and C_4-phases, or more polar material such as cyano-, diol- or amino-phases as demonstrated in Fig. 5.5. In addition, even more sophisticated systems enabling the separation of chiral molecules have been developed, and the most recent improvement in high-throughput analysis was achieved by so-called monolithic columns consisting of a single piece of an organic polymer or silica with flow-through pores prepared by direct polymerization of

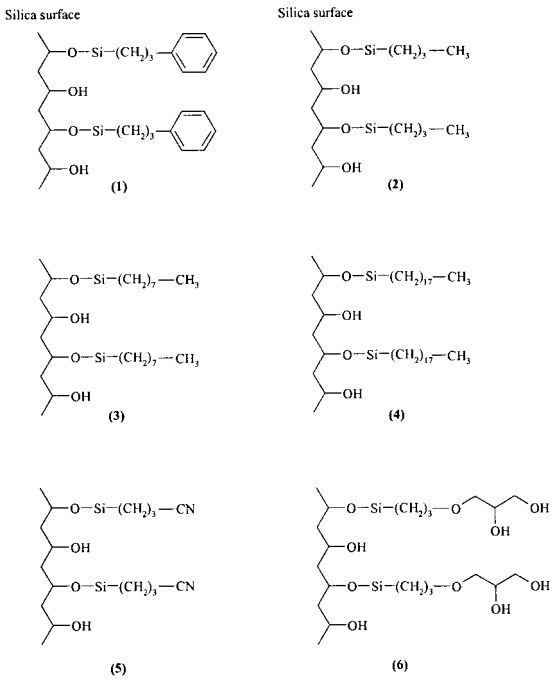


Fig. 5.5 Commonly used stationary phases in reversed phase chromatography (RPC): (1) Phenyl-, (2) C_4-, (3) C_8-, (4) C_{18}-, (5) cyano- and (6) diol-phases.

appropriate monomers. These materials provide better stability and, more importantly, higher performance than conventional columns packed with particles.

Mobile phases. In case of RPC, the stationary phase is the non-polar and the mobile phase the polar component. Conventional RPC employs buffer systems consisting of, for example, KH_2PO_4/H_3PO_4 or NaH_2PO_4 in combination with organic solvents such as methanol or acetonitrile, which are very suitable for analyzers such as ultraviolet (UV) -detectors. But the coupling of HPLC to MS requires different considerations regarding the eluents as there are restrictions on pH, solvent choice, solvent additives and flow rates for LC in order to accomplish optimal mass spectrometric results (Wheeler 1955). In general, atmospheric pressure ionization (e.g. electrospray ionization, ESI) demands volatile solvent additives to prevent ion source contaminations; hence commonly used phosphate, borate or sulfate additives are not recommended but, for instance, ammonium acetate, ammonium formate or tetraethy-lammonium hydroxide (TEAH) are. Moreover, ESI is not compatible with ingredients favoring the generation of strong ion pairs resulting in neutralization of ions after desorption. Control of pH is of paramount importance in particular with ESI, because of its enhancing effect on analyte ionization. Compounds of basic character should be analyzed under acidic conditions utilizing additives such as acetic acid or formic acid in the 0.1–1.0% range, while components of acidic nature (e.g. carboxylic acids) are preferably negatively ionized under alkaline conditions; for example, by means of addition of ammonium hydroxide. Commonly employed organic solvents are comparable to conventional HPLC without interfacing to MS, i.e. methanol, ethanol and acetonitrile.

Detectors

The detection and identification of chromatographically separated compounds is of paramount importance in doping control analysis. Many different types of detectors have been developed such as FID, nitrogen phosphorus detector (NPD), UV-detector and mass selective detectors such as quadrupole, time-of-flight, ion trap, Fourier-Transform ion cyclotron resonance (FT-ICR), triple-stage quadrupole and sector mass analyzers. In addition, hybrid instruments composed by two or more of these units are commercially available, and numerous applications enabling the determination of administration of prohibited compounds are based on a combination of analyte-specific sample preparation, chromatography and sensitive as well as selective detectors.

FLAME IONIZATION DETECTOR

One of the oldest and universal detectors interfaced to GC is the flame ionization detector (FID) (Fig. 5.6). Here, the gas mixture eluting from the GC column is aerated, fortified with hydrogen and ignited electrically. The hydrogen–air flame itself creates only very few ions, but most organic compounds generate an increased number of ions and

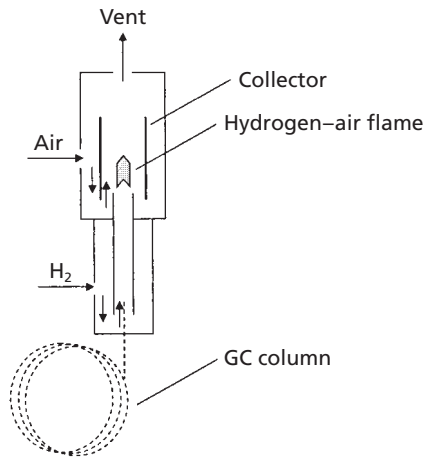


Fig. 5.6 Principal design of flame ionization detectors (FIDs).

electrons upon pyrolysis in the hydrogen–air flame, and thus enable an improved conduction of electricity. To a so-called *collector* located near the flame, a polarizing voltage is applied, which attracts generated ions producing a current that is proportional to the amount of sample being burned after elution from the GC column. This particular current is sensed by an electrometer, converted to a digital signal, and plotted as peak in a chromatogram.

NITROGEN PHOSPHORUS DETECTOR

The nitrogen phosphorus detector (NPD) is a variant of the FID. The main difference is a glass or ceramic bead that is located right above the jet of the hydrogen–air plasma. A low hydrogen/air ratio can not sustain a flame, resulting in decreased hydrocarbon ionization. But the alkali ions on the bead surface facilitate the ionization of nitrogen- or phosphorus-containing compounds, thus favoring the detection of those compounds while suppressing the abundance of other, mainly hydrocarbon-based substances.

ULTRAVIOLET ABSORBANCE DETECTOR

While FID and NPD can only be interfaced to GC, the UV absorbance detector has proven to be one of the most popular analyzers for high performance LC since the late 1960s. The principle is based on conventional spectrophotometry and the Beer–Lambert law:

$$\text{Log } I_0/I = \varepsilon c d$$

with I_0 = intensity of the incident light, I = intensity of the transmitted light, ε = extinction coefficient, c = concentration of the absorbing analyte and d = path length of the cell. In practice, the eluent of a HPLC analysis is passed through a cell of fixed length, a light source emitting in a distinct UV range (e.g. deuterium lamp, 190–600 nm) and, providing light of defined intensity is focused on the cell through a monochromator, the intensity of the transmitted light of selected wave length is detected by means of photo diodes.

The detectors described so far enable the recognition of compounds chromatographically separated either by GC or HPLC. The main drawback of these analyzers is the low specificity and correlated lack of detailed information on the analytes. As a consequence, instruments based on MS have become the first choice in doping control analysis being employed in combination with both GC and HPLC, complementary to the 'traditional' instrumentation utilizing FID, NPD and UV detectors. In order to identify substances by MS, ionization is required, which can be accomplished by many different techniques in consideration of the respective chromatographic system. Nowadays, GC-MS frequently employs techniques such as electron ionization and chemical ionization, while HPLC is mainly interfaced to mass spectrometers by ESI, atmospheric pressure chemical ionization (APCI) or also atmospheric pressure photo ionization (APPI). In the following, we briefly describe the principles of electron ionization and ESI as well as quadrupole and ion trap analyzers, the most commonly used tools in doping control laboratories.

Ionization techniques

ELECTRON IONIZATION

Electron ionization (EI) is widely used to generate positive ions of analytes separated by GC. Here,

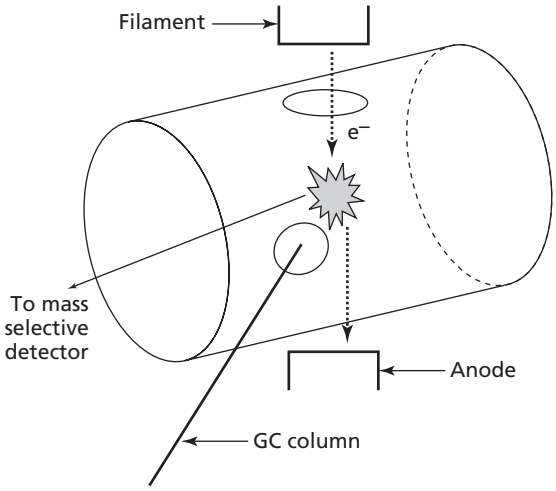


Fig. 5.7 Schematic assembly of an ion source with electron ionization.

electrons emitted from a cathode, the so-called filament, are accelerated to an anode, and collide by orthogonal orientation within the ion source with compounds eluting from the GC column as demonstrated in Fig. 5.7. The collision of electrons released with commonly utilized 70 eV (potential between filament and anode is set to 70 V) with analytes in an ion source gives rise to a number of processes; for example:

(1) $AB + e^- \rightarrow AB^+ + 2e^-$

(2) $AB + e^- \rightarrow AB^{2+} + 3e^-$

(3) $AB + e^- \rightarrow AB^-$

For positive ionization, the most important mechanism is (1) with the generation of a positively charged molecule (AB^+) by liberation of an electron upon impact of the accelerated electron released from the filament. In addition, also two electrons can be removed from the analyte AB giving rise to a doubly charged molecule AB^{2+} (2). Moreover, electrons can be incorporated into the molecule AB resulting in a negatively charged analyte AB^- (3). Besides these examples of effects of EI on compounds introduced into an ion source by GC, the consequences of this ionization technique also have to be taken into consideration. As a major result of EI, highly energetic radical cations are generated inclining to dissociate into product ions, which are detected in the mass selective analyzer giving rise to a characteristic mass spectrum of the respective substance. These product ions can be radicals as well as even-electron ions, depending on rearrangement and elimination processes.

ELECTROSPRAY IONIZATION

The development of the comparably soft ionization technique utilizing electrospray was recently awarded with the Nobel Prize for chemistry in 2002. John Fenn was honored for fundamental research regarding the ionization of macromolecules that was already introduced by Yamashita and Fenn in 1984 (Yamashita & Fenn 1984b; Voyksner 1997). With this technique, liquids containing protonated or deprotonated molecules are sprayed by means of a capillary tip at high voltages (1 kV and higher), forming charged droplets that shrink by solvent evaporation and repeated droplet disintegration leading to very small and highly charged droplets. Finally, gas-phase ions are produced from these very small droplets as discussed in two different theories, namely *charged residue model* (Dole *et al*. 1968) and *ion evaporation* (Iribarne & Thomson 1976). In Fig. 5.8, the main processes of ESI are presented. First, the penetration of imposed electric field into the liquid of the capillary leads to an enrichment of positive charges at the surface of the liquid, causing destabilization of the meniscus, formation of a cone and a so-called jet-emission of droplets bearing an excess of positive ions. The charged droplets shrink by solvent evaporation while the charge remains constant. Hence, an increase of the electrostatic repulsion occurs, resulting in fission of the droplets as they reach the Rayleigh stability limit. This phenomenon continues with ongoing evaporation of solvent until very small and highly charged droplets are created. Finally, as proposed by Dole *et al*. (1968), only one ion remains in a singly charged droplet, and the evaporation of solvent gives rise to a gas-phase ion (*charged residue model*). Alternatively, the direct ion emission from droplets with a radius smaller than 10 nm was postulated, also generating gas-phase ions (*ion evaporation*) (Kebarle & Ho 1997).

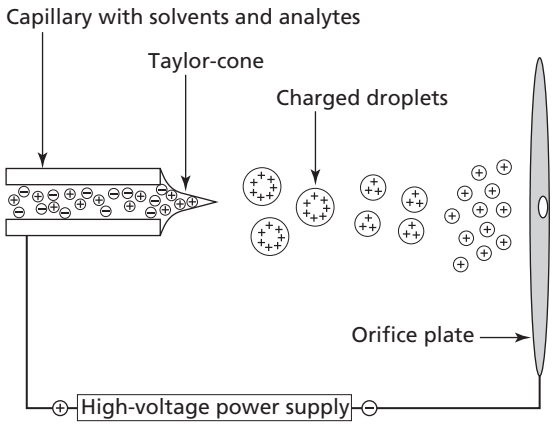


Fig. 5.8 Processes in positive electrospray ionization. Application of high voltage to a liquid-filled capillary leads to enrichment of positive ions at the liquid surface, destabilization of the meniscus and generation of a cone that emits droplets containing an excess of positive charges.

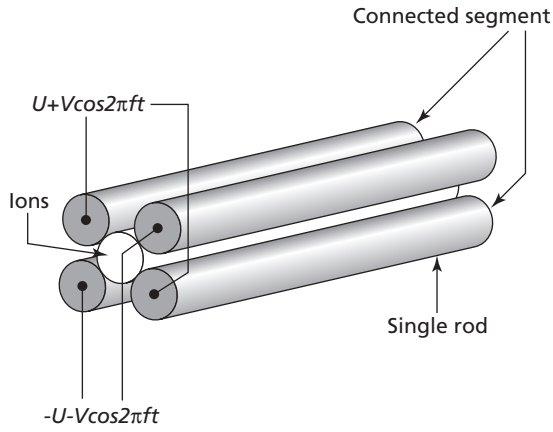


Fig. 5.9 General assembly of a quadrupole mass selective analyzer. Two opposing metal or gold-coated fused silica rods are electrically connected while adjacent rods are isolated. Ions are accelerated from the ion source into the center of the quadrupole analyzer and their trajectories are influenced by application of ac and dc voltage. Only ions of defined mass/charge ratios pass through the quadrupole at distinct voltages, which enables mass filtering.

Mass analyzers

The ions generated from substances by various ionization techniques are analyzed by different mass selective detectors such as quadrupole or ion trap instruments. Both analyzers operate under high vacuum and can be interfaced to GC (and thus EI) as well as LC (including ESI) by respective coupling systems. As EI is performed under high vacuum, no special interface is necessary in order to control different pressure levels, but ESI operates at atmospheric pressure. Hence sophisticated vacuum barriers are present in modern LC-ESI-MS(/MS) systems maintaining the required reduced pressure in mass selective detectors.

QUADRUPOLE ANALYZERS

A quadrupole mass spectrometer is composed by four rods consisting of various materials; for example, fused silica coated with a gold layer. Opposing rods are connected while adjacent segments are electrically isolated, as demonstrated in Fig. 5.9. Initially, ions that are generated in the ion source are accelerated into the center of the quadrupole. While applying alternating current (ac) to the segments, positive or negative fields are established towards the centerline of the quadrupole and, thus, positive ions passing through the rods are pushed away with positive and attracted with negative polarization. The extent of ion deflection is directly depending on the applied voltage, its frequency (i.e. the duration of exposure to alternating fields) and the mass of ions. In addition, to one segment positive direct current (dc) is applied while the other segment is provided negative dc. As a result, ions of a distinct mass to charge ratio can travel through the two-dimensional quadrupole field if the applied direct current and alternating current with a defined frequency are appropriate for stable oscillating moving. These parameters can be modified for an optimized mass selection, enabling the isolation of a single ion or the recording of a full spectrum by scanning a distinct range of mass/charge ratios over a given time period (Budzikiewicz 1998; Lottspeich & Zorbas 1998).

Ions isolated by quadrupole instruments can be subjected to further experiments as frequently employed in triple stage quadrupole mass

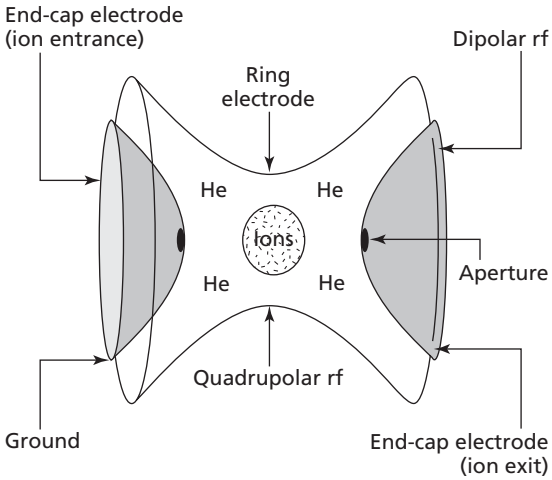


Fig. 5.10 Principal design of an ion trap mass spectrometer. The ring electrode is edged by two end-cap electrodes establishing an rf field upon voltage application that enables the storage of ions in the center of the ion trap.

spectrometers. Here, three quadrupoles are present, the first one of which is used to pick a single ion from the ion beam introduced into the mass spectrometer. In the second quadrupole, the so-called collision cell, the isolated ion hits molecules of a collision gas (e.g. argon, nitrogen) and dissociates with respect to the applied accelerating voltage and collision gas pressure. The resulting product ions are finally measured in a third quadrupole, either by full scan or selected ion monitoring, providing information on fragmentation processes as well as structure of the analytes.

QUADRUPOLE ION TRAP ANALYZERS

A frequently employed alternative to quadrupole mass spectrometers are (quadrupole) ion trap analyzers. Already in 1953, Paul and co-workers described the applicability of a three-dimensional quadrupole to 'trap' ions, and in the last decades, sophisticated ion trap mass spectrometers were developed based on this invention (Louris *et al*. 1987, 1989; Patterson *et al*. 2002). In Fig. 5.10, the principal composition of an ion trap instrument is shown, including two end-cap electrodes (one with an ion inlet and one with an ion exit aperture) and a ring electrode, in the center of which ions are stored by application of appropriate rf field established between the ring electrode and the two end-cap electrodes. Ions that are generated by an external ion source (e.g. EI or ESI) are transferred into the ion trap, which contains a gas (e.g. helium, argon) at approximately 1 mtorr. A commonly employed gas is helium that damps the trajectories of ions to the center of the ion trap, and moreover it reduces the kinetic energy of ions through collisions favoring the storage of the ions within the trap. The storage of ions of a broad range of mass/charge ratios is directly depending on the ac voltage applied to the ring electrode. Hence, the consecutive ejection of ions enabling their mass selective detection is accomplished by the so-called instability mode, which is based on the successive increase of the ac voltage applied to the ring electrode in combination with an ac voltage applied to the end-cap electrodes causing resonant motion. Ions of defined mass/charge ratios develop instable trajectories, are ejected through the perforations of the end-cap electrode (ion exit) and detected with an electron multiplier.

In addition to an efficient scan operation mode, ion trap mass spectrometers offer possibilities of MS^n experiments. With the selective removal of ions from the trap, storage of a precursor ion of interest and its resonant activation, collisionally activated dissociation (CAD) is obtained, giving rise to product ion spectra with a comparable amount of information as obtained with triple stage quadrupole MS/MS experiments. But an important advantage of the ion trap technique is that trapping of ions allows the selection, storage and subsequent dissociation of a product ion generated by MS/MS experiments, namely MS^3, providing information on the fragmentation pathway and composition of product ions, which is of great interest, in particular in ESI-MS.

Sample preparation and purification strategies

In doping controls, mainly urine specimens are obtained from athletes in order to be analyzed regarding prohibited compounds according to established anti-doping rules. In addition, also samples

of whole blood, plasma/serum and hair are obtained for certain purposes of drug identification. In order to accomplish utmost specificity and required sensitivity in analyses, sample pretreatment has become a very important part of doping controls, and various strategies are present to purify specimens from salts and interfering compounds, and to isolate the target analyte(s) in particular.

Liquid–liquid extraction

One of the oldest and most frequently utilized sample preparation steps is liquid–liquid extraction (LLE). The very first publications on the identification of benzedrine (a racemic mixture of amphetamine sulfate) and metabolites in human urine employed LLE in order to determine excretion rates (Richter 1938). Here, urine samples were alkalized by means of 2 N sodium hydroxide and extracted with petroleum ether. In the following decades, numerous applications using LLE for the purification of biological fluids were presented, all of which were based on the same principle of adjusting pH and extraction of acidic, neutral or alkaline compounds (Keller & Ellenbogen 1952; Axelrod 1954; Beckett & Rowland 1965; Beckett & Wilkinson 1965; Kolb & Patt 1965; Cartoni & Cavalli 1968). The knowledge about pI of analytes provides the option of multiple extraction of specimens and/or re-extraction of compounds at various pH values into different solvents. With the desire for more detailed, specific and sensitive analytical assays, LLE procedures were improved and extended in order to remove coextracted interfering substances, and these principles have been utilized for up-to-date screening and confirmation methods in doping control analysis (Donike *et al*. 1970; Spyridaki *et al*. 2001; Van Eenoo *et al*. 2001; Thevis *et al*. 2003b). For instance, stimulants such as amphetamines, ephedrines and related derivatives are commonly extracted from urinary matrices into ethers under alkaline conditions, and the organic layer is concentrated and subsequently analyzed by GC-MS and/or GC-NPD. Moreover, LLE is employed for the analysis of corticosteroids (Fluri *et al*. 2001), diuretics, β-receptor blocking agents and anabolic steroids under various conditions (i.e. salt additives, organic solvents and pH values) (Geyer *et al*. 1997; Thevis *et al*. 2001; Deventer *et al*. 2002).

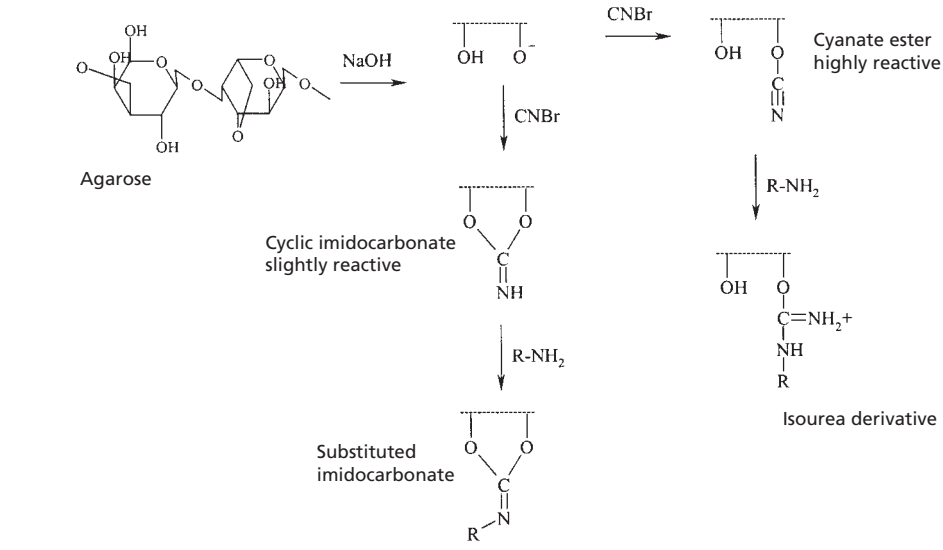
A very powerful tool turned out to be the so-called re-extraction, which was already employed in 1952 (Keller & Ellenbogen 1952) and applied to doping control analysis in order to enhance purity of samples to be analyzed. In particular, the extraction of analytes under alkaline conditions into an organic solvent (e.g. diethyl ether) and subsequent retransfer of components of interest from the organic into an acidified aqueous layer (e.g. 0.06 N HCl) proved to be very useful for confirmation measurements, for instance for the β_2-agonist clenbuterol, by highly efficient reduction of biological background (Sigmund *et al*. 1998).

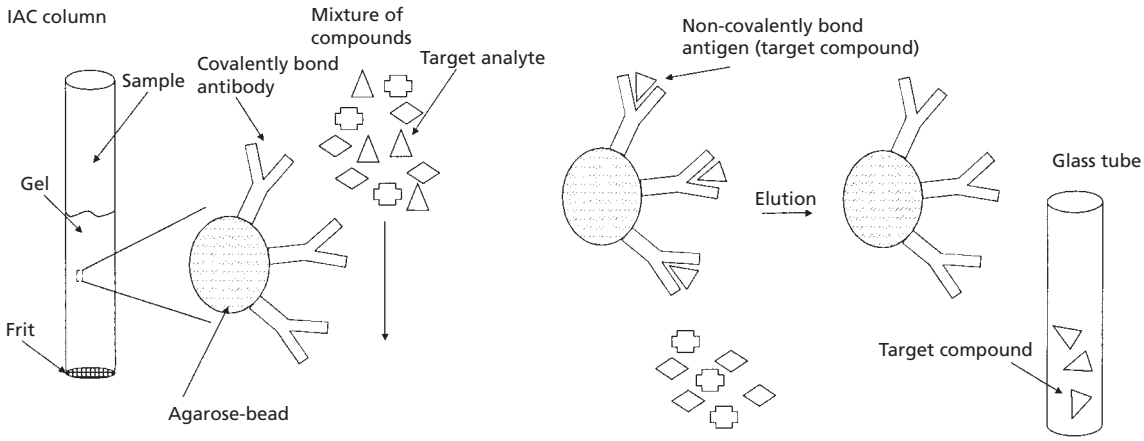
Solid-phase extraction

For a series of compounds, an alternative approach to purify biological samples and concentrate analytes is solid-phase extraction (SPE). Based on various adsorbing materials such as polystyrene, C_{18} or ion exchange phases, several screening and confirmation procedures in doping control analysis have been established. In 1968, Bradlow demonstrated the possibility to extract steroid conjugates with neutral resins (Bradlow 1968), and further improvements in material and methods have provided tools for the development of procedures for doping control purposes, for example, for anabolic steroids (Donike *et al*. 1984) and diuretics (Thieme *et al*. 2001). A major advantage of SPE is the feasibility of full automation of sample extraction, including conditioning of cartridges, sample loading, washing and subsequent elution. Moreover, derivatization of analytes can be accomplished during SPE (Lisi *et al*. 1991).

Immunoaffinity chromatography

A more recently introduced way of analyte isolation from biological matrices is immunoaffinity chromatography (IAC). In general, mono- or polyclonal antibodies able to bind non-covalently to distinct parts of target molecules are linked to a carrier such as spherical agarose particles. The resulting 'gel' is placed in a column bearing a frit, urine or plasma is





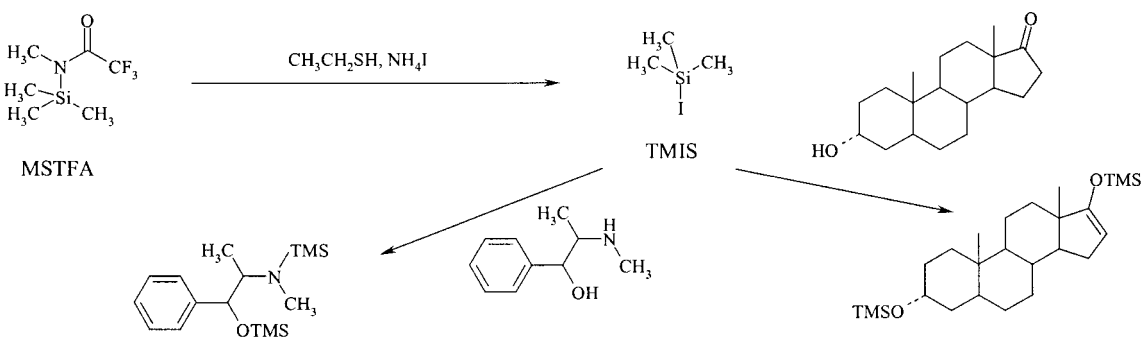
Scheme 5.5 Activation of agarose and subsequent immobilization of antibodies. IAC, immunoaffinity chromatography.

loaded onto the column and passed through the gel. The carrier-linked antibodies recognise target analytes as they flow through the column and capture these compounds by generating non-covalent complexes, while other, non-antigenic substances missing the antibody–antigen reaction, are eluted. Under various conditions, the non-covalent complex can be broken without harming the antibody or analyte of interest and the purified antigen is analyzed, for example by GC-MS, basically without any interfering signals. The principal production of an IAC column and its use is shown in Scheme 5.5. Commonly employed carriers are commercially available agarose-beads. The surface of these beads is activated in order to chemically couple peptides or proteins, which is accomplished by various techniques. One of the most frequently employed methods is cyanogen bromide (CNBr) activation that was introduced in 1967 by Axen *et al*. (1967). Under alkaline conditions, CNBr introduces cyanate

esters and imidocarbonates into the matrix of agarose by reaction with its surface hydroxyl functions to which antibodies are chemically connected as isourea or substituted imidocarbonate derivatives (Hermanson *et al*. 1992). The beads bearing covalently fixed antibodies are placed in a column, which recognise and retain only specific analytes or defined classes of compounds present in biological fluid. With appropriate eluents, for example mixtures of organic solvents and water, the captured analytes are released from the antibodies, concentrated and analyzed by conventional techniques such as MS (Schänzer *et al*. 1996; Machnik *et al*. 1999). The reduction of biological background results in improved signal/noise ratios, and thus in better detection limits of GC-MS and LC-MS/MS instruments.

Derivatization techniques

GC-MS is one of the primary tools for screening and confirmational analyzes in doping control laboratories. These systems provide specificity and sensitivity for numerous compounds that are prohibited according to the list of banned substances of IOC and WADA. But a principle requirement to identify drugs by means of GC-MS is volatility. As many analytes are heavy volatile, for example β_2-agonists, various diuretics and β-receptor blocking agents, they are derivatized to more volatile analogs by means of different reagents. Here, hydroxyl and primary or secondary amino functions are target groups because of their ability of hydrophilic interactions causing decreased volatility. One of the first modifications of analytes was accomplished by acetylation utilizing acetic anhydride. The resulting molecules have to be purified from remainders of acetic acid and acetic anhydride, commonly achieved by LLE. Because of this laborious and time-consuming way of preparing compounds for GC-MS analysis, several derivatization agents were developed, preferably including trimethylsilyl (TMS) residues. There are for instance hexamethyldisilazane (HMDS), trimethylsilyl-imidazole (TMSImi) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Donike 1969), the latter one of which is nowadays the most frequently employed derivatization reagent, in particular in combination with ammonium iodide and ethanethiol, which gives rise to *in situ* generated trimethyliodosilane (TMIS), a highly reactive trimethylsilylation reagent (Donike 1973; Donike & Zimmermann 1980). With TMIS, hydroxyl and amino groups are modified and also keto functions are converted to their enol-TMS ethers, as demonstrated in Scheme 5.6. Several other powerful reagents have been used that introduce trifluoroacetyl (TFA)- or heptafluorobutyl (HFB) -residues into molecules, for example *N*,*N*-bistrifluoroacetamide, *N*-methyl-*N*-bistrifluoroacetamide (MBTFA) (Donike & Derenbach 1976) or *N*-methyl-*N*-bisheptafluoroburtyramide (MBHFB), respectively. The trifluoroacetylation results in more stable derivatives of amino functions than trimethylsilylation, hence selective modification of analytes with TMS and TFA groups has also become possible, enabling the gas chromatographic separation of stereoisomers



Scheme 5.6 Derivatization of doping-relevant analytes by means of trimethyliodosilane (TMIS).

of stimulants such as ephedrines (Opfermann & Schänzer 1996; Thevis *et al*. 2003b).

Qualitative analysis of doping-relevant compounds

For most of the prohibited compounds in elite sports, qualitative evidence of their presence in urine samples of athletes is sufficient for a positive test result. As many assays for screening and confirmation purposes are based on chromatography interfaced to MS, guidelines for the identification of substances by means of GC-MS or LC-MS(/MS) systems have been established. The identification of a compound is accomplished if the relative abundances of a specified amount of characteristic ions (depending on MS techniques) agrees, with respect to allowed variations, with those obtained by analysis of the corresponding reference material. In addition, retention times of signals observed in urine samples of athletes and in appropriately fortified reference urine specimens are compared and may shift only within a defined time frame. Hence, knowledge about chromatographic and, in particular, mass spectrometric properties of respective analytes is of utmost importance in order to characterize and identify compounds in a complex matrix such as urine. Numerous investigations regarding the mass spectrometric behavior of performance enhancing or masking agents and their detection for doping control purposes have been performed (Masse *et al*. 1989; de Boer *et al*. 1991; Donike *et al*. 1995; Ayotte *et al*. 1996; Shackleton *et al*. 1997; Bowers 1998; Aguilera *et al*. 1999; Thevis *et al*. 2000, 2001, 2002, 2003a; Ventura *et al*. 2000; Leinonen *et al*. 2002), and the principles of some procedures will be described in the following.

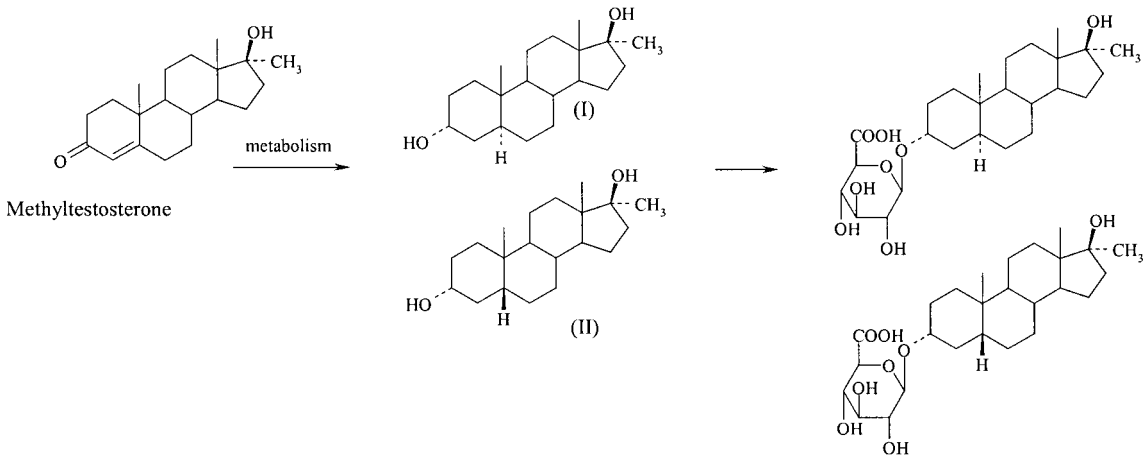
Anabolic steroids

Considering statistical data regarding conducted doping controls and classes of abused drugs, anabolic steroids are the most frequently detected prohibited compounds. In 2001, for example, more than 40% of the banned substances identified by 25 IOC-accredited laboratories were related to anabolic agents. One representative of this category is methyltestosterone, a derivative of testosterone obtained by introduction of an additional methyl group at C-17. Most anabolic steroids are subjects of extensive metabolism, giving rise to a series of metabolites, for instance by reduction of keto functions, oxidation of hydroxyl groups, introduction of hydroxyl residues and oxidation/reduction of carbon-carbon bonds of the steroid nucleus (Schänzer 1996). Following this phase-I metabolism, conjugation to glucuronides and/or sulfates is observed as common phase-II modification prior to renal excretion. In Scheme 5.7, the principal metabolism of methyltestosterone is demonstrated, generating 17α-methyl-5α-androstan-3α,17β-diol (I), 17α-methyl-5β-androstan-3α,17β-diol (II) and corresponding glucuronic acid conjugates.

Commonly employed strategies to identify metabolites of anabolic steroids are based on the enzymatic hydrolysis of phase-II metabolites to the corresponding phase-I metabolites, purification, concentration, derivatization and subsequent GC-MS analysis. For most anabolic steroid metabolites, no endogenous production in humans is possible, except for nandrolone, which will be discussed later. Hence, in case of qualitative determination of these compounds in urine samples of athletes elected for doping controls, a positive test result will be reported. In Fig. 5.11a, the EI-mass spectrum of 17α-methyl-5α-androstan-3α,17β-diol after bistrimethylsilylation is presented, and Fig. 5.11b shows a typical GC-MS chromatogram with characteristic ion traces of the methyltestosterone metabolites found in a urine sample.

DESIGNER STEROIDS

The problem of so-called designer steroids triggered an avalanche in the sport as well as the scientific world in October 2003 (Knight 2003) when the doping control laboratory of the University of California, Los Angeles (UCLA) identified a compound related to gestrinone, a drug administered in cases of endometriosis. Its hydrogenation at the ethinyl residue at carbon 17 results in a steroid hormone termed *tetrahydrogestrinone* (THG), which can be considered as an analog to the highly efficient anabolic steroid trenbolone, but the physiological



Scheme 5.7 Metabolism of methyltestosterone to 17α-methyl-5α-androstan-3α,17β-diol (I), 17α-methyl-5β-androstan-3α,17β-diol (II) and their glucuronides.

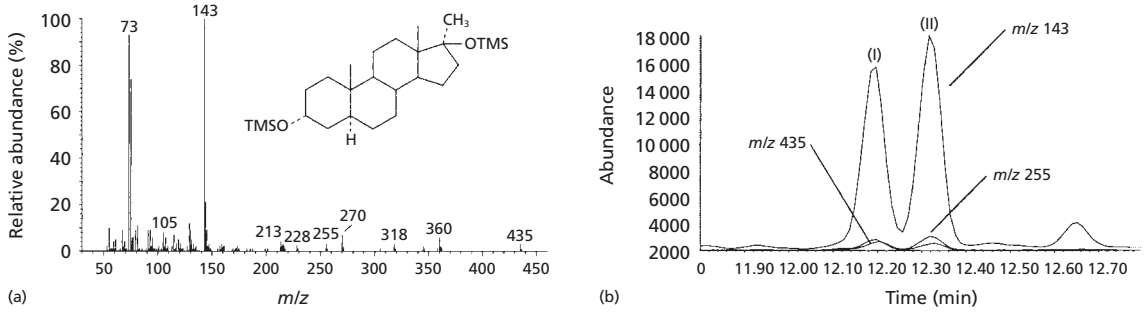


Fig. 5.11 (a) Electron ionization-mass spectrum of the bis-trimethylsilylated 17α-methyl-5α-androstan-3α,17β-diol (mol. wt. = 450); (b) gas chromatography-mass spectrometry (GC-MS) chromatogram of characteristic ion traces of bis-trimethylsilylated 17α-methyl-5α-androstan-3α,17β-diol (I) and 17α-methyl-5β-androstan-3α,17β-diol (II).

effects and side effects of THG have never been clinically investigated. The commonly employed strategy of doping control laboratories analyzing pharmaceutically produced and clinically tested remedies is obviously not sufficient to cope with the willingness of some athletes to cheat and to risk their health in order to win by a short head against competitors. Owing to the fact that many drug screening procedures are based on the comparison of reference compounds to urine samples by means of mass spectrometric techniques such as selected ion monitoring (SIM) or multiple reaction monitoring (MRM), unknown derivatives of remedies, such as THG, are invested with invisibility for these conventional methods. Hence, more flexible assays have been established enabling the detection of known as well as unknown drugs by common structures such as a principal steroid nucleus, which is possible in particular by means of modern LC-MS/MS systems.

Endogenous steroids

While the administration of anabolic steroids causes the presence of metabolites normally not occurring in human urine samples, doping by means of

testosterone is more difficult to uncover as it is also produced endogenously. Here, different approaches have been established to determine applications of testosterone, and the two most frequently utilized tools are the testosterone/epitestosterone (T/E) ratio and the so-called isotope ratio mass spectrometry (IRMS) of carbons. The profile of endogenous steroids varies under numerous influences (Geyer *et al*. 1996), but the ratio of T and E has proven to be a reliable parameter that indicates abuse of testosterone as the production of epitestosterone is independent from testosterone. A threshold ratio of six is utilized that does not immediately entail a positive test result but triggers follow-up studies to test for an abnormal but naturally elevated testosterone concentration.

With the availability of IRMS instruments, numerous investigations have been published demonstrating the possibility to distinguish between endogenously generated and chemically synthesized testosterone by MS. Naturally produced testosterone comprises different $^{13}C/^{12}C$ ratios than chemically synthesized analogs that are used for medical supplementation. By means of GC, combustion of eluting analytes and mass spectrometric measurement of resulting carbon dioxide provides information about the origin of testosterone by different contents of ^{13}C and ^{12}C (Horning *et al*. 1998; Aguilera *et al*. 1999).

Diuretics and β_2-agonists

Two representatives of classes of compounds frequently analyzed by means of LC-MS(/MS) are diuretics and β_2-agonists. In particular the category of diuretics demonstrates the chemical heterogeneity of drugs administered for comparable or identical purposes. Here, primarily negative ionization is performed (Thieme *et al*. 2001; Thevis *et al*. 2002, 2003a) owing to the acidity of drugs belonging to the group of diuretics, but few compounds such as triamterene require positive ionization. For β_2-agonists, protonation of analytes and detection of positively charged molecules is the method of choice. As described in Analytical techniques above, ion sources interfacing LC to MS mainly generate protonated or deprotonated molecules without any considerable fragmentation. Thus, structure information about analytes, specificity and selectivity of mass analyzers are provided by CAD of ionized drugs and analysis of derived fragments. This requires the knowledge of proton affinity and dissociation behavior after efficient activation of target molecules by CAD, which differs significantly from fragmentation routes observed with EI. For diuretics as well as for the majority of β_2-agonists (except for salbutamol, see Quantitative analysis of prohibited drugs below), qualitative analysis of these drugs is sufficient, and typical ESI product ion spectra of epithiazide and fenoterol are presented in Fig. 5.12. Commonly, extracted ion chromatograms enable the sensitive detection of these compounds in biological matrix, and confirmation of their presence is obtained by comparison of relative abundances of product ions. In 2001, approximately 17.5% of the banned substances detected in 25 IOC-accredited laboratories in doping control samples were drugs related to β_2-agonists, and 5% were classified as diuretics.

Quantitative analysis of prohibited drugs

For several compounds, including stimulants such as ephedrines, metabolites of anabolic steroids such as nandrolone, and β_2-agonists such as salbutamol, threshold levels have been established, which are the basis of the decision as to whether a sample is reported positive or negative. Various reasons are given for this regulation. Ephedrines are ingredients of many remedies frequently used in cold therapy, hence their use is legal in the sense of anti-doping rules as long as their urinary concentration does not exceed 5, 10, or 25 $\mu g \cdot mL^{-1}$ for the derivatives norephedrine, ephedrine and pseudoephedrine, respectively. Salbutamol, a sympathomimetic agent, is one of four permitted β_2-agonists (in addition to salmeterol, terbutaline and formoterol), as long as the application is conducted via inhalation. As the differentiation between orally administered pills and pressurized aerosol dosage is very complicated, the presence of salbutamol in competition samples is reported to the corresponding federation if the urinary level exceeds 100 $ng \cdot mL^{-1}$, and in out-of-competition testing a threshold of 1 $\mu g \cdot mL^{-1}$ has been established owing to anabolic effects observed

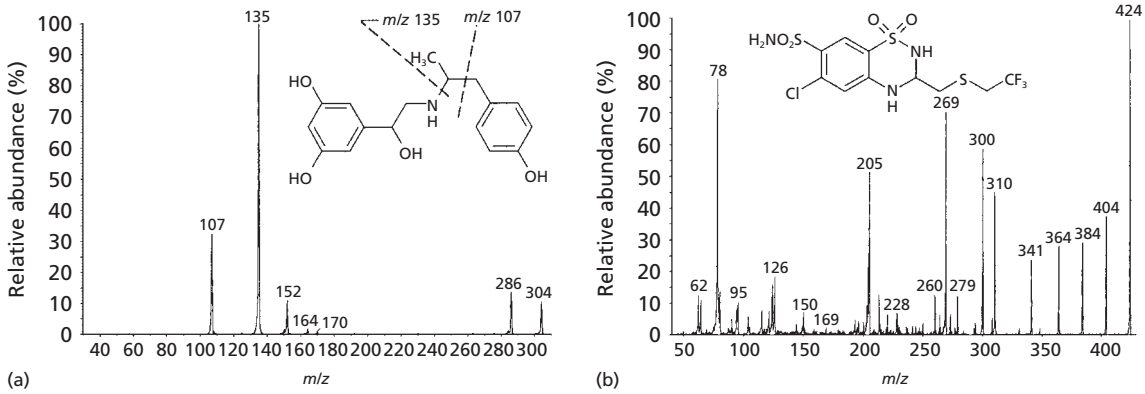


Fig. 5.12 (a) Electrospray ionization (ESI) product ion spectrum of the β_2-agonist fenoterol (mol. wt. = 303); (b) ESI product ion spectrum of the diuretic agent epithiazide (mol. wt. = 425).

with some sympathomimetics if administered in doses much higher than therapeutically used. The presence of the nandrolone metabolite 5α-estran-3α-ol-17-one (norandrosterone) in urine samples of elite athletes is observed to certain extents based on endogenous production. Thus, also here threshold values have been introduced with 2 ng·mL^{-1} for male and 5 ng·mL^{-1} for female athletes. Numerous studies were performed in order to substantiate these levels, and various 'parameters' influencing the endogenous generation of this metabolite were taken into consideration, for example, high physiological stress or pregnancy, which cause significantly elevated concentrations in urine specimens. Quantitation of these substances is accomplished by calibration curves utilizing regular sample preparation procedures and appropriate internal standards with comparable or identical physicochemical properties (Schänzer & Donike 1995).

Summary

Doping control analysis of low molecular weight drugs is generally based on chromatographic and mass spectrometric techniques that enable the detection and identification of remedies and their metabolites in body fluids such as urine and blood. While early procedures employed mainly GC, various analyzers such as FIDs and NPDs, as well as MS, recently published assays utilize primarily LC interfaced to MS by means of atmospheric pressure ionization techniques due to a faster sample preparation as no derivatization of analytes is required. In addition, more flexible mass spectrometric experiments to determine and characterize known therapeutics and also unknown designer drugs have become possible by frequently employed triple quadrupole or ion trap analyzers supporting the fight against doping and the illegal use of drugs. The range of substances important for doping control analysis has changed ever since lists of prohibited compounds and methods of doping have existed, and laboratories permanently expand or modify analytical procedures within this dynamic process in order to improve performance regarding sensitivity, specificity and flexibility to limit the misuse of drugs in sport as well as to protect athletes from false suspicion. Here, new developments concerning high speed and high resolution chromatography, high resolution and high sensitivity MS as well as modern ionization techniques provide valuable tools for analytical laboratories to obtain even more detailed information on the administered therapeutics, for example their chemical structures and metabolism, and enable extended time frames for the determination of drug abuse. As many therapeutics, such as anabolic steroids, are applied during out-of-competition periods but preserve performance-enhancing effects for several weeks, doping control analysis requires in- as well as out-of-competition tests and the utmost specificity and sensitivity of analytical procedures.

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Chapter 6

The Reproductive Axis

JOHANNES D. VELDHUIS AND ARTHUR L. WELTMAN

Abstract

The reproductive axis is largely stable in the face of intensive physical training in healthy men and women. However, altered reproductive-hormone outflow is associated with any of numerous doping protocols, exhaustive or non-escalating training schedules, concomitant weight loss, psychosocial stress, inadequate caloric intake for workload and sustained strenuous exertion in adolescence. Exposure to anabolic steroids induces loss of menstrual cyclicity in women, a decrease in spermatogenesis in men, and a significant reduction in protective (high-density lipoprotein) cholesterol concentrations in both genders. More subtle endocrine and metabolic adaptations are detectable in the absence of the foregoing risk factors, but their medical significance is not established. For example, one longitudinal investigation showed that the only detectable effect of supervised long-distance running training to complete a marathon in healthy young women is slight abbreviation of the post-ovulatory (luteal) phase of the menstrual cycle within the normal range. Cross-sectionally based reports describe an array of reproductive abnormalities in athletes, but such data are confounded by one or more known comorbid factors. Accordingly, supervised, graded, non-exhaustive, voluntary strenuous exercise in healthy adults maintaining an adequate caloric intake has minimal potential for adverse reproductive consequences.

Introduction

Critical assessment of the impact of vigorous exercise training and strenuous sports engagements on the endocrine system is a recent medical accomplishment. Indeed, the majority of earlier clinical reports cited in textbooks or forecast in contemporary advertisements of health-related supplements are flawed by significantly confounding issues, which render definitive interpretation impossible. Scientific requirements for valid inference currently include (non-exclusively): (i) prospective evaluation of demonstrably normal healthy individuals; (ii) supervised, graded, escalating training schedules of quantifiable intensity and duration; (iii) concurrent longitudinally monitored gender and age-matched control cohorts without exercise intervention; (iv) verifiable adequacy of concomitant nutritional support to match individual energy demand; (v) documented absence of covert drug or hormone use (doping); (vi) baseline and anterograde assessment of perceived psychosocial stress; and (vii) comprehensive family history to identify genetic factors known to predict reproductive disorders in the general population. At present, no single study fulfills the foregoing ensemble expectations. More subtle understanding of metabolic and endocrine implications of vigorous and competitive sports will thus require further clinical and physiological studies.

Principles of reproductive physiology

Contemporary knowledge of human reproductive physiology embraces an integrative view of

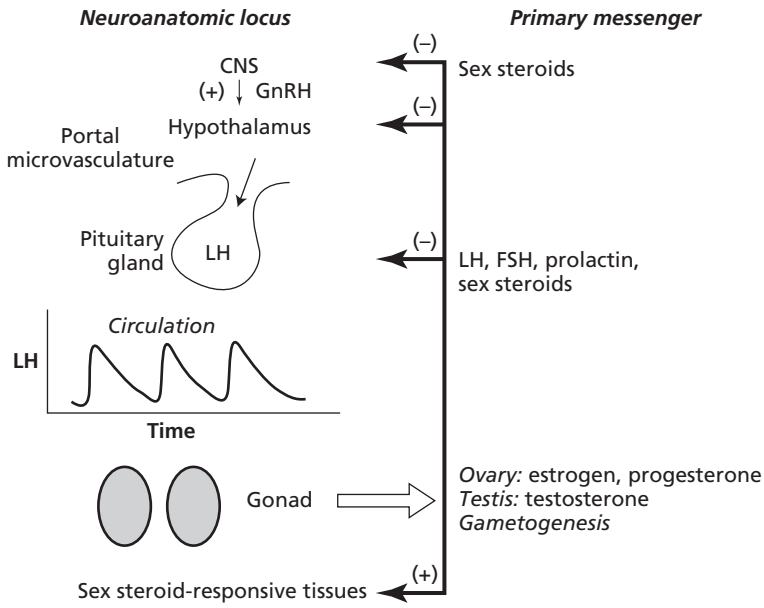

Fig. 6.1 Schematized illustration of basic elements maintaining homeostasis of the male and female reproductive axes. Signals arising in the central nervous system and peripheral tissues converge on an ensemble of about 1200 hypothalamic gonadotropin-releasing hormone (GnRH) -secreting neurons. Synchrony of neuronal excitation releases pulses of GnRH into a microvascular portal-venous system (solid arrows [+]), which connects the mediobasal hypothalamus to the pituitary gland. GnRH drives transcription, translation, processing and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by gonadotrope cells. The general circulation delivers LH to gonadal (ovarian or testicular) compartments. LH stimulates the synthesis and secretion of testosterone (and thereby estradiol) in men and women, and progesterone in women after ovulation. In women, FSH promotes Graafian follicle development, oocyte fertilizability and ovarian responsiveness to LH. In men, FSH synergizes with LH-stimulated intratesticular testosterone to promote early stages of spermatogenesis in seminiferous tubules. Sex steroids act on peripheral target tissues, such as muscle, fat, breast, bone, skin, sexual organs, the hypothalamus and pituitary gland (open arrow [–]). In women, estrogen and progesterone exert sequential feedback (inhibition) and feedforward (enhancement), which cycle mediates the preovulatory LH surge. The same sex hormones stimulate uterine endometrial growth and maturation, which are necessary for implantation of a (fertilized) blastocyst in pregnancy.

hormonal signal exchange among the central nervous system (hypothalamus), anterior pituitary gland (gonadotrope cells) and gonad (testis and ovary) (Fig. 6.1). The foregoing set of neuroendocrine glands (the reproductive axis) is linked to signals associated with pubertal development, somatic growth, body composition, stress responses, appetite, energy expenditure and insulin action (Urban *et al*. 1988; Evans *et al*. 1992; Giustina & Veldhuis 1998). An integrative perspective of the reproductive axis is necessary to reconstruct the pathogenesis of endocrine disturbances and discern the mechanisms of normal adaptations to exercise. For example, adequate caloric repletion of expended energy is a minimal (necessary, but not sufficient) prerequisite for successful reproductive function during strenuous training (Loucks 2003).

Endocrine adaptations to physical exertion are transduced via multiple levels of non-exclusive control: (a) central-neural and blood-borne inputs to the hypothalamus; (b) hypothalamic integration to yield convergent signal outflow to the anterior pituitary gland; (c) feedback to the hypothalamus via core body temperature, inflammatory mediators, tissue-derived metabolites (e.g. lactate, free fatty acids) and secreted products of target cells (insulin-

like growth factor stimulated in the liver by growth hormone); (d) exogenous substrates (glucose, fat and amino acids); and (e) stress-adaptive thyroidal and adrenal hormones (e.g. thyroxine, cortisol and epinephrine). This minimal network of interactions governs hypothalamic stimulation of gonadotrope cells by intermittent (burst-like) release of the decapeptide, gonadotropin-releasing hormone (GnRH). GnRH induces (feeds forward on) the pituitary synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two proteins are key effectors of the ovary and testis. The size and time-pattern of GnRH pulses jointly determine the amounts of LH and FSH secreted (Urban *et al*. 1988; Evans *et al*. 1992; Veldhuis 1999). LH drives sex-steroid production (below). FSH controls sperm and egg development and the production of a feedback protein, inhibin. No clinically significant alterations in FSH or inhibin secretion have been attributable to physical training (Veldhuis *et al*. 1998). This statement is also true of prolactin, a lactational hormone that increases transiently with a bout of exercise and in response to diverse psychological and physical stressors.

In men, LH acts on testicular Leydig cells to promote the synthesis and secretion of 4–6 mg of testosterone each day (Urban *et al*. 1988). In women, LH stimulates ovarian theca and postovulatory luteal cells to secrete about 0.15 mg of testosterone (a precursor of estrogens) and 10–20 mg of progesterone per day (Evans *et al*. 1992). Testosterone, estrogen and progesterone reversibly suppress (feed back on) the hypothalamo–pituitary unit, thereby supervising intermittent secretion of GnRH, LH and FSH (Veldhuis 1999). Time-delimited inhibition by sex-steroid hormones is a fundamental regulatory mechanism that preserves reproductive homeostasis. In general, homeostasis is the aggregate outcome of reciprocal feedforward (stimulatory) and feedback (repressive) interactions that enforce repeated, reversible incremental adjustments to maintain hormone concentrations within the physiological range for age, gender and species. Autoregulation is analogous to a cruise-control device, which holds a vehicle's speed within the desired preset range. Cascades of signal exchange in healthy individuals thereby ensure life-supporting concentrations of substrates (e.g. glucose), metabolites (e.g. lactate) and hormones (sex steroids, cortisol, insulin, growth hormone, etc). Homeostasis requires deterministic physiological mechanisms, low background randomness, a normal genetic endowment, adequate psychosocial support and successful adaptation to internal and external stress (Veldhuis 1996).

Hypothalamo–pituitary–gonadal homeostasis in strenuous physical training

Longitudinal clinical studies

Few clinical investigations have entailed prospective randomization of a demonstrably healthy cohort of volunteers into strata of unequal training intensity, duration and/or type. One longitudinal study assigned young menstruating women randomly to either low (sublactate threshold) or high (supralactate threshold) intensity physical training (Rogol *et al*. 1992). The program comprised supervised long-distance running for 18 months with the individual goal of completing a marathon. Reproductive hormones were measured at baseline and after 1 year of gradually escalating training volume at the preassigned exercise intensity. LH, FSH, prolactin and estradiol did not change detectably in the low-intensity exercise cohort or in a concurrent longitudinal control group (recreationally active women). Women who trained consistently above the individually determined lactate threshold for an identical total running distance of > 500 miles (> 800 km) manifested a small (1.8-day) abbreviation of the luteal phase of the menstrual cycle with no evident difference in mean progesterone concentrations (Rogol *et al*. 1992). Shortening of luteal-phase length did not exceed normal month-to-month differences recognized in healthy premenopausal women.

The importance of adequate nutritional intake to match total caloric expenditure is illustrated in a recent prospective randomized intervention. In this 2-month-long study, women undertaking weight reduction during training were more likely to develop disturbances in ovulatory or luteal-phase timing than individuals training identically with

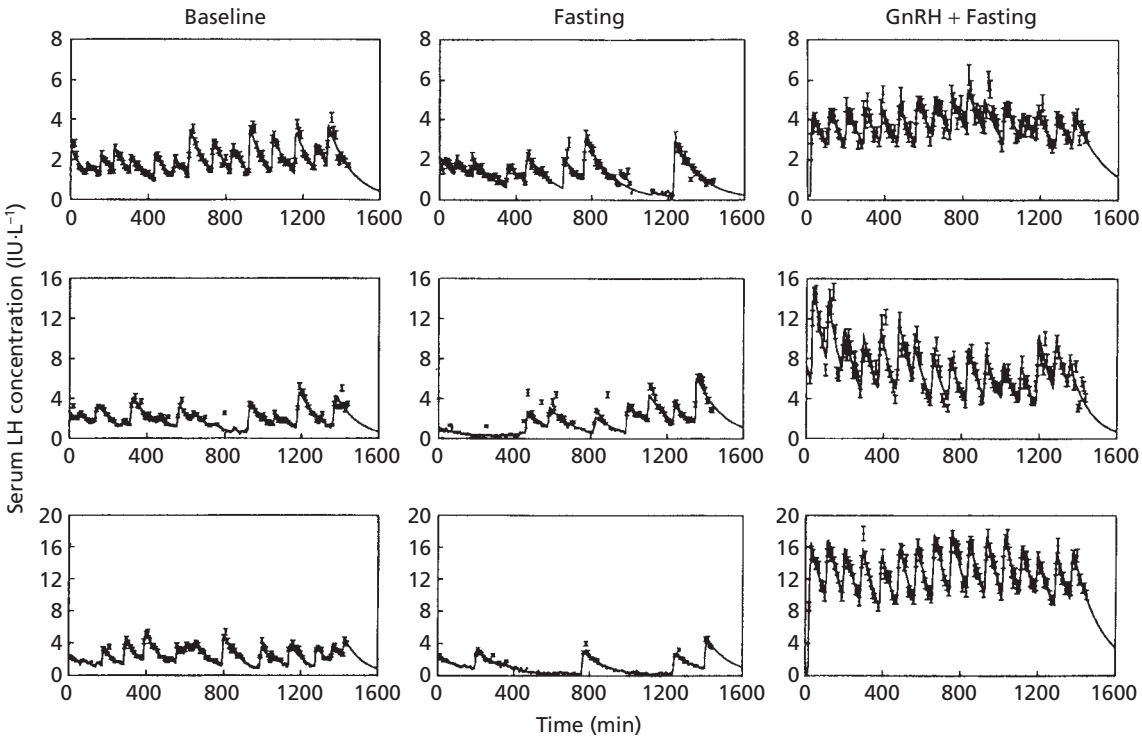

Fig. 6.2 Short-term fasting suppresses, and gonadotropin-releasing hormone (GnRH) infusion restores, pulsatile luteinizing hormone (LH) release in healthy young men. Profiles are shown in three individuals (top, middle, bottom). LH concentrations were sampled every 10 min for 24 h. The triptych illustrates the fed session (left); day 3 of fasting with saline injections (middle); and day 3 of fasting accompanied by intravenous injection of synthetic GnRH (100 ng·kg^{-1} every 90 min) (right). Repeated GnRH pulses restore LH secretion. These data signify that caloric deprivation represses hypothalamic GnRH outflow to pituitary gonadotrope cells, which retain responsiveness to GnRH stimulation. (Reprinted with permission from Aloi *et al*. 1997.)

unrestricted caloric ingestion (Bullen *et al*. 1985). In each case, menstrual cycles became normal within 6 months of completing the study.

Observational studies

Cross-sectional appraisal of hypothalamo–pituitary–ovarian hormones in collegiate long-distance runners has revealed a subset of subjects with irregular menstrual cyclicity due presumptively to reduced hypothalamic GnRH release (Rogol *et al*. 1983; Veldhuis *et al*. 1985; MacConnie *et al*. 1986) (Fig. 6.2). Blunted GnRH secretion in this setting is inferred from decreased LH pulse frequency in the presence of normal or accentuated pituitary LH release induced by the injection of synthetic GnRH peptide (Fig. 6.3). Precisely why LH pulsatility is disrupted in some but not all long-distance runners is not known. Epidemiological correlations indicate that one or more apparent reproductive risk factors often prevail (see below).

Table 6.1 highlights several factors that may increase the risk of developing irregular menstrual cycles in strenuously training young women. Among diverse considerations, deficient caloric intake in relation to any given level of energy expenditure is an established risk for oligomenorrhea in prospectively randomized analyses (*vide infra*) (Bullen *et al*. 1985; Loucks 2003). Albeit not definitive, several other inferred clinical associations are

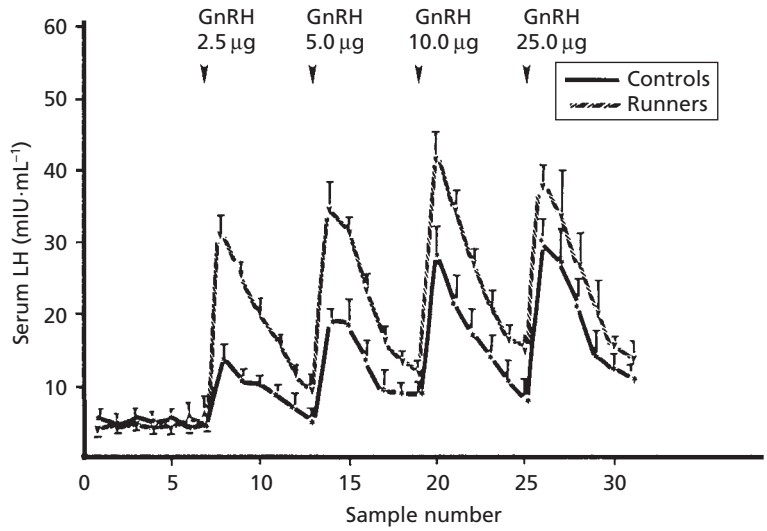

Fig. 6.3 Accentuation of low-dose gonadotropin-releasing hormone (GnRH) -stimulated luteinizing hormone (LH) release in cross-sectionally recruited female collegiate long-distance runners with irregular menstrual cycles (see Fig. 6.2). Synthetic GnRH peptide was injected intravenously in escalating doses at the indicated times. LH concentrations were monitored by sampling blood from the forearm every 20 min. Greater LH secretion stimulated by low doses of GnRH in women athletes than controls indicates that the pituitary gland is capable of producing LH in response to an adequate (hypothalamic) GnRH stimulus. Clinically inferred risk factors associated with menstrual dysfunction in such individuals are highlighted in Table 6.1. (Adapted with permission from Veldhuis *et al*. 1985.)

Table 6.1 Factors associated with increased prevalence of menstrual irregularity in athletes studied cross-sectionally and/or by self-referral to a physician.

Apparent risk factor	Putative mechanism
Self-imposed dieting, heavy laxative use, anorexia or bulimia	Metabolic and stress-adaptive reduction in hypothalamic GnRH release
Anabolic steroids, testosterone and progestins	Direct feedback suppression of LH and GnRH secretion
High doses of injected human growth hormone	Prolactin-like effect to limit brain GnRH secretion
Psychosocial stress	Central nervous-system inhibition of GnRH outflow
Personal or family history of reproductive disorder	Unrelated primary disease; self-selection for study

GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

helpful in adumbrating possible avenues for further investigation (Veldhuis *et al*. 1998).

Insufficient total caloric intake, anorexia nervosa, bulimia, binge eating, surreptitious vomiting and excessive use of purgatives all activate the stress-adaptive hypothalamo–pituitary–adrenal axis. Complex central-neural responses to metabolic stress inhibit LH pulsatility by repressing hypothalamic GnRH release, thereby reducing feedforward stimulation of LH secretion by the anterior pituitary gland (Bergendahl *et al*. 1996, 2000; Loucks 2003). For example, short-term fasting suppresses LH pulses consistently in healthy adults. A study in young men showed that the 50% fall in LH and testosterone concentrations induced by fasting was prevented by injecting pulses of synthetic GnRH every 90 min intravenously (Aloi *et al*. 1997) (Fig. 6.4). The outcome of this intervention demonstrates

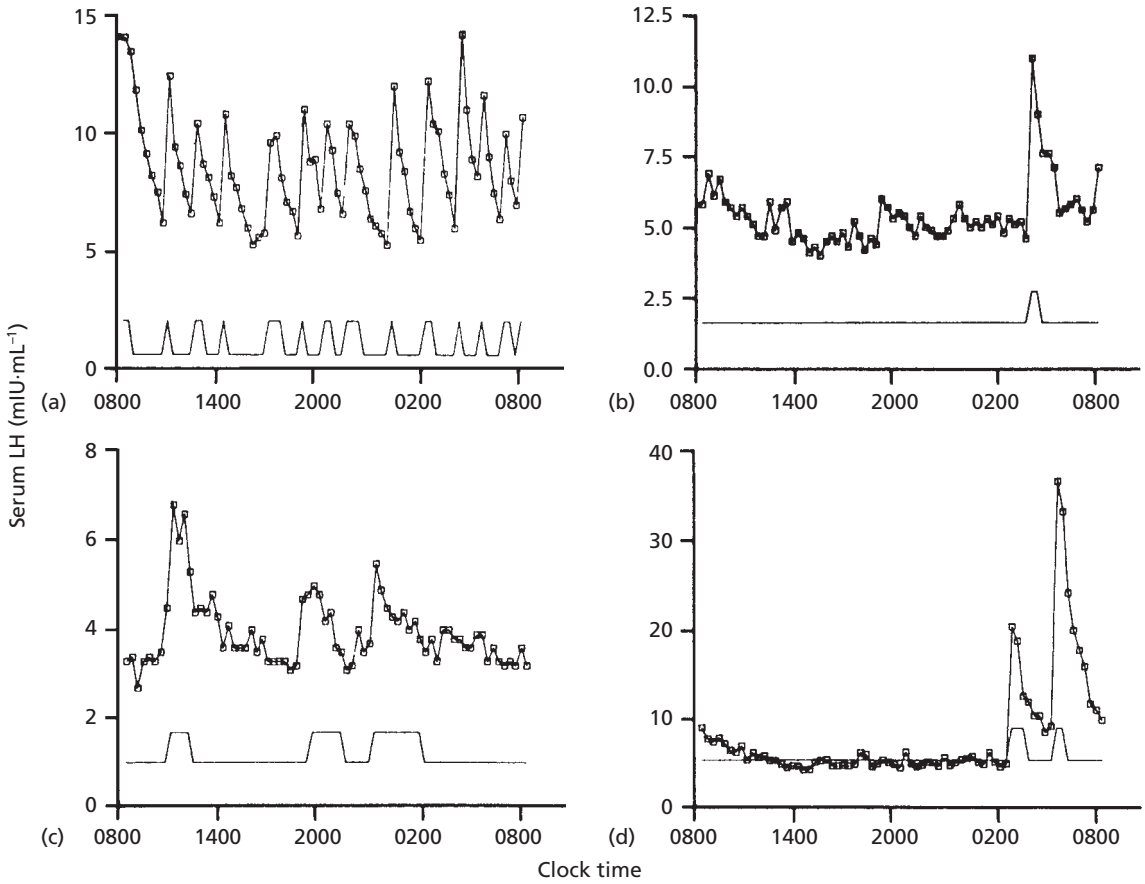

Fig. 6.4 Illustrative patterns of pulsatile luteinizing hormone (LH) release monitored every 20 min over 24 h in four young women identified cross-sectionally. Subjects were intercollegiate long-distance runners who reported irregular menstrual cycles. Some participants had a subnormal frequency (daily number) of LH pulses. The causal relationship, if any, to strenuous physical training is unknown. As discussed in the text, an independent longitudinal investigation demonstrated normal LH pulsatility before and after 1 year of endurance running in young women, each of whom ran at least 500 miles (800 km) (Rogol *et al*. 1992). (Reprinted with permission from Veldhuis *et al*. 1985.)

that caloric deficiency limits brain outflow of GnRH, but does block the pituitary gland.

Abuse (non-therapeutic administration) of anabolic steroids, testosterone, synthetic progestins and human growth hormone (which exerts a prolactin-like inhibitory effect on GnRH secretion) can cause oligo- or amenorrhea (fewer than normal or no menstrual cycles, respectively) and oligo- or azoospermia (reduced or undetectable sperm counts). Erythropoietin injections are not known to do so. A high degree of perceived psychosocial stress, whether originating from or independently of the training and competition environment, may also disrupt menstrual function (Evans *et al*. 1992; Veldhuis *et al*. 1998). In addition, some athletes begin intensive training with pre-existing (unrecognized) hormonal abnormalities and/or a strong genetic potential for endocrine disease. Both factors may be unmasked by strenuous athletic activity or detected independently of the training experience.

Unresolved issues

A significant and unresolved clinical issue is how two or more factors that individually heighten the risk of altered menstrual function interact adversely in the setting of vigorous physical training. This question is particularly difficult to address, because possible combinatorial factors are nearly illimitable. For example, what would be the overall effect on menstrual function of strenuous training in a woman with a family but not a personal history of polycystic ovarian syndrome (present in 5.0–8.5% of otherwise healthy young women of diverse ethnicities), occasional prior menstrual irregularity and minimal weight loss during the exercise program? And how is the foregoing aggregate likelihood further influenced by the choice of a particular sport; for example, competitive fencing, volleyball, pole vaulting, the 500-m run or 100-m free-style swimming. Observational inferences suggest that menstrual dysfunction is less common among participants in cycling and swimming than other athletic events (Evans *et al*. 1992). And under what circumstances are risk factors that are inferable in women applicable to men? There are no explicit data at present to address such multifactorial queries. In fact, bias of ascertainment, self-referral to physicians, exercise modality (e.g. greater or lesser cooling of core body temperature), perceived stress, probability of hormone abuse, weight loss or maintenance, age of training onset and other unknown confounding factors vitiate definitive interpretation of cross-sectional data.

In relation to the medical care of competitive athletes, doctors should be cognizant that physical exertion alone is an unlikely proximate cause of clinically significant endocrine changes. Thus, physician-directed review of dietary habits, weight loss, caloric intake, medical records, family history, systemic symptoms, physical signs and screening laboratory tests can protect the athlete's personal health. Thorough assessment is necessary to exclude or identify remediable associated illness.

Whether strenuous physical training affects sperm function or female fecundity (ability to conceive) or fertility (carrying a healthy infant to full-term delivery) has not been established rigorously. Relevant investigations would comprise longitudinal, unbiased monitoring of FSH, inhibin and activin subunits, follistatin, spermatogenesis, Graafian follicle maturation, ovulation, luteinization, and lifetable analyses of fecundity, fertility and live births *inter alia*. Anecdotal clinical observations predict that changes in fecundity or fertility would be subtle rather than clinically significant.

The impact, if any, of physical exertion on the postpartum state is unknown. Illustrative unexplored issues include the time required for recovery of menstrual cyclicity after pregnancy; lactational efficiency, milk volume and nutritional content for the infant; and infant–maternal bonding.

Greater clinical understanding is required of the relatively neglected domain of sexual libido, potentia and satisfaction in athletes pursuing elite physical training. And, medical assurance is needed that all athletes have full access to instructional guides concerning risks of sexually transmissible disease and communicable respiratory and gastrointestinal illness. Both necessities arise from the wide spectrum of sociocultural, nutritional and geographic venues engaged in international competition.

Summary

Strenuous physical training in the healthy young adult (albeit not necessarily in children) does not disrupt reproductive function significantly. This conclusion does not apply when training is accompanied by deficient caloric intake, weight loss, aberrant eating habits, concomitant disease, high genetic risk of a primary reproductive disorder, undue psychosocial stress and/or abuse of alcohol, drugs or hormonal agents. Additional clinical research is needed in behalf of the athletic and medical communities to clarify more subtle questions related to the impact of strenuous physical exertion on fecundity, fertility, postpartum health and sexual function.

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Chapter 7

Growth Hormone Variants and Human Exercise

WESLEY C. HYMER, RICHARD E. GRINDELAND, BRADLEY C. NINDL AND WILLIAM J. KRAEMER

Introduction

Exercise stimulates release of human growth hormone (GH) from the anterior pituitary gland and elevated concentrations of circulating GH ultimately help contribute to the exercise-induced increases in muscle hypertrophy and fat breakdown as well as other physiological responses. A review of the exercise/GH literature permits the generalization that it is largely the duration and intensity of the exercise bout that regulates plasma concentrations of circulating GH.

It seems far less certain, however, that many are fully aware of the complexities underlying the GH production 'system' in the pituitary gland itself. Issues underlying these complexities often appear as studies published in specialty journals in which the major focus of the effort is *not* that of exercise-induced release of GH. This situation has started to change.

For the reader primarily interested in exercise physiology/medicine, the primary purpose of this chapter is to bring the issue of GH system complexity into sharper focus. What do we mean by 'system complexity'? Elements of that complexity reside in signals delivered to the pituitary from other parts of the body, for example muscles. Still other elements reside in the GH producing cells themselves. The final product of that complexity is the well-known textbook form of GH molecule, as well as variants of that molecule, that circulate in human blood. We contend that it is this 'army of molecules' that participates in bringing about the physiological responses that have been ascribed to GH for many years.

In this chapter we consider the biochemistry and physiological activities of the GH molecule and its variant forms. We examine the literature that shows that GH variants in the circulation change after exercise. We also briefly review aspects of the cellular biology of the pituitary with the hope that these aspects help offer a fundamental basis for appreciating how production of GH variants may relate to the issue of what could happen after bouts of aerobic/resistance exercise. And finally we offer data that strongly suggest the existence of a novel feedback loop from the muscle to the pituitary. The authors believe that this newly discovered feedback loop might help explain one mechanism(s) underlying exercise-induced release of GH.

Measurement of human growth hormone

In today's world, plasma concentrations of circulating GH are almost always measured using immunoassay procedures. However, other detection systems exist and the method chosen to measure GH in the blood is important. Before the introduction of GH immunoassays investigators usually relied on biological assays that often required the use of rats; certain of these bioassays continue to be used in the authors' laboratories (Roth *et al*. 1963; Hunter & Greenwood 1964). Because they often yield interesting data that may at times conflict with those obtained by immunoassay, we believe a review of such bioassay techniques is warranted.

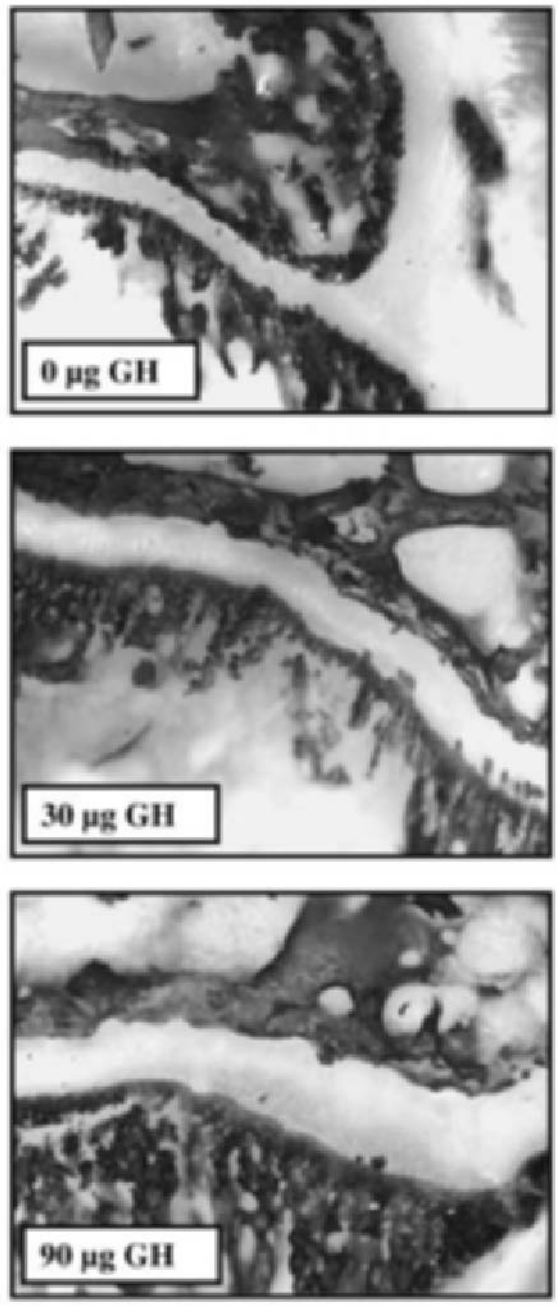
Growth hormone bioassays: perspectives from the older literature

Tests that were developed over 50 years ago reflected the growing awareness at that time of the anabolic, lipolytic and diabetogenic actions of GH. A 1962 review (Papkoff & Li 1962) nicely summarized the essential details of some of these tests available at that time.

In terms of body weight tests the following generalizations apply: large numbers of rats (10/dose level) are required; daily injections can be subcutaneous or intraperitoneal; either intact or hypophysectomized rats are used; weight gain tests are relatively insensitive (50 $\mu g \cdot day^{-1}$ in the intact mature female rat, 10 $\mu g \cdot day^{-1}$ in the immature hypox rat); indices of precision (calculated by dividing the standard deviation of the responses by the slope of the line) are > 0.2; and finally other hormones contaminating a native GH preparation (e.g. thyroxine) may synergize with GH resulting in augmented weight gain. In spite of these disadvantages, the traditional rat weight gain bioassay, that measures the weight of hypox rats after subcutaneous administration of GH for 10 days, is still required by regulators in the USA for individuals assessing the bioidentity and potency of GH preparations produced by recombinant technologies.

In terms of the rat tibial line GH bioassay proposed by Greenspan *et al*. (1949), a major advantage over the weight gain bioassay was the marked increase in assay sensitivity (a response can be detected with a total dose of 5 µg administered over a 4-day period). This assay quantifies the width of the uncalcified epiphyseal cartilage plate delineated from the silver nitrate stained calcified portions of the plate (Fig. 7.1). This test has been used by the authors of this chapter in many studies, including those investigating effects of exercise/bed rest on circulating GH.

The list of biological effects of human GH increases steadily. As pointed out by Strasburger (1994), it has been known for a long time that GH is anabolic protein that promotes longitudinal bone growth. It is also lactogenic, has both insulin agonistic and antagonistic properties, is lipolytic, stimulates ornithine decarboxylase in the liver, promotes



sodium and water retention, and modulates immune system function among others.

The IM-9 lymphocyte cell line and the 3T3-F422A adipocyte assays are more recent, noteworthy examples of *in vitro* cell based biological assays. To our knowledge they have not been used to evaluate GH activities of plasma after exercise. Space limitations prohibit their consideration in this chapter.

Growth hormone bioassays: new perspectives

In an excellent study published by Roswall *et al*. (1996), careful comparisons are offered between two new GH bioassays developed in their laboratories and the hypox rat weight gain bioassay described in section earlier. In order to fully appreciate the basis for these new assays it is necessary to consider: (a) details of the structure of GH molecule produced by recombinant technology (recombinant human growth hormone, rhGH); (b) its structural variants and degraded forms; and (c) molecular interactions between these well-defined forms and the human growth hormone (hGH) receptor.

The primary structure of recombinant human growth hormone and its associated molecular landmarks

The linear sequence of the 191 amino acid (22 kDa) form of rhGH is shown in Fig. 7.2. This form, of course, is identical to the native 22 kDa GH molecule that is synthesized in the pituitary gland and released into the bloodstream on physiological demand. A distinguishing structural feature is the position of the cystine residues responsible for formation of the large internal disulfide loop and the smaller loop at the c-terminus. Also shown in Fig. 7.2 is an enzymatic cleavage site between residues threonine-142 and tyrosine 143; a cleavage that results in a two-chain structure held together by these disulfide bridges. Generation of this two-chain form may result from the action of a membrane bound protease during secretion from the pituitary gland. On long-term storage of GH in solution, deamidation of asparagine residues 149 and 152, as well as loss of the first two residues at the N-terminus, can occur.

Many structural variants of the GH molecule are present in biological samples. In further studies Roswall *et al*. (1996) generated two of these naturally occurring variants using rhGH as their starting preparation. One was the covalent dimer of methionyl GH; the other was the 20 kDa transcriptional variant produced by deletion of residues 32–46. These variants were used in studies later described.

How rGH molecules interact with membrane bound tissue GH receptors (GHRs) is obviously important for a more complete understanding of the importance and physiological consequences of exercise-induced elevations in circulating GH. Studies by Cunningham and his colleagues ~ 15 years ago not only established the complete amino acid sequence of membrane bound GHR, but also showed that the extracellular domain was essentially identical to the glycosylated form of the receptor isolated from human serum (Cunningham & Wells 1989; Cunningham *et al*. 1991). These investigators learned that one molecule of 22 kDa GH complexed with two molecules of the extracellular domain of GHR. At low GH concentrations receptor binding occurs sequentially at two distinct sites on the hormone molecule. Figure 7.3 shows details underlying receptor dimerization that will lead to signal transduction in GH responsive tissues.

Understanding the basis of this detailed molecular physiology enabled Roswall *et al*. (1996) to develop two different types of GH bioassays. One, termed high performance receptor binding chromatography (HPRBC), compares the ability of a test sample of

Fig. 7.1 (*opposite*) Rat epiphyseal cartilage plates prepared from hypophysectomized female rats after subcutaneous injection of saline or growth hormone (GH) standard for 4 days prior to sacrifice. The total dose of GH administered is shown in the lower left corner of the panels. In this assay cartilage plates are stained with silver nitrate and plate widths measured with an ocular micrometer. Ten measurements are taken across the plate and averaged. Plates from control animals typically average 150 μm and those from GH injected rats increase to > 200 μm in dose-dependant fashion. The assay is usually done in double-blind fashion. Photographs kindly provided by Dr. Scott Gordon.

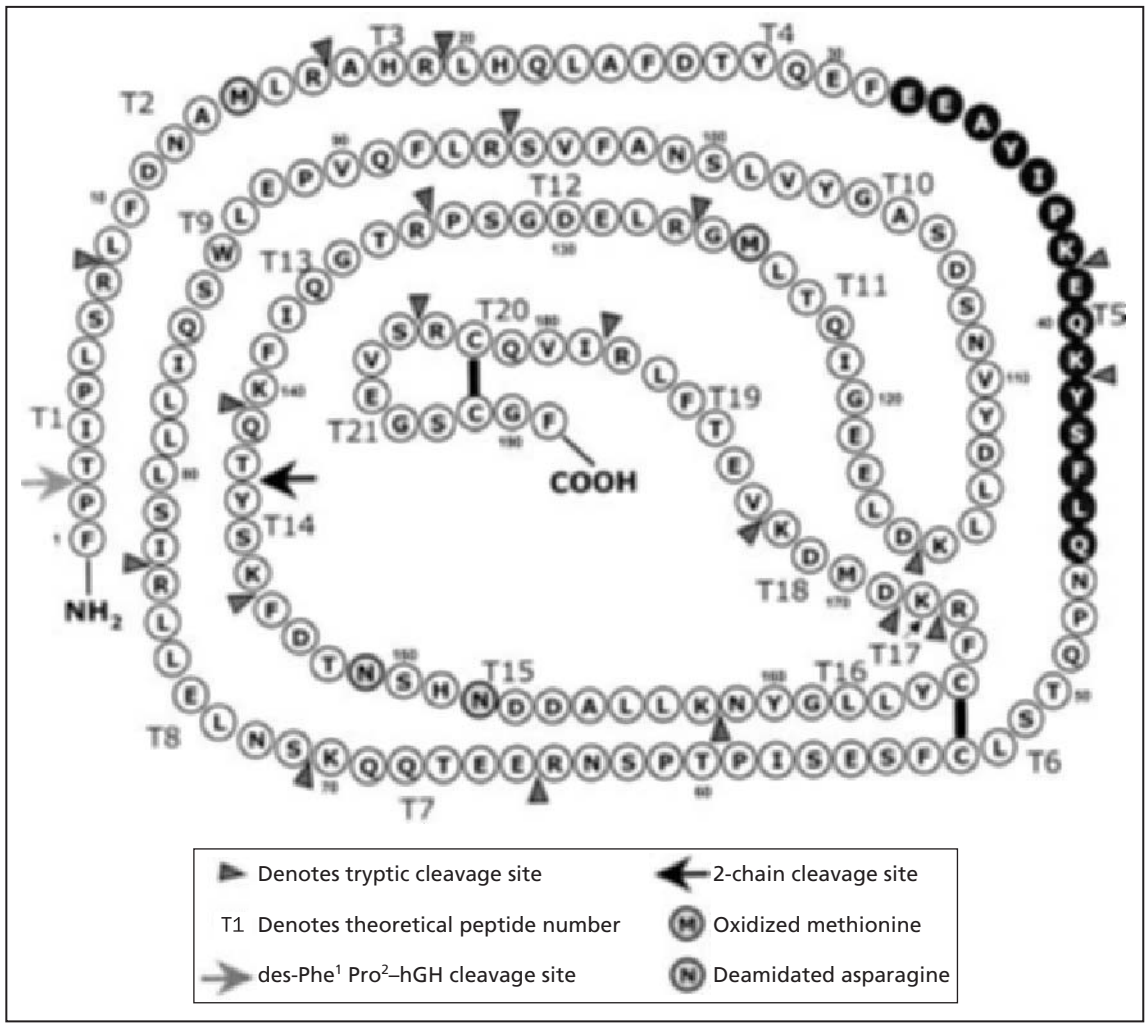


Fig. 7.2 The linear sequence of recombinant human growth hormone (rhGH). The diagram indicates: (a) tryptic cleavage sites; (b) theoretical peptide number (T1–T21); (c) the two-chain enzymatic cleavage site; (d) methionine residues susceptible to oxidation; and (e) asparagines residues susceptible to deamidation. Residues 32–46, marked in black, are deleted in the 20 kDa splice variant of GH. (Adapted from Roswall *et al*. 1996.)

GH with that of a rhGH standard to form a stable 2 : 1 receptor/rGH complex with soluble GHR. Non-denaturing size exclusion chromatography is used to analyze the resulting complex. Strasburger and colleagues have recently developed the immuno-functional enzyme linked immunoassay (IFA) that is based on these earlier findings of Cunningham and his colleagues (Strasburger *et al*. 1996). This assay uses a GH monoclonal antibody and a biotinylated rGH binding protein (BP) to assess functional activity of the GH-containing preparation. The IFA has been used by to measure circulating levels of GH after exercise (Nindl *et al*. 2000)

In the second assay described by Roswall *et al*. (1996), termed the cell proliferation assay (CP), cells from a mouse myeloid leukemia cell line (FDC for ~ 20 h prior to -P1) are transfected with full length receptor and subsequently incubated with test

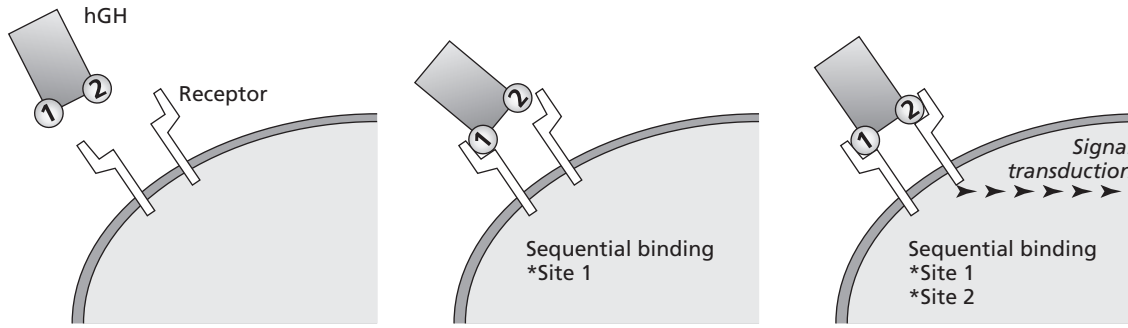


Fig. 7.3 Idealized model of a 22 kDa human growth hormone (hGH) molecule in plasma interacting with receptors on the plasma membrane of a target cell. Proper signal transduction requires receptor dimerization via two binding sites on the hormone molecule.

samples measuring 3H-thymidine uptake into DNA as a measure of biological activity. A similar strategy was used by Wada *et al*. (1998) to measure GH activity of certain variants using Ba/F3-hGHR cells. These cell based assays measure a response (i.e. DNA synthesis) that is several steps distal to receptor dimerization. Roswall *et al*. (1996) suggest that this fact puts the assay: 'several steps closer to the *in vivo* biological response' (p. 36).

Assay comparisons

A comparison between the activities of genetic and chemical rGH variants assessed by the rat weight gain, HPRBC and CP assays are informative and important to consider before assessing similar parameters in human plasma after exercise. Data in Table 7.1, reproduced from the Roswall *et al*. (1996), show full activity and good correspondence between results in some samples (e.g. deamidated rGH and oxidized rGH); poor activity with preparations of dimer or trypsin treated rhGH; and 'super-potency' in the rat bioassay when the two-chain rhGH variant is tested. As indicated by Roswall *et al*. (1996) and others, increased biological activity two-chain GH has been reported previously.

Even though the cell assays described by Roswall *et al*. (1996) and Wada *et al*. (1998) have yet to be used for studies using blood samples collected before and after exercise stress, it seems quite likely that they will in the near term become important for understanding GH activity.

Table 7.1 Biological activities of genetic and chemical variants of recombinant human growth hormone (rhGH) by the rat weight gain assay; the high performance receptor binding chromatography (HPRBC) assay and the cell proliferation (CP) assay. (Data taken from Roswall *et al*. 1996.)

Sample description	Rat bioassay	HPRBC assay	CP assay
Deamidated rhGH	0.88 (n = 1)	1.01 (n = 3)	0.86
Two-chain rhGH	1.78 (n = 2)	0.75 (n = 2)	0.77
Aspartic acid mutant rhGH N149D	0.80 (n = 1)	1.00 (n = 4)	0.87
Aspartic acid mutant rhGH N152D	1.22 (n = 1)	0.95 (n = 2)	—
Aspartic acid mutant rhGH N149D + N152D	0.85 (n = 1)	0.96 (n = 4)	0.93
Des-Phe^{1}Pro^{2}-rhGH	0.46 (n = 1)	0.60 (n = 4)	0.94
Oxidized rhGH	0.79 (n = 4)	0.98 (n = 2)	0.77
Met-hGH covalent dimer	0.10 (n = 1)	0.07 (n = 5)	0.36
Aged rhGH	0.73 (n = 1)	0.96 (n = 3)	—
Trypsin-treated rhGH	0.47 (n = 1)	0.30 (n = 1)	—

See Roswall *et al*. (1996) for a discussion of the principles underlying these data. Note that the two-chain variant, relative to the 22 kDa form, is highly potent in the rat weight gain assay and less so in the other *in vitro* biological assays.

GH form	Proportion of total GH (%)
Monomeric	
22 kDa total	43.0
22 kDa free	21.0
22 kDa in high affinity complex	20.0
22 kDa in low affinity complex	2.0
20 kDa total	8.0
20 kDa free	5.5
20 kDa bound in high affinity complex	0.5
20 kDa bound in low affinity complex	2.0
Acidic GH (desamido- and acyl-GH) total	5.0
Acidic GH bound fractions	Unknown
Dimeric	
22 kDa non-covalent dimer total	14.0
22 kDa disulfide dimer total	6.0
22 kDa dimers bound fraction	Unknown
20 kDa non-covalent dimer total	5.0
20 kDa disulfide dimer total	2.0
20 kDa dimers bound fraction	Unknown
Acidic GH non-covalent dimer total	1.5
Acidic GH disulfide dimer total	0.5
Acidic dimers bound fractions	Unknown
Trimeric to pentameric	
22 kDa non-covalent oligomers total	7.0
22 kDa disulfide oligomers total	3.0
22 kDa oligomers bound fraction	Unknown
20 kDa non-covalent oligomers total	1.0
20 kDa disulfide oligomers total	0.5
20 kDa oligomers bound fraction	Unknown
Acidic GH oligomers (non-covalent and S–S) total	1.0
Acidic GH oligomers bound fraction	Unknown
Non-S–S-linked covalent oligomers total	1.0
Fragments	
16 kDa, 12 kDa, and 30 kDa immunoreactive fragments	Variable

Table 7.2 Estimated proportions of growth hormone (GH) variant forms in human plasma 15 min after secretion. (From Baumman 1991b.)

The wide array of growth hormone forms in the blood

It has been suggested by Baumann (1991b) that there may be as many as 100 different forms of GH present in the human circulation. The concept that numerous molecular forms of the hormone arise from post-transcriptional/translational modification of the single pituitary *GH-N* gene is certainly not new. Pioneering work from the laboratories of Lewis, Sinha, Kostyo and Baumann to name but a few, provide the foundation for the information offered in Table 7.2 that is taken from a 1991 Baumann paper (Baumann 1991b). It shows the estimated proportions of GH variant forms in the plasma 15 min after a secretory episode. Many of these studies were done before techniques of recombinant technology became widely available. Not surprisingly, this type of information therefore was obtained using traditional biochemical procedures.

The literature is rich with observations concerning the biochemical nature of GH variants. A brief but non-comprehensive review of this literature is important for the innate understanding of the heterogeneity and complexity of the GH mechanisms potentially involved with exercise responses and

adaptations. The fact that plasma GH immunoreactivity consists of several molecular weight species that can be separated by size exclusion column chromatography has been known for > 30 years. In the past it was useful to categorize the three main isomers (variants) of GH as little, big or big–big; a designation based upon their elution positions from the column. The physical nature of these size variants is not nearly as well defined as those in studies using GH made by recombinant means. Nevertheless, the careful studies by the Baumann (Baumann 1991a, 1991b, 1999; Baumann *et al*. 1994) and Lewis groups (Lewis *et al*. 2000), among others, that have attempted to characterize the big and big–big variants, permit the conclusion that these variants represent an oligomeric series. Similar oligomers in extracts of human pituitary tissue further support this view. Most investigators therefore believe that aggregation states up to at least pentameric hGH exist and the distinction of big and big–big GH is arbitrary. The authors prefer to categorize the oligomers in terms of their apparent molecular mass based on elution profiles following sephadex chromatography. In addition to these oligomeric size variants charge variants of the GH molecule are known. These are thought to be reflected in acetylated, deamidated or cleaved GH (see Fig. 7.2).

The careful study by Stolar *et al*. (1984) also showed that a majority of the big and big–big GH variants converts to little hGH (22 kDa) during extraction and storage (e.g exposure to 4 mol potassium thiocynate [KSCN] and two freeze–thaw cycles result in ~ 70% conversion to little GH). The variants surviving this harsh treatment migrate as distinct species with apparent molecular weights of 45, 62, 80 and 110 kDa. These latter forms covert quantitatively (and mainly) to little GH after sulfhydryl reduction. Acidic GH comprises a smaller component. The 20 kDa variant may tend to dimerize preferentially.

What is known about the biological activity of these oligomeric species? In general, the big forms (dimer) are thought to have reduced activity in radioreceptor and rodent based assays. However, in the IFA, Strasburger *et al*. (1996) report that dimers, on a molar basis, have slightly higher reactivity in the IFA (110%) relative to the 22 kDa monomer.

A recent review by the Lewis and Sinha group summarizes the properties of five variant forms of GH (Lewis *et al*. 2000). Two of these are the short and larger peptides generated from proteolytic cleavage between residues 43 and 44 of the GH molecule (see Fig. 7.2). Their evidence favors the concept that the short peptide (GH [1–43]) potentiates the physiological effects of insulin; the larger peptide (GH [44–191]) has anti-insulin properties. In fact these authors indicate that: '. . . we believe that this (larger peptide) is the long sought after diabetogenic substance of the pituitary gland' (p. 58).

A ~ 3 kDa peptide, isolated from both human post-mortem pituitary tissue and human plasma, is active in the rat tibial line bioassay (Hymer *et al*. 2000). This peptide is not a fragment of the GH molecule. The relationship of this finding to variant forms described by Baumann is unclear (Baumann 1999). Limited amino acid sequence data, including residues 9–25 in the middle of the peptide, indicate that this peptide is not a breakdown product of the GH molecule. Most interesting is the finding that many of these residues are non-polar and bear striking similarity to the c peptide that is contained in the proinsulin molecule. Similar to the c peptide, this human pituitary tibial peptide, obviously has biological activity. Unpublished data from one of our laboratories (R.G.) shows that the rat pituitary also contains a small peptide that is active in the tibial bioassay.

Variant forms of growth hormone: an emerging data base

In spite of the fact that it has been known for many years that exercise is a potent stimulator of circulating GH, it is only recently that the topic of possible exercise-induced generation of circulating GH variant forms been addressed at all (Nindl *et al*. 2003). In this section we consider some of our prior information in the context of the background given previously. To summarize and analyze this information in a logical way requires an appreciation of the following types of variables in the analysis: human subject choice; exercise type (intensity/duration); GH assay type; methodology used to isolate the variant forms; and special treatments of blood samples.

Table 7.3 Summary of recently published studies in which different types of growth hormone (GH) assays have been used to measure concentrations of GH in human plasma after aerobic or resistance exercise.

Reference	Exercise type/duration	GH immunoassay		GH bioassay		Chemical reduction
		IRMA	Poly	Tibial	IFA	
Rubin *et al*. 2003	Treadmill: 60, 75, 90, 100% $\dot{V}o_{2max}$ for 10, 10, 5 & 2 min, respectively	X			X	X
Hymer *et al*. 2001	Squats: 6 sets of 10-RM	X	X	X	X	X
Wallace *et al*. 2001	Cycle: 80% $\dot{V}o_{2max}$ for 20 min	X			X	
Nindl *et al*. 2001	Resistance: high volume, multiset	X	X		X	
Bigbee *et al*. 2000	Treadmill: 27 $m \cdot min^{-1}$ for 15 min		X	X		
McCall *et al*. 2000	Vibration: stimulus-muscle afferents for 10 min		X	X		
Nindl *et al*. 2000	Squats: 6 sets of 10-RM	X			X	
McCall *et al*. 1997, 1999	Isometric plantar flexion: 30, 80, 100% MVC		X	X		

Poly: assay using a polyclonal antiserum.
Tibial: rat tibial line bioassay.
Chemical reduction: those studies in which a reducing agent has been added to the plasma sample before assay.
IFA, immunofunctional assay (as described by Strasburger *et al*. 1996); IRMA, immunoradiometric assay; RM, repetition maximum.

Table 7.3 provides a summary by subcategorizing studies (only human) according to our arbitrary requirement that the blood sample has been studied by at least two procedures for the purposes of further understanding exercise-induced rises in circulating GH in terms of GH molecular heterogeneity. Only the Hymer study (Hymer *et al*. 2001) used fractionated plasma to measure GH variants; all the rest in Table 7.3 used only neat plasma.

Data from studies to date thus far show that exercise can modify either the activity or molecular character of GH in the circulation. Wallace *et al*. (2001) utilized seven different assays to measure GH in 17 aerobically trained men before and after 20 min of cycle ergometry at 80% $\dot{V}o_{2max}$ to further characterize the response of GH molecular isoforms to exercise. Serum was assayed with antibodies specific for total, pituitary, 22 kDa, recombinant, non-22 kDa, 20 kDa and immunofunctional (IF) GH. Salient findings from this study were: (a) all forms of GH increased during and at the end of exercise; (b) 22 kDa GH was the predominant isoform (73%) at the cessation of exercise; (c) the ratios of non-22 kDa/total GH and 20 kDa/total GH increased and those of recombinant/pituitary GH decreased. Wallace *et al*. (2001) attributed the increase in non-22 kDa isoforms to slower disappearance rates of 20 kDa and perhaps non-22 kDa GH isoforms. Collectively, Wallace's findings demonstrate that the proportion of GH isoform changed across acute exercise and into recovery. Although the 22 kDa was the predominant isoform detected at peak concentrations, isoforms of GH other than 22 kDa increased during the post-exercise period. These results suggest that the proportion of 20 kDa, 17 kDa and possibly other non-22 kDa isoforms (dimers, oligomers and GH bound to serum proteins) increase after exercise. The authors postulated that the increase in the proportion of isoforms other than 22 kDa after exercise may be attributed to differential pituitary isoform secretion, the appearance of isoforms from non-pituitary sources, generation of fragments, dimers and oligomers in the circulation, and differences in clearance rates of the different isoforms. The authors also speculated that the biological consequences of their findings might potentially reside in enhanced diabetogenic effects of smaller GH

isoforms, which may serve to prevent post-exercise hypoglycemia.

Extending on Wallace's work (Wallace *et al*. 2001), we (Hymer, Kraemer and Nindl) next conducted a study in which we fractionated human plasma in 35 women before and after acute resistance exercise (six sets of 10-repetition maximum [RM] squats, separated by 2-min rest periods) using size exclusion chromatography into three size classes (Hymer *et al*. 2001). Fraction A contained molecules > 60 kDa (presumably oligomers and/or monomeric GH bound to receptor); fraction B contained molecules 30–60 kDa (presumably homodimers and heterodimers); and fraction C contained GH molecules < 30 kDa (presumably a mixture of 22, 20, 16, 12, and 5 kDa forms). All samples were then assayed using the Diagnostic Systems Laboratory (DSL) IFA, the Nichols radioimmunometric assay (IRMA) and the National Institutes of Diabetes and Digestive and Kidney Diseases radioimmunoassay (NIDDK RIA). Additionally, we assayed all samples before and after glutathione (GSH) treatment in order to determine the effects of chemical reduction of disulfide linked bonds. Recovered immunoreactivities were 4–11% in fraction A, 22–45% in fraction B and 44–72% in fraction C. Significant exercise-induced increases were observed for the lower molecular weight GH moieties (30–60 kDa and < 30 kDa isoforms), but not for the higher molecular weight GH moieties (> 60 kDa). Another important finding was that chemical reduction of the post-exercise samples increased immunoassayable GH as measured by the Nichols and NIDDK assays more than pre-exercise samples, suggesting that exercise may specifically increase the release of disulfide-linked hormone molecules and/or fragments. From these data, the most important effect of acute resistance exercise appears to be on dimeric hormone. Because complexes of GH and BPs have longer half-lives than free GH, it is possible that dimeric GH might also have a longer half-life. Therefore, the net effect of the increase in GH isoforms within this molecular weight range would be to prolong the biological activities of these forms after exercise.

In a study by Nindl *et al*. (2000) the first data comparing the effects of exercise on IF versus immunoreactive (IR) GH was presented. Comparisons were made between the IF versus IR GH concentrations in men and women before and after acute resistance exercise (i.e. six sets of 10-RM squats separated by 2-min rest periods). IF GH concentrations were determined by an enzyme linked immunosorbent assay (ELISA) purchased from Diagnostics Systems Laboratories (DSL, Webster, TX), which was based on Strasburger's work (Strasburger *et al*. 1996), and IR GH concentrations were determined by a monoclonal IRMA purchased from Nichols (San Juan Capistrano, CA). In this study, both men and women demonstrated similar increases for IR (men: 1.47 vs. 25.0 $ng \cdot mL^{-1}$; women: 4.0 vs. 25.4 $ng \cdot mL^{-1}$) and IF (men: 0.55 vs. 11.7 $ng \cdot mL^{-1}$; women: 1.94 vs. 10.4 $ng \cdot mL^{-1}$) GH following exercise. However, post-exercise IF GH was significantly less than IR GH for both men and women. The ratio of IR/IF after exercise was ~ 2 and similar for both men and women. The correlation between post-exercise IR and IF GH was $r = 0.83$. This study initially indicated that about half of the GH isoforms measured by the Nichols IRMA released after exercise did not possess intact sites 1 and 2 required for receptor dimerization, thus suggesting biological inactivity.

A following study considered the fact that GH is released in an episodic, pulsatile manner. IF GH was measured in 10 men who underwent two overnight blood draws with sampling every 10 min from 1700 to 0600 h. The overnight serial sampling was performed in both a control and an acute heavy resistance exercise condition (Nindl *et al*. 2001). For the exercise condition, subjects performed a high-volume, multiset resistance exercise bout from 1500 to 1700 h. IF GH was compared to the Nichols IRMA and NIDDK's polyclonal RIA. The Pulsar peak detection system was used to evaluate the pulsatility profile characteristics of GH. Even though the results from all three immunoassays were highly correlated (correlations ranged from 0.85 to 0.95), the Nichols IRMA again yielded higher mean GH concentrations than did IF GH (3.98 vs. 1.83 $ng \cdot mL^{-1}$, respectively). The results from the Nichols IRMA also yielded higher pulse amplitudes compared to IF GH (8.0 vs. 4.63 $ng \cdot mL^{-1}$, respectively).

The consistent finding in these studies (Nindl *et al*. 2000, 2001, 2003) was that IF GH measured from the same sample was approximately one half

that of the Nichols IRMA, one of the most widely used GH assays in clinical practice in the USA. Since the IF assay purports only to measure biologically active forms of GH (i.e. only those forms of GH capable of inducing receptor dimerization are translated to an assay signal), the additional GH isoforms detected by the Nichols IRMA are likely fragments whose potential biological actions are not mediated by the GHR. It has been reported that the GH fragment 44–191 is detectable in substantial levels in human serum and may even antagonize GH action (Rowlinson *et al*. 1996). Since this fragment lacks part of the N-terminus, it is unlikely to be detected by the IF assay; however, the fragment could be detected by the IRMA, depending on the targeted epitopes. Alternatively, the additional GH isoforms detected by the Nichols IRMA could also be high molecular weight variant forms of GH (Baumann 1991a; Lewis *et al*. 2000).

Our findings conclusively show that at least some of the molecules released during secretory bursts are able to dimerize GHRs. In that sense, these molecules are biologically active. On the other hand, our data also demonstrated that GH isoforms are released, both in exercise and non-exercise conditions, that are not capable of initiating signal transduction through the GHR. Based on the high correlations among the immunoassays and the similar detection of the number of peaks and interpeak intervals, it appears that the immunoassays report qualitatively comparable pictures of the GH response. The quantitative differences among the immunoassays have yet to be fully explained, but are likely due to the existence of various molecular isoforms. Other factors that contribute to the differences in GH measurement could include the assay equilibrium conditions, buffer, tracer and standard used (Wood 2001).

It is important to consider the impact of BPs in the IFA (Strasburger *et al*. 1996; Nindl *et al*. 2001). The IFA uses an rGHBP to bind site 1. One could infer that a GH molecule already complexed to a GHBP would not be detected in this assay system, as site 1 would not be freely accessible. Also, a GH–BP complex might be configured such that site 2 is not exposed to the monoclonal antibody (mAb7B11). It has been reported that the high-affinity GHBP inhibits GH binding to receptors and *in vitro* bioactivity via competition for ligand (Strasburger *et al*. 1996). If it is true that GH complexed to BP is too large a molecule to traverse the capillary endothelium in order to bind to cellular receptors, the lack of detection of the GH complexed in the IFA provides further support for the functional selectivity of the IFA.

Rubin *et al*. (2003) also compared the Nichols IR versus the DSL IFA in six endurance-trained men during intermittent treadmill running at progressively increasing intensities (60% $\dot{V}O_2$ max for 10 min, 75% for 10 min, 90% for 10 min and 100% for 2 min). Samples were assayed before and after the addition of glutathione (GSH; 10 mmol for 18 h at room temperature) in order to break disulfide bonds between possible oligomeric GH complexes. For the IRMA, GH was elevated after the 75% exercise intensity and remained elevated through 30 min of post-exercise. After adding GSH, the IRMA indicated elevations in GH as early as 60% exercise intensity and remained elevated 45 min into recovery. At 75%, the GSH assay run was higher than the non-GSH assay run. With the IFA, GH was elevated at 60% in the non-GSH conditions, whereas the GSH assay run indicated elevations at 75%. Both GSH and non-GSH conditions remained elevated through 30 min of recovery. These data indicate that the addition of GSH to serum samples prior to assay via an IRMA may break existing disulfide bonds aggregated to GH molecules, thus altering the apparent assays signal to reveal total GH.

The complexity of the anterior pituitary cell system

The GH-producing cell system of the anterior pituitary gland is tremendously complex. How might knowledge of that complexity help in unraveling mysteries and mechanisms underlying the relationships between exercise and GH variants? Human pituitary tissue obtained after surgery or death is difficult to obtain, so the rat pituitary gland has been the tissue of choice to study biology of the GH cell. Space limitations do not permit full review of this topic. Instead, results from highly selected studies are summarized here, in the format of a few sentences for each point considered.

Table 7.4 The effect of chemical reduction on concentrations of rat growth hormone (GH) variants contained in alkaline extracts separated on non-reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) rat pituitary prior to immunoassay. (From Farrington & Hymer 1990.)

	GH (μg)		
Apparent mol. wt. (kDa)	–βME	+βME	Fold increase
< 22	4.7 + 0.9	6.1 + 0.7	1.3
22–57	1.8 + 0.2	3.2 + 0.5	1.8
57–77	2.1 + 0.2	8.1 + 0.4	3.9
77–100	1.2 + 0.2	4.7 + 0.5	3.9
100–150	0.9 + 0.15	5.1 + 0.3	5.6
< 150	0.9 + 0.3	4.0 + 0.4	4.4

Hormone contained in different regions of the gel were eluted and reduced with mercaptoethanol prior to GH using a polyclonal antibody. Recovery of GH from the gel averaged 103% (n = 3 experiments).

1 Human pituitary glands weigh ~ 350 mg; those of rats 8–12 mg. Human pituitary glands contain 4–8 mg GH/gland; rats ~ 200 μg GH/gland. GH accounts for ~ 80% of the total hormone in the gland.
2 Quantitative extraction of GH requires homogenization at alkaline pH (9–10). Non-reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of pituitary extracts shows multiple GH forms with apparent molecular weights of 14–88 kDa. After chemical reduction there is a four to sixfold increase in the GH immunoreactivity in those forms with mass > 50 kDa (Table 7.4). High molecular weight forms of GH are aggregates that are linked by disulfide bridges resulting from antiparallel alignment of cystine residues between two GH monomers (Lewis *et al*. 1975). GH aggregates are released from the pituitary and are present in human plasma.
3 Aggregates of GH are packaged in ~ 300 μ diameter secretion granules. These granules contain biologically active GH (Hymer & McShan 1963). According to Dannies (1999) 'not a great deal' is known about molecular packaging of the hormone into granules and, '. . . how cells concentrate hormones is a major unanswered question in endocrinology' (p. 3). In some hormone-packaging cell systems there is good evidence that different secretory granule proteins are packaged *in separate granules in the same cell*. It is probable that heterogeneity in GH packaging is directly related to the issue of GH variants and exercise.
4 Subpopulations of rat GH containing granules are separable by continuous flow electrophoresis; and GH containing granules isolated from the post-mortem human pituitary also yield subpopulations as well. Preliminary evidence indicates that the more rapidly migrating particles are rich in tibial line bioactivity and relatively poor in IR GH.
5 At least two types of rat pituitary GH cells can be routinely separated by density gradient centrifugation. GH that is rich in tibial line activity is preferentially released from the more dense cell; and this hormone is oligomeric (Farrington & Hymer 1990).
6 Implantation of GH cells into the cerebral ventricles of hypophysectomized rats show that receipients gain weight and have longer tibial, femoral and pelvic bones (Weiss *et al*. 1978). Intraventricular implantation of the denser GH cells not only increases body weight, it also increases width of the epiphyseal plate and weight of the gastrocenimus muscle (Grindeland & Hymer unpublished).

Collectively these findings support the contention that not only is there a molecular basis underlying GH variants, but that there is an underlying cellular biology basis as well.

Tibial line assay and exercise responses

The issue of the dichotomy between measurements of GH concentrations made by tibial line bioassay and those made by immunoassay is important to consider in future experiments addressing the relationship between GH variants and exercise. It is important to consider GH variants in terms of those molecular forms which react with high affinity antisera to the 22 kDa 'native', textbook form of GH

(immunoassayable GH; iGH) or to those form(s) that do not react with such antisera, but which elicit growth as revealed by the tibial line bioassay (bioassayable GH; bGH). It is possible that the pituitary secretes variant forms of GH which have other functions (e.g. lipolytic activity), but literature on this possibility is somewhat limited.

At the moment the tibial assay appears to be the assay of choice if one wishes to know the GH 'status' of the subject. In spite of the fact that the assay is labor intensive, costly and lengthy it clearly offers information that is simply not obtainable any other way. There is absolutely no question that today's scientist or clinician is comfortable and familiar with data that report plasma GH concentrations measured by immunoassay. However, apparent concentrations of bGH reported in the literature are often hundreds or even thousands of nanogram per milliliter! Why is this so? Because this bioassay measures *the biological activity and not nanograms of purified hormone*. In this context it is important to realize that purified GH from a number of different species (human, bovine, murine), and their bGHs, yield parallel dose response curves in the tibial bioassay. Similar tibial growth curve responses elicited by these hormone preparations enable one to express biological potency in terms of a standard 22 kDa hormone preparation.

In any event it is important that, at the very least, some appreciation of the difficulty and importance of establishing the most meaningful quantification of plasma GH in a given physiological context of the subject is determined. Moreover any possible skepticism regarding measurement of bGH by tibial assay may be alleviated by the following two examples of the dichotomy issue between bGH and iGH.

Dichotomy between bioassayable growth hormone and immunoassayable growth hormone

The rat has played a major role in the evolution of our current understanding of the iGH–bGH dichotomy. For example, many investigators report that stimuli (e.g. cold exposure, hypoglycemia, exercise) that elicit increased plasma iGH concentrations in humans have no effect on circulating iGH in the rat. Investigations by one of us (R.G.) revealed that the rat responded to these stimuli by secretion of GH in form(s) not recognized by antisera to the 22 kDa rat GH molecule (Ellis & Grindeland 1974). However, these circulating immunologically unreactive forms promoted significant growth of the assay rat (Ellis & Grindeland 1974).

These types of results permit the general, *yet still speculative*, conclusion that while there is no obvious relationship between iGH and bGH in the rat, variations in bioactive/immunoactive human GH concentrations in biological samples tend to change in the same direction. However, because titers of human iGH and bGH are not directly proportional we believe that one should not use iGH measurements as an index of total circulating GH.

Almost coincidental with these early studies on rat and human bGH was the finding that plasmin, a protease, was active on subprimate GH (rat, bovine) and either reduced or abolished the immunological activity of the 22 kDa hormone (Ellis *et al*. 1968). However, this treatment yielded peptides with normal or even enhanced biological activity. Other laboratories have shown that human iGH, treated with human plasmin, does not appear to lose immunological activity but to gain in biopotency (Singh *et al*. 1974; Lewis *et al*. 1975; Nguyen *et al*. 1981). Clearly the immunological/biological ratio of the 22 kDa GH molecule can be significantly altered after enzyme treatment.

Human bed rest studies

Humans performing a moderate exercise (i.e. plantar flexion) lasting several minutes show a one to twofold increase in plasma bGH but no effect on iGH when done under the usual 1 G conditions. However, when subjected to absolute, head-down bed rest the identical exercise regimen does not evoke an increase in bGH secretion and has no effect on iGH release (McCall *et al*. 1997). Interestingly, a few days after bed rest the increased bGH secretion in response to exercise returns (Table 7.5).

What bearing might these data from have on GH variants and human exercise? In the exercise physiology literature metabolic regulators are usually invoked as a dominant mechanism by which

Table 7.5 Plasma immunoassayable and bioassayable growth hormone concentrations. Pre- and post-exercise during bed rest (ng·mL^{-1}). (Adapted from McCall *et al*. 1997.)

Day	Hormone	Pre	Post
Before bed rest			
–13/12	bGH	2146	0 3565*
	iGH	5.3	4.7
–8/7	bGH	2162	0 4161*
	iGH	5.0	5.3
During bed rest			
2/3	bGH	2350	2203
	iGH	5.1	5.8
8/9	bGH	2433	2105
	iGH	4.8	5.2
13/14	bGH	2594	2085
	iGH	4.7	5.2
After bed rest			
+2/3	bGH	1807	2379
	iGH	2.0	4.5
+10/11	bGH	1881	0 4160*
	iGH	4.9	5.3

*$p < 0.05$ between pre- and post-exercise values. Growth hormone measured by immunoassay did not differ at any time or condition between pre- and post-exercise samples. bGH, bioassayable growth hormone; iGH, immunoassayable growth hormone.

increased muscle activity increases iGH secretion. It is interesting to find that none of the commonly cited metabolic factors (e.g. lactate, plasma glucose) appear to explain reduced plasma concentrations of bGH. This obvious inconsistency led one of the authors (R.G.) to ask the following question: 'Is there a neural mechanism that controls GH secretion?' The answer seems to be yes; and it is relevant to exercise physiologists.

Muscle afferents regulate release of bioassayable growth hormone in rats

Initial studies used animals in which nerves serving hind-limb muscles were severed. When the *distal* ends are stimulated electrically for 15 min, a treatment that generates a pattern resembling that of a rat walking at 1.5 miles per hour (2.4 km per hour) (Gosselink *et al*. 1998, 2000; McCall *et al*. 2000), the stimulation has no effect on concentrations of either plasma or pituitary bGH or iGH.

On the other hand, if the *proximal* end of the severed nerve of a fast twitch muscle is stimulated, there is a massive (one to twofold) increase in plasma bGH within 5 min after stimulation! Significantly, this *increase* in plasma bGH is mirrored by a large *decrease* in pituitary gland concentrations of bGH. However, there is no effect on iGH concentrations in either plasma or the pituitary gland. Equally interesting is the finding that stimulation of the proximal end of the soleus nerve results in *reduced* plasma concentrations of bGH. This observation implies specificity of muscle groups in this afferent pathway.

These results are interesting from two points of view. First, they argue that there is a neural control component to the pituitary GH system in addition to a metabolic component. Secondly, we would argue that these nerve stimulation studies provide insight into the physiological significance of bGH. If one assumes that one of GH's most critical functions is assuring a constant glucose supply to the heart and brain, the results appear to make good sense. Massive GH release in response to high metabolic demand (e.g. fasting, hypoglycemia or cold exposure) and release of GH in response to activation of a quiescent muscle of locomotion would both offer mechanisms for increased carbohydrate uptake by tissues. These are protective mechanisms for the organism.

Of course it is well established that human iGH is secreted in response to exercise, but this response begins only after 15 or 20 min of exercise. We propose that during quiescence the soleus and other postural muscles, which are perhaps 80% active at rest, signal the pituitary via muscle afferents to decrease bGH secretion and thereby permit non-neural and non-cardiac tissues to use glucose. As the muscles of locomotion become active the muscles would signal the pituitary gland to secrete bGH. The net effect would presumably be to inhibit utilization of glucose by active muscle and provide an alternate energy supply by mobilizing fatty acids from the fat depots.

In Fig. 7.4 we offer a model, adapted from our published study (McCall *et al*. 2001), that closes a

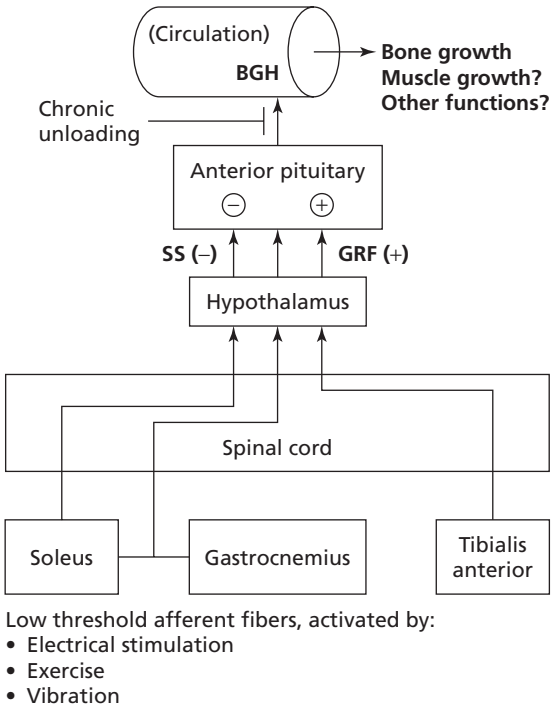


Fig. 7.4 Model of proposed muscle afferent–pituitary feedback axis that is postulated to regulate release of bioactive growth hormone (BGH) from the anterior pituitary gland. BGH is defined as those form(s) of the hormone molecule that stimulate widening of the epiphyseal cartilage plate (see Fig. 7.1). This model is suggested by the studies of McCall *et al*. (2001) in which stimulation of afferents from ankle flexors of the rat (e.g. tibialis anterior) or the entire posterior compartment of ankle extensors (e.g. soleus and gastrocnemius) stimulates release of BGH but inhibits BGH when only afferents from the soleus muscle are stimulated. Chronic unloading inhibits the exercise-induced increase of plasma BGH. Afferents may activate pituitary GH cells directly. Possible physiological functions of BGH, other than stimulation of the tibial epiphyseal growth plate, have not been identified. GRF, growth hormone releasing factor; SS, somatostatin. (Adapted from McCall *et al*. 2001.)

muscle/neural feedback loop to the pituitary GH system. In Fig. 7.4 the neural inputs are received by hypothalamic neurons. On the other hand, it is conceivable that neural inputs might be received by the anterior pituitary gland directly. There are a few reports in the literature that describe nerve inputs to the pituitary. One of the more recent is that of Paden *et al*. (1994) that describe '. . . a surprisingly extensive innervation of the anterior lobe of the pituitary' (p. 503). It is interesting that they are also frequently associated with blood vessels and do not have the appearance of vasomotor fibers. Their distribution is uneven and they appear to contact only a subset of glandular cells (GH/adrenocorticotropic hormone; ACTH).

Acute versus chronic resistance exercise and bioassayable growth hormone

In a recent study we (Kraemer, Hymer and Nindl) evaluated the effects of an acute resistance exercise bout (i.e. six sets of 10-RM squats separated by 2 min of rest) on bGH before and after 6 months of periodized resistance training in young, healthy women. Results from this study indicated that while acute resistance exercise did not alter circulating bGH, 6 months of chronic resistance training clearly potentiated bGH concentrations (Fig. 7.5). These results suggest that one of the benefits of chronic resistance training is to increase the biological activity of circulating GH. This new finding potentially represents one of the mechanisms by which muscle and bone can benefit from resistance training.

Summary

The main points we have tried to emphasize in this chapter can be summarized in the following way.

1 *GH molecules are heterogeneous*. In this chapter we define and consider GH heterogeneity in different ways. These include: (a) size and charge variants of the molecule(s) resulting from the single pituitary GH gene; (b) activities of the molecule(s) in terms of the biological (*in vivo*) versus immunological (*in vitro*) signals they generate; and (c) cells in the pituitary that produce and release GH molecules.

2 *Either aerobic or resistance exercise can result in differential release of GH variants into the circulation*. To date there are only a handful of studies that address the issue of GH variants after human exercise. Thus far they support the idea that release of oligomeric forms of the 22 kDa GH monomer is increased after exercise. The activities of circulating GH, measured

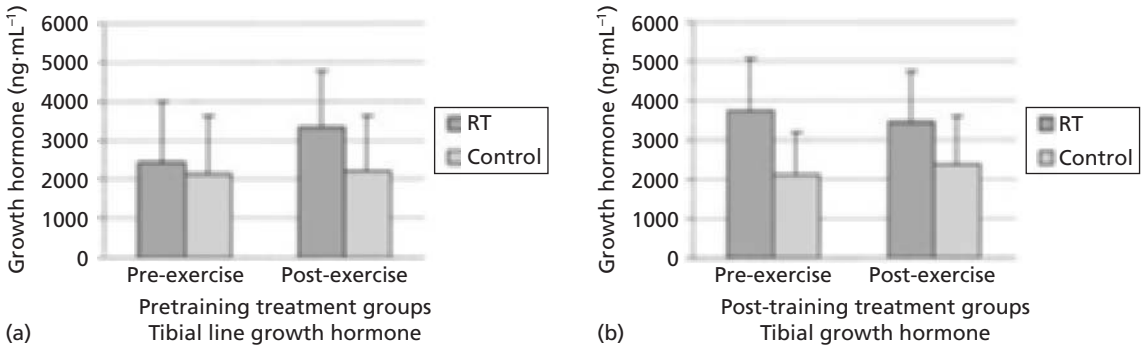


Fig. 7.5 Bioassayable growth hormone in control versus exercise groups sampled pre and post an acute resistance exercise test consisting of six sets of 10-RM squats separated by a 2-min rest period before (a) and after (b) 6 months of periodized resistance training (RT) (unpublished data).

by biological and immunological assays, are often not parallel. The intensity and duration of the exercise appear to play a major role in this dichotomy of activity. After training, resting concentrations of GH active in the rat bone growth assay are increased.

3 *The GH-producing cell system in the rat pituitary gland is heterogeneous. GH cellular heterogeneity may also be present in the human pituitary gland, but these types of studies are difficult to do*. Evidence supports the view that GH-containing secretion granules and GH-producing cells are heterogeneous. This heterogeneity appears to have physiological relevance. Additional work is required to establish a link between these components in exercising rats and humans.

4 *Regulatory mechanisms responsible for releasing GH variants from the pituitary may involve signals from neural pathways originating in muscles activated by exercise*. In this chapter we offer evidence to support the existence of a novel feedback loop from certain muscle groups to the pituitary gland. This loop appears to exist in humans as well as rats. It may be an important factor in controlling the production and/or release of GH variants from the pituitary gland.

In conclusion, readers of this chapter know that the information explosion we are experiencing in today's biological sciences results not only from past studies but from a rapidly expanding technology and data base as well. This is obvious. In this chapter the authors have considered seminal works done some 50 years ago and tried to show that they are relevant today and help in a fuller appreciation of the role that variant forms of the GH molecule might play in the beneficial effects of human exercise. We have tried to show that a successful marriage between the experimental approaches used in endocrinology, endocrine biochemistry/cell biology and exercise physiology may lead to new insights into the role that GH molecular heterogeneity plays in human exercise. Obviously a start has been made, but much more needs to be done.

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Chapter 8

Growth Hormone Binding Proteins

GERHARD BAUMANN

Growth hormone binding proteins (GHBPs) are soluble, circulating proteins that form complexes with growth hormone (GH). They are integral parts of the somatotropic axis and have activities as modulators of GH action and GH transport in blood. An overview of the growth hormone–insulin-like growth factor (GH–IGF) axis is shown in Fig. 8.1.

History

The presence of GHBPs in blood was first postulated in the 1960s (Irie & Barrett 1962; Touber & Maingay 1963; Collipp *et al*. 1964; Hadden & Prout 1964), but these observations were not generally accepted as physiological phenomena at that time (Berson & Yalow 1966a, 1966b). In 1977, Peeters & Friesen (1977) described a GH binding factor in pregnant mouse serum. However, this observation was also largely ignored. It was not until Baumann and Herington independently described, characterized and partially purified GHBPs from human and rabbit serum (Ymer & Herington 1985; Baumann *et al*. 1986; Herington *et al*. 1986b) that the GHBPs were generally accepted as authentic. Two GHBPs, one with high affinity and the other with low affinity for GH were described at that time (Baumann *et al*. 1986). While the high affinity GHBP was easy to work with and soon became recognized as the GH receptor (GHR) ectodomain (Leung, D.W. *et al*. 1987; Baumann *et al*. 1988), it took some years to characterize the low affinity GHBP (Baumann *et al*. 1990; Tar *et al*. 1990), ultimately leading to its recognition as transformed α_2-macroglobulin (Kratzsch *et al*. 1995b). In general, the term 'GHBP' is used for the high affinity GHBP—a convention that will also be followed in this chapter unless stated otherwise.

Nature and chemical properties

The high affinity GHBP represents the extracellular domain of the GHR (Leung, D.W. *et al*. 1987; Spencer *et al*. 1988). It is a single chain glycoprotein with a molecular weight that varies widely depending on the species from 28 kDa (chicken) to 65 kDa (humans), with most of the variation due to differences in glycosylation. The polypeptide backbone accounts for approximately 28–30 kDa, with minor differences among species. The GHBP is evolutionarily conserved from teleost to humans; it has been found in the blood of all vertebrates examined. In some species it is derived directly from the GHR by proteolysis, in others (rodents), it is synthesized as a separate gene product (see below). The precise structure of the GHBP is known in only a few species, with the carboxy terminus unknown in many. Two subdomains, each composed of beta-pleated sheets, have been identified: an amino-terminal subdomain 1 containing the GH binding site; and a carboxy-terminal subdomain 2 that is involved in dimerization of the GHR. An approximately 10 amino acid linear stem region extends between subdomain 2 and the transmembrane helix of the GHR (Baumann & Frank 2002). The precise cleavage site in the GHR giving rise to the GHBP has recently been mapped in the rabbit: cleavage occurs in the extracellular stem region between subdomain 2 and the transmembrane domain, with the GHBP carboxy terminus at residue no. 238, i.e. eight residues

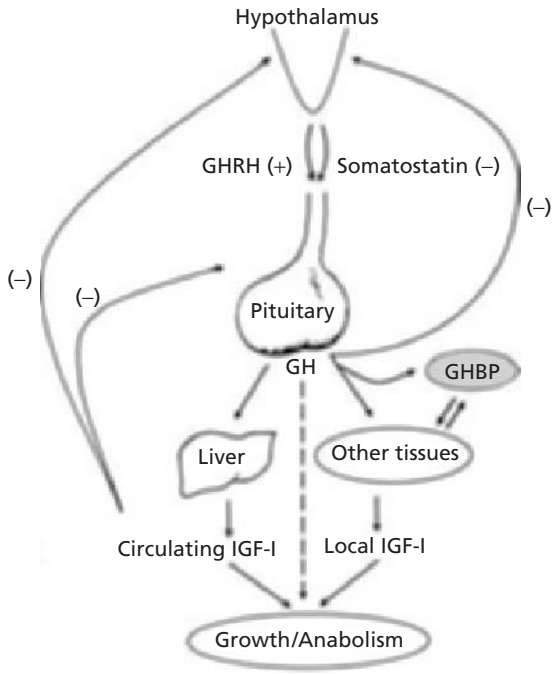

Fig. 8.1 The hypothalamo–pituitary–somatotropic axis, also known as growth hormone–insulin-like growth factor (GH–IGF) axis. Pituitary GH secretion is under positive (stimulatory) control from the hypothalamus via growth hormone releasing hormone (GHRH) and under negative (inhibitory) control via somatostatin. After secretion, GH binds to receptors in liver and virtually all other tissues, and to growth hormone binding proteins (GHBPs) in the circulation. There is interchange between free GH, GHBP-bound GH and receptor-bound GH. Peripheral tissues produce IGF-I in response to GH. The liver is responsible for 60–70% of circulating IGF-I; other tissues are responsible for the rest. In tissues other than the liver, local (paracrine/autocrine) IGF-I action is very important. Direct (non-IGF-dependent) GH actions are indicated by the dashed arrow. IGF-I feeds back negatively on the hypothalamus and pituitary gland to inhibit GH secretion. GH itself also inhibits its own production via negative feedback at hypothalamic level (short-loop feedback).

outside the plasma membrane (Wang *et al*. 2002). Based on the sequence similarity of the rabbit and human GHRs in the extracellular stem region, it appears likely that the human GHBP has the same length, though this still needs direct confirmation. In rodents, the GHBP is the product of an alternatively spliced GHR mRNA and is synthesized *de novo*. It contains a carboxyterminal 'tail' of 27 (mouse) or 17 (rat) amino acids in lieu of the transmembrane domain in the GHR (Baumbach *et al*. 1989; Smith *et al*. 1989). The mouse and rat GHBP contain 273 and 255 amino acids, respectively. The extent of GHBP glycosylation differs among species, but there is only limited information for rodent GHBP about the nature of the sugar moieties. Mouse serum GHBP is glycosylated at three asparagine residues, wheras tissue-associated GHBP (see below) contains less carbohydrate and is glycosylated at two asparagine residues (Cerio *et al*. 2002). Rat serum GHBP contains sialic acid, whereas tissue-associated GHBP is rich in mannose (Frick *et al*. 1998). The details of the carbohydrate side chain structure are not known. In humans, two high affinity GHBPs exist (one containing and the other lacking the sequence encoded by exon 3 of the *GHR* gene) (Kratzsch *et al*. 1997b). This is the consequence of *GHR* polymorphism with respect to exon 3 (Pantel *et al*. 2000; Seidel *et al*. 2003). The presence or absence of the exon 3-encoded sequence in the GHR or the GHBP has no significant functional consequence with respect to GH binding. However, small differences in the correlations between serum GHBP level and anthropometric/metabolic parameters have been reported for the two GHBP isoforms (Seidel *et al*. 2003).

The high affinity GHBP binds GH with dissociation constants ranging from 10^{-8} to 10^{-9} mol (Ymer & Herington 1985; Baumann *et al*. 1986; Herington *et al*. 1986b; Smith *et al*. 1988; Massa *et al*. 1990). It exhibits somewhat lower affinity (K_d 10^{-6} to 10^{-7}) for the 20 000 kDa (20 K) variant of human GH (Baumann *et al*. 1986). Like the GHR, the GHBP has the capacity to form ternary (2 GHBP : 1 GH) complexes with GH, but due its low concentration in biological fluids, the 1 : 1 GHBP–GH complex predominates under physiological conditions (Baumann *et al*. 1994). The association rate for the human GHBP is very rapid ($\sim 2 \times 10^7 \cdot mol^{-1} \cdot min^{-1}$ at 37°C, 80% maximum binding reached in 5 min), the dissociation rate is $3.7 \times 10^{-2} \cdot min^{-1}$ at 37°C (dissociation half-time ~ 20 min) (Baumann *et al*. 1986; Veldhuis *et al*. 1993, Baumann 1995).

The low affinity GHBP is a heterogeneous plasma component that binds GH with a K_d in the micromolar

range (Baumann *et al*. 1986, 1990; Massa *et al*. 1990; Tar *et al*. 1990; Leung, K.C. *et al*. 2000). It has high binding capacity and in humans has been shown to represent a modified form of α_2-macroglobulin ('transformed α_2-macroglobulin') (Kratzsch *et al*. 1995b). Relatively little is known about the molecular nature of low affinity GHBPs in animals.

Generation and tissues source(s)

As indicated above, the high affinity GHBP is generated by different mechanisms depending on species. In humans, rabbits and several other species, the GHBP is produced by proteolytic cleavage of the juxtamembranous stem region of the GHR, a process named 'shedding' (Fig. 8.2). The enzyme involved in this process has been recently identified: it is a zinc metalloproteinase of the ADAM family named TACE (tumor necrosis factor-α converting enzyme), also known as ADAM-17 (Black *et al*. 1997; Zhang *et al*. 2000). Mature, catalytically active TACE is a plasma membrane-resident enzyme that associates with the GHR, followed by cleavage and shedding of GHBP and a truncated GHR 'remnant', which has its own intracellular fate. TACE is responsible for cleavage of a number of transmembrane proteins in their extracellular domain, with shedding of soluble ectodomains akin to the GHBP. It is possible that other enzymes in the same class also contribute to GHBP shedding, but this remains to be investigated. The regulation of TACE activity and the shedding process are still poorly understood. The conformational change induced in the GHR by binding of GH (dimerization or change in predimerized GHR) renders it less prone to proteolysis than the monomeric, unliganded GHR (Zhang *et al*. 2001). GHBP shedding is thought to occur principally if not exclusively at the cell surface, based on the localization of active TACE and the fact that a GHR variant with a long plasma membrane residence time (devoid of the cytoplasmic/internalization domain) is a particularly good source of GHBP (Dastot *et al*. 1996).

Rodents generate GHBP by an entirely different mechanism. Both mouse and rat *ghr* genes contain a special exon (exon 8A) encoding the hydrophilic GHBP tail (see above) interposed between exons 7 and 8 (Edens *et al*. 1994; Zhou *et al*. 1994, 1996). Exon 8 encodes the transmembrane helix. Differential mRNA splicing of exon 7 to either exon 8A or exon 8 yields the GHBP or the GHR, respectively (Fig. 8.3). Both transcripts are expressed in the same tissues, but it is unknown what regulates their relative expression. It should be noted that the mouse GHR is not completely resistant to TACE proteolysis, at least when induced by phorbol ester. However, the

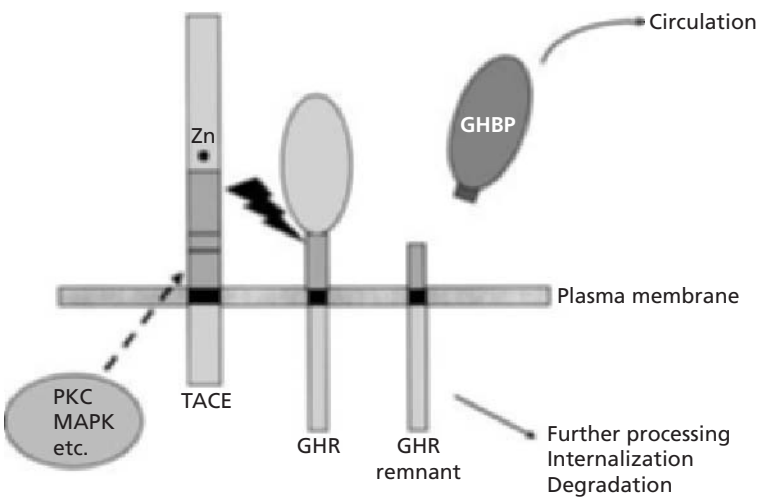

Fig. 8.2 The generation of growth hormone binding protein (GHBP) via proteolytic cleavage of the growth hormone receptor (GHR). A membrane-bound zinc-dependent metalloproteinase (tumor necrosis factor-α converting enzyme [TACE]) cleaves the GHR in its juxtamembranous stem region 8–9 amino acid residues outside the plasma membrane. The GHR ectodomain is shed as the GHBP, which reaches the circulation. The GHR remnant protein, consisting of the transmembrane and intracellular domains, undergoes additional processing and may have its own biological role. TACE is activated by MAP kinase (MAPK) and protein kinase C (PKC) dependent pathways.

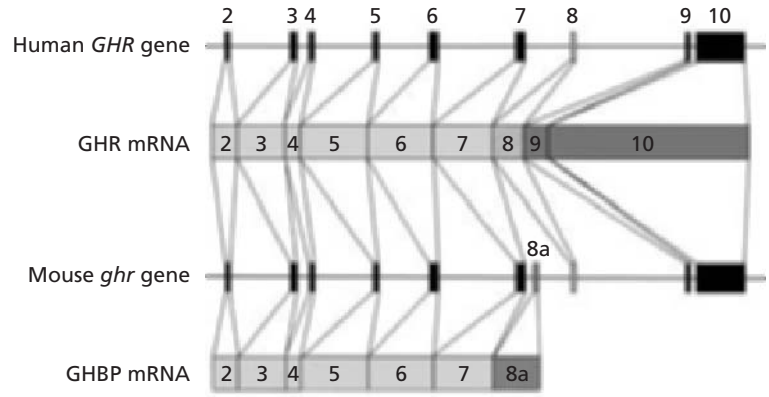

Fig. 8.3 The human and mouse *GHR* genes and their products are shown. In humans and most other species, the only gene product is the growth hormone receptor (GHR). In rodents, an additional embedded exon (exon 8a) encodes the hydrophilic tail of the growth hormone binding protein (GHBP). Splicing exon 8a to exons 2–7 yields the GHBP mRNA (bottom). Splicing exon 8–10 to exons 2–7 yields the GHR mRNA (top). Exon 8a is not spliced to downstream exons because it contains a cleavage/polyadenylation site and lacks a canonical splice donor site at its 3′ end. Exon 1 of the *GHR* gene is not shown because it is not part of the coding region (it encodes the 5′ untranslated region[s]). Light tint denotes the extracellular, darker tint the intracellular domain. denotes the transmembrane domain, the hydrophilic GHBP tail.

cleavability of the mouse GHR is about two orders of magnitude lower than that of the rabbit GHR (G. Baumann, unpublished). *In vivo*, it appears that most if not all of the circulating rat GHBP is derived from the alternative mRNA splicing mechanism (Sadeghi *et al*. 1990). The two different mechanisms of GHBP generation are illustrated in Fig. 8.4.

Rhesus monkeys use both the proteolytic and an alternative mRNA splice mechanism for GHBP generation (Martini *et al*. 1997). In that case, the alternative mRNA encoding the GHBP derives from a readthrough into intron 7, which results in a 7 amino acid 'tail' replacing the transmembrane domain due to an intronic stop codon. It is not known which mechanism predominates in generating the GHBP in the monkey.

The tissue sources of the GHBP are well-defined in the rodent, where the GHBP can be specifically recognized and differentiated from the GHR at both the mRNA and protein level by its unique carboxyterminal tail. GHBP is expressed ubiquitously, and generally is coexpressed with the GHR (Carlsson, B. *et al*. 1990; Lobie *et al*. 1992). However, their expression is not necessarily regulated in a parallel fashion (Walker *et al*. 1992). Of interest, a substantial portion of rodent GHBP remains associated with the plasma membrane (and intracellular membranes) by an as yet unknown link (Frick *et al*. 1994, 1998); it has been suggested that an Arg-Gly-Asp sequence in the GHBP may interact with membrane integrins to provide a tether (Cerio *et al*. 2002). GHBP in the circulation differs from the tissue-associated form in its glycosylation moiety. Membrane-associated GHBP forms have not been described in non-rodent species. The tissue source of the GHBP in species employing the proteolytic shedding mechanism is less clear as it is more difficult to differentiate the GHBP from the GHR. Since both the GHR and TACE are expressed ubiquitously, all tissues can theoretically contribute to GHBP generation. However, the quantitative aspects of GHBP generation by individual tissues are not clearly established. Based on the relative abundance of GHRs in the liver, that organ is generally thought to be a major source. However, it should be noted that this concept has not been directly validated. Studies of venous gradients in visceral organ effluents have not identified a major organ source (Segel *et al*. submitted for publication). It appears likely that multiple tissues contribute

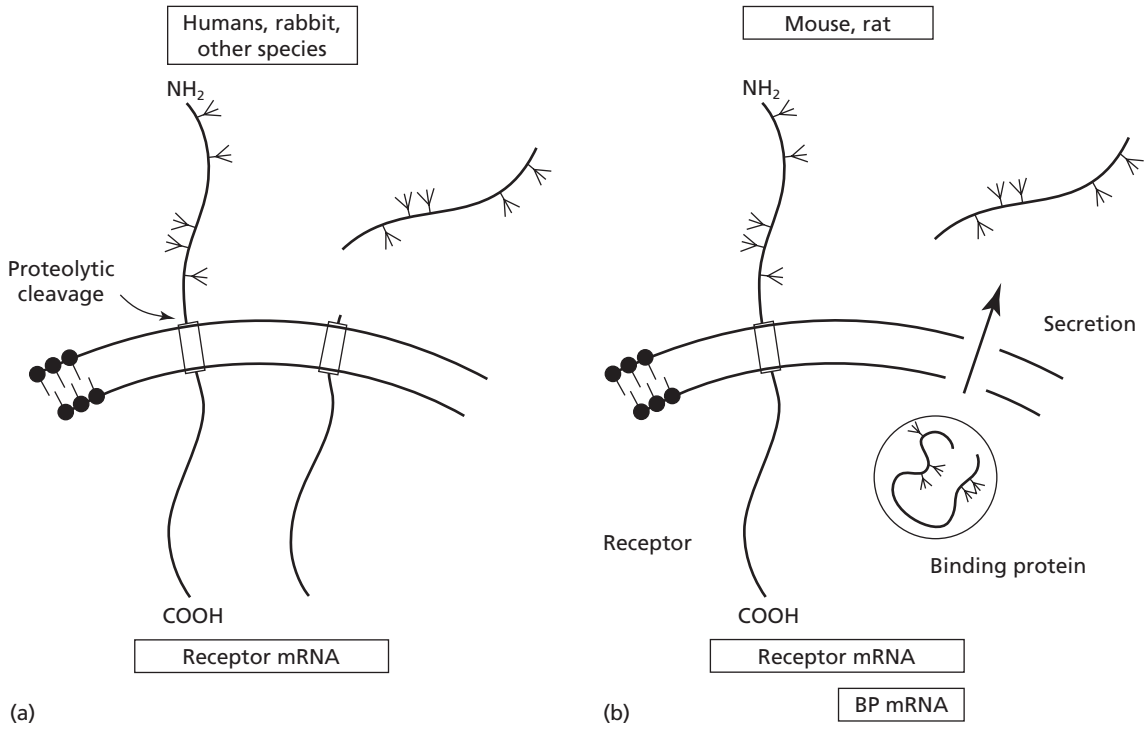

Fig. 8.4 The two mechanisms of growth hormone binding protein (GHBP) generation are shown. (a) The proteolytic shedding mechanism. (b) The splice variant/direct synthesis and secretion mechanism. (Adapted from Baumann, G. (1990) Growth hormone binding proteins. *Trends in Endocrinology and Metabolism* **1**, 342–347. Copyright 1990, with permission from Elsevier.)

to the circulating GHBP pool, though their relative contributions remain to be determined.

Growth hormone binding proteins in biological fluids

The high affinity GHBP is found in blood and most other biological fluids, such as urine, lymph, milk, semen, follicular fluid and amniotic fluid (Hattori *et al*. 1990; Postel-Vinay *et al*. 1991a; Amit *et al*. 1993; Maheshwari *et al*. 1995; Harada *et al*. 1997). Cerebrospinal fluid contains no detectable GHBP (Nixon & Jordan 1986). Unlike in rabbit milk, the GHBP found in *human* milk appears to related to the prolactin receptor rather than the GHR (Mercado & Baumann 1994). The concentration of GHBP in blood varies over a 10-fold range; it is generally present at nanomolar to subnanomolar concentrations. This concentration, together with its affinity, allows the GHBP to act as a buffer and dynamic modulator for circulating free GH. Under physiological and basal conditions, approximately 45% of circulating GH in human blood is bound to the high affinity GHBP (Baumann *et al*. 1988, 1990). This proportion changes dynamically after a GH secretory spike (Veldhuis *et al*. 1993).

GHBP is also present within the cell (Herington *et al*. 1986a; Lobie *et al*. 1991; Frick *et al*. 1994), but the source, destination and function of this intracellular GHBP is not clear.

The *low affinity* GHBP has only been described in blood, where it circulates at micromolar concentrations (Baumann *et al*. 1990; Leung, K.C. *et al*. 2000). In humans, approximately 8% of circulating GH is bound to this GHBP; in rats it can be calculated that about 20% of GH is bound to the low affinity GHBP

(Barsano & Baumann 1989; Baumann *et al*. 1989a; Leung, K.C. *et al*. 2000).

Functional aspects

The principal known function of the GHBPs is to form complexes with GH. Quantitatively, this is more important for the high affinity GHBP than the low affinity GHBP. An indirect 'function' of GHBP generation is the inactivation of GHRs by cleaving and shedding its ectodomain, a process that can be viewed as receptor 'decapitation'. GH binding has multiple consequences. At the local (cellular/tissue) level, GHBP competes with the GHR for GH ligand, the consequence of which is decreased GH action (Fig. 8.5). This effect can be clearly shown *in vitro*, where GHBP inhibits GH binding to GHRs and GH action in a dose-dependent manner (Lim *et al*. 1990; Mannor *et al*. 1991). An additional likely reason for decreased GH action is the formation of unproductive, non-signaling GHR/GHBP dimers (Fig. 8.5). Dimerization and proper GHR dimer conformation is necessary for signal transduction by the GHR. A GHR/GHBP dimer cannot perform that function. Formation of such heterodimers by GH binding would inhibit GH action in a GHBP concentration-dependent fashion. Indeed, this effect has been demonstrated for naturally occurring and mutant forms of the GHR that lack the intracellular domain (Ayling *et al*. 1997; Ross *et al*. 1997; Iida *et al*. 1999). It has not directly been proven that the same phenomenon occurs with soluble GHBP, though this would be predicted. The short GHR, in contrast to the GHBP, contains a transmembrane domain and is membrane-anchored. If the GHR exists in the membrane in a predimerized form even in the absence of GH binding (Ross *et al*. 2001), the membrane-resident short GHR form is not necessarily

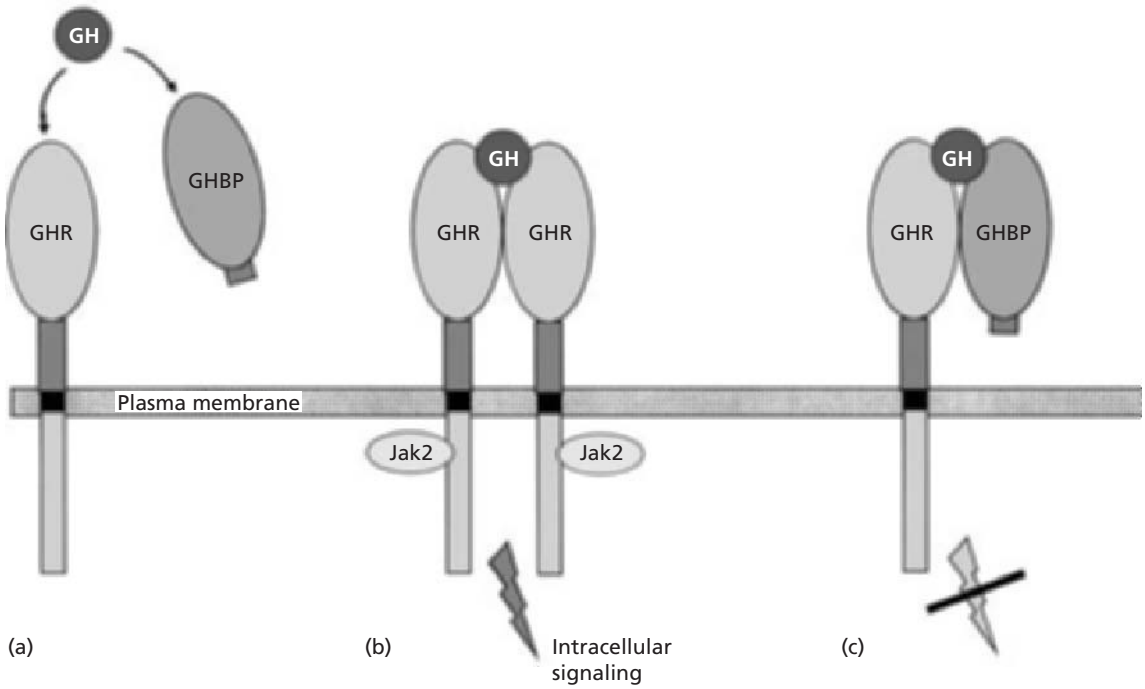

Fig. 8.5 Inhibitory actions of the growth hormone binding protein (GHBP) on GH bioactivity. (a) GHBP competes with the GHR for GH. (b) Normal GH signaling through the growth hormone receptor (GHR). Binding of GH to the GHR induces GHR dimerization or activation of a preformed GHR dimer. The conformationally appropriate GHR-dimer binds Jak2 and initiates intracellular signaling. (c) A GHR–GHBP heterodimer is unable to initiate signaling, with the GHBP acting as a dominant negative inhibitor.

representative for the extracellular, soluble GHBP. Therefore, the concept of GHR/GHBP heterodimers remains to be formally validated.

In contrast to its inhibitory action *in vitro*, the GHBP tends to enhance GH action *in vivo*. GHBP prolongs the plasma half-life of GH through formation of a complex that is too large for efficient glomerular filtration and renal elimination—the principal route of GH clearance (Baumann *et al*. 1987a, 1989b). The complex also diminishes GH clearance through GHR-mediated mechanisms, such as cellular internalization, and delays chemical degradation. In rats, the metabolic clearance of complexed GH is 10-fold lower than that of free GH (Baumann *et al*. 1989b). In humans, the GH–GHBP complex has been estimated to have a plasma half-life of 25–29 min, versus 4–9 min for free GH (Veldhuis *et al*. 1993). The GH–GHBP complex in blood serves as a circulating GH reservoir, which dynamically dampens GH oscillations resulting from secretory pulses. GHBP, when given in large doses, has been shown to enhance GH bioactivity *in vivo*, despite its inhibitory actions *in vitro* (Clark *et al*. 1996). The net effect of the high affinity GHBP on GH action in the intact organism is thus complex, concentration- and compartment-dependent, and difficult to predict.

Little is known about the effect of the low affinity GHBP on GH action. Based its low affinity, it is likely to form a loose complex with GH that easily dissociates. Therefore, it probably has only a limited impact on GH dynamics and GH action.

Regulation of growth hormone binding protein production

In species that generate GHBP by the proteolytic mechanism, GHBP production depends both on GHR expression and on the regulation of TACE activity. Both the GHR and TACE are expressed ubiquitously. GHR expression is regulated by developmental stage, gender, species, metabolic state, and differentially by tissue. Little is currently known about the regulation of TACE activity. In rodents, GHBP production is linked to expression of the GHBP mRNA variant. This is also regulated in a complex, tissue and metabolic state-dependent manner, and no systematic studies exist that comprehensively address this issue. Because of these limitations, the following discussion is primarily focused on the regulation of GHBP levels in serum.

In humans, the principal factors affecting serum GHBP as part of normal physiology are development, gender, aging and nutrition. For reasons that are not known, serum GHBP concentrations in normal subjects vary over a 10-fold range (~ 0.3–3.0 nmol) (Rajkovic *et al*. 1994; Maheshwari *et al*. 1996); it is not clear whether this normal variablity has biological significance. There is no significant diurnal variation in serum GHBP (Snow *et al*. 1990; Carmignac *et al*. 1992; Carlsson, L.M. *et al*. 1993), but a minor seasonal variation with nadir in August has been reported in children (Gelander *et al*. 1998). GHBP levels are very low in the fetus, rise rapidly during early childhood, stay constant through adolescent and adult life, and decline in old age after the age of 60 years (Daughaday *et al*. 1987; Holl *et al*. 1991; Martha *et al*. 1993; Maheshwari *et al*. 1996). A similar ontogenetic pattern is present in the rat (Mulumba *et al*. 1991). GHBP levels are higher in females than males, both in humans and more markedly in rodents (Massa *et al*. 1990; Hattori *et al*. 1991; Rajkovic *et al*. 1994). This is probably in large part an estrogen effect. Maternal GHBP levels during pregnancy show a very marked species difference. In humans, there is only a small increase in GHBP levels in early pregnancy (Blumenfeld *et al*. 1992), whereas in mice, GHBP levels in serum (and membrane-associated GHBP in the liver) show a very large increase (Cramer *et al*. 1992; Camarillo *et al*. 1998). It is the latter phenomenon that led to the first description of the GHBP (Peeters & Friesen 1977). Rats also increase their GHBP during pregnancy, though to a lesser degree than mice (Frick *et al*. 1998). An important factor regulating GHBP levels is nutrition. Malnutrition decreases and overnutrition increases serum GHBP; and a highly significant correlation exists between body mass index and GHBP levels, and especially between visceral fat and GHBP levels (Hochberg *et al*. 1992; Martha *et al*. 1992; Roelen *et al*. 1997b). These changes parallel those seen for IGF-I levels and probably reflect the effect of insulin on GHR expression and consequently GHBP levels (Baxter & Turtle 1978; Mercado *et al*. 1992; Kratzsch *et al*. 1996).

GH up-regulates GHBP in rodents (Sanchez-Jimenez *et al*. 1990; Carmignac *et al*. 1992), but data on this subject in humans are variable and inconsistent (see Baumann 2001 for review). It may be concluded from this that GH does not have a major effect on GHBP levels in humans. Of interest, acromegaly, a disease of chronic GH excess, is associated with low to low normal GHBP in most studies (Amit *et al*. 1992; Roelen *et al*. 1992; Mercado *et al*. 1993; Kratzsch *et al*. 1995a; Fisker *et al*. 1996). This is not necessarily a direct function of GH, but may be a result of other adjustments occurring in acromegaly. Thyroid hormone up-regulates GHBP levels (Amit *et al*. 1991; Romero *et al*. 1996). Estrogens, especially when given by the oral route, increase GHBP levels in humans and rodents, but lower serum GHBP in the rabbit (Weissberger *et al*. 1991; Carmignac *et al*. 1993; Yu *et al*. 1996). Androgens lower serum GHBP levels (Postel-Vinay *et al*. 1991b; Keenan *et al*. 1996; Yu *et al*. 1996). Glucocorticoids lower GHBP in humans and rodents, but increase GHBP in rabbit blood (Heinrichs *et al*. 1994; Miell *et al*. 1994; Gabrielsson *et al*. 1995). Insulin up-regulates GHBP levels (Mercado *et al*. 1992; Massa *et al*. 1993; Kratzsch *et al*. 1996), whereas IGF-I, in a single report, was shown to lower GHBP levels (Silbergeld *et al*. 1994).

Exercise and physical training have an effect on plasma GHBP levels. Acute exercise, such as cycle ergometry, induces a short-lived and mild increase in GHBP (Wallace *et al*. 1999). Chronic endurance or fitness training has been shown to lower serum GHBP by 10–40% in most studies (Roemmich & Sinning 1997; Eliakim *et al*. 1998b, 2001; Scheett *et al*. 2002), but at least one study showed a small rise in GHBP (Roelen *et al*. 1997a). GHBP is inversely related to peak oxygen uptake and fitness (Eliakim *et al*. 1998a). This is in part linked to the above-mentioned relatienship between adiposity and GHBP. The physiological significance of these exercise/training related GHBP changes is not fully understood.

Growth hormone binding protein and disease

Several pathological conditions are associated with abnormal serum GHBP levels. In most cases, the change in GHBP parallels altered GH sensitivity; the GHBP level is therefore thought to mirror GHR abundance in tissues. Foremost among the disorders with abnormal GHBP is the genetic GH insensitivity syndrome due to inactivating mutations in the *GHR* gene (Laron syndrome), which results in severe growth retardation and dwarfism (Rosenfeld *et al*. 1994). Absence or malfunction of the GHBP is generally a direct result of the mutant GHR, which is either not expressed (e.g. gene deletions, nonsense mutations), prematurely degraded or not properly directed to the plasma membrane (e.g. some missense mutations), or unable to bind GH (certain missense mutations) (see Baumann 2002 for an updated listing of known *GHR* mutations). Absence of GHBP activity in the serum of patients with Laron syndrome was the first strong indication that the GHBP is a GHR fragment (Baumann *et al*. 1987b; Daughaday & Trivedi 1987). About 80% of cases with Laron syndrome have low or undetectable GHBP in their blood (Woods *et al*. 1997). The others have either normal serum GHBP or in rare cases even elevated GHBP. The underlying *GHR* mutations in GHBP-positive cases include those that prevent proper receptor dimerization or lack the intracellular signaling domain (Duquesnoy *et al*. 1994; Ayling *et al*. 1997; Kaji *et al*. 1997; Iida *et al*. 1998; Gastier *et al*. 2000). A mutant receptor lacking the transmembrane helix results in massively elevated serum GHBP activity, which represents the mutant, soluble GHR rather than the normal GHBP (Woods *et al*. 1996; Silbergeld *et al*. 1997).

Several disorders associated with acquired GH insensitivity are also characterized by abnormally low GHBP levels. Catabolic conditions, such as malnutrition, insulinopenic uncontrolled diabetes, post-traumatic states and critical illness are examples of acquired GH-resistance, characterized by low IGF-I levels despite normal or elevated GH secretion. Growth failure may occur in severe cases (Mauriac syndrome, a condition of poorly controlled diabetes, hepatomegaly and growth retardation) (Mandell & Berenberg 1974; Mauras *et al*. 1991). The fact that serum GHBP is decreased in GH-resistant states lends credence to the concept that serum GHBP concentration reflects tissue GHR abundance. In animal models, catabolic states are

associated with decreased hepatic GHR levels and GH resistance (Baxter & Turtle 1978; Postel-Vinay *et al*. 1982; Massa *et al*. 1993). Upon resolution of the underlying disease process, GH sensitivity, GHR expression and GHBP levels return to normal. The changes in GHR expression and consequent GHBP release are thought to be largely mediated by insulin (Mercado *et al*. 1992; Hanaire-Broutin *et al*. 1996).

The counterpart of GH resistance, GH hypersensitivity, is associated with elevated GHBP levels. The only well-recognized condition in this category is overnutrition/obesity, which is characterized by normal or elevated IGF-I levels despite suppressed GH secretion. It has long been known that overweight children grow faster than lean children (Forbes 1977). Obesity is associated with elevated serum GHBP levels, probably reflecting increased tissue GHR expression (Hochberg *et al*. 1992; Kratzsch *et al*. 1997a; Roelen *et al*. 1997b). Thus, the biochemical parameters as well as the functional aspects of the GH–IGF axis in obesity are the exact opposite from those in malutrition.

With regard to the low affinity GHBP, little is known about changes in its serum level in either normal physiology or in disease.

Assays for the growth hormone binding protein (Figs 8.6 and 8.7)

The classical assay for both the high and the low affinity GHBPs measures their functional property of radiolabeled GH binding, with separation of bound from free GH by size exclusion chromatography (Baumann *et al*. 1986; Herington *et al*. 1986b). This assay is quantitative under most circumstances because under physiological conditions the GHBPs in serum are largely unoccupied. Correction for occupation of the high affinity GHBP by endogenous GH must be made above a GH concentration of 10 ng·mL^{-1} (Baumann *et al*. 1989a). Variations of this fundamental GH binding assay employ other methods for separation of bound from free GH, such as charcoal or immunoprecipitation with anti-GHR antibodies (Barnard *et al*. 1989; Amit *et al*. 1990;

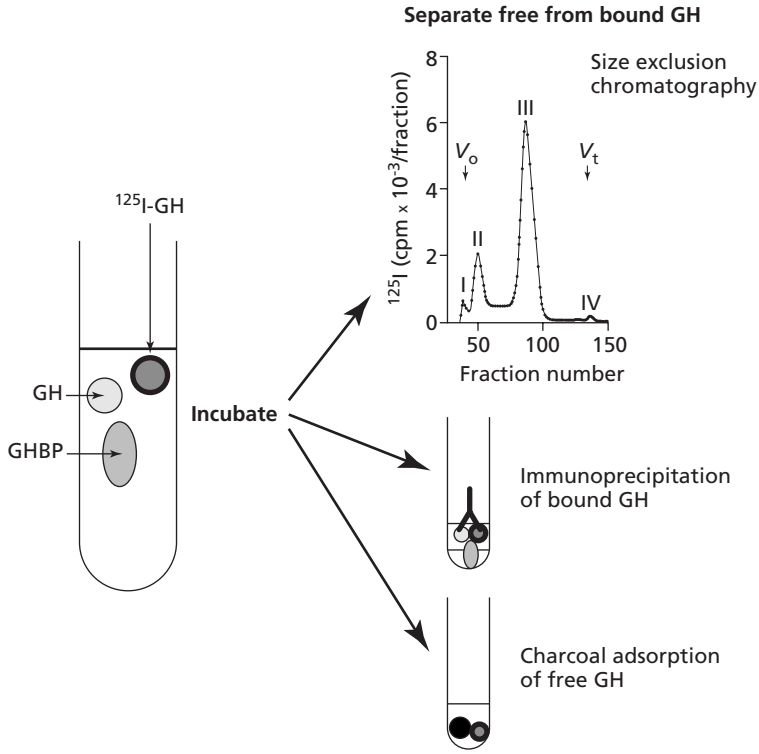

Fig. 8.6 Growth hormone binding protein (GHBP) assays based on the principle of GH binding. To serum containing GHBP and endogenous GH radioiodinated GH is added, and GHBP–GH complex formation is allowed. Bound GH is then separated from free GH by either size exclusion chromatography (top), immunoprecipitation with anti-GH antibodies (center), or adsorption of free GH with activated charcoal (bottom). The assay is quantitative because under most conditions, GHBP is essentially unoccupied by endogenous ligand. At high endogenous GH levels (> 10 ng·mL^{-1}), correction for GHBP saturation by unlabeled GH is necessary. Different variations of this binding assay exist, using other modes of separating free from bound GH.

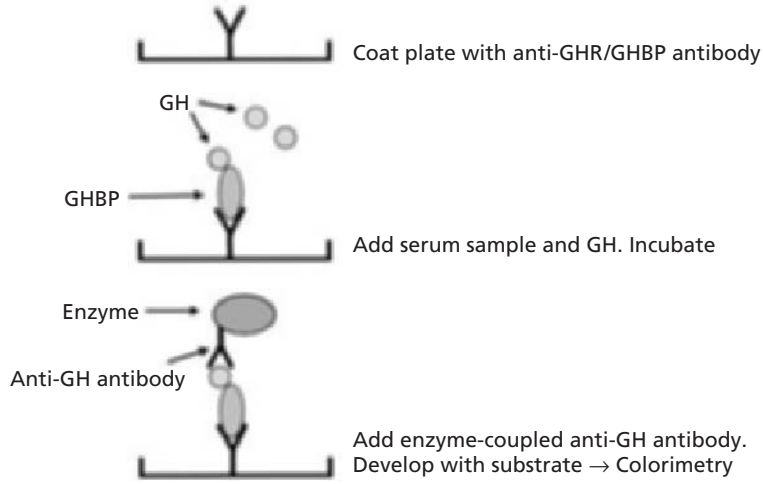

Fig. 8.7 Ligand-mediated immunofunctional assay (LIFA) for growth hormone binding protein (GHBP). This is a solid-phase, sandwich-type, two-site assay. A monoclonal antibody directed against the extracellular domain of the growth hormone receptor (GHR) (the GHBP), yet not interfering with its GH-binding site, is adsorbed to a microtiter plate. After washing, serum and an excess of exogenous GH are added, and formation of a ternary complex consisting of the antibody, GHBP and GH is allowed to form. After washing, an anti-GH-antibody coupled to an enzyme (e.g. horseradish peroxidase) is added. The antibody recognizes the solid-phase complex. Signal amplification is then achieved by the enzymatic reaction, using a color-yielding substrate.

Ho *et al*. 1993). Specific assays that differentiate the GHBP from the GHR have been devised by using antibodies directed against the unique hydrophilic tail of the rodent GHBP (Barnard *et al*. 1994); this strategy cannot be used for GHBP in species that use the proteolytic shedding mechanism for GHBP generation (e.g. humans, rabbits). A two-site sandwich-type assay with enzyme linked immunosorbent assay (ELISA) design ('ligand-mediated immunofunctional assays [LIFAs]') has been developed for the human high affinity GHBP (Carlsson, L.M. *et al*. 1991). Its results correlate well with the conventional GH-binding assay, but for unknown reasons the absolute GHBP values are lower than those obtained in other assays (Mercado *et al*. 1993). One assay with classical radioimmunoassay design (radiolabeled GHBP tracer and anti-GHBP antiserum) has been reported for human GHBP (Kratzsch *et al*. 1995a). This type of assay is independent of GH binding and able to measure dysfunctional GHBP with abnormal GH binding properties (such as mutant GHBP in certain cases of Laron syndrome). A radioimmunoassay specific for the exon 3-containing human GHBP has also been developed (Kratzsch *et al*. 1997b). Other assays with variations on these methodological themes have been reported for several species, including human, rat and mouse GHBP. Unfortunately, relatively little information is available about the correlation among these assays. Commercial assays are available, but also suffer from a relative lack of crossvalidation with established assays. GHBP measurement is still primarily a research tool; its main practical application in clinical medicine is in the diagnosis of GH insensitivity (Laron) syndrome.

No standardized assays for the low affinity GHBP are available. This plasma component has been measured by GH binding assay followed by size exclusion chromatography (Baumann *et al*. 1989a; Tar *et al*. 1990) or immunoprecipitation with anti-α_2-macroglobulin antibody (Kratzsch *et al*. 1995b).

Effect of the high affinity growth hormone binding protein on growth hormone measurement in serum

The high affinity GHBP can interfere with GH immunoassays in serum because of competition with

antibodies for GH. In general, antibodies, especially when polyclonal, have higher affinity for GH than the GHBP. Nevertheless, depending on the assay design, interference can be significant. Assays particularly vulnerable are those employing relatively low affinity monoclonal anti-GH antibodies, quick turnaround assays that are run under brief incubation, non-equilibrium conditions and insensitive assays employing a high serum volume. The non-equilibrium assay design is problematic because time is required to transfer GHBP-bound GH from the GHBP to the antibody. Reports of interference in GH assays by the GHBP range from negligible to significant (Jan *et al*. 1991; Chapman *et al*. 1994; Jansson *et al*. 1997; Fisker *et al*. 1998). It is important to carefully examine each GH immunoassay for GHBP interference as not all factors responsible for interference are known, and assay methodology should be optimized to minimize GHBP effects.

Conclusions

Two GHBPs exist in blood and other biological fluids. The high affinity GHBP is the ectodomain of the GHR; it is either shed from cells by the action of TACE, a member (ADAM-17) of the ADAM family of metalloproteinases, or directly secreted (in rodents) as a soluble GHR mRNA splice variant. Cleavage by TACE occurs in the juxtamembranous, extracellular stem region of the GHR. The GHBP has complex effects on GH blood transport, GH clearance and GH action, with both enhancing and inhibitory modulation of GH bioactivity. The physiological significance of the GHBP for the somatotropic axis is still incompletely understood. Serum GHBP levels appear to reflect GH responsivity of the organism, presumably by reflecting GHR status in tissues. Regulation of serum GHBP levels is complex and in part variable among species; the principal regulators are ontogeny and development, nutrition, gender/estrogen, and in rodents pregnancy. Diagnostic use of the high affinity GHBP is currently limited to genetic GH insensitivity (Laron) syndrome. GHBP can interfere in GH immunoassays, and GH assays need to be optimized in this regard.

The low affinity GHBP is transformed α_2-macroglobulin. It appears to have limited importance for GH biology.

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Chapter 9

Resistance Exercise: Acute and Chronic Changes in Growth Hormone Concentrations

WILLIAM J. KRAEMER, BRADLEY C. NINDL AND SCOTT E. GORDON

Introduction

Resistance exercise is the most prolific form of exercise producing anabolic effects in muscle and connective tissues (Kraemer *et al*. 1996). Human growth hormone (GH), also called somatotropin, a pleiotropic polypeptide, exerts a multitude of effects upon the metabolic state of the human body, in part mediating a myriad of metabolic and growth processes. The complexity of pituitary release and production of GH, aggregates, and binding proteins is just beginning to be elucidated with the interpretation of the literature on resistance exercise and training remaining a puzzle with parts of the picture still requiring study (Nindl *et al*. 2003). Nevertheless, understanding the responses and adaptations of the 22 kDa GH will continue to play an important part of our understanding of the adaptations to resistance training. The *hGH-N* gene expresses the main pituitary molecular weight variant in the GH family which is the 22 kDa form. This protein is 191 amino acids in length (monomeric 22 kDa represents ~ 21% of all circulating plasma GH). The next most prevalent form is the 20 kDa molecule (monomeric 20 kDa represents ~ 6% of all circulating plasma GH) formed through alternative mRNA splicing during which amino acid residues 32–46 are cleaved out. Human GH can also undergo post-translational modification and peripheral tissue proteolytic cleavage at its site of action to form variants and aggregates (i.e. dimers, trimers, pentamers) and fragments which exist in the circulation. The existence of high- and low-affinity GH binding proteins (released from the pituitary and also cleaved from the extracellular domain of the GH receptor) adds further complexity to the nature and spatial arrangement of circulating GH moieties. Thus, within the limitations for the scope of this chapter we will examine some of the basic responses and adaptations of the 22 kDa GH to resistance exercise and training.

Growth hormone release and control

Many pituitary hormones are under hypothalamic control. The hypothalamic hormones (e.g. releasing factors) growth hormone releasing hormone (GHRH) and somatostatin (SS) serve to stimulate and inhibit GH release, respectively. These releasing factors are secreted from neurons originating in the arcuate, periventricular and paraventricular nuclei (i.e. hypothalamic nuclei) whose axon terminals extend toward the median eminence. The releasing factors are delivered into a portal system of veins which acts as a humoral pathway for direct delivery to the anterior pituitary. The portal blood system originates from capillary loops in the median eminence of the tuber cinereum and blood drains in a parallel fashion down the pituitary stalk. In the anterior pituitary, the portal blood vessels break up into the sinusoids which provide the nutrient supply. This vascular supply is very small and the capillary permeability is quite high. In this manner, releasing factors from the hypothalamus may reach secreting cells (i.e. somatotrophs) of the anterior pituitary (Fig. 9.1). Functionally distinct types (i.e. type I and II or band 1 and band II cells) of somatotrophs have been identified using density gradient

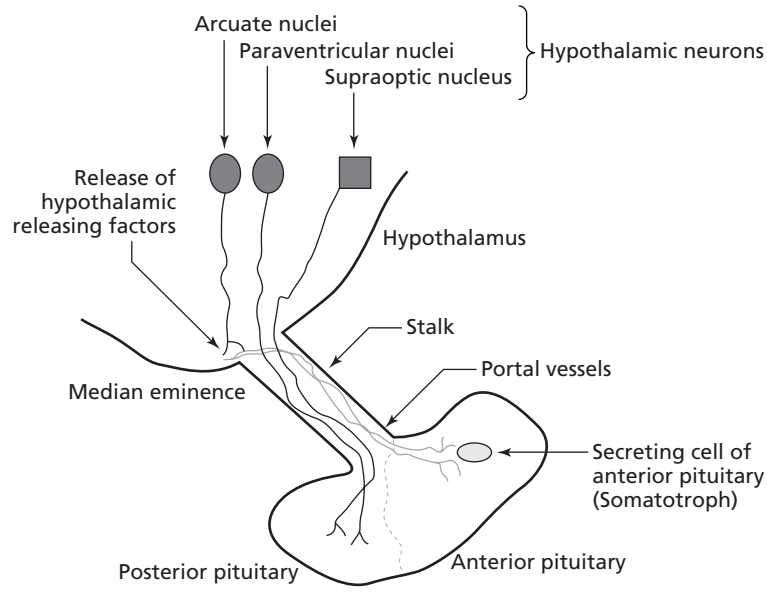

Fig. 9.1 Hypothalamic–pituitary neural interface regulating growth hormone (GH) secretion.

centrifugation. The somatotrophs in these two fractions can be distinguished morphologically by their staining characteristics and ultrastructure, and have been shown to vary in their responsiveness to GH secreting stimuli with the type I being more responsive (Snyder *et al*. 1977). The physiological significance of this cellular heterogeneity and whether exercise can differentially influence these cells remains unknown, but presents a provocative area of future study as the complexities of exercise-induced release and adaptations interact with the heterogeneity of the different molecular weight forms of GH and GH aggregates.

The central mechanisms underlying pulsatile GH release are classically thought to involve elevated secretion of GHRH into the hypophysial portal blood during troughs or nadirs of SS secretion. The precise molecular mechanisms responsible for this pulsatility remain speculative. Superimposed on this basic mechanism are other control and modulatory mechanisms that determine somatotroph responsiveness at any give point in time. There is evidence that GHRH affects both GH biosynthesis and release, and SS inhibits release without affecting biosynthesis. It is also thought that GHRH is required for the initiation of GH pulses, while SS dictates the amplitude of the pulse. SS control over GH pulse amplitude is supported by studies that have reported that the GH response to GHRH is enhanced by administration of SS antagonists (viz. pyridostigmine [an acetylcholinesterase inhibitor] and hexapeptide GH-releasing peptides) (Cappa *et al*. 1993). The cloning of a GH secretagogue receptor has also demonstrated that the GH secretagogue, the previously elusive endogenous ligand of the receptor (recently identified as ghrelin), is part of a new physiological regulating system in GH secretion.

It has been well established that GH is secreted from the anterior pituitary gland in an episodic, *pulsatile* manner throughout the day with a dramatic surge of release during slow-wave sleep (Fig. 9.2). This pulsatile release is under the regulatory control of the two hypothalamic hormones that serve to stimulate (i.e. growth factor releasing hormone) or inhibit (i.e. SS) GH release. The balance between these two hormones determines the relative magnitude of initial release from the anterior pituitary.

It does appear that GH can exert acute negative feedback on its own release. When subjects are administered a single dose of GH, subsequent GH responses to GHRH are diminished or abolished (Scanlon *et al*. 1996). This 'somatotroph desensitization' mechanism can be reversed by prior activation of cholinergic pathways. Currently, it appears that

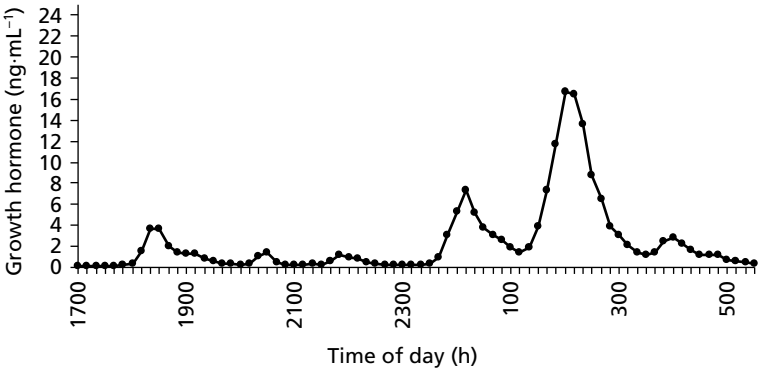

Fig. 9.2 Example 13-h growth hormone (GH) pulsatility profile for a young health male. Note that the greatest pulse amplitude occurred during sleeping hours (approximately 0200 h).

alterations in GH release are mediated through an inhibition of SS secreting cells via cholinergic pathways (Giustina & Veldhuis 1998). As noted above SS inhibits GH release, but not its synthesis. This is an important concept as it may explain 'rebound' GH secretion after SS priming and withdrawal (Giustina & Veldhuis 1998).

There are many neuromodulators (i.e. neuropeptides, neurotransmitters, physiological conditions) that have putative roles in regulating GH secretion via GHRH and SS have been an intense area of regulatory mechanism research. Veldhuis *et al*. (2004) have postulated 'that sex-steroid-specific control of SS and GHRH outflow may mediate the former gender contrasts, whereas unknown (gender-independent) factors may determine the capability of exercise to significantly antagonize GH autoinhibition'. Table 9.1 lists a summary of these known modulators. These factors act to stimulate (α_1-adrenergic, amino acids, dopamine, muscarinic cholinergic, GABA(-B), galanin, growth hormone releasing peptide (GHRP), histamine, hypoglycemia, neuromedin C, opiates, serotonin, diabetes, acute and chronic exercise, starvation and stress) or inhibit (α_1-adrenergic, GHRH immunization cortisol/glucocorticoids, glucose, hypothyroidism, obesity, aging) GH release. Peripheral feedback regulation of the somatotroph is mediated by the array of GH target actions. Namely, insulin-like growth factor I (IGF-I), glucose and free fatty acids can each exert feedback influences at hypothalamic/pituitary levels. The array of multivariate neuroregulatory factors shown in Table 9.1 that influence GH release emphasize the concept that GH release is a complex issue. The interplay between exercise and many of these factors in the control of GH release is not fully understood.

Growth hormone release patterns

Two unique characteristics of GH are its pulsatile release and the degree of molecular heterogeneity. The *GH-N* gene expresses the main pituitary molecular weight variant in the GH family, which is the 22 kDa form. This protein is 191 amino acids in length with two disulfide cross-linkages (monomeric 22 kDa represents ~ 21% of all circulating plasma GH). The next most prevalent form is the 20 kDa molecule (monomeric 20 kDa represents ~ 6% of all circulating plasma GH) formed through alternative mRNA splicing during which amino acid residues 32–46 are cleaved out. Human GH can also undergo post-translational modification and peripheral tissue proteolytic cleavage at its site of action to form variants and aggregates (i.e. dimers, trimers, pentamers) and fragments which exist in the circulation. The existence of high- and low-affinity GH binding proteins (released from the pituitary and also cleaved from the extracellular domain of the GH receptor) adds further complexity to the nature and spatial arrangement of circulating GH moieties. This molecular heterogeneity appears to have physiological significance as the different forms have been shown to possess different biological activities (e.g. relative potency in bioassays) as well as their ability to be detected in immunoassays. The contents of the band I and band II secretory cells in the pituitary can impact the

Table 9.1 Neuroregulatory modulators of growth hormone (GH) secretion in humans.

Effector	Effect
Adrenergic (α_1, α_2 & β_2)	No effect, ↑, ↓
Age	↓
Amino acids	↑
Autofeedback at hypothalamus by IGF-I	↓
Bombesin	No effect basally, ↓ hypoglycemia effect
Dopamine	↑
Muscarinic (chlolinergic & nicotinic)	↑ & ↓
Cortisol/glucocorticoids	↓
Diabetes mellitus (type 1 & type 2)	↑ & ↑ or ↓
Estrogen	↑ Amplitude
Excitatory amino acids	Unknown
Exercise (acute & chronic)	↑ & ↑
Fatty acids	↓
GABA(-B)	↑ Basal ↓ (stim)
Galanin	↑
GHRP	↑
Glucose	↓
Histamine	↑
Hypoglycemia	↓
Hypothryrodisim	↓
IGF-I (pituitary inhibition)	Yes
Immunization (or antagonist) (GHRH & SS)	↓ Amplitude & unknown
Leptin	Inversely correlated with GH
Neuromedin C	Unknown
Neuropeptide Y	↓?
Nitrc oxide	No effect
Obesity	↓
Opiates	↑
Senescence/aging	↓
Serotonin	↑
Starvation	↑
Stress (shock, restraint, endotoxin, psychological)	↑
Testosterone	↑
TRH	No effect
DHT	No effect

DHT, dihydrotestosterone; GHRH, growth hormone releasing hormone; GHRP, growth hormone releasing peptide; GNRH, growth hormone releasing hormone; IGF-I, insulin-like growth factor I; SS, somatostatin; TRH, thyrotropin releasing hormone.

profile of the composition of the circulatory concentrations of various GH isoforms and may eventually provide the insights into the vast array of physiological mechanisms related to GH, yet not explained by the 22 kDa molecule alone (Hymer *et al*. 2001).

Growth hormone secretion

Many factors affecting GH secretion including age, gender, diet and nutrients, stress, other hormones (e.g. gonadal steroids, thyroid hormones and IGF-I), adiposity, fitness level and exercise have all been implicated as factors that influence GH concentrations in the blood (see Fig. 9.1). Since the initial observations of Hunter *et al*. (1965), it is well recognized that physical activity is a naturally occurring stimulator of GH release into the circulation. GH has been linked to the promotion of anabolism in both muscle and connective tissue. Specifically, it enhances cellular amino acid uptake and protein

synthesis in skeletal muscle, resulting in hypertrophy of both muscle fiber types (Noall *et al*. 1957; Ullman & Oldfors 1989; Crist *et al*. 1991). Cartilage growth and bone deposition can also be stimulated by GH, increasing bone mineral density (BMD) and other markers of bone formation (Isaksson *et al*. 1990; van der Veen & Netelenbos 1990; Parfitt 1991; Bikle *et al*. 1995; Orwoll & Klein 1995). While debatable with the re-emergence of the larger family of GH(s) and GH binding proteins, classic dogma has proposed that many of the anabolic effects of GH are mediated via IGF-I secreted by the liver and other tissues (Florini *et al*. 1996). While this may be true if GH is only defined as the 22 kDa isoform, such a paradigm may have to be re-examined if the GH superfamily of polypeptides and binding proteins are now considered. Nevertheless, exercise-induced GH release is in part responsible (either directly or indirectly) for the anabolic effects of exercise. Furthermore, there is evidence in rats that GH may stimulate the autocrine/paracrine production of IGF-I by skeletal muscle, cartilage, and bone cells themselves (Turner *et al*. 1988; Isaksson *et al*. 1990; Bikle *et al*. 1995). Lastly, GH may interact both directly and indirectly with androgens (Jørgensen *et al*. 1996), estrogens (Holmes & Shalet 1996) and thyroid hormone (Weiss & Refetoff 1996) with respect to secretion and target tissue actions.

Resistance exercise-induced changes in growth hormone concentrations

It has become apparent over the past 15 years that different exercise protocols will result in different concentrations of GH. To understand the composite factors that may mediate such differential response to the myriad of different exercise protocols possible with resistance training, one must look at some of the key factors that result from choices made in the acute program variables in workout design (Fleck & Kraemer 2004). It is also apparent that interaction of various exercise factors is vital in determining the magnitude of the GH response. The key external factors that interact and produce an increased concentration of GH in the circulatory biocompartment are:

1 The amount of muscle mass recruited.
2 The resistance loading used in the exercise.
3 The volume of exercise performed.
4 The amount of rest between sets and exercises.

The activation of an adequate amount of muscle tissue is vital to increase plasma concentrations of GH. The amount of tissue that is activated is influenced by the resistance used, the total work and the type of exercise performed (e.g. small muscle group exercise vs. large muscle group exercise). The first data supporting this paradigm were observed by Vanhelder *et al*. (1984). In their study, GH significantly increased above rest after performing seven sets with a resistance of 85% of the 7-repetition maximum (RM) in the squat exercise. However, when the resistance was reduced to 28% of the 7-RM while keeping rest periods the same and equating the total work, no changes in circulating concentrations of GH were observed after the exercise protocol. Based on 'size principle', the number of motor units required to perform the 28% load was less than needed for the 85% load. Thus, activation of enough tissue appears to be a vital element of the exercise stimulus to elicit a significant GH response.

The volume of exercise or total work has also been implicated in the magnitude of response. When each of the acute program variables—choice of exercise, order of exercise, rest periods, resistance used—are kept constant and only when the number of sets performed is increased thereby producing more total work, is the response of GH increased. This effect has been demonstrated in both men and women using whole body, multiexercise resistance protocols and 10-RM loadings (Mulligan *et al*. 1996; Gotshalk *et al*. 1997). In addition, a higher level of strength fitness can also allow an individual to perform greater amounts of total work resulting in a higher GH response as well (Ahtiainen *et al*. 2003). Little is known about the thresholds of total work to elicit an increase in GH in the circulation, but likely would be interactive with the other elements of a workout protocol (e.g. length of rest periods amount of tissue activated, and resistance used).

The interactive effects of different choices made for the acute program variables (i.e. choice of exercise [e.g. large or small muscle groups], order of exercise [e.g. large muscle group first or small muscle groups first], rest period length [short duration

or long duration], resistance used [e.g. 5-RM or 10-RM] and number of sets or total work [low J or high J]) in designing a resistance exercise session can result in a different GH response. While the combinations that could be used are many, prior research has shown that when comparing multi-exercise, total body resistance protocols the three factors that appear to impact GH most dramatically are the combination of high total work, short rest period length (1 min between sets and exercise) and a moderately heavy 10-RM resistance in both men and women (Kraemer *et al*. 1990, 1993).

Since any resistance training session uses a wide array of different combinations of the acute exercise variables, GH responses are a function of these acute program variable choices. These appear to be the underlying governing principles for the creation of the exercise stimulus in resistance training. The most dramatic finding related to these variable structures and GH is the impact they have on acid-base balance, which in turn appears to have a major role in stimulating the release of GH into the circulation. Each of the above four factors can be manipulated to produce an effect on metabolism that either limits or promotes the accumulation of hydrogen ions and decreases in blood pH, which in turn account for almost half of the shared variance with GH production. This makes acid-base decreases (i.e. increase in ATP hydrolysis, decrease in pH, increase in hydrogen ion) prime determinants of the amount of 22 kDa isoform in the circulation (Gordon *et al*. 1994). Reductions in the rest periods between sets of exercises with whole body workouts have been shown to produce the most dramatic increases in lactate responses with resistance exercise (Kraemer *et al*. 1990, 1993). However, reducing the rest period length will also impact the amount of resistance that can be lifted (Kraemer *et al*. 1987). Thus, there appears to be a crucial modulation of the amount of resistance used and then amount of tissue activated that drives the GH response. However, Takarada *et al*. (2000) has shown that occlusion of the arm can have a dramatic effect on GH resulting in significant increases with relatively low intensity (20% of 1-RM) while no changes in GH were observed without occlusion. One may conclude that for the 22 kDa molecule, hypoxia and disruption of acid-base balance plays a major regulatory role in the stimulation of GH (Sutton 1983). Resistance exercise workouts that have short rest periods (1 min rest between sets and exercises, moderate intensities [8–10-RM load ranges] and whole body workout protocols [8–10 exercises]) can produce such physiological demands and results in the most dramatic GH changes in the blood (Fig. 9.3).

The majority of these studies have examined the short-term recovery responses (typically < 2 h) of GH. The impact of GH pulsatile release at different times of day and GH roles over different phases of recovery from resistance exercise remains to be elucidated. McMurray *et al*. (1995) presented data which utilized a resistance exercise protocol as the

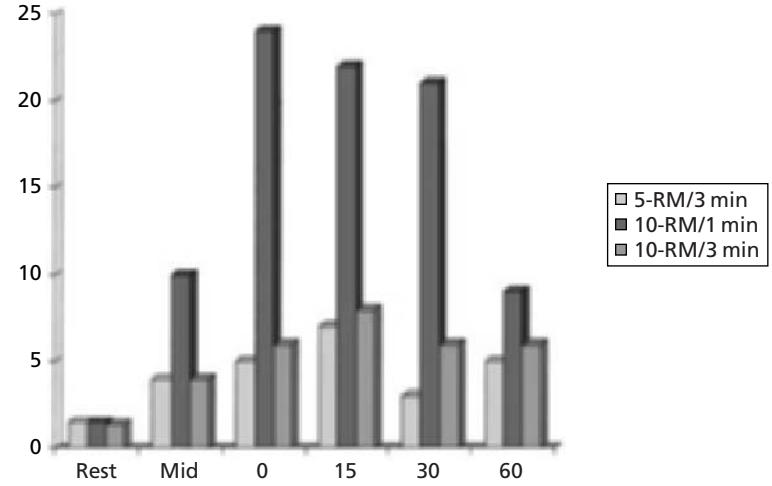

Fig. 9.3 GH responses to different rest period lengths with different resistance exercise protocols. The 10/1 post-exercise responses $p < 0.05$ from the other protocols at each time point. All post-exercise concentrations are significantly ($P < 0.05$) above resting values.

exercise perturbation on nocturnal GH responses. The exercise protocol consisted of performing 3 × 6–8-RM sets of six different exercises (18 sets total). Blood samples were obtained from 2100 to 0700 h. The investigators did not observe any effect of resistance exercise on nocturnal GH release. Conversely, Nindl *et al*. (2001) examined GH pulsatile GH release over a longer period of time. The acute heavy resistance exercise protocol began at 1500 h and was designed to be a high-volume workout that included 50 total sets and recruited and activated a large amount of muscle tissue. Blood was sampled every 10 min from 1700 to 0600 h under control and resistance exercise conditions. The results of the study demonstrated that heavy resistance exercise in the late afternoon decreased overnight maximum GH concentrations and GH pulse amplitude; however, overall mean GH concentrations were not significantly reduced. Acute heavy resistance exercise differentially influenced the temporal pattern of the overnight release because GH was lower during the first half of sleep, but greater during the second half for the exercise versus control conditions. The mean maximum GH concentration and mean pulse amplitude were lower in the exercise vs. the control condition.

Several possible explanations for this finding were speculated in that such results could be mediated by an increase in SS tone after exercise. Although SS inhibits GH release, it does not negatively affect GH biosynthesis. This may be an important concept because it may explain a 'rebound' in GH secretion after SS priming/withdrawal. The nocturnal peaks were lower for the exercise vs. control conditions during 2300–0300 h, but higher during 0300–0600 h. Thus, even though the mean GH concentration was similar between the control and exercise conditions, the temporal pattern of GH release was clearly influenced by daytime exercise. From a mechanistic perspective, the acute heavy resistance exercise bout may have resulted in an elevated SS tone during 2300–0300 h. During this time, GH release was inhibited to some degree and concurrently; GH biosynthesis was not inhibited. At ~ 0300 h, this SS tone was withdrawn, and GH molecules biosynthesized and stored during the time when GH release was inhibited were then available and readily released. These data also demonstrated that enhanced IGF-I inhibitory feedback on pituitary GH release was unlikely because serum IGF-I concentrations did not differ between the control and exercise conditions. It is well know that an array of other metabolic and hormonal signals, such as changes in GHRH, hexapeptides, or Ghrelin release, could also have mediated the observed GH response.

Influence of age

It has been shown that the acute GH response is somewhat limited in older individuals (Craig *et al*. 1989; Pyka *et al*. 1992; Kraemer *et al*. 1999). A major factor contributing to this limited GH response maybe the magnitude of exertion displayed and the inability to perform as much total work in an exercise protocol. It was reported in an investigation by Pyka *et al*. (1992) that lower blood lactate concentrations in the elderly during resistance exercise supported the theory that the amount of effort exerted in a resistance exercise session may impact the resulting GH response. With the negative influence of aging on buffering capacities and toleration of acidosis a combination of factors could help explain the reductions in exercise-induced GH following acute resistance exercise (Godfrey *et al*. 2003). Short-term training (10–12 weeks) does not appear to alter this response (Craig *et al*. 1989; Kraemer *et al*. 1999). Ten-weeks periodized resistance training program showed no significant changes in GH for resting or exercise-induced concentrations for younger or older subjects (Kraemer *et al*. 1999). The lactate response to the acute resistance exercise was lower in the older than in the younger men with use of the same relative resistance, while increases in lactic acid do not change the acid-base balance (Robergs *et al*. 2004), it acts as an indication of reduced metabolic demands and a lower disruption of acid-base status. This may partially explain the lower post-exercise GH values in older individuals. Reductions observed in resting and exercise-induced concentrations of the 22 kDa GH concentrations do not appear to be changed with short-term training (Fig. 9.4).

There is only a rudimentary understanding of the effects of exercise and aging on the physiological mechanisms underlying 'somatopause' (i.e. the

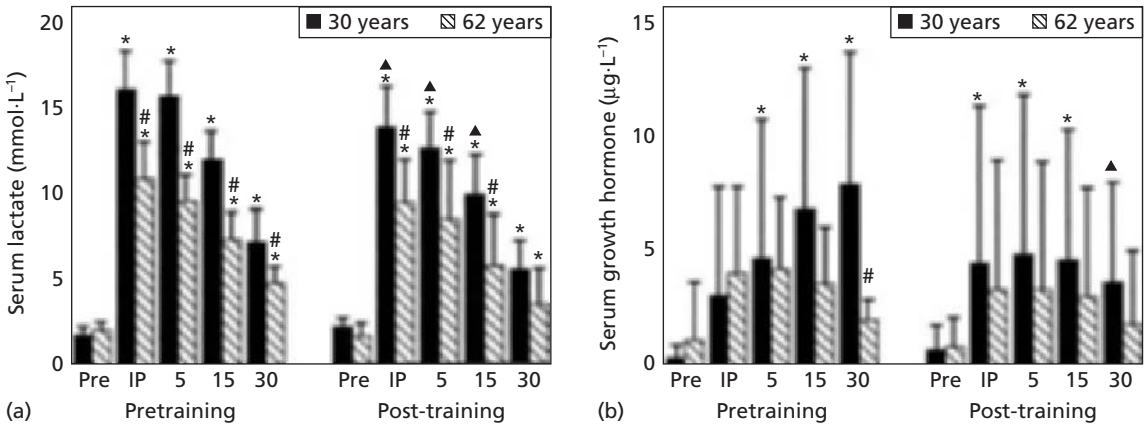

Fig. 9.4 Responses (means + SD) of lactate (a) and growth hormone (b) after acute heavy resistance exercise test (AHRET) before and after 10 weeks of periodized strength and power training for 30-year-old and 62-year-old men. *, Significantly different ($P \leq 0.05$) from corresponding pre-exercise value. #, Statistically significant difference ($P \leq 0.05$) between 30-year-old and 62-year-old men. ▲, Significantly different ($P \leq 0.05$) from corresponding pretraining value.

decrease within the GH–IGF-I system). Because this endocrine axis is considered to be of great importance in maintaining the integrity of the musculoskeletal system, it is conceptually pragmatic to suggest that resistance exercise regimens should attempt to target it. Keeping in mind that GH also exhibits a great deal of molecular heterogeneity, and the standard radioimmunoassay (RIA) focuses on the 22-kDa variant, this is an especially important point for future studies, when it is considered that the higher molecular weight species of GH could possess greater biological activity and that the lack of changes or reductions in the immunoreactive GH may not present the complete picture of the adaptational responses of GH variants to resistance exercise training. In other words, measurement of just the 22 kDa isoform may not tell the whole story of the adaptive response of pituitary secretions of GH.

Training adaptations

To date, resistance training does not appear to affect resting concentrations of the 22 kDa GH isoform. Surprisingly, no changes in the resting concentrations of GH have been observed in response to resistance training in men and women of various ages (Kraemer *et al*. 1999; McCall *et al*. 1999; Häkkinen *et al*. 2000; Marx *et al*. 2001). Even long-term training in competitive lifters and body builders have shown the same responses of the 22 kDa GH molecule and show no changes in the hypopituitary axis for resting concentrations (Häkkinen *et al*. 1988; Ahtiainen *et al*. 2003) when compared to untrained or lesser trained individuals. These findings are consistent with dynamic feedback mechanisms and pulsatility of the GH molecule and the many roles it may play in the homeostatic control of other metabolic and repair processes. McCall *et al*. (1999) did observe a correlations between resting GH and the magnitude of type I and type II muscle fiber hypertrophy (r = 0.62 to 0.74, respectively). These relationships could be indicative of a role for repeated acute resistance exercise-induced GH elevations on cellular adaptations in trained muscle. Changes in receptor sensitivity, differences in feedback mechanisms, IGF-I potentiation, binding protein interactions, stimulation of other molecular weight variants in the pituitary somatotrophs as well as diurnal variations may all interact to mediate 22 kDa GH responses.

Gender effects

The exercise-induced responses of women compared to men appear to be similar in the absolute magnitude of the value achieved but the relative changes from rest are smaller (Kraemer *et al*. 1991).

This also impacts the acute exercise responses. Some protocols (e.g. 5-RM, 3 min rest periods or low total work protocols) which may only marginally elevate GH concentrations in the circulation in men result in no significant exercise-induced elevations in women due to the higher resting concentrations (Kraemer *et al*. 1991, 1993). How such lower magnitudes of GH responses to acute exercise influence the adaptations of target tissues remains unknown.

It has been postulated that women's higher resting concentrations potentially compensate for lower levels of other anabolic hormones which may minimize the role of the acute elevation with exercise stress. These have been observed in the early follicular phase of the menstrual cycle. Our recent data indicate that estrogen in the form of oral contraceptives may have minimal effects on the GH response to resistance exercise response (unpublished data). While overt influences of the menstrual cycle may mediate some of these responses, differential patterns of pituitary GH release may also explain the gender differences as the GH mass and mode of GH secretion may be regulated differently in men and women (Pincus *et al*. 1996).

Specificity of exercise

One of the underlying principles in biological science as well as a principle of resistance training is 'specificity'. This may be seen when examining the response of GH to resistance exercise as well. In a study by Kraemer *et al*. (2001) four different groups of subjects trained for 19 weeks performing all concentric repetitions, concentric repetitions with double the volume, or a typical program of concentric and eccentric repetitions or no training (control group). Subjects performed two exercise tests consisting of three sets of 30 isokinetic concentric contractions in one testing session and three sets of 30 isokinetic eccentric contractions with knee extensions. The tests were separated by 48 h. The acute response to a concentric stimulus was similar, but when an eccentric protocol was performed the group that had trained with the typical concentric and eccentric contraction style had the highest GH response to the eccentric challenge, indicating sensitivity to the specific eccentric stimulus that was contained in the type of repetitions they were performing with training. With detraining the responses were muted to a similar degree across all groups. These data indicate that GH secretion may be sensitive to the specific muscle actions used during resistance training. Such a response is supported by the relatively new construct as it has been shown that the anterior pituitary may be directly innervated by nerve fibers mostly with synapses on corticotroph and somatotroph cells (Ju 1999). It has also been suggested that 'neural–humoral' regulation of GH secretion may take place such that a rapid neural response is observed during the initial stress with the humoral phase subsequently occurring (Ju 1999). If this is the case, then it may be possible for higher brain centers (e.g. motor cortex) to play an active role in regulating GH secretion during resistance exercise and this regulatory mechanism may be sensitive to specific muscle actions used during resistance training.

Physiological impact

Due to the many actions of GH and its complexity as a biological effector, its influence on skeletal muscle hypertrophy and other tissue anabolism has just started to be dissected beyond simplistic direct and indirect hypotheses. A multitude of important interacting factors are thought to contribute to resistance training-induced skeletal muscle hypertrophy, and some investigators have raised the possibility that the pronounced rise in blood GH concentration elicited by acute heavy resistance exercise may be included with these mechanisms. This hypothesis is supported by observations that optimal heavy resistance training-induced skeletal muscle hypertrophy is compromised in hypophysectomized rats unless synthetic or pituitary-extracted GH is administered (Goldberg & Goodman 1969; Grindeland *et al*. 1994). However, directly contradicting this theory is the fact that an acute bout of aerobic exercise also produces an equally large increase in blood GH concentrations as an acute bout of heavy resistance exercise while aerobic exercise training has little or no net anabolic effect on skeletal muscle tissue (Kraemer *et al*. 1995). Therefore, assuming the GH response to exercise *is* important to muscle tissue hypertrophy

and other tissue anabolism, it may be speculated that acute heavy resistance exercise and acute aerobic exercise differ not in their effect on serum *immunoreactive* GH concentrations but in their effect on serum *bioactive* GH concentrations, an hypothesis that has recently received much attention (Hymer *et al*. 2001). Furthermore, the difference may be due to activation of different motor neuron pools and subsequent sequence of receptor events that are in part stimulated by the electromechanical activation process of activated muscle fibers. Alternatively, immunoreactive GH may stimulate pituitary release of higher molecular weight binding proteins and aggregates into the circulation; each of these hypotheses remains to be substantiated with direct evidence (Nindl *et al*. 2003).

In addition, the pulsatile release of GH is important for mediating linear growth. The growth process is episodic and discontinuous, whether measured during a period of a day or over longer periods of time. For example, in rodents, linear growth is highest when GH secretory bursts are separated by about 3 h of very low GH levels, as in the case in the male rat, and is reduced when GH levels show small deviations around a relatively baseline, as in the case of the female rat. Thus, sexually dimorphic mechanisms regulating the pulsatile release of GH between males and females (high amplitude GH pulses and low interpulse [GH]) characterize GH profiles in males, whereas female rats have less regular pulses and higher interpulse [GH] are partly responsible for differences in growth rate (Slob & Van der Werff Ten Bosch 1975; Jaffe *et al*. 1998). Additionally, the temporal pattern of GH release is also coupled to key enzymes responsible for longitudinal bone growth and it may be important to view the GH release on a pulsatile basis rather than a single static measure and this is well illustrated by considering the sexual dimorphism in GH release.

However, there are some animal data to suggest that exercise-induced GH is important for somatic and muscle growth. Using the Hamster model, Katarina Borer has pioneered work into the role of GH in exercise modulated growth by demonstrating that exercised golden hamsters (an animal continual growth model) exhibited increased basal pulsatile GH, skeletal elongation and permanent increase in body mass and reductions in fat mass when compared to sedentary controls (Borer 1989, 1995). Since the pulsatile release of GH is an integral component of agonist function, it would appear that GH pulsatility in humans cannot be ignored as well as the heterogeneity of the GH super family of molecules and aggregates. However, these were female hamsters and development takes place only at certain windows of a developmental time period leading to maturation.

Summary

Acute resistance exercise can be a potent stimulus for the 22 kDa GH isoforms with increases related to the type of exercise protocol utilized. Training appears to have no significant impact on resting concentrations of these isoforms in men or women of different ages. However, changes in pulsatile release with acute exercise may be one mechanism by which an altered secretory profile may be mediated and such systems appear to differ between men and women. Feedback loops and interactions with other hormonal systems (e.g. IGF, GH binding proteins, stimulation of molecular aggregates) remain complex and an avenue for future research as it relates to target tissue responses. It is evident that the responses and adaptations of GH will become a more integrated variable as the physiological context for its function and roles becomes further delineated.

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Chapter 10

The Growth Hormone Response to Acute and Chronic Aerobic Exercise

ARTHUR L. WELTMAN, LAURIE WIDEMAN, JUDY Y. WELTMAN AND

Introduction

Growth hormone (GH) is secreted by the anterior pituitary in a pulsatile pattern. Multiple GH isotypes and oligomers exist in plasma in addition to the predominant 22 kDa protein (Baumann 1991; Lewis *et al*. 2000; Nindl *et al*. 2003). Minor isoforms do not change uniquely in response to exercise. GH activates cells by dimerizing receptors and triggering a cascade of phosphorylation reactions that signal to the nucleus.

The amount of GH secreted in each pulse is under physiological control by peptidyl agonists and antagonists (Arvat *et al*. 1998; Mueller *et al*. 1999; Farhy *et al*. 2001; Bowers 2002). Brain (hypothalamic) growth hormone releasing hormone (GHRH) stimulates GH synthesis and secretion and somatostatin (SS) inhibits GH release without affecting its synthesis (Giustina & Veldhuis 1998; Hartman 2000). A growth hormone releasing peptide (GHRP), ghrelin, expressed in the stomach, anterior pituitary gland and hypothalamus amplifies GH secretion via cognate receptor codistributed with the peptide (Giustina & Veldhuis 1998; Kojima *et al*. 1999; Hartman 2000). Transgenic inactivation of central–neural GHRP receptors reduces GH secretion by one-third in the mouse (Shuto *et al*. 2002). These three effector molecules govern GH secretion by convergent mechanisms (Veldhuis & Bowers 2003b). Many of the metabolic effects of GH are mediated by insulin-like growth factor I (IGF-I), which is synthesized in the liver and all nucleated cells under the control of GH and tissue-specific hormones (Giustina & Veldhuis 1998).

GH secretion declines by approximately 14% per decade after the age of 40 years (Rudman *et al*. 1981; Zadik *et al*. 1985; Iranmanesh *et al*. 1991) and is markedly reduced in obesity, even in younger individuals (Veldhuis *et al*. 1991, 1995). Whereas GH production falls by 50% every 7 years in men beginning in young adulthood (Iranmanesh *et al*. 1991, 1998; Veldhuis *et al*. 1995), the decrease is nearly twofold less rapid in premenopausal women (Asplin *et al*. 1989; Winer *et al*. 1990; Weltman, A. *et al*. 1994; van den Berg *et al*. 1996). Many age-related physical adaptations resemble those recognized in GH-deficient adults, including reduced muscle mass and exercise capacity, increased body fat especially abdominal visceral fat, unfavorable lipid and lipoprotein profiles, reduction in bone mineral density and cerebro and cardiovascular disease. Which is cause and which is effect is difficult to ascertain, in that intra-abdominal adiposity and limited exercise also predict reduced GH production (Vahl *et al*. 1997; Clasey *et al*. 2001). Below we highlight the effects of exercise on GH release. Several comprehensive reviews discuss other physiological factors (Veldhuis 1996a, 1996b; Veldhuis *et al*. 1997; Giustina & Veldhuis 1998; Hartman 2000; Veldhuis & Bowers 2003a, 2003b).

Acute aerobic exercise and growth hormone secretion

Aerobic exercise stimulates GH release within approximately 15 min and induces peak values at or near the end of exertion (Lassarre *et al*. 1974; Sutton & Lazarus 1976; Kozlowski *et al*. 1983; Raynaud *et al*.

1983; Bunt *et al*. 1986; Chang *et al*. 1986; Felsing *et al*. 1992; Luger *et al*. 1992; Weltman, A. *et al*. 1992, 1994; Cappon *et al*. 1994; Chwalbinska-Moneta *et al*. 1996; Pritzlaff *et al*. 1999, 2000; Wideman *et al*. 1999, 2000a). The intensity and duration of the aerobic stress, physical fitness, gender and age all influence the GH response to exercise. Although the threshold of exercise intensity was envisioned earlier (Chang *et al*. 1986; Felsing *et al*. 1992; Chwalbinska-Moneta *et al*. 1996), randomly ordered separate day studies affirm that exercise intensity predicts GH secretion in a linear dose–response fashion (Pritzlaff *et al*. 2000; Pritzlaff-Roy *et al*. 2002).

Women maintain higher GH concentrations than men at all ages, and manifest less orderly patterns of pulsatile GH release (Hartman *et al*. 1990; Veldhuis 1995; van den Berg *et al*. 1996; Pincus *et al*. 1996; Engstrom *et al*. 1998; Giustina & Veldhuis 1998; Wideman *et al*. 1999). The time course of exercise-induced GH release is similar by gender (Lassarre *et al*. 1974; Bunt *et al*. 1986), but women differ in several specific features; viz: anticipatory GH release before exercise and more rapid attainment of peak GH concentrations during exercise (Wideman *et al*. 1999, 2000a; Pritzlaff-Roy *et al*. 2002). The effect of exercise is largely independent of circadian rhythmicity, since the time of day does not influence the responses, at least in young men (Kanaley *et al*. 2001). Young women and men achieve equivalent absolute GH concentrations during exercise (Wideman *et al*. 1999), but the fractional increase over baseline is higher in men (Bunt *et al*. 1986; Wideman *et al*. 2000a).

Figure 10.1 illustrates the impact of gender on exercise intensity-dependent GH release in young adults (Pritzlaff *et al*. 1999; Pritzlaff-Roy *et al*. 2002). In this study, men and women each undertook six randomly ordered sessions (one control resting [C] and five exercise conditions [Ex]). Exercise comprised 30 min of treadmill running at one of the following graded intensities (normalized to the individual lactate threshold [LT]): 25% and 75% of the difference between LT and rest (0.25 LT and 0.75 LT, respectively), LT, and 25% and 75% of the difference between LT and $\dot{V}O_{2peak}$ (1.25 LT and 1.75 LT, respectively). GH responses to exercise increased progressively with increasing exercise intensity (Pritzlaff *et al*. 1999; Pritzlaff-Roy *et al*. 2002). By simple linear regression analysis, women had a higher intercept value (greater baseline GH secretion) and slope (accentuated sensitivity) to aerobic exercise (Fig. 10.2).

The extent that these acute relationships apply to graded chronic exercise training intensities is not known.

Exercise stimulates less GH secretion in healthy older individuals than in young individuals (Weltman, A. *et al*. 2000b, 2001). Analogous exercise intensity–GH response comparisons indicate that aging in man attenuates the graded effectiveness of exercise (slope term by 3.9-fold). An unresolved question raised by these outcomes is whether higher relative training intensities would be able to drive young adult-like GH release in older men (Weltman, A. *et al*. 2000b).

Exercise stimulates GH secretion 5.7–7.3-fold less in post-menopausal women than in premenopausal women, whether or not post-menopausal individuals were receiving hormone replacement (Marcell *et al*. 1999; Weltman, A. *et al*. 2001). Plausible mechanistic bases for the consistent deficit in GH secretion in the older adult include excessive SS release and diminished GHRH or possibly ghrelin drive. In fact, prolonged stimulation with GHRH or a synthetic GHRP (ghrelin surrogate) will elevate GH secretion over 1–3 months in the elderly individual (Evan *et al*. 2001; Richmond *et al*. 2001).

The GH response to exercise may also be blunted in middle-aged men (Zaccaria *et al*. 1999). This inference derives from study of a small group of young (N = 5, age = 21 years) and middle-aged (N = 7, age = 42 years) men who undertook incremental exercise (50 watts every 3 min) until volitional exhaustion (Fig. 10.3).

Middle-aged men evidence lower and delayed peak GH release. If verified, diminutive GH responsiveness before later life would encourage investigations of earlier interventional strategies to maintain favorable GH drive of anabolism.

Relative adiposity and frank obesity suppress baseline and stimulated GH production (Veldhuis *et al*. 1995; Weltman, A. *et al*. 2000b; Weltman, J.Y. *et al*. 2002). When compared to various pharmacologic and physiologic stimuli, the magnitude of the

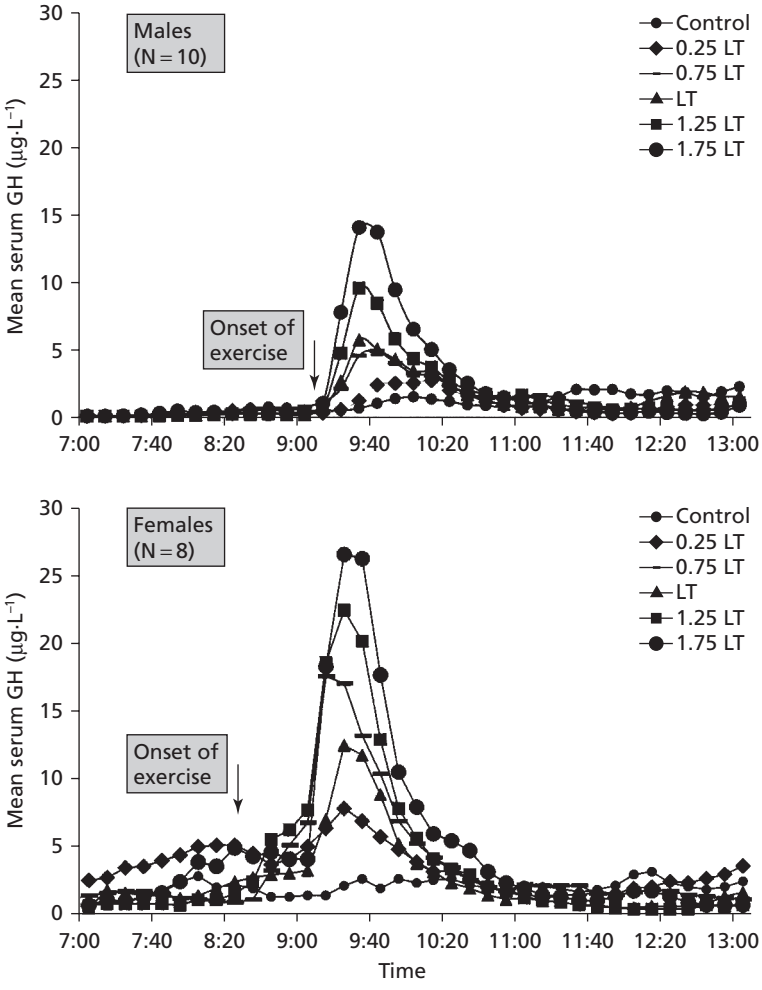

Fig. 10.1 Mean serum growth hormone (GH) concentrations monitored by sampling blood every 10-min for 6 h during rest (Control), and graded exercise defined as fractions (0.25, 0.75, 1.0, 1.25 and 1.75) of the individual lactate threshold (LT). Values are the mean ± SEM (N = 10 young men and eight young women). (From Pritzlaff *et al*. 1999 and Pritzlaff-Roy 2002, used with permission.)

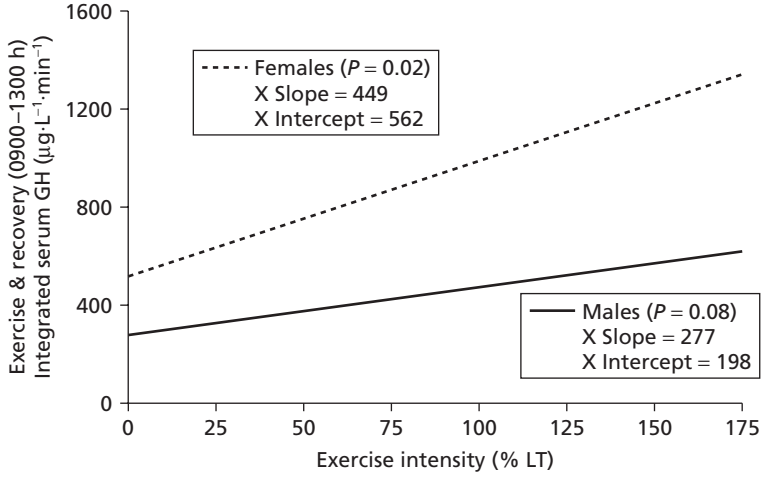

Fig. 10.2 Impact of gender on baseline (unstimulated) and exercise-induced growth hormone (GH) secretion assessed over a graded range of intensity (see Fig. 10.1). The higher slope of this relationship in women denotes a greater sensitivity of the GH response to exercise, and the elevated intercept signifies higher baseline (rest) GH secretion. (From Pritzlaff-Roy 2002, used with permission.)

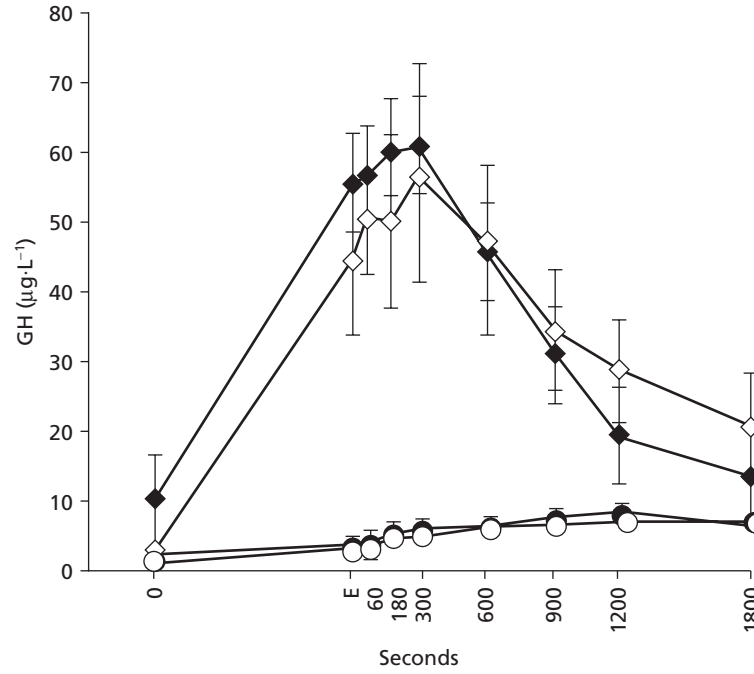

Fig. 10.3 Growth hormone (GH) responses to maximal exercise at exhaustion (E) and during recovery in young (diamonds) and middle-aged (circles) athletes before (open) and after (filled) a 4-month period of training (Adapted from Zaccaria *et al*. 1999.)

GH response to exercise in obese subjects may exceed that induced by L-dopa or clonidine (Cordido *et al*. 1990; Tanaka *et al*. 1990), but not necessarily GHRH, pyridostigmine or L-arginine (Williams *et al*. 1984; Cordido *et al*. 1990, 1993; Maccario *et al*. 1997; Kelijman & Frohman 1998). Only GHRP and combined secretagogues remain moderately (but not maximally) effective (Cordido *et al*. 1990, 1993; Maccario *et al*. 1997). Limited analyses of the interaction among gender, obesity and exercise suggest that abdominal visceral fat may be a key determinant of stimulated GH release (Clasey *et al*. 2001). Kanaley *et al*. (1999) examined exercise induced GH secretion in cohorts comprising of non-obese, lower-body obese and upper-body obese (e.g. viscerally obese) women (Fig. 10.4).

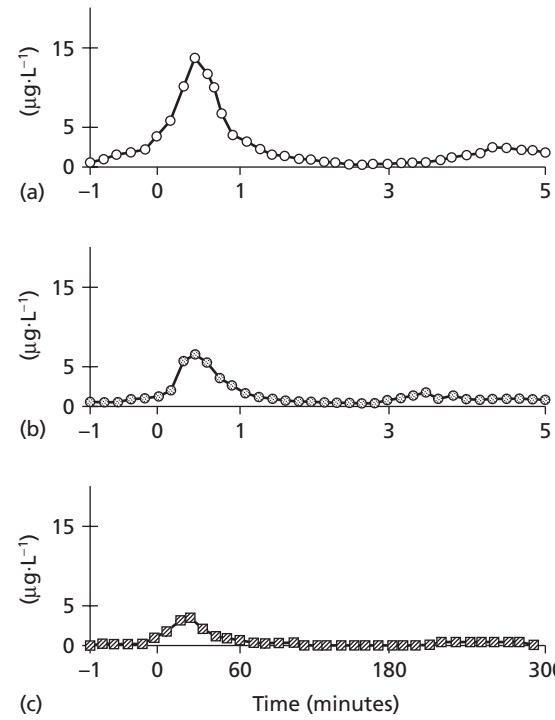

Fig. 10.4 (*right*) Mean serum growth hormone (GH) concentrations for non-obese (a), lower body obese (b), and upper body obese (c) groups over the 6-h period of blood sampling. Exercise began at time zero and continued for 30 min at an intensity of 70% $\dot{V}O_{2peak}$. (Adapted from Kanaley *et al*. 1999.)

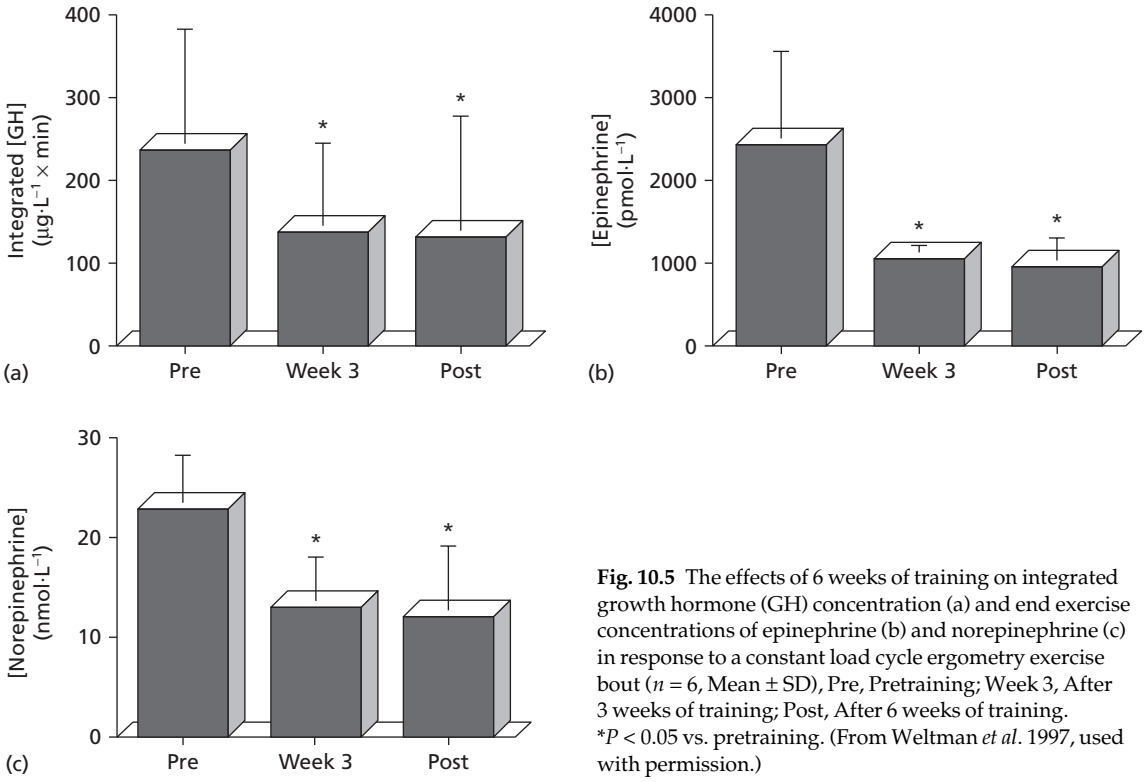

Fig. 10.5 The effects of 6 weeks of training on integrated growth hormone (GH) concentration (a) and end exercise concentrations of epinephrine (b) and norepinephrine (c) in response to a constant load cycle ergometry exercise bout ($n = 6$, Mean ± SD), Pre, Pretraining; Week 3, After 3 weeks of training; Post, After 6 weeks of training. *$P < 0.05$ vs. pretraining. (From Weltman *et al*. 1997, used with permission.)

The rank order of peak GH concentrations was 13.7 μg·L^{-1} (non-obese), 6.8 μg·L^{-1} (lower body obese) and 3.5 μg·L^{-1} (upper body obese). Definitive radiological measurement of adipose-tissue topography will be needed to verify these anthropometric inferences.

Chronic endurance training and growth hormone release

Sustained endurance training limits acute exercise-induced GH release at the same absolute intensity (Hartley *et al*. 1972; Weltman, A. *et al*. 1997). In

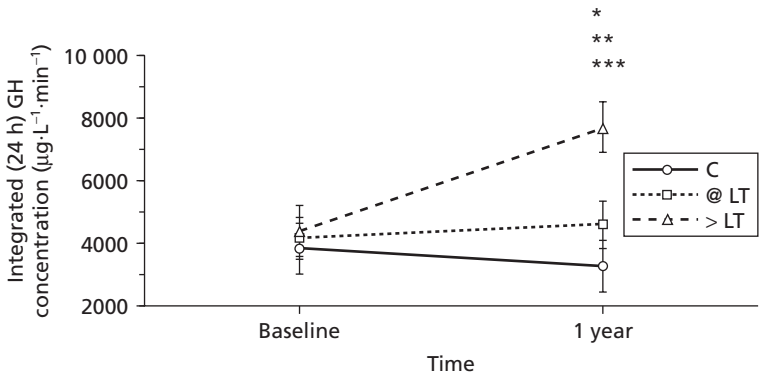

Fig. 10.6 Effects of 1-year of run training on 24-h integrated serum growth hormone (GH) concentrations. *1 year > baseline in > lactate threshold (LT) group ($P < 0.05$); **at 1 year > LT group > control (C) group ($P < 0.05$); ***at 1 year > LT group > @LT group ($P < 0.05$). (From Weltman *et al*. 1992, used with permission.)

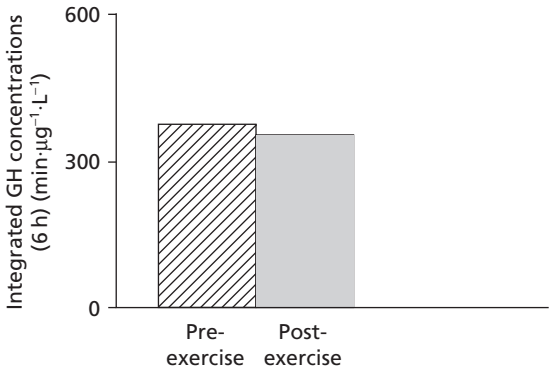

Fig. 10.7 The 6-h integrated serum growth hormone (GH) concentrations in 10 obese subjects before and after 16 weeks of aerobic exercise in response to an exercise bout at 70% $\dot{V}O_{2peak}$ for 30 min. (Adapted from Kanaley *et al*. 1999.)

young men, response down-regulation is evident within the first 3 weeks of training (Fig. 10.5) (Weltman, A. *et al*. 1997).

On the other hand, total (24-h) GH secretion appears to increase in trained individuals even on non-training days when some aerobic training was targeted at an intensity above the lactate threshold (Fig. 10.6) (Weltman, A. *et al*. 1992).

How healthy aging, gender and increased adiposity modulate long-term training effects is not yet established. In one study in older men (ages 50–78 years), serum IGF-I concentrations did not differ between marathon runners and age-matched sedentary subjects (Deuschle *et al*. 1998). In another investigation, 1 year of exercise training (either supervised aerobic training [4 days a week] or supervised strength training [3 days a week]) in healthy older (aged 59–79 years) adults did not alter 24-h integrated GH concentrations (Hartman *et al*. 2000). Apparent unresponsiveness might be due to: (i) an insufficient training stimulus; (ii) lack of change in percentage body fat and abdominal visceral fat which correlate negatively with GH release and increase with age; and/or (iii) intrinsically diminished responsiveness of the aging GH–IGF-I axis.

Sixteen weeks of aerobic training in obese women yielded a significant training effect (e.g. increased $\dot{V}O_{2peak}$), but did not alter GH responses to acute exercise at the same relative intensity (Fig. 10.7) (Kanaley *et al*. 1999). However, training for 4 months did not affect body weight, fat weight and fat free weight (estimated by skinfolds and bioelectrical impedance). Whether daily GH secretion rates increase in this context is not known. Indeed, the precise relationship between acute exercise-stimulated

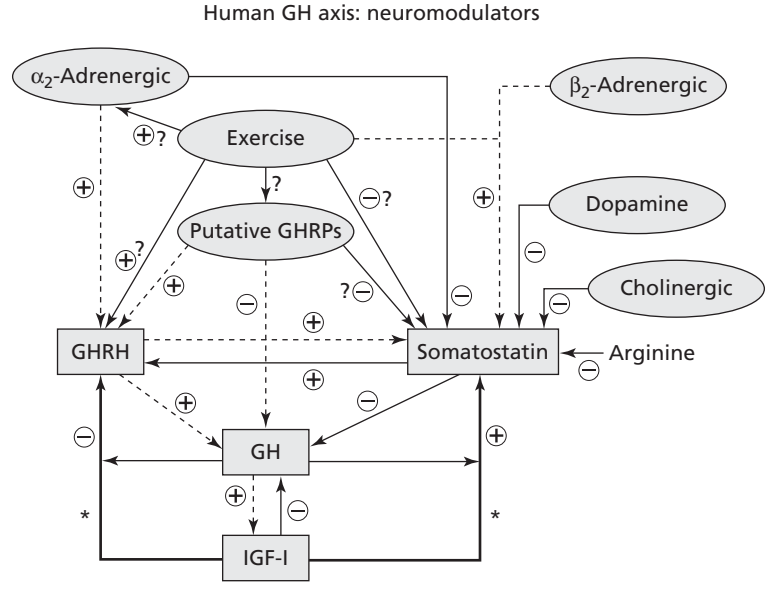

Fig. 10.8 Schematic representation of the possible interactions or mechanisms that control exercise-induced growth hormone (GH) release. *, indicates the possibility that exercise modifies the normal autonegative feedback control of GH on growth hormone releasing hormone (GHRH) and somatostatin; +, denotes stimulation; –, denotes inhibition. (From Giustina & Veldhuis 1998. © 1998, The Endocrine Society.)

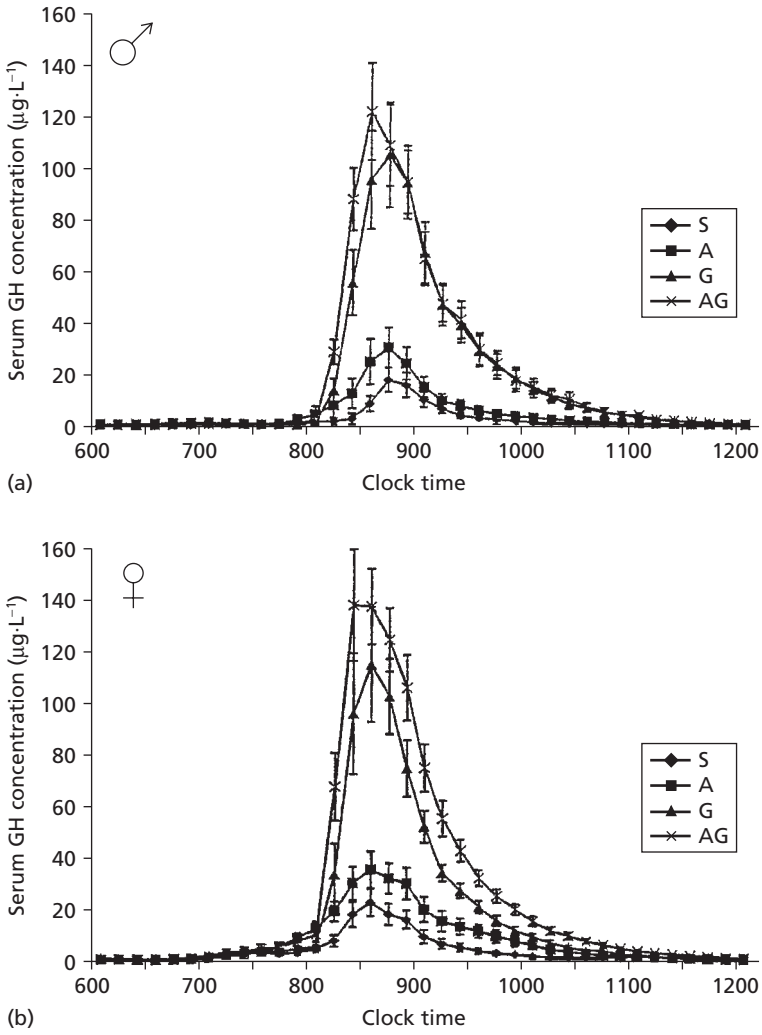

Fig. 10.9 Mean serum growth hormone (GH) concentration ($\mu g \cdot L^{-1}$) profiles basally and in response to saline (S), GH releasing peptide-2 (G), and/or L-arginine (A) infusion at rest in men (a) and women (b). Data are means ± SEM. Clock time (hours) is shown. (From Wideman *et al*. 2000b, used with permission.)

and training-enhanced (mean) GH production on non-training days remains unclear.

Neuroendocrine control of exercise-induced growth hormone release

The neuroendocrine basis underlying exercise-induced GH release is complex and remains enigmatic (Giustina & Veldhuis 1998; Wideman *et al*. 2000a). Presumptive mechanisms involve GHRH, SS and/or ghrelin (Fig. 10.8) (Veldhuis & Bowers 2003b).

Putative modulators include (non-exclusively) catecholamines, muscarinic agonists, opiatergic peptides, GABA and possibly excitatory amino acids (Thompson *et al*. 1993; Giustina & Veldhuis 1998; Weltman, A. *et al*. 2000a). Albeit correlational, plasma norepinephrine concentrations peak before and are proportionate to exercise-stimulated GH concentrations in young men with and without exercise training (Weltman, A. *et al*. 1997, 2000a). Such data are consistent with, but not proof of, central noradrenergic (α_2-adrenorecptor) drive. Serum ghrelin concentrations are higher in lean than obese adults (Veldhuis & Bowers 2003b), but do not change during 45 min of exercise and 3 h of recovery (Dall *et al*. 2002).

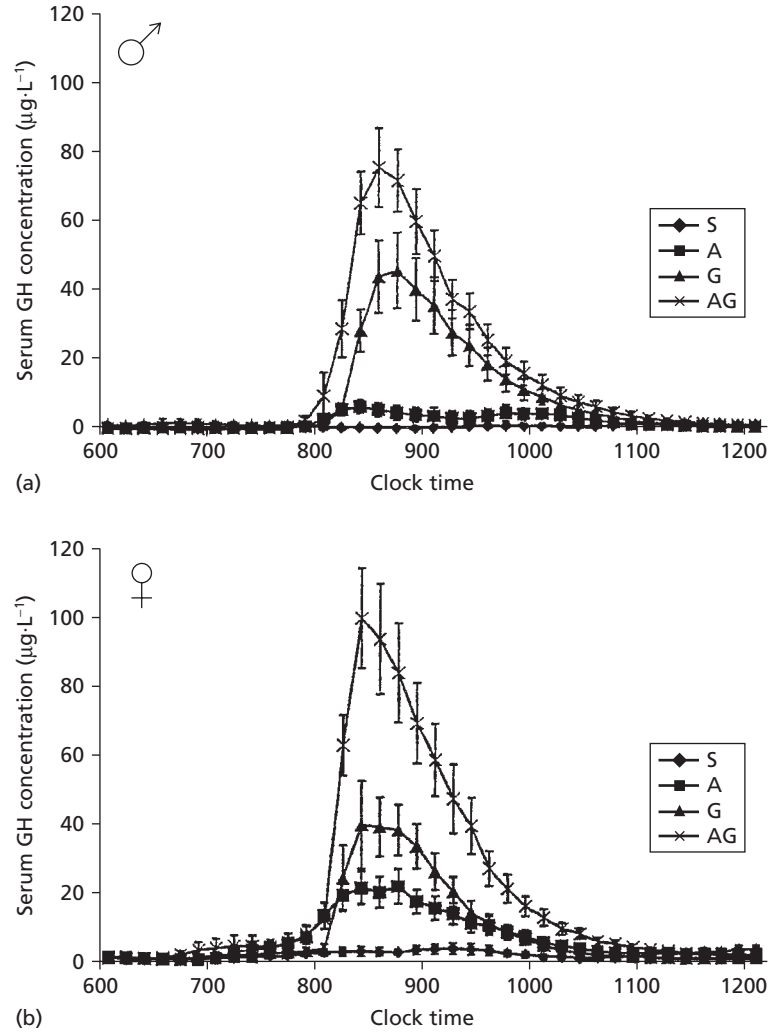

Fig. 10.10 Mean serum growth hormone (GH) concentration (μg·L^{-1}) profiles basally and in response to saline (S), GH releasing peptide-2 (G), and/or L-arginine (A) infusion with exercise in men (a) and women (b). Data are means ± SEM. Clock time (hours) is shown. (From Wideman *et al*. 2000a, used with permission.)

Two probes of neuroendocrine pathways mediating GH secretion are L-arginine and GHRP-2, which induce SS withdrawal and mimic ghrelin action respectively. GHRP-2 also potentiates the effect of GHRH and mutes that of SS (Bowers *et al*. 1994; Pihoker *et al*. 1995; Popovic *et al*. 1995; Giustina & Veldhuis 1998; Bowers & Granda-Ayala 1999; Mueller *et al*. 1999; Veldhuis & Bowers 2003b). All three agonists show sensitivity to gender (Merimee *et al*. 1969; Benito *et al*. 1991; Bercu *et al*. 1991; Bowers 1993; Penelva *et al*. 1993; Veldhuis 1996a, 1998, 2003; Giustina & Veldhuis 1998; Jaffe *et al*. 1998; Veldhuis *et al*. 2001; Veldhuis & Bowers 2003a). At rest, basal and L-arginine (but not GHRP-2) stimulated GH release is higher in women than men (Fig. 10.9) (Wideman *et al*. 2000b).

The synergy is equivalent in absolute terms in the female and the male before exertion. Exercise potentiates maximal GH concentrations driven by L-arginine or GHRP-2 alone as well as together, and absolute responses are compatible by gender (Fig. 10.10) (Wideman *et al*. 2000a).

Fractional responses to exercise (fold-increase above rest) are twofold higher in men than women administered combined stimuli. Since GHRH expressly facilitates GHRP-2 and L-arginine actions,

synergy of the latter two secretagogues during exercise strongly supports the inference that exertion releases hypothalamic GHRH. This concept will need to be tested by selective GHRH-receptor antagonists.

In complementation, estimates of stimulated GH secretory-burst mass (Veldhuis *et al*. 1990) corroborate the impact of L-arginine and GHRP-2 (Wideman *et al*. 2000a, 2000b). However, GHRH, SS and putatively endogenous ghrelin are comodulated by inhibitory (β-adrenergic and α_1-noradrenergic) and stimulatory (cholinergic and opiatergic) signal (Ghigo *et al*. 1993, 1994; Thompson *et al*. 1993; Giustina & Veldhuis 1998; Mueller *et al*. 1999; Veldhuis & Yoshida 2000). Exercise appears to stimulate multiple central–neural neurotransmitters (Sutton & Lazarus 1974; Uusitupa *et al*. 1982; Moretti *et al*. 1983; Bowers *et al*. 1984; Thompson *et al*. 1993; Giustina & Veldhuis 1998). The integration of such responses is a daunting challenge in exercise physiology.

An important emerging issue is the degree to which specific GH secretagogues can amplify the effects of exercise in aging individuals. In one preliminary study in older men, combined stimulation with GHRP-2 and aerobic exercise elicited greater GH secretion (summed amplitude) than either intervention alone, but did not act with true synergy (supra-additive effect) (Weltman, A. *et al*. 2002). The later outcome could denote excessive somatostatinergic restraint, impaired outflow of endogenous GHRH (which synergizes with GHRP), and/or down-regulation of the GHRP signaling pathway as inferred independently in the aging human and experimental animal (Veldhuis *et al*. 2001, 2002; Veldhuis 2003). A corollary question posed by current data is how all three of exercise, age and gender govern GH secretion (Brill *et al*. 2002).

Conclusions

Aerobic exercise is a potent stimulus for GH release, particularly in young men and women. Age and obesity blunt responsiveness markedly, putatively by modifying metabolic signals to and effects of all three of GHR (stimulatory), SS (inhibitory) and ghrlein (GHRP, amplifying). Gender further determines GH secretion driven by exercise at any age. In principle, impoverishment of physiological GH secretion in sedentary, obese and aged individuals would exacerbate visceral fat accumulation, dyslipidemia, relative insulin resistance, diminished bone and muscle mass and (possibly) reduced quality of life.

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Chapter 11

Proopiomelanocortin and Exercise

HEINZ W. HARBACH AND GUNTER HEMPELMANN

Introduction

The pituitary proopiomelanocortin (POMC) system is activated under physical, psychological and immunological stressors, which induce the release of POMC fragments into the cardiovascular compartment. The response to physical stress includes the release of a variety of neuronal or humoral signals from nervous, endocrine or immune systems ending up with morphological or functional alterations, for example of muscular tissue or of the cardiovascular system (Teschemacher 2003).

Adrenocorticotropic hormone (ACTH) and β-endorphin are the compounds mostly studied under physical stress conditions, whereas further POMC fragments, such as β-lipotropin (β-LPH), are also released upon physical exercise. The secretion of POMC derivatives during exercise is an adaptive attempt of the athlete's organism to cope with different stress situations. It is intimately linked to a variety of psychological strategies that facilitate the organism's navigation through a stressful environment (McCubbin 1993). ACTH and β-endorphin appear to be released during exercise in a sufficient intensity and duration. The exercise response may also be affected by the training status of the individual and the population being investigated (Goldfarb & Jamurtas 1997). Aerobic activities (endurance exercise) are quite different from anaerobic activities, such as resistance exercise. In case of situations of overtraining challenge, a reduced ACTH response may reflect the athlete's impaired ability to cope with the stress situation. Whereas short-term overtraining (overreaching) can be reversed by a more prolonged period of recovery, further exposure to stressors induces overtraining syndrome. Changes in circulating hormonal concentrations have been proposed as indicators of overtraining, although such hormonal changes are not always observed (Fry *et al*. 1998).

However, it remains to be elucidated, which purpose for POMC fragments are released into the cardiovascular compartment related to exercise conditions. This has to be emphasized despite of a frequently offered answer suggesting the opposite by referring to an 'adaptation to stress'. The clarification of the functional significance of the POMC system, which is obviously activated by the human organism every day and under multiple stress conditions, still remains a target of interest (Teschemacher 2003). The exact mechanism(s) responsible for exercise-induced increases in β-endorphin, ACTH and cortisol remain speculative. They may also differ in dependence of the duration of exercise stress or in dependence of the exercise stress intensity (i.e. submaximal and maximal) exercise stress, when high-intensity exercise at supramaximal oxygen (O_2) consumption ($\dot{V}o_{2max}$) does not longer produce significant increase in plasma β-endorphin (Kraemer, W.J. *et al*. 1993). This review summarizes the current knowledge about POMC in exercise physiology.

POMC and its occurrence in the history of life

POMC—phylogenetic ontogeny and POMC-derived peptides

Proopiomelanocortin (POMC) is older than 500 million years. This protein has not only been found in

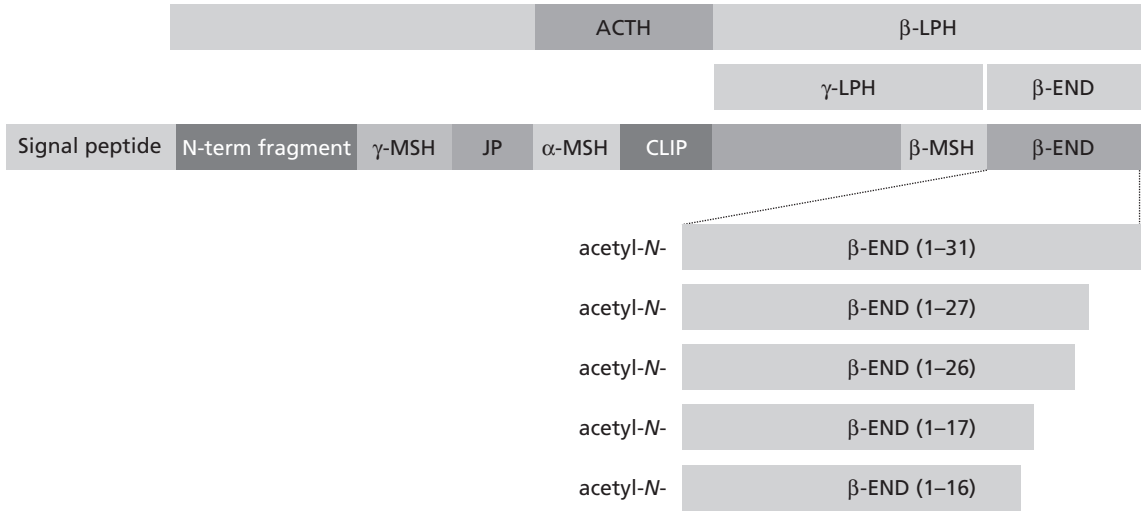

Fig. 11.1 Schematic representation of known products of enzymatic processing of proopiomelanocortin (POMC). Signal peptide; N-terminal fragment; adrenocorticotropic hormone (ACTH); β-lipotropin (LPH); γ-LPH; α-melanotropin (MSH); β-MSH; γ-MSH; corticotropin-like intermediate lobe peptide (CLIP); joining peptide (JP); β-endorphin (1–31) (β-END (1–31)); β-END (1–27); β-END (1–26); β-END (1–17); β-END (1–16); acetyl-*N*-β-END (1–31); acetyl-*N*-β-END (1–27); acetyl-*N*-β-END (1–26); acetyl-*N*-β-END (1–17); acetyl-*N*-β-END (1–16).

mammalians but already in invertebrates (Denef & Van Bael 1998), and is also expressed in unicellular organisms (Salzet *et al*. 1997). So far, POMC might be of significance both for the primitive living beings and for the highest developed living systems. Much about the ontogeny of the human POMC system is not known, but it is known that POMC fragments are prepared in the human fetal organism already from the 5th week of gestation on (Facchinetti *et al*. 1987). POMC and two other major prohormones, proenkephalin and prodynorphin, give rise to the three 'families' of opioid peptides: the endorphin, the enkephalin and the dynorphin families.

Peptide hormones and neuropeptides are synthesized as part of a large protein precursor molecule POMC from which they are post-translationally liberated and modified through enzymatic processing (Fig. 11.1). In humans, there is one *POMC* gene per haploid genome located on chromosome 2 (p23). It is 7665 base pairs long and consists of three exons separated by two large introns (3–4 and 2–3 kb respectively). The first exon is about 100 nucleotides in length and contains the 5′-untranslated region of the POMC mRNa. Exon 2 consists of about 150 nucleotides and contains, besides a small portion of the 5′-untranslated sequence, the sequence of the signal peptide and the first 18 amino acids of POMC. The third exon encodes the rest of the POMC precursor with the sequences of ACTH, β-LPH and β-endorphin. Biological active peptides arising from POMC are subjects to further processing, thus yielding smaller peptide products with distinct biological activity. So far, POMC is processed to pro-γ-melanocyte stimulating hormone (pro-γ-MSH) (also called N-POMC), corticotropin-like-intermediate protein (CLIP), joining peptide (JP), ACTH and β-LPH (Fig. 11.1). There are at least 10 β-endorphin derivatives known to exist in the mammalian organism, β-endorphin (1–27), (1–26), (1–17) and (1–16), as well as their *N*-acetylated forms, and in addition *N*-acetyl- β-endorphin (1–31) (Teschemacher *et al*. 1990a; Höllt 1993; Young *et al*. 1993). Pro-γ-MSH is cleaved to give an N-terminal peptide (N-POMC (1–49)) ACTH to give α-MSH and CLIP, and β-LPH is cleaved to β-endorphin and γ-LPH. The JP has no biological activity, although it contains a single cysteine residue, unique in humans, which allows it to dimerize, and it is thought that POMC may also dimerize within the cell during biosynthesis (Bicknell *et al*. 1996).

N-POMC is involved in adrenal mitogenesis and α-MSH controls pigmentation in lower vertebrates.

The mRNA in the pituitary is 1072 bases long, whereas the transcript in the brain is longer and most of the transcripts in the periphery are shorter, i.e. about 800 bases long. POMC is produced in particularly high amounts in the pituitary gland. The POMC mRNA in the pituitary, in the brain and in the periphery as far as comparable with pituitary mRNA length is translated into the amino acid sequence of 'pre-proopiomelanocortin', 'pre-POMC'. After cleavage of the N-terminally located signal sequence from pre-POMC, POMC is left, which is a protein of 241 amino acid residues, from which POMC fragments such as ACTH or β-endorphin are released (for review see Höllt 1993; Bertagna 1994).

POMC localization in the human organism

The main site of expression of the *POMC* gene is the pituitary gland, in particular the corticotropic cells of the anterior lobe and the melanotropic cells of the intermediate lobe. The intermediate lobe as the genuine location of the melanotropic cells in lower species is vestigial in the human pituitary gland. So far the human organism lacks a well-defined intermediate lobe containing melanotropic cells. However, the POMC-expressing cells in the anterior lobe are able to process POMC according to either type of enzymatic cleavage pattern, the melanotropic as well as the corticotropic one. These enzymatic systems, however, apparently are still regulated separately (Evans, V.R. *et al*. 1994).

Outside the pituitary POMC transcripts have been found as well, for example in the arcuate nucleus of the hypothalamus and other regions of the brain. Moreover, POMC-like transcripts have been detected in a number of peripheral tissues. These are the thyroid gland, thymus, adrenal medulla, gonads, placenta, pancreas, kidneys, spleen, liver, gastrointestinal wall, skin, monocytes, macrophages and T-cells.

POMC expression in the human organism

In the corticotropic cells POMC is glycosylated and phosphorylated and subsequently enzymatically cleaved into three big fragments, a so-called 16 K-fragment, ACTH and β-LPH (Fig. 11.1); the latter POMC fragment consists of the sequences of γ-LPH and β-endorphin, which are released in small amounts also. In the melanotropic cells, the same POMC fragments are released from POMC as in the corticotropic cells; however, they are further processed to release a series of smaller fragments, which are in part N-terminally acetylated or C-terminally amidated. α-Melanocyte stimulating hormone (α-MSH) or acetylated β-endorphin fragments are typical examples for this type of POMC derivatives (Loh 1992; Young *et al*. 1993; Castro & Morrison 1997).

POMC—the precursor of ACTH and β-endorphin—and the endocrine regulation of the response to exercise stress

POMC release under physical stress conditions

The 'corticotropic part' of the POMC system is activated under the conditions of physical exercise, which results in the release of ACTH and β-endorphin immunoreactive material (IRM). ACTH was studied as the main representative of the POMC system for a long time under the aspect of stress-induced activation of the 'hypothalamic–pituitary–adrenal axis' (HPA axis) (Ganong *et al*. 1987; Tache & Rivier 1993). ACTH is regarded as a main valid parameter of the endocrine stress response besides epinephrine, norepinephrine and cortisol (Adams & Hempelmann 1991). Later β-endorphin and β-LPH were recognized to be released from the pituitary under identical or similar stress conditions (Owens & Smith 1987; McLoughlin *et al*. 1993).

ACTH and β-endorphin IRM concentrations in the plasma mostly were secreted in equimolar quantities in response to exercise (Akil *et al*. 1984; De Meirleir *et al*. 1986; Farrell *et al*. 1987; Rahkila *et al*. 1988; Strassman *et al*. 1989; Schwarz & Kindermann 1990; Heitkamp *et al*. 1993; Schulz *et al*. 2000). However, as shown in female marathon runners, basal levels of ACTH and β-endorphin IRM can be different and the increase of ACTH levels under

certain conditions, for example upon running to exhaustion, can exceed the increase of β-endorphin IRM by a factor of five (Heitkamp *et al*. 1996).

Acute stress can be defined biochemically by measuring an increase in catecholamine release. Chronic stress has proved more difficult to define in biochemical terms, although in psychological terms it has been considered as the lack of ability to cope with the environment or loss of control in a long-term situation (McLoughlin *et al*. 1993). In the case of athlete's feeling subjective symptoms of stress, combined with accelerated fatigability or diminished physical performance, a state of overtraining stress is given.

POMC response to acute aerobic and endurance exercise

Assuming that the stress response is a neuroendocrine mechanism that occurs in anticipation of physical exercise, De Vries *et al*. (2000) investigated whether an incremental exercise protocol could be used as a model stressor. Subjects cycled at 40–100% of the power output at the maximal O_2 consumption ($\dot{V}O_{2max}$) each up to exhaustion. Their results showed that increases in heart rate, lactate, epinephrine and norepinephrine reflect the relative workload, in contrast to increases in ACTH hormone and β-endorphin, which were observed only after exercise reached an intensity of 80% $\dot{V}O_{2max}$. Their results demonstrated that activation of stress hormones occur at different time points, the delayed response of the HPA axis during incremental exercise contrasted with the non-delayed HPA axis response observed during psychological stress (De Vries *et al*. 2000). Farrell *et al*. (1983) compared ACTH levels before and after submaximal (80% of maximal O_2 consumption [$\dot{V}O_{2max}$]) and exhaustive isotonic exercise (100% $\dot{V}O_{2max}$) and also concluded that exercise-induced increases in plasma ACTH and their correlation with circulating cortisol depend on the intensity of isotonic exercise. At workloads of 40–60% $\dot{V}O_{2max}$ β-endorphin was not significantly elevated, but it was significantly elevated at 80% $\dot{V}O_{2max}$ (Donevan & Andrew 1987; Langenfeld *et al*. 1987; McMurray *et al*. 1987; Sforzo 1989). In a study by Duclos *et al*. (1997) ACTH levels after running exercise proved to be elevated in marathon-trained athletes in comparison with untrained volunteers. However, since cortisol levels did not reflect this difference, the authors of this study hypothesized a decreased HPA axis sensitivity to cortisol negative feedback. Results of a following study from Duclos *et al*. (1998) discarded this hypothesis of a decreased adrenal sensitivity of the ACTH targets after pituitary adrenal stimulation.

Marquet *et al*. (1999) administered low and high doses of dexamethasone. After the last dose the subjects performed a maximal cycling exercise, and blood was sampled just before and after each exercise bout. Blood levels of ACTH, β-endorphin, cortisol and sex steroids except testosterone were lowered by dexamethasone at rest and after exercise. These effects were interpreted as an impairment of the adaptation to intense physical loads.

Controversy exists regarding the effects of duration of exercise and exercise dosage on β-endorphin release during exercise. Angelopoulos (2001) demonstrated an increased concentration of β-endorphin maintained over time during intense exercise (trials at 80% $\dot{V}O_{2max}$ for 10–30 minutes), whereas Goldfarb *et al*. (1991) reported a gradual increase in β-endorphin concentration over time at 80% $\dot{V}O_{2max}$, and Taylor *et al*. (1994) observed a linear increase in β-endorphin concentration with time. The disparity among the results may be due to differences in the exercise dosage or a match between the rate of β-endorphin release and disappearance at 50% $\dot{V}O_{2max}$ conditions. The secretion of β-endorphin and ACTH were also described in dependence of the intensity of exercise (Rahkila *et al*. 1987, 1988). However, results from Petraglia *et al*. (1990) at marathon race conditions and submaximal exercise at 50% $\dot{V}O_{2max}$ indicate that the duration but not the workload were responsible for β-endorphin and β-LPH release.

Goldfarb and Jamurtas (1997) reviewed significant increases in β-endorphin at workloads of 60% and 90% $\dot{V}O_{2max}$ only at the higher workload and dependant on the duration of exercise, respectively. Graded or incremental exercise of an aerobic nature appears to increase β-endorphin. Exercise at 66% and 57% $\dot{V}O_{2max}$ was reported to increase plasma β-endorphin in the untrained and trained individuals,

respectively. The authors indicated that the β-endorphin levels did not differ significantly between the endurance-trained and untrained participants either prior to or during exercise.

Lac *et al*. (1999) present reactions of cortisol to a dexamethasone treatment via ACTH blockade and to an exercise bout at $\dot{V}O_{2max}$. After exercise, plasma ACTH rose to 600% of basal value, whereas cortisol was unaffected, which was explained by differences in peripheral metabolism.

Changes of plasma ACTH and β-endorphin were observed under high-intensity cycle exercise and exposure to a simulated altitude of 4300 m (hypobaric and hypoxic) and under sea level conditions: Exercise at acute hypobaric hypoxia elicited no significantly different responses in plasma β-endorphin, ACTH or cortisol and no changes in β-endorphin/ACTH molar ratio than those elicited under normobaric conditions (Kraemer, W.J. *et al*. 1991). A significant increase in serum ACTH was observed in response to a 21 km non-competitive race at low altitude (350 m below sea level) in comparison to 620 m above sea level; it was proposed that ACTH may play a role in acclimatization to exercise at low altitudes (el Migdadi *et al*. 1996).

Repeated physical exercise including the following recovery period may be a useful model to study the effect of various rest intervals on subsequent stress reactions. Therefore, Ronsen *et al*. (2002) designed a study that compared neuroendocrine and immune responses during days with two equal bouts of high-intensity endurance exercise, but different periods of rest between the first and second bout. They demonstrated that when a second bout of exercise was performed after 3 h compared with 6 h of rest, increases in epinephrine, ACTH and cortisol were augmented. They showed that recovery time as such is a significant factor in achieving homeostasis between repeated sessions of endurance exercise.

β-Endorphin and ventilatory responsiveness. To investigate the hypothesis that endurance exercise may lead to a decrease in ventilatory chemosensitivity as possibly mediated by an increase in endogenous β-endorphin, the hypercapnic ventilatory responsiveness and circulating β-endorphin immunoreactivity was measured at marathon race. All runners experienced a rise in β-endorphin activity from pre-marathon to immediate post-marathon. However, hypercapnic ventilatory responsiveness showed no significant change. Mahler *et al*. (1989) concluded that the natural increase in endogenous β-endorphin activity associated with marathon running does not modulate central chemosensitivity. Adult male students were given $\dot{V}O_{2max}$ tests to confirm their aerobic fitness levels. The 'fit group' showed significantly lower heart rate responses than the 'non-fit group', and these fitness group differences were abolished by the opioid antagonist naltrexone. McCubbin (1993) suggested that regular aerobic exercise conditioning augments release of inhibitory opioids.

POMC response to acute resistance exercise

Aerobic activities (endurance exercise) are quite different from anaerobic activities, such as resistance exercise. A decrease in β-endorphin plasma concentration following resistance exercise was observed as compared with pre-exercise (Pierce *et al*. 1994), and in addition, no relationship between affect and β-endorphin response to exercise was observed (McGowan *et al*. 1993). As Goldfarb and Jamurtas (1997) also described, it appeared there is no definitive response of β-endorphin to resistance exercise.

In heavy-resistance exercise, it appeared that the duration of exercise, length of the rest periods between exercise sets and blood lactate were key exercise variables that influence increases in plasma β-endorphin concentration (Kraemer, W.J. *et al*. 1993). Their data—only one of six heavy-resistance exercise protocols examined resulted in a significant increase in plasma β-endorphin and serum cortisol concentrations—demonstrated that the exercise stimulus for β-endorphin increase was characterized by longer-duration sets and shorter rest periods between sets and exercises. The number of repetitions that the resistance (i.e. intensity) allows in the set (i.e. duration) and the rest period length are the primary determinants of the physiological stress (Kraemer, W.J. *et al*. 1989b). β-Endorphin increased at the end of resistance exercise during energy balance, but significantly only during negative energy

balance (Walberg-Rankin *et al*. 1992). β-Endorphin or whole blood lactate were not influenced by resistance training experience in elite weightlifters (Kraemer, W.J. *et al*. 1992). It appears that there is no definitive response of β-endorphin to resistance exercise. Non-exhaustive performance such as heavy resistance exercise, although requiring high muscular activity for short time periods, did not lead to an increase of β-endorphin levels at all (Pierce *et al*. 1993b), even decreased them (Pierce *et al*. 1994), or only allowed an increase under approximately exhaustive conditions (Kraemer, W.J. *et al*. 1993). Schulz *et al*. (2000) demonstrated the increase of ACTH and β-endorphin IRM concentrations in the plasma upon anaerobic exercise, correlated with the increase of lactate levels observed upon anaerobic exercise. Authentic β-endorphin (1–31) was only found in two plasma samples containing minor concentrations of the peptide. They concluded that the β-endorphin IRM released into blood under anaerobic exercise was identical with authentic β-endorphin (1–31) only to a minor extent. The major part of the material in fact released into the blood upon anaerobic exercise was probably identical with β-LPH.

The results examining resistance exercise and β-endorphin response are equivocal: partly due to the selection of participants; partly due to the intensity, duration and rest periods of exercise utilized (Kraemer, W.J. *et al*. 1989; Goldfarb & Jamurtas 1997); and partly due to the specificity of assays utilized (Harbach *et al*. 2000; Schulz *et al*. 2000).

Ethanol did not increase circulating cortisol concentration above that caused by the resistance exercise, but it appeared to have a more prolonged effect without a change in circulating ACTH (Koziris *et al*. 2000). Unchanged resting concentrations of cortisol during short periods of heavy strength training were also observed by Fry *et al*. (1998) and Raastad *et al*. (2001).

Chronic training effects and POMC response: chronic aerobic and endurance exercise versus chronic resistance exercise

POMC response to chronic aerobic and endurance exercise. Buono *et al*. (1987) observed a significant increase in $\dot{V}O_{2max}$ of volunteers but a blunted ACTH response to an absolute submaximal work rate in a 12-week training program. In addition, Luger *et al*. (1987) observed decreased ACTH levels in trained subjects under consideration of absolute workload. In contrast, Ronkainen *et al*. (1986), who investigated the effects of endurance exercise on the function of adrenal cortex of female runners, supposed no alteration of the function of the adrenal cortex by chronic endurance exercise, when responses of cortisol to intravenous ACTH injections were estimated.

POMC response to chronic resistance exercise. Data from Kraemer, W.J. *et al*. (1992) on effects of resistance training experience demonstrated an increase of β-endorphin, testosterone and lactate, whereas only testosterone was influenced by 2-years' training experience.

Deschenes *et al*. (2002) tested whether muscle unloading were attributable to adaptations of the neural system rather than modifications of myofiber size and confirmed that the loss of muscular strength could primarily be attributed to a decreased capacity of the nervous system to excite the muscle. In contrast to resistance training, which results in strength gains and muscle hypertrophy, muscle unloading evokes reductions in muscle performance. In addition, they observed that muscle unloading affects the hormonal milieu in a manner that promotes muscle atrophy: increase of the catabolic hormone cortisol in the absence of a concomitant elevation of ACTH.

Sleep deprivation did not alter the resting β-endorphin response, nor did it affect the β-endorphin response to high-intensity exercise (McMurray *et al*. 1988).

Influence of training. The influence of training on basal plasma levels or on levels under exercise conditions has been reviewed (Goldfarb & Jamurtas 1997). A number of studies differing in the composition of volunteer groups as well as using different protocols obviously revealed controversial results: Training has been reported to have increased, made no difference to or decreased β-endorphin levels following exercise. Goldfarb and Jamurtas (1997)

interpreted the discrepancy in the literature as in part related to the type of training and its intensity, the methods used to measure β-endorphin and the mode of exercise utilized. A clear-cut experimental design was presented by Engfred *et al*. (1994), which did not just compare trained and untrained volunteers but looked at the same subjects in a state before and after training as conducted and controlled in the frame of their own study. Training-induced changes in response to exercise of norepinephrine, epinephrine, growth hormone, β-endorphin and insulin were similar in the groups. Incremental exhausting and prolonged exhausting endurance exercise such as marathon running induced an increase of similar magnitude in both β-endorphin and ACTH concentration (Heitkamp *et al*. 1993). ACTH and β-endorphin response to exercise workload proved to be dramatically reduced after a 5 weeks' training protocol. In a similarly designed study, untrained females were subjected to 8 weeks' endurance training and the effects of running 30 minutes three times a week on ACTH and β-endorphin levels were measured (Heitkamp *et al*. 1998). Basal β-endorphin levels were not altered by the training program, but basal ACTH levels increased. Both, ACTH as well as β-endorphin levels, were dramatically increased immediately after the exercise; this increase, however, was found to be attenuated in case of β-endorphin. Thus, training appeared to provoke a POMC fragment-specific response, which was difficult to recognize just upon acute exercise challenge of the POMC system. Results obtained with untrained and trained volunteers also showed differences between the two groups concerning different ACTH and β-endorphin responses, which can be interpreted in the same way (Diego Acosta *et al*. 2001). Training also appears to influence β-endorphin metabolism in the resting state and during exercise in dependence of training previously performed; mild endurance exercise 50% $\dot{V}o_{2max}$ resulted in no change of β-endorphin, whereas exercise at 66% was reported to increase β-endorphin (Viru & Tendzegolskis 1995).

Luger *et al*. (1987) examined plasma ACTH, cortisol and lactate responses in sedentary subjects, moderately trained and highly trained runners. Basal concentrations of plasma ACTH were elevated in the highly trained subjects. Like the levels of plasma ACTH and cortisol, the level of plasma lactate was coupled to exercise intensity, since all groups had similar plasma lactate responses at matched exercise intensities. Trained subjects required much higher absolute workloads to produce comparable elevations in plasma lactate concentrations; they had much less activation of the HPA axis as compared with untrained subjects at matched absolute workloads. Daily strenuous exercise was interpreted to lead to chronic ACTH hypersecretion and adrenal hyperfunction—physical conditioning associated with adaptation mechanisms such as the ability to increase the capacity to handle a higher workload with less pituitary–adrenal activation (Luger *et al*. 1987). Data of the same group showing elevated basal concentrations of ACTH and cortisol and a blunted response to exogenous corticotropin-releasing hormone (CRH) were compatible with mild sustained hypercortisolism in highly trained runners (Luger *et al*. 1988).

In studies comparing sedentary with trained subjects, no training-induced adaptation of POMC derivative release was observed under different exercise conditions (Kraemer, R.R. *et al*. 1989; Goldfarb *et al*. 1991). Three training groups were elected for maximal treadmill exercise. Sprint intervals, endurance training and combination training were observed over 10 weeks. No training-induced hormonal changes were observed for the endurance group. While exercise-induced increases were observed, the combination group exhibited significant post-training reductions in plasma responses of β-endorphin and ACTH (Kraemer, W.J. *et al*. 1989a).

In comparison with men, female marathon runners showed higher baseline concentrations, lesser increases in β-endorphin, lower baseline concentrations and larger increases in ACTH concentration after marathon running and treadmill (Heitkamp *et al*. 1996). In addition, the same group observed a tendency to elevated basal ACTH in endurance training (Heitkamp *et al*. 1998).

POMC release under extreme physical stress conditions. Ultramarathon foot race is extreme physical stress; resting serum ACTH and β-endorphin levels were significantly elevated above normal range (altered

baseline hormonal state), ACTH remained elevated, and β-endorphin plasma concentration was within the normal range without significant change and was interpreted as a hormonal adaptation to prolonged stress (Pestell *et al*. 1989). A more pronounced response in trained subjects was only seen under extreme exercise conditions (Farrell *et al*. 1987).

Tharp already in 1975 made the following statements concerning the role of glucocorticoids in exercise and training: Exhaustion produces a decrease in plasma glucocorticoid, which may represent a defense mechanism to prevent depletion of body resources. Chronic exercise training produces adrenal cortex hypertrophy and usually a smaller rise in plasma glucocorticoids during an acute exercise bout than that obtained with non-trained subjects. The changes in glucocorticoid response during training appear to be produced by decreased responsiveness to the adrenal cortex itself to ACTH stimulation and possibly by adaptation of the HPA axis which reduces the ACTH released in response to stress (Tharp 1975).

Data from Viru and Tendzegolskis (1995) demonstrated that levels of γ_{1-17}-endorphin and α_{1-16}-endorphin and the ratios to β-endorphin were significantly higher in untrained individuals, indicating an alteration of the metabolism of β-endorphin in the resting state and during exercise as dependent on previous training.

Overtraining and addiction to exercise

This chapter refers to exercise physiology in a state of imbalance between strain of exercise training and the athlete's tolerance of stress which leads to overtraining. The literature on overtraining is confusing because of a lack of universal terminology (Fry & Kraemer 1997). Overtraining can be defined as any training performed with incomplete recovery between bouts of exercise, leading to physical performance decrements (Fry & Kraemer 1997; Raastad *et al*. 2001). A further lack in the literature is that studies of overtraining do not consequently utilize adequate changes in exercise volume or intensity, or do not carefully consider rest and recovery characteristics of the training programme, or do not deal with actual decreases in performance (Fry & Kraemer 1997). Whereas short-term overtraining (overreaching) can be reversed by a more prolonged period of recovery, further exposure to stressors induces overtraining syndrome. Substrates such as lactate, urea, enzymes (e.g. creatine kinase) or hormones (e.g. cortisol, testosterone, growth hormone) are of interest in overtraining research, but we will only report on ACTH and β-endorphin as representative POMC derivatives in the blood in overtraining analysis.

Urhausen *et al*. (1995) in parallel with Barron *et al*. (1985) and Fry and Kraemer (1997) (for review see Urhausen *et al*. 1995; Fry & Kraemer 1997) observed an impaired exercise-induced increase in ACTH after exhausting short-endurance test at 110% of the individual threshold or in response to insulin-induced hypoglycemia in overtrained marathon runners, which was ascribed to a negative feedback through cortisol, depletion of the pituitary ACTH pool or change of the intracellular homeostasis (Urhausen *et al*. 1995). Kraemer *et al*. (1989b) also reported an impaired exercise-induced rise of ACTH in athletes during overtraining. Repeated acute or chronic exposure to a particular stress results in adaptation, whereby the HPA axis becomes less responsive to subsequent or continued exposure to that particular stress. Data from Wittert *et al*. (1996) show that intense physical training leads to adaptive changes in basal HPA function, including a phase shift and increased pituitary in basal HPA function, a phase shift and increased pituitary ACTH secretion, but also a blunted cortisol response.

During heavy endurance training or overreaching periods, the majority of findings give evidence of a reduced adrenal responsiveness to ACTH, compensated by an increased pituitary ACTH release (Lehmann *et al*. 1998). A decreased β-endorphin response was also reported in overtrained athletes (Urhausen *et al*. 1995). There is additionally evidence for decreased intrinsic sympathetic activity and sensitivity of target organs to catecholamines (Lehmann *et al*. 1998).

The parasympathetic, Addison type represents the dominant modern type of the overtraining syndrome. In an early stage, despite increased pituitary ACTH release, the decreased adrenal responsiveness

is no longer compensated and the cortisol response decreases. In an advanced stage of overtraining syndrome, the pituitary ACTH release also decreases. However, this complete pattern is only observed subsequent to high-volume endurance overtraining at high caloric demands. The functional alterations of pituitary–adrenal axis and sympathetic system can explain persistent performance incompetence in affected athletes (Lehmann *et al*. 1998).

An exercise effect to be rather elicited in the central nervous system than in the periphery is addiction to exercise. However, in a study targeting this question, scores on exercise-dependence survey were not correlated with β-endorphin plasma levels (Pierce *et al*. 1993a).

Neuroendocrine responses to high volume resistance exercise overtraining can be grouped with highly aerobic activities, whereas excessive resistance training intensity (anaerobic activity) produces a distinctly different neuroendocrine profile (Fry & Kraemer 1997). Cortisol concentrations in response to resistance exercise at increased training volume or increased training intensity were compared: Cortisol increased at rest and acute increased training volume, whereas no change or a slight decrease was observed at increased training intensity. However, there was a lack of increased cortisol levels with high-intensity resistance exercise overtraining. This might be of interest for overtraining research, since it has been shown that increased circulating levels of cortisol may be associated with psychological depression (Fry & Kraemer 1997).

It appears quite different when anaerobic activities, such as resistance exercise, are compared with aerobic activities. Many of the overtraining symptoms identified for aerobic exercise have not been reported for anaerobic overtraining protocols, and altering some of the acute training variables for resistance exercise results in a variety of different physiological responses (Fry *et al*. 1998). Exercise-induced cortisol levels decreased when training volume was doubled, arguing that the presence of increased resting levels of cortisol contributes an exhaustion of the HPA axis, thus preventing an adequate cortisol response to acute stress. In the presence of maximal intensity resistance exercise overtraining, no changes were observed for resting cortisol levels. Such responses are contrary to high volume resistance exercise and overtraining with highly aerobic activities. It becomes apparent that some of the classical signs of overtraining, based on data from endurance athletes, cannot necessarily be applied to overtraining resulting from highly anaerobic activities (Fry & Kraemer 1997).

With ACTH and resistance exercise overtraining, acute ACTH levels are attenuated with increased exercise intensity. When strength performance has been decreased via maximal intensity resistance exercise overtraining, circulating ACTH levels did not change either at rest or after exercise (Fry & Kraemer 1997).

Previous research has attempted to identify endocrine markers ('overtraining markers') of an impending or concurrent overtraining syndrome for both aerobic and anaerobic activities. Data from McGowan *et al*. (1993) support research showing resistance exercise does not produce the significant increase in β-endorphin immunoreactivity widely reported after endurance exercise. In addition, they showed no relationship between affect (mood) and β-endorphin response to resistance exercise. Despite speculations on an essential participation of hypothalamic–pituitary hormonal changes in the pathogenesis of overtraining, only few results with respect to athletes who were actually in a state of overtraining are available (Urhausen *et al*. 1995). Training intensity effect monitoring for resistance exercise was only addressed by Fry and colleagues (Fry & Kraemer 1997; Fry *et al*. 1998). Because of the lack of association between endocrine and performance alterations, they argued that the type of exercise does not readily permit use of hormonal alterations and endocrine adaptations, respectively, to monitor impending overtraining. In addition, it appeared that short-term, high-relative-intensity resistance exercise overtraining may not be successfully monitored via circulating hormonal concentrations.

Implications of these neuroendocrine responses to overtraining for adequate training should be managed by dosing the training stresses in a variable manner to avoid overtraining with long-term disruption of homeostasis (Fry & Kraemer 1997). The magnitude of temporary fatigue and recovery

rate (after heavy resistance exercise) may be an indication of effectiveness for long-term adaptations of the neuromuscular system, which make it necessary to start the next training session after complete recovery (Ahtiainen *et al*. 2003).

Cardiovascular effects

Exercise can be regarded as one of many stressors provoking cardiovascular reactions of the organism and therefore activating the pituitary POMC system. Shen *et al*. (1992) observed an exercise-induced rise of β-endorphin plasma concentrations also in the presence of naloxone. Their results confirm a rise in intrinsic heart rate (heart rate following autonomic blockade) and β-endorphin concentrations with acute exercise, but indicate that the changes in intrinsic heart rate are not β-endorphin related.

The β-endorphin response to exercise is of special interest in patients with coronary artery disease (CAD). Letizia *et al*. (1996) observed patients with suspected CAD and those with definite CAD under cycloergometric stress tests. At peak exercise plasma levels of β-endorphin and ACTH increased concomitantly in subjects that did not exhibit clinic and electrocardiogram (ECG) signs of ischemia during stress test. β-Endorphin, ACTH and cortisol increased further during recovery. β-Endorphin significantly increased at peak exercise in patients with CAD and negative stress test, but ACTH and cortisol plasma levels were not significantly modified. On the contrary in those patients with a positive stress test, the plasma levels of β-endorphin and POMC-correlated peptides were not modified. As an interpretation of this behavior they assumed the rise in β-endorphin concentration observed at peak in patients with CAD and negative stress test as associated with painless ischemia.

Patients with asymptomatic ischemia on exercise had a significantly greater β-endorphin response than those with angina. Public speaking elicited a significantly larger β-endorphin increase than did exercise. Patients with silent versus painful ischemia experience had a greater β-endorphin response to exercise. However, the β-endorphin response to a speech stressor was greater than to exercise. Increased β-endorphin response to a speech stressor was interpreted as a partial predominance of silent ischemia during psychological stress (Miller *et al*. 1993). In patients with CAD and exercise-induced ischemia, public speaking produces psychological stress manifested by increased cardiovascular reactivity and causes an increase in plasma β-endorphin levels that was significantly correlated with pain thresholds (Sheps *et al*. 1995). Sex differences in chest pain at exercise conditions were observed by the same authors. Women reported chest pain more often than men during daily activities and during laboratory mental stressors but not during exercise. Men had lower scores than women on measures of depression or trait anxiety. Women had significantly lower plasma β-endorphin levels at rest and at maximal mental stress. They concluded that their results reflect sex differences in the affective and discriminative aspects of pain perception, which may help to explain sex-related differences in clinical presentations (Sheps *et al*. 2001).

Plasma β-endorphin levels were studied due to exercise-induced ischemia in patients with CAD. There was no significant difference in plasma β-endorphin levels during or after exercise between symptomatic and asymptomatic patients; thus, the differences in circulating levels of β-endorphin and also ACTH were not associated with the presence or absence of pain (Heller *et al*. 1987; Marchant *et al*. 1994). This observation is in accordance to the above mentioned observation of Droste *et al*. (1991) that exercise-induced elevation in pain threshold was not related to plasma β-endorphin levels.

Influence of age, race and gender

Effects of chronic exertion on β-endorphin and the relationship to melatonin secretion were studied in well-trained athletes by Appenzeller and Wood (1992): β-endorphin and melatonin increased after exercise. Interestingly, the magnitude of this increase was age-dependent. Chronic exertion was associated with a decrease in exercise induced opioid release and in such individuals whose melatonin secretion was not β-endorphin related. The first study demonstrating that older men can make physiological adaptations in the endocrine system with resistance training was made by Kraemer, W.J.

et al. (1999). They examined the adaptations of the endocrine system to heavy-resistance training in younger versus older men in a strength-power training program. The amount of cortisol produced at resting levels was reduced and the response to the resistance exercise stress was lower in the older men. The decrease in resting concentrations of cortisol throughout the training program in the older men without significant changes in the ACTH concentrations indicated that the ACTH receptors in the adrenal gland may have been 'down-regulated'. Changes in exercise-induced cortisol responses observed after exercise were apparently mediated by a reduction in ACTH responses to exercise stress. The inability to engage similar hormonal mechanisms in response to heavy-resistance exercise training was interpreted as meaning that the plasticity of the endocrine system in older men was altered or impaired.

Exhausting bicycle exercise in post-menopausal women induced a strong release of ACTH, cortisol and prolactin, and natural killer cell activity. Vander-Pompe *et al*. (2001) assumed that low vigor in post-menopausal women interferes with the endocrine and immune responses to exhausting exercise.

Yanovski *et al*. (2000) have shown that plasma ACTH of African-American women was significantly greater than that of Caucasian women, but that this was not accompanied by greater plasma cortisol concentrations. Plasma ACTH of African-American women contained many 'non-intact' ACTH fragments which were not found in Caucasian women. Plasma ACTH, measured after intense exercise, was significantly greater in African-American women than in Caucasian women, but plasma cortisol after exercise was not different. The ACTH of African-American and Caucasian women did not appear to be equipotent at adrenal melanocortin-2 receptors because the greater ACTH of African-American women did not lead to greater cortisol secretion.

Data from Goldfarb *et al*. (1998) suggested that women cycling at 80% $\dot{V}O_{2max}$ had a similar β-endorphin response to men independent of their menstrual cycle. Comparing ACTH or β-endorphin plasma levels of female athletes with those of male athletes or volunteers under basal or exercise conditions, no major differences have yet been demonstrated, with the exception of a slightly lower basal β-endorphin IRM concentration in women as compared to men independent of the time of the women's menstrual cycle.

ACTH, cortisol and β-endorphin were measured in a treadmill run at 80% of a previously determined maximum heart rate of male and female runners and compared to the concentrations of sedentary men and women by Kraemer, R.R. *et al*. (1989). The run resulted in no rise in β-endorphin, ACTH and cortisol. β-Endorphin values were significantly higher in men than in women. No sex or training differences were seen with respect to change of hormone concentrations over the course of the run. Their data indicated that gender and training do not affect ACTH and cortisol concentrations before, during and after treadmill running at 80% of maximum heart rate, whereas β-endorphin concentrations were higher in men under these conditions.

Plasma ACTH response to exercise was significantly attenuated in lactating women performing graded treadmill exercise finally to elicit 90% $\dot{V}O_{2max}$ uptake in comparison to non-lactating women. These results were interpreted to mean that stress-responsive neurohormonal systems were restrained in lactating women (Altemus *et al*. 1995).

HPA and hypothalamic–pituitary–gonadal (HPG) axis modification, combined with cognitive impairments, have been reported in elderly subjects and related to physical training status. Data from Struder *et al*. (1999) suggest that elderly endurance athletes reveal a prolonged secretion of glucocorticoids.

Physiological and pathophysiological aspects of exercise induced POMC release and consequences to homeostasis of energy and nutrition

Exercise can be regarded as one of many stressors provoking metabolic reactions of the organism and therefore activating the pituitary POMC system. However, in contrast to most of the other stressors, there is no doubt that the organism tries to adapt to this stressor not by reduction of morphology and functions to the original state before exposition to exercise, but by conversion of morphology and functions to an altered, i.e. to a stressor-adapted

state, thus counteracting future stress situations of the same type.

Sustained hyperglycemia was observed to not be a stimulus to enhanced β-endorphin secretion into plasma and this lack of response was not effected by prior exercise (Farrell *et al*. 1986). Lower β-endorphin levels were found in patients with diabetes and silent myocardial ischemia at rest, following exercise, as compared with those with silent myocardial ischemia who were not diabetic. Hikita *et al*. (1993) assumed a less significant role of β-endorphin in diabetic patients than in non-diabetic ones.

Angelopoulos (2001) observed comparatively higher β-endorphin concentrations during exercise under opioid antagonism (naloxone) as compared to placebo trial and assumed a positive feedback loop for β-endorphin on plasma glucose regulation. Tabata *et al*. (1991) observed an abolished corticotropin-releasing factor and ACTH increase after exercise at workloads of 50% $\dot{V}O_{2max}$ until exhaustion of healthy young men when their blood glucose concentrations were maintained at the pre-exercise level. The critical level for triggering the pituitary–adrenal–cortical axis was 3.3 mmol. Inder *et al*. (1998) demonstrated a significant rise in peripheral CRH at submaximal exercise, but this was not associated with hypoglycemia. Cortisol rise in physical exercise or during recovery may be initiated by the drop in blood glucose and may prevent the inflammatory and immune reactions from overshooting and so may stabilize homeostasis of the organism (De Vries *et al*. 2000).

Numerous studies (for review see Steinberg & Sykes 1985; Cumming & Wheeler 1987; Sforzo 1989; Schwarz & Kindermann 1992; Hoffmann *et al*. 1996; Goldfarb & Jamurtas 1997; Vassilakopoulos *et al*. 1999) have shown that activation of the pituitary POMC system is achieved by a certain degree of metabolic demand, which is characterized by blood lactate levels beyond the anaerobic threshold, which again are reached upon incremental or short-term anaerobic exercise, or which also can be reached after prolonged aerobic endurance exercise. Apparently the anaerobic state leads to the release of CRH and arginin–vasopressin, known to be released from the hypothalamic paraventricular nucleus or from the posterior pituitary, respectively, for enhancement of POMC fragment release (Inder *et al*. 1998).

Thus, although the prerequisites or the consequences of anaerobic metabolism are apparently closely related to the activation of the POMC system, lactate by itself does not seem to directly stimulate the hypothalamic structures responsible for pituitary POMC system activation (Petrides *et al*. 1999). Taylor *et al*. (1994) assumed base excess as the best indicator of β-endorphin release or the primary stimulus for activation of the pituitary POMC system. In contrast, endurance exercise (marathon and steady-state cycling) reported that constant lactate levels were not related to β-endorphin increase (Goldfarb *et al*. 1991; Heitkamp *et al*. 1996). Blood lactate levels do not appear to be related to HPA hormone plasma concentrations at high exercise intensities (Kraemer, W.J. *et al*. 1989b). It might be associated with stimulation of testosterone secretion or plasma testosterone elevation during exercise, but this requires further investigation (Raastad *et al*. 2000). However, lactate was significantly correlated with β-endorphin, ACTH and cortisol (Kraemer, W.J. *et al*. 1989a). Low volume resistive exercise elevates lactate concentrations without altering endorphin (Kraemer, R.R. *et al*. 1996).

Metabolic response to exercise stress should be envisaged as a possible candidate for POMC fragment function (Weissman 1990). In view of the fact that the release of POMC fragments is obviously dependent on anaerobic state with lactate levels beyond the anaerobic threshold, and that metabolic acidosis probably is a direct stimulus for POMC fragment release, the most likely functional target of POMC fragment release would be counterbalancing the obvious metabolic derailment, exercise is responsible for, by increased energy supply of the skeletal muscle (Knudtzon 1986; Evans, A.A. *et al*. 1997). Since an anaerobic, acidotic state appears to be the stimulus for β-endorphin release, β-endorphin targets might well be parts of a peripheral system responsible for metabolic homeostasis or acid/base equilibrium (Schulz *et al*. 2000).

Acute amino acid supplementation enhanced the ACTH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) response to CRH and

gonadotropin-releasing hormone (GnRH) (Di Luigi *et al*. 1999), whereas acetylsalicylic acid influences ACTH, β-endorphin and cortisol responses to exercise-related stress in humans. Their data confirm a role of prostaglandin in these responses. It might be of interest, because of the large use of anti-inflammatory drugs in athletes, whether the interaction between acetylsalicylic acid and hormones might positively or negatively influence health status (Di Luigi *et al*. 2001).

POMC and exercise and immunological influence

Exercise can be regarded as one of many stressors provoking immunologic reactions of the organism and therefore activating the pituitary POMC system. Effects on the immune system elicited by β-endorphin (Teschemacher *et al*. 1990b) are still a matter of speculation.

An attractive candidate to be considered would be a POMC fragment influence on the immune system. Interactions of β-endorphin (Sibinga & Goldstein 1988) or ACTH (via corticosteroid release from the adrenals) with cells of the immune system are well testified, such as effects of stress in general (Fricchione & Stefano 1994) and of physical exercise in particular (Jonsdottir *et al*. 1997). These effects could contribute to both activation as well as suppression of separate functions of the immune system responsible for defense against infections or necessary for morphological alterations in terms of elevated capability to cope with future exercise stress.

It is suggested that exercise can be employed as a model of temporary immune suppression that occurs after severe physical stress. The increase in catecholamines and growth hormone mediate the acute effects of exercise on neutrophils, whereas cortisol may be responsible for maintaining lymphopenia and neutrocytosis after exercise of long duration. Lastly, the role of β-endorphin is less clear, whereas the cytokine response is closely related to muscle damage. However, POMC does not seem to be directly involved in the elevated cytokine level (Pedersen *et al*. 1997; Pedersen & Hoffmann-Goetz 2000) or elevations of basal natural killer cell activity (Dishman *et al*. 2000). However, observations from Nagao *et al*. (2000) suggest that exercise-induced catecholamines modulate the expression of adhesion molecules on natural killer cells, resulting in a mobilization of natural killer cells into the circulation.

The mechanisms underlying exercise-associated immune changes are multifactorial. Altered plasma glucose has also been implicated in decreasing stress hormone levels and thereby influencing immune function (Nieman & Pedersen 1999).

A further candidate related to stress, immunological function, insulin sensitivity and cardiovascular disorders is dehydroepiandrosterone, secreted by the adrenal cortex in response to ACTH (for review see Kroboth *et al*. 1999).

Strenuous exercise recruits neutrophils and lymphocytes into the circulation and, during recovery, lymphocyte concentration rapidly decrease, resulting in lymphocytopenia if exercise intensity and duration are of sufficient magnitude. A novel finding of a study by Ronsen *et al*. (2002) was the more pronounced neutrophilia and lymphocytopenia during and after a second bout of exercise in the short-rest trial compared to the long-rest trial. They showed that repeating exercise with only a few hours rest results in magnified neuroendocrine stress responses and conceived that performing daily repeated exercise sessions, thus imposing substantial physical, psychological and metabolic stress, could lead to both adaptive and maladaptive alterations in the immune system (Ronsen *et al*. 2002).

Correlations between POMC derivatives and gonadal steroids or gonadotropins under exercise conditions

To investigate the mechanisms of blunted adrenocortical responsiveness to exercise and mild hypercortisolism in eumenorrheic and amenorrheic runners in comparison to eumenorrheic sedentary women ACTH stimulation, tests were performed in the presence and absence of dexamethasone suppression. The cortisol response to stimulation in amenorrheic runners was blunted in the presence of their mild hypercortisolism and appeared to be due to a normal limitation in maximal adrenal secretory capacity (De Souza *et al*. 1994).

Ovulatory and eumenorrheic runners underwent exercise in the follicular and luteal phases and after GnRH agonist desensitization. Baseline peripheral β-endorphin and cortisol levels were not different between the eumenorrheic and oligomenorrheic groups. A significant increase in β-endorphin levels in response to exercise occurred only in the eumenorrheic group after GnRH agonist desensitization. They concluded that alterations in menstrual cyclicity and ovulation in conditioned runners were not due to an increase in opioid tone. The hypothalamic–gonadotropic axis appeared to be intact in oligomenorrheic runners. Sex steroid administration had no effect on basal β-endorphin levels, but this probably was not due to pre-existing increased opioid tone (Meyer *et al*. 1999).

Menstrual cycle alterations in athletes are accompanied by shortening of the luteal phase and secondary amenorrhoea. So far it appears that the reduced LH secretion might be caused by an increased CRH secretion inhibiting GnRH release. In addition, increased CRH tone leads to increased β-endorphin levels which also inhibits GnRH release (Keizer & Rogol 1990).

Effects of oral contraceptives on plasma β-endorphin. Plasma immunoreactive β-endorphin levels at rest were higher in non-pill users than in pill users. Corticotropin levels at rest did not differ between the pill and non-pill users. At 60% $\dot{V}o_{2max}$ a slight increase was found in the concentrations of corticotropin and β-endorphin in the non-pill but not in the pill users. At 90% $\dot{V}o_{2max}$, plasma β-endorphin and corticotropin levels increased significantly in both groups. A threshold elevation of the intensity of exercise required to increase β-endorphin and corticotropin secretion by the use of oral contraceptives was concluded (Rahkila & Laatikainen 1992). To examine individual hormonal responses to extreme physical stress, blood samples were taken from highly trained athletes before and within finishing a 1000-km ultramarathon foot race. ACTH levels were significantly elevated above the normal range. Immunoreactive β-endorphin, growth hormone, prolactin, testosterone, cortisol and cortisol-binding globulin were within the normal range. Catecholamines and ACTH remained significantly elevated above the normal mean and β-endorphin was within the normal range, without significant change. A significant increase in cortisol was seen, with no change in cortisol-binding globulin. As a model of chronic physical stress, the results demonstrated a significantly altered baseline hormonal state as reflected in the primary mediators of the stress response, the catecholamines and the HPA axis. The athletes' response to severe exercise was distinct from that of untrained individuals in who conjugated catecholamines decrease and ACTH increase. This was interpreted as a possible hormonal adaptation to prolonged stress (Pestell *et al*. 1989).

New aspects of the functional significance of POMC and interpretation of stress definitions

Functional significance of POMC derivatives

The functional significance of most of the POMC systems scattered over the organism is unknown. So far this holds for the pituitary POMC system. It has been known for a very long time that the cleavage products of POMC are released into the blood under 'stress' conditions. The pituitary gland as an endocrine organ is destined for sending hormonal messages to tissues of the whole organism via the cardiovascular compartment, receiving the orders for this kind of message transfer mainly from the central nervous system, but also, to a considerably smaller extent, over the cardiovascular compartment itself. Apparently, the pituitary contains several message transfer systems thought for specific transfer tasks such as the gonadotropic or the thyreotropic system. For each of these specific message transfer systems, specific orders based on specific stimuli can be postulated—as well as specific targets in the periphery to be met by the hormonal messengers released from the pituitary into the blood (Mooren *et al*. 2005).

In fact, for the pituitary POMC system a big number of stimuli triggering its activation have been demonstrated, which can all be comprised under the term 'stress'. Physical as well as emotional stressors have been shown to induce the release of

POMC fragments into the cardiovascular compartment. These stressors range from fear to pain, from physical exercise and training to exhaustion exercise, from metabolic shifts to serious injury due to parturition, surgery or even accidents. Peptides derived from POMC are, in function, as hormones when they are secreted from pituitary cells into the bloodstream.

However, it should be mentioned that physical and psychological stressors are processed in the organism in a different way: the information launched upon peripheral tissue injury, in principle carrying the character of life-threat, is immediately signaled via spinal cord to the brain and is directly linked to the pituitary via hypothalamic structures. In contrast, emotional stressors or the psychological components of physical stressors are thought to be received and processed by supraspinal structures such as the limbic system in a pretty complicated way before the resulting information is transferred via hypothalamic structures to the pituitary for activation of the POMC system (Herman & Cullinan 1997), i.e. the activation of the HPA axis.

Since the isolation of the first endogenous opioids in 1975, interest has focused on deducing the analgesic properties of β-endorphin because the amino acid sequence of its N-terminus is able to bind with opiod receptors. However, exercise-induced elevation in pain threshold was not related to plasma endorphin levels (Droste *et al*. 1991), whereas elevated serum β-endorphin concentrations induced by exercise have been linked to altered pain perception (Harber & Sutton 1984). β-Endorphin has been correlated with several psychological and physiological changes, including 'exercise-induced euphoria', 'runners' high', exercise dependence, negative mood state changes, food intake suppression, immune suppression and reproductive dysfunction. But the insulation function provided by the blood brain barrier precludes any association between endorphin and central nervous activity, and, in addition, an increase of β-endorphin associated with an intensive bout of aerobic exercise appears to be independent of a tendency towards exercise dependence (Pierce *et al*. 1993a).

Methodological aspects of determination of POMC derivatives

A large individual variation in the β-endorphin/β-LPH response was noted by Farrell *et al*. (1982) and Sheps *et al*. (1988). In addition, some methodological difficulties concerning the determination of β-endorphin still exist. Whereas the determination of ACTH always revealed clear-cut results, in many studies β-endorphin IRM, in addition to β-endorphin, was determined to may be contain at least 10 β-endorphin derivatives. In fact, recent findings showed that β-endorphin IRM was more identical with β-LPH than with β-endorphin. In studies, wherein β-LPH was determined in comparison with ACTH or β-endorphin IRM using β-LPH specific determination methods (Oleshansky *et al*. 1990, Petraglia *et al*. 1988), β-LPH reached about the same plasma concentrations as β-endorphin IRM or ACTH. In contrast, further studies with selective or even highly specific assays for authentic β-endorphin showed that under exercise conditions the plasma concentrations of authentic β-endorphin were low (Farrell *et al*. 1987; Engfred *et al*. 1994) to minimal (Harbach *et al*. 2000; Schulz *et al*. 2000) in comparison with ACTH or β-LPH. Fragments from different regions of β-endorphin (1–31) might have quite different functions, as demonstrated on immune cells: β-endorphin interacts through its N-terminal fragment with opioid receptors whereas its interactions with binding sites on complement or on thymocytes occur via its C-terminus. Therefore the identity of 'β-endorphin' claimed to occur in the plasma upon exercise is of considerable importance for correct conclusions in terms of a possible function (Schulz *et al*. 2000).

Also, POMC fragment release from the adrenal medulla under physical exercise cannot be excluded (Evans, C.J. *et al*. 1983). Besides ACTH and β-LPH, further POMC derivatives, which apparently represent small-sized β-endorphin fragments (Wiedemann & Teschemacher 1983), may be released upon physical exercise under certain conditions. The proportions of the plasma concentrations of defined β-endorphin fragments may also vary as dependent on the state of fitness of the volunteers (Viru & Tendzegolskis 1995).

In addition to catecholamine release, there is evidence for an acute response of the pituitary POMC system to all kinds of stressors. However, there is no clear-cut information about the functional significance of the POMC fragments released under stress into the cardiovascular compartment. Although there are well-known targets such as the adrenal gland for ACTH, the functional significance of the effects elicited by the POMC fragments at peripheral targets is not clear at all; effects by ACTH via corticosteroids, for example in response to fear, are still a matter of speculation. Thus, the pituitary POMC system clearly plays the role of a 'stress-responder' but as yet it cannot be classified as a 'stress-adapter', since it is unclear how it might contribute to the reduction of a disturbed function to a homeostatic state (Teschemacher 2003). Although a couple of well-known exercise effects have been linked to POMC fragments, in particular to opioid peptides (for review see Cumming & Wheeler 1987), the functional significance of the POMC fragments released under exercise conditions is still a matter of speculation. Some of the functions discussed for POMC derivatives released into the cardiovascular compartment appear to be unlikely, since the respective effects are not thought to be elicited in peripheral tissues but in the central nervous system; for example, reduction of depressive state, reduced anxiety, improved self-esteem, improved well being—all in all, an improvement of mood occasionally becoming manifest as 'runner's high'. Experimental data indicate that aerobic exercise can activate central nervous system endogenous opioid systems, as shown by altered brain opioid levels and by increased levels of cerebrospinal fluid β-endorphin in running rats (Hoffmann *et al*. 1996).

The most likely hormonal role for circulating β-endorphin is the modulation of the adrenal response to stress, by controlling the release of cortisol in response to ACTH stimulation (McLoughlin *et al*. 1993). Further candidates for POMC fragment functions in the periphery are certainly influences on food uptake or reproduction, since fat tissue or gonads represent peripheral targets. However, central POMC systems in hypothalamic areas, in either case, appear to be more important candidates for influence on reproductive dysfunction (Rivier & Rivest 1991).

β-Endorphin and psychological effects during exercise

No biochemical link was observed for β-endorphin that might explain the possible influence of physical activity on depression (Williams & Getty 1986). However, lower β-endorphin resting plasma levels of trained subjects were related to an adaptation to exercise training and a greater emotional stability and lower depression (Lobstein *et al*. 1989). Endurance training of 8 months duration appeared to decrease the resting plasma β-endorphin concentrations and the depression scores (Lobstein & Rasmussen 1991).

Singh *et al*. (1999) observed different plasma ACTH and cortisol responses to psychological and exercise stress tests after dexamethasone treatment. Subjects were classified as responders based on ACTH responses to exercise. A psychological stress test raised heart rate, blood pressure, plasma ACTH and cortisol levels in both high responders (HRs) and low responders (LRs). HRs tended to have higher heart rates and blood pressures in anticipation of the psychological stress test than LRs. ACTH responses of HRs were higher, although not significantly, throughout the psychological stress test than LRs. HRs had a significantly greater cortisol response to the psychological stress than LRs. They suggested that the adrenal cortex of the HRs were hypersensitive to ACTH and concluded that men who are highly responsive to exercise stress were also highly responsive to psychological stress.

Post-traumatic stress disorder may be associated with changes in endogenous opioid peptide function. To test this hypothesis, Vietnam combat veterans with post-traumatic stress disorder underwent a standard exercise stress test. Resting plasma β-endorphin levels were comparable between veterans and controls. However, post-exercise plasma β-endorphin levels were significantly higher than resting levels only in the post-traumatic stress disorder patients. These data suggested a differential alteration in plasma β-endorphin response to exercise in post-traumatic stress disorder (Hamner & Hitri 1992). Healthy adolescent women received psychological and endocrine examinations. Anxiety scores and frustration tests were used. On the basis

of the results of these tests, subjects were divided into two groups: normal subjects and subjects with evidence of anxiety and/or frustration. Plasma levels of ACTH and β-endorphin were measured under basal conditions and after physical exercise. Basal concentrations of ACTH and cortisol were similar in the two groups, whereas basal β-endorphin levels were significantly higher in the anxiety/frustration group than in the control group. A striking increase in plasma ACTH and a slight increase of β-endorphin levels were observed in the anxiety/frustration group after exercise. Absolute levels of ACTH and β-endorphin after physical exercise were significantly higher in this group than in the control group. These findings indicate increased levels of adrenocorticotropic and opioid activity in adolescent women with high scores on psychological measures of anxiety and frustration (Gerra *et al*. 1992). In addition, significant elevations of β-endorphin and CRH were observed after running and meditation and were associated as a positive influence (Harte *et al*. 1995).

However, in one study by McGowan *et al*. (1993) no significant relationship was observed between pre- or post-exercise plasma β-endorphin and either total mood disturbance.

Conclusions

The secretion of POMC or POMC derivatives during exercise is regarded as an adaptive attempt of the athlete's organism to cope with different stress situations and is intimately linked to a variety of psychological strategies which facilitate its navigation through a stressful environment (McCubbin 1993). The secretion of ACTH and β-endorphin and their increase occurs in relation to the intensity and the duration of the physical exercise. But the exercise response may also be affected by the training status of the individual and the population being investigated (Goldfarb & Jamurtas 1997).

Efforts to clarify the question of β-endorphin involvement in exercise effects by the blockade of opioid receptors during exercise (Strassman *et al*. 1989; Angelopoulos 2001) revealed controversial effects on β-endorphin plasma levels but did not provide further insight into the mechanisms under question.

In case of situations of overtraining challenge, a reduced ACTH response reflects the athlete's impaired ability to cope with the stress situation. ACTH and cortisol responses of maximal intensity resistance exercise overtraining are contrary to high-volume resistance exercise and overtraining with highly aerobic activities. Thus, it becomes apparent that signs of overtraining, based on data from endurance athletes, cannot necessarily be applied to overtraining resulting from highly anaerobic activities. Future research must address the possible mechanism(s) of such a cortisol response, whether it is due to adrenal cortex exhaustion or sympathetic or other control (Fry & Kraemer 1997).

POMC fragments may have influence on the immune system, in particular at physical exercise stress (Jonsdottir *et al*. 1997). It is suggested that exercise can be employed as a model of temporary immune suppression that occurs after severe physical stress. The increase in catecholamines (epinephrine and norepinephrine) and growth hormone mediate the acute effects of exercise on neutrophils, whereas cortisol may be responsible for maintaining lymphopenia and neutrocytosis after exercise of long duration (Pedersen *et al*. 1997). However, the role of β-endorphin is less clear, but the cytokine response is closely related to muscle damage, and stress hormones do not seem to be directly involved in the elevated cytokine level. Lastly, these phenomena make it unlikely that β-endorphin plays a major immune modulatory role in the immediate recruitment of natural killer cells (for review see Pedersen & Hoffman-Goetz 2000).

The metabolic response to exercise stress might be a candidate for POMC fragment function (Weissman 1990) in support mechanisms for energy and homeostasis (Knudtzon 1986). POMC is acting in a complex interrelationship between the endocrine, metabolic, cardiac, neurologic and immune systems under physical exercise conditions. What can be concluded from the diverse evidence about exercise and the involvement of POMC? Given the inherent difficulties, the following ideas are summarized:

1 Among POMC derivatives, the most information —related to exercise conditions—is available on ACTH, which was studied as the main representative of POMC under the aspect of stress-induced

activation of the HPA axis. Later β-endorphin and β-LPH were recognized to be coreleased from the pituitary into the cardiovascular compartment under exercise conditions.

2 It is strictly the 'corticotropic' part of the pituitary POMC system that is activated under physical stress conditions.

3 β-Endorphin plasma levels have universally been reported to increase with exercise. However, the large individual variation in the β-endorphin/β-LPH response and the methodological difficulties concerning the determination of authentic β-endorphin are involved in the problem, and the biological significance of β-endorphin is still not elucidated.

4 Training and extreme physical stress lead to different ACTH and β-endorphin responses. The stimulus responsible for β-endorphin release under anaerobic exhaustive exercise conditions is probably acidosis, whereas non-exhaustive performance, such as heavy resistance exercise, does not lead to an increase of endorphin levels.

5 Age, gender and race have an important influence on POMC derivatives under exercise conditions, but further investigation of this influence on adaptation to acute and chronic exercise is necessary.

6 Glucose and lactate are substrates, which were intensively observed in exercise physiology because of changes under physical and extreme stress. ACTH, cortisol and β-endorphin seem to have an influence. However, it is not yet clear in which way they may contribute in stabilizing the homeostasis of the organism.

7 Correlations between POMC derivatives and gonadal steroids or gonadotropins are well testified.

8 Exercise is one of many stressors provoking immunologic reactions and therefore activating the pituitary POMC system. Effects of β-endorphin and ACTH can contribute to both activation as well as suppression of the immune system.

The response of POMC fragments, such as ACTH and β-endorphin, to exercise stress is complex and, in spite of multiple possible solutions, not yet elucidated. Despite evidence for an acute response of the pituitary POMC system to exercise stress ('stress sensor'), it cannot be classified as a 'stress regulator' or 'stress adapter'. Further investigation is necessary—in addition to the tremendous attempts of recent years—to define the functional significance of the POMC fragments released at exercise stress.

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Chapter 12

Introduction to the Insulin-Like Growth Factor Signaling System

CHARLES T. ROBERTS, JR

Introduction

The insulin-like growth factor (IGF) signaling system plays a critical role in the growth and development of many tissues, and is an important mediator of overall pre- and postnatal growth (reviewed in Rosenfeld & Roberts 1999). The IGF system is also implicated in pathophysiology, and plays a particularly important role in tumorigenesis. As described in more detail below, the IGF system is comprised of the IGF ligands (IGF-I and IGF-II), a series of cell-surface receptors that mediate the biological effects of the IGFs, and a family of insulin-like growth factor binding protein (IGFBP) that modulate the half-lives and bioavailability of the IGFs in the circulation and in extracellular fluids (Fig. 12.1). This review will provide an overview of the IGF system, its various components and signaling mechanisms, and its role in growth and development, with an emphasis on human data. As other chapters in this volume will describe the effects of exercise on specific components of the IGF system, such as the IGF ligands and the IGFBPs, this chapter will also discuss the potential effects of exercise on IGF signaling through the IGF receptors per se.

Insulin-like growth factor system components

The insulin-like growth factor ligands

IGF-I AND IGF-II STRUCTURE

The IGF-I and IGF-II ligands are encoded by large

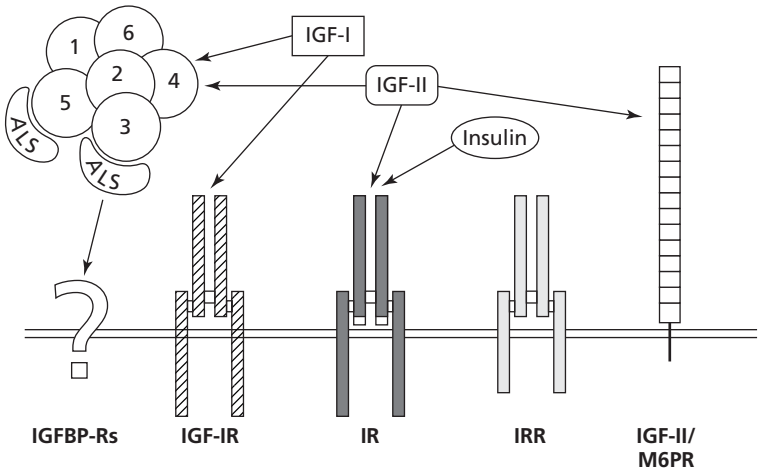

Fig. 12.1 Schematic overview of the insulin-like growth factor (IGF) signaling system.

genes, which have been extensively characterized in humans and rodents. The mature IGF-I peptide consists of B and A domains that are homologous to the B and A chains of insulin. Unlike the situation with insulin, they are not proteolytically cleaved, but remain linked in the mature peptide by a C domain analogous to the C peptide of insulin. Both IGF-I and IGF-II contain an additional short D domain that is not found in insulin. Additionally, the IGF-I and IGF-II prohormones contain a C-terminal E peptide that is cleaved in the Golgi apparatus during secretion. Alternative splicing of exons 5 and 6 of the human, rat and mouse *IGF-I* genes produces alternative E peptides, but the physiological significance of this prohormone diversity is unclear. The presence of multiple leader exons in mammalian *IGF-I* genes also results in variation in the signal peptide of the preprohormones, but the physiological consequences of this are also unclear. The organization and splicing of the human and rodent *IGF-II* genes is also complex, but involves non-coding exons and, therefore, does not affect the structure of the mature IGF-II molecule or its precursors.

IGF-I and IGF-II expression

The expression of the *IGF-II* gene in rodents is widespread prenatally, but diminishes drastically after birth, with the choroid plexus and the leptomeninges being the persistent sites of synthesis in adult animals. Murine expression of IGF-I, on the other hand, is low prenatally and significantly increases during puberty, with hepatic production being a major contributor to overall IGF-I levels in the circulation. IGF-I is produced by numerous other adult organs, however, including kidney, lung and bone, and exerts endocrine, paracrine and autocrine effects. This inverse pattern of IGF-I and IGF-II expression in rats and mice initially led to the concept of IGF-II as a fetal growth factor and IGF-I as an adult growth factor. This is not the situation in humans, however, since both IGF-I and IGF-II are produced throughout life by multiple tissues. In fact, the circulating levels of IGF-II are consistently several-fold higher than those of IGF-I, which supports that concept that there are potentially divergent roles for the two IGFs in human physiology.

The insulin-like growth factor receptors

The IGF-I and IGF-II ligands interact with an array of cell-surface receptors that may be present singly or in combination on target cells. It was initially thought that IGF-I primarily activated the type 1 IGF or IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase structurally and functionally related to the insulin receptor (IR) (LeRoith *et al*. 1995; Adams *et al*. 2000). IGF-II, on the other hand, was known to interact with high affinity with the type 2 IGF, or IGF-II receptor (IGF-IIR). Subsequent studies have shown that both IGF-I and IGF-II interact with the IGF-IR, albeit with a ~ threefold difference in affinity (IGF-I > IGF-II). The cloning of the IGF-IIR cDNA revealed that it was identical to the previously characterized cation-independent mannose-6-phosphate (M6P) receptor involved in endocytosis and intracellular trafficking of M6P-tagged proteins. Although some early studies proposed an active role for the IGF-IIR in IGF-II signaling, based upon apparent sequence homology between the cytoplasmic domain of the IGF-IIR and the intracellular loops of G-protein-coupled receptors, subsequent studies ruled out the ability of the short intracellular domain of the IGF-IIR to mediate signal transduction. The function of this molecule in IGF-II action is thought to reflect its ability to serve as a clearance receptor for the IGF-II, thereby influencing the extracellular concentration of IGF-II.

The biological effects of IGF-I result primarily from its activation of the IGF-IR. IGF-I does not cross-react with the IR, except at pharmacological doses, since the relative affinity of IGF-I for the IGF-IR versus the IR differs by at least an order of magnitude. It was initially thought that IGF-II, like IGF-I, only bound appreciably to the IGF-IR as compared to the IR. Studies in knockout mice lacking various combinations of the IGF system and the IR suggested that IGF-II acted through the IR in early development, prior to detectable *IGF-IR* gene expression (Louvi *et al*. 1997). The molecular basis for this phenomenon was revealed when it was discovered that a splice variant of the IR displayed high affinity for IGF-II. Specifically, the IR transcript is subject to alternative splicing of exon 11, which encodes a 12-amino acid segment at the C terminus

of the extracellular β subunit. Previous studies had shown that the IR isoform encoded by the mRNA lacking the exon-11 sequence (IR-A) displayed a twofold higher affinity for insulin than the IR-B isoform specified by the exon 11-containing mRNA. It has been established that the IR-A isoform, in fact, functions as a high-affinity receptor for IGF-II and produces predominantly proliferative effects as compared to the principally metabolic effects elicited by insulin stimulation of IR-B (Frasca *et al*. 1999). Thus, IGF-I functions primarily by activating the IGF-IR, while IGF-II can act through either the IGF-IR or through the A form of the IR.

Hybrid receptors and the insulin receptor-related receptor

The scope of IGF signaling is made significantly more complex by the existence of hybrid receptors that result from the dimerization of IGF-IR and IR hemireceptors, each consisting of a single α and β subunit linked by disulphide bonds (Fig. 12.2). These hybrid receptors are formed by the formation of intra-α subunit disulphide bonds in the Golgi apparatus of cells expressing both the *IGF-IR* and *IR* genes. While originally considered to represent a small proportion of the total number of IGF-IR and IR in a given cell, some reports have suggested that the formation of hybrids is preferred over the formation of classical IGF-IR and IR heterotetramers. This could result from the preferential formation of disulphide bonds between cysteine residues in IGF-IR and IR α subunits themselves. Thus, in some circumstances, hybrid receptors may outnumber 'pure' IGF-IR or IR molecules at the cell surface.

With respect to ligand binding, IGF-IR/IR hybrid receptors retain high affinity for IGF-I, but exhibit severely reduced affinity for insulin. It is thought that this reflects the ability of IGF-I to efficiently bind to either IGF-IR α subunit, while tight insulin binding requires its interaction with both of the β subunits found in the IR. As a consequence, the existence of significant number of hybrid receptors may preferentially diminish cellular responsiveness to insulin, but not IGF-I. This has been proposed as a mechanism through which up-regulation of IGF-IR

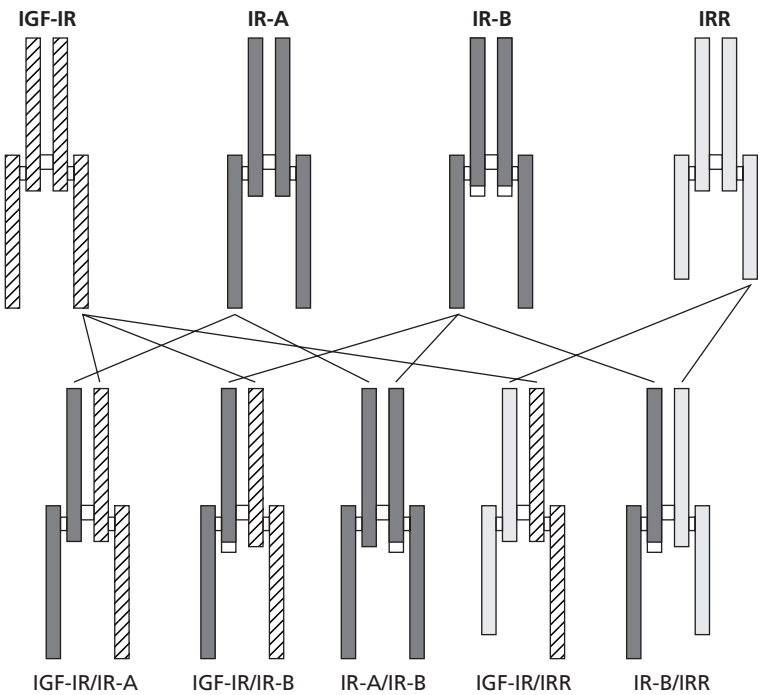

Fig. 12.2 Insulin-like growth factor (IGF) system receptors and hybrids.

expression could result in insulin resistance in cells expressing the IR. The situation with hybrid receptors has been further complicated by the existence, and the IGF-II-binding characteristics, of IR-A and IR-B. IR-A/IR-B hybrid receptors undoubtedly occur, since most cell express both splice variants. The difficulty in distinguishing these variants experimentally has precluded an examination of the binding characteristics and signaling capabilities of this particular class of hybrid receptors. It has been demonstrated, however, that IGF-IR/IR-A hybrids bind IGF-I, IGF-II and insulin, whereas IGF-IR/IR-B hybrids bind IGF-I with high affinity, IGF-II with low affinity, and do not bind to insulin (Pandini *et al*. 2002). Thus, the relative expression of the *IGF-IR* and *IR* genes and the degree of alternative splicing of exon 11 of the *IR* gene governs the ability of a given cell to respond to IGF-I, IGF-II and insulin.

The insulin receptor-related receptor (IRR) is the third member of the IGF-IR/IR family and does not exhibit binding to IGFs or to insulin (Watt *et al*. 1993). Although still considered an orphan receptor, it has been shown to form hybrids with the IR when both entities are overexpressed in NIH-3T3 fibroblasts. The formal possibility exists, therefore, that IGF-IR/IRR, IR-A/IRR, or IR-B/IRR hybrids may occur in the restricted set of tissues that express the *IRR* gene, and that the formation of such hybrids could, like the formation of IGF-IR/IR hybrids, influence cellular IGF and insulin responsiveness. In fact, a recent study that analyzed double and triple knockouts of the *IR*, *IGF-IR* and *IRR* genes demonstrated a role for the IRR in testis development, presumably through its modulation of IR and IGF-IR action through hybrid formation (Nef *et al*. 2003).

Insulin-like growth factor binding proteins

The biological activities of the IGF ligands are modulated by a family of high-affinity IGFBPs (IGFBP-1 through -6) that are found in the circulation and in extracellular fluids (Jones & Clemmons 1995). IGFBP-3 is the predominant IGFBP in serum, and most circulating IGF-I and IGF-II is not found in a free form, but in a ternary complex with IGFBP-3 and a third component, the acid-labile subunit (ALS), in a 1 : 1 : 1 molar ratio. IGFBP-5 also forms ternary complexes with IGFs and ALS. While IGFBP-1 through -4 exhibit generally similar affinities for IGF-I and IGF-II, IGFBP-5 and -6 bind IGF-II with 10- and 100-fold greater affinities, respectively, than their binding to IGF-I. The IGFBPs do not bind insulin. The IGFBPs control IGF action by increasing the half-lives of circulating IGFs, by controlling their availability for receptor binding, and, in the case of cell surface-associated IGFBPs, by potentially influencing their direct interaction with receptors. Each of the IGFBPs is subject to limited and potentially regulated proteolysis by various proteases. Thus, ligand–receptor interactions in the IGF system are subject to complex regulation as a result of IGFBP levels, expression profile, degree of cell–surface association and extent of proteolysis.

A series of studies performed over the last several years has established the concept of IGF-independent actions of some of the IGFBPs (Oh 1998). IGFBP-3 and 5, in particular, have been shown to exhibit effects on proliferation, migration and sensitivity to apoptosis that are independent of their effects on IGF signaling per se. Some of these 'IGF-independent' effects are still modulated by IGF binding to the responsible IGFBP, so that 'IGF receptor-independent action' may be a more accurate term for these novel functions. The cell-surface or intracellular molecules that participate in those effects are poorly characterized, but IGFBP-3 and -5 have been identified in the nuclei of cells following exposure to exogenous recombinant protein. This aspect of IGFBP action, when clarified, will add an important dimension to our understanding of the IGF signaling system in general.

IGF-I receptor and insulin receptor signaling pathways

The signaling pathways that mediate IGF action are, in large part, represented by those identified to date for the IGF-IR (Fig. 12.3). Upon binding of IGF-I or IGF-II to the extracellular α subunit (specifically, a binding region comprised of the internal cysteine-rich region and the adjacent C-terminal L2 domain of the α subunit), a conformational change is induced in the trans-membrane β subunits, resulting in

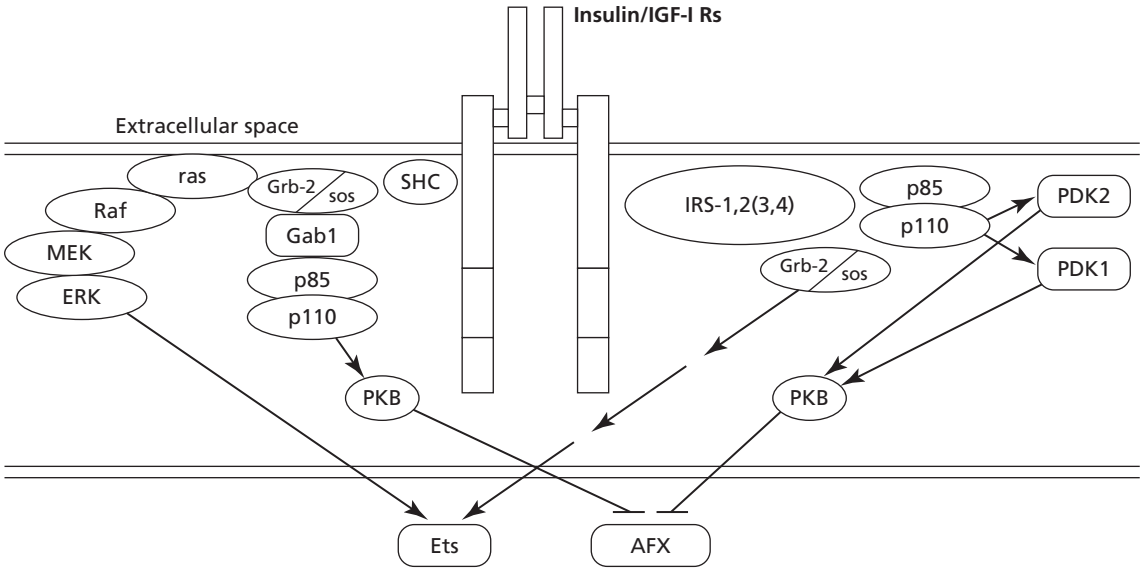

Fig. 12.3 Insulin-like growth factor I receptor (IGF-IR) signal transduction pathways.

trans-autophosphorylation of the tyrosine kinase domain intrinsic to the cytoplasmic portion of the β subunit. This process fully activates the receptor tyrosine kinase, which autophosphorylates the additional tyrosine residues in the juxtamembrane and C-terminal domains that flank the tyrosine kinase domain. These residues, particularly tyrosine 950 in the juxtamembrane domain, then serve as docking sites for members of the insulin receptor substrate (IRS) and the Shc adaptor protein families. Subsequent phosphorylation of these proteins by the receptor tyrosine kinase allows IRS and Shc proteins to engage factors such as Grb-2/SOS and the p85 regulatory subunit of PI3 kinase, leading to activation of the MAP kinase and PI3 kinase cascades that constitute the major signal transduction cascades emanating from the activated IGF-IR. Among the ultimate targets of the MAP kinase and PI3 kinase cascades are members of the Ets and forkhead transcription factor families, which provides a mechanism for IGF action at the cell surface to effect the changes in gene expression that underlie the effects of IGF signaling on cellular proliferation, differentiation and apoptotic sensitivity.

There is a general notion that the MAP kinase pathway exerts effects on proliferation, and that the PI3 kinase pathway modulates differentiation and sensitivity to apoptosis. However, as is the case with almost every aspect of the IGF system, nothing is simple. In MCF-7 breast cancer cells, IGF-I-induced mitogenesis requires the PI3 kinase pathway, but not the MAP kinase pathway (Dufourny *et al*. 1997), while in H19-7 neuronal cells, the PI3 kinase pathway is also required for IGF-I-stimulated mitogenesis, and the MAP kinase cascade is necessary for IGF-I-induced differentiation (Morrione *et al*. 2000). In terms of anti-apoptotic signaling by the IGF-IR, protection of Rat-1 fibroblasts from ultraviolet-B-induced apoptosis required PI3 kinase pathway activation, but not the MAP kinase pathway (Kulik *et al*. 1997), while IGF-IR-mediated protection of PC12 cells from serum withdrawal-induced apoptosis involved both pathways acting in a synergistic fashion (Parrizas *et al*. 1997). These and other examples suggest that the specific pathways involved in, and their relative contribution to, the effects of IGF-IR signaling on growth, differentiation and apoptosis are cell type-specific.

While IGF action can clearly be controlled by the levels of extracellular ligand and the number (and types) of receptors at the cell surface, the relative abundance of receptor targets may be an important

factor in determining the effects of IGFs in a given target cell. For example, there are four members of the IRS family, IRS-1 through 4, each of which has a similar, yet unique, structure. The presence of different combinations of IRS proteins may result in differential responses to IGF-IR activation. In fact, recent studies have suggested that IRS-3 and IRS-4 can inhibit processes activated through IRS-1 and IRS-2 (Tsuruzoe *et al*. 2001). The relative levels of Shc and IRS proteins may also be an important factor influencing IGF action, in that they have been shown to compete for binding to tyrosine 950 of the activated IGF-IR.

The general characteristics of signaling and the regulatory possibilities described above for the IGF-IR also apply to the IR (with IR-A and IGF-IR/IR hybrid receptors being relevant to our topic), but there are important differences between the IGF-IR and IR that may have important ramifications for differential actions of IGF-I and IGF-II. In the first place, apart from the conserved tyrosine 950/960 in the IGF-IR and the IR, the position and number of tyrosine residues in the juxtamembrane and C-terminal domains that are subject to autophosphorylation differ between the IGF-IR and IR. In addition, the IGF-IR and the IR have been shown to utilize different heterotrimeric G proteins as part of their signaling mechanisms (Dalle *et al*. 2001; Kuemmerle & Murthy 2001), and other proteins have been identified that interact specifically with the C terminus of the IGF-IR, but not with the IR. A final difference in signaling pathways engaged by the IGF-IR and the IR is the involvement of STAT-3 (Zong *et al*. 1998, 2000; Prisco *et al*. 2001) and STAT-5 (Okajima *et al*. 1998) in IGF-IR signaling. These differences, in conjunction with the existence of different classes of hybrid receptors, make IGF signaling in cells that express the IR (or the IRR), in addition to the IGF-IR, extremely complicated.

The role of the insulin-like growth factor system in growth and development

The contributions of IGF action to growth and development have been discerned from studies in transgenic mice in which various components of the IGF signaling system have been ablated or overexpressed, and from human studies of populations such as Pygmies and rare individuals with mutations affecting the *IGF-IR* and *IGF-I* genes. These findings are discussed below.

Evidence from transgenic animals

PRENATAL GROWTH

The role of IGF action in prenatal growth has been inferred from the phenotypes of transgenic and knockout mice in which the expression of the *IGF-I*, *IGF-II*, *IGF-IR* and *IGF-II*/*MGP* receptor genes have been manipulated (DeChiara *et al*. 1991; Baker *et al*. 1993; Liu *et al*. 1993; Powell-Braxton *et al*. 1993). IGF-I and IGF-II deficiency each results in a 40% decrease in birthweight, with IGF-II knockout mice also exhibiting placental growth retardation. Double knockouts exhibit an additive growth deficiency of 80%. IGF-I knockouts can exhibit general perinatal lethality, depending on the genetic background. IGF-IR knockout mice exhibit a 55% decrease in growth rate, which is less than that seen in the IGF-I/IGF-II knockouts, and invariably die of suffocation at birth due to inadequate development of the musculature of the diaphragm. Additional deletion of the *IGF-I* gene in IGF-IR knockout animals does not further decrease birth weight, suggesting that IGF-I functions exclusively through the IGF-IR. In contrast, IGF-II and IGF-IR double knockouts are more growth-retarded than single IGF-IR knockouts, suggesting that IGF-II effects can be mediated by another receptor during embryogenesis. Analysis of IGF-IR/IR knockouts (Louvi *et al*. 1997) suggested that the IR was responsible for IGF-II signaling not mediated by the IGF-IR. As mentioned above, it was subsequently found that alternative splicing of exon 11 produces an IR isoform that exhibits high affinity for IGF-II. An indirect role for the IGF-II/M6P receptor in prenatal growth (Lau *et al*. 1994; Wang *et al*. 1994; Ludwig *et al*. 1997) was inferred from the phenotype of IGF-II/M6P receptor knockout mice, which exhibit modest fetal and placental overgrowth (25–40%). This phenotype has been interpreted as resulting from the excess IGF-II that is seen in the serum and tissues of these animals due to lack of the clearance function of the IGF-II/M6P receptor.

POSTNATAL GROWTH

IGF-I-deficient mice that do not die perinatally exhibit severely reduced postnatal growth, while IGF-II-deficient mice, although smaller than normal at birth, have normal growth velocities postnatally. These findings support the concept that IGF-I is the principal mediator of postnatal growth. The lack of a postnatal phenotype in IGF-II-deficient mice is not surprising in light of the normal shutoff of IGF-II expression in almost all mouse tissues postnatally.

Global overexpression of IGF-I in transgenic mice produces generalized hyperplasia and organomegaly, resulting in adult animals that are 30% larger than normal (Mathews *et al*. 1988). Conversely, postnatal overexpression of IGF-II does not result in increased somatic growth (Rogler *et al*. 1994; Wolf *et al*. 1995). Again, the lack of a growth phenotype in IGF-II transgenics may reflect the lack of postnatal IGF-II expression in postnatal rodents.

To date, no convincing phenotype has been observed in knockouts of any of the six IGFBPs, including several double knockouts. This puzzling lack of an effect may reflect the redundancy of function among the six IGFBPs.

Insulin-like growth factor system effects in humans

The role of IGF action in human growth and development has come from several lines of evidence, including analysis of African Pygmies, a patient with mutational loss of the *IGF-I* gene and a series of patients with hemizygosity of the *IGF-IR* gene resulting from loss of the distal arm of chromosome 15.

EFE PYGMIES

Initial cross-sectional studies of Mbuti and Babinga Pygmies concluded that the short stature of these populations was due to the lack of the pubertal growth spurt (Mann 1987). Subsequent longitudinal studies of Efe Pygmies demonstrated growth retardation at birth that increased in the first 5 years of life (Bailey 1990, 1991). More recently, it has been shown that immortalized T- and B-cell lines from Efe Pygmies are IGF-I-resistant (Geffner *et al*. 1993, 1995; Cortez *et al*. 1996). The molecular basis for this IGF-I resistance and, potentially, the growth phenotype of the Efe population, appears to be a defect in *IGF-IR* gene expression (Hattori *et al*. 1996). Thus, decreased IGF action due to decreased IGF-IR levels causes pre- and postnatal growth retardation in humans.

HUMAN MUTATIONS AFFECTING THE *IGF-I* AND *IGF-IR* GENES

One patient has been described who was homozygous for a partial deletion of the *IGF-I* gene (Woods *et al*. 1996). This patient made no active IGF-I, exhibited severe pre- and postnatal growth retardation, and also presented with deafness, mental retardation and microcephaly, characteristics not found in patients with growth hormone deficiency or resistance. This patient's parents were heterozygous for the *IGF-I* gene mutation, had extremely low circulating IGF-I levels and also exhibited short stature. These findings provide additional support for a role of IGF-I in both pre- and postnatal growth and development.

A number of patients have been described that are hemizygous for the *IGF-IR* gene as the result of deletion of the distal arm of chromosome 15 (Roback *et al*. 1991; Siebler *et al*. 1995) or ring chromosome 15 syndrome (Tamura *et al*. 1993; Peoples *et al*. 1995). All of these patients exhibited intrauterine growth retardation and postnatal growth failure, as well as other developmental abnormalities. Although the growth-deficiency phenotype consistently manifested by these patients is consistent with decreased IGF-IR levels, the fact that no direct demonstration of IGF resistance in cells derived from these patients has been reported makes this data supportive, but not definitive, evidence for a role of IGF-IR action in human pre- and postnatal growth and development.

Potential effects of exercise on insulin-like growth factor signaling and action

The possible consequences of exercise on the functioning of the IGF signaling system and IGF-regulated physiology can occur at many levels, including effects on local and circulating concentrations of IGF

ligands and IGFBPs (as well as proteolysis of the latter) or more subtle effects on IGF receptor expression and function at a cellular level. The relationship between exercise and the IGFs and their binding proteins are described in companion chapters, so the following discussion will focus on exercise and IGF signaling. There are, to date, no data on the relationship between exercise and expression or the intrinsic activity of the IGF-IR that mediates the majority of IGF signaling.

An alternative possibility is that changes in IR expression or activity could modulate IGF signaling. This could be a direct effect if these changes involved the IR-A isoform of the IR, since, as discussed above, this IR isoform is a functional receptor for IGF-II. Alteration of expression or activity of either IR isoform could also indirectly affect IGF-I or IGF-II signaling through the IGF-IR as a result of the formation of hybrid receptors. While the evidence to date supports an effect of exercise on general insulin action and glucose metabolism in particular (reviewed in Wojtaszewski *et al*. 2002; Zierath 2002), the extent of exercise-induced effects on IR levels or activity, particularly in humans, is unclear. One study reported an effect of exercise on IR autophosphorlyation (Youngren *et al*. 2001), but most studies have described effects on more distal signaling components such as IRS-1 and IRS-2 (Chibalin *et al*. 2000; Nagasaki *et al*. 2000) and the PI3 kinase pathway (Kim *et al*. 1998). These latter effects, however, would be rather non-specific, as they affect signaling factors utilized by a multitude of hormones and growth factors.

Conclusions

Despite decades of intensive investigation, there remain basic aspects of the IGF system that are poorly understood. Principal among these is the role of IGF-II in human physiology. Additionally, the effects of IGF-I and IGF-II in combination at a cellular level are entirely unknown, although most human tissues are routinely exposed to a combination of endocrine, paracrine and, often, autocrine IGF-I and IGF-II. In the context of this volume, the complexity of the IGF system provides a wide spectrum of targets through which the effects of exercise on IGF-regulated physiology and pathophysiology may be mediated.

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Chapter 13

Exercise, Training and the GH–IGF-I Axis

ALON ELIAKIM, DAN NEMET AND DAN M. COOPER

Introduction

Physical activity plays an important role in tissue anabolism, growth and development, yet little is known about the mechanisms that link patterns of exercise with tissue anabolism. Considerable anabolic stimulus arises even from the relatively modest physical activity of daily living (Leblanc *et al*. 1990). Therefore, anabolic effects of exercise training are not limited to individuals participating in competitive sports who are particularly focused on improvement of muscle strength and endurance. On the other hand, participation of young athletes in intense training, especially if associated with inadequate caloric intake, exposes the young athletes to several health risks and hazards, and may lead to a reduction in growth potential (Theintz *et al*. 1993).

The exercise-associated anabolic effects are age and maturity dependent. It is remarkable that naturally occurring levels of physical activity, energy expenditure and muscle strength exhibit some of their most rapid increases during childhood and adolescence. The combination of rapid growth and development, high levels of physical activity and spontaneous puberty-related increases in anabolic hormones (growth hormone [GH], insulin-like growth factor I [IGF-I] and sex steroids) suggest the possibility of integrated mechanisms linking exercise with a variety of anabolic responses. This chapter will focus on the effect of physical activity and exercise training on components of the GH–IGF-I axis and on differences between systemic and local (i.e. muscle) responses to exercise. Finally, the effect of exercise on the GH–IGF-I axis has been studied more thoroughly in recent years and, therefore, the GH–IGF-I response to exercise and training can assist competitive athletes and coaches in the evaluation of the training load.

The GH–IGF-I axis

The GH–IGF-I axis is composed of hormones, growth factors, binding proteins (BPs) and receptors that regulate many essential life processes, including growth and development, metabolic and reparative processes and aging. Therefore, the understanding of the axis must consider each individual component and the interaction between them under both physiologic and pathological conditions. The axis starts in the central nervous system (CNS) where several neurotransmitters (chatecholamines, serotonin and cholinergic agents, etc.) stimulate the hypothalamus to synthesize growth hormone releasing hormone (GHRH) and somatostatin (SS). GHRH stimulates the anterior pituitary to synthesize and secrete GH. In contrast, SS directly inhibits GH secretion.

GH is the major secretory product of the axis. One of GH most important actions is the stimulation of hepatic IGF-I synthesis. However, other effects of GH on metabolism, body composition and tissue differentiation are independent of IGF-I. GH exerts direct feedback effect on the two hypothalamic hormones that control its secretion. Tissue GH effects result from the interaction between GH and the GH receptor. The GH receptor contains intracellular and extracellular transmembrane domains. The extracellular domain is identical in structure to growth

hormone binding protein (GHBP) (Leung *et al*. 1987); therefore, a unique feature of this axis is that GH receptor number and activity can be determined easily by measurements of circulating GHBP levels.

IGF-I is part of the insulin-related peptides. IGF-I can act as a hormone, and these effects are GH dependent. However, the majority of IGF-I actions occur primarily as a result of paracrine or autocrine secretion and regulation, which are only partially GH-dependent. IGF-I is responsible for most, but not all, of the anabolic and growth promoting effects of GH. IGF-I stimulates SS secretion, and inhibits GH by a negative feedback mechanism (Berelowitz *et al*. 1981), but it is not clear whether circulating or local central IGF-I is responsible to this feedback mechanism.

The majority of circulating IGF-I is bound to several IGFBPs. The most important circulating BP during adult life is IGFBP-3, which is synthesized mainly in the liver, and is GH dependent. When bound to IGF-I, it complexes with an acid-labile subunit to form the circulating complex that carries most of the IGF-I in the serum. Some of the IGFBPs are GH dependent (e.g. IGFBP-3), but others, such as IGFBP-1 and -2, are insulin dependent (being high when insulin levels are low). The interaction between IGF-I and its BPs is even more complicated since some BPs stimulate (e.g. IGFBP-5), while others inhibit (e.g. IGFBP-4) IGF-I anabolic effects (Rajaram *et al*. 1997).

The effects of IGF-I result from its interaction with two different receptors. Type I receptor has tyrosine-kinase activity, and mediates most of the IGF-I effects. This receptor exhibit similarities to the insulin receptor and, therefore, may bind also insulin which has known anabolic effects as well. The type II receptor is identical to the mannose-6-phosphate receptor, and binds IGF-II as well.

Some hormones in the GH–IGF-I axis (i.e. GHRH, SS and GH) have a pulsatile pattern of secretion, and it has been shown that the pulsatility of GH secretion is significantly important for accelerated growth rate (Clark *et al*. 1985). In contrast, IGF-I and IGFBPs level are relatively stable during the day.

In addition to the important effect on growth, GH and IGF-I have a marked effect on body composition. Both hormones stimulate increases in muscle mass and bone mineral density, and reduce fat distribution.

Several components of the axis are age dependent. GHRH, GH, GHBP, IGF-I and IGFBP-3 reach their peak circulating levels during puberty (Mauras *et al*. 1987) and decrease with aging (Corpas *et al*. 1993). These changes are partially sex-hormone dependent. Nutritional state has also a remarkable influence on the GH–IGF-I axis. For example, fasting and malnutrition increase GH secretion, but despite the elevated GH, IGF-I levels are reduced (Marimme *et al*. 1982), probably due to a decrease in GH receptors. In this chapter we will focus on physical activity, another environmental regulator of the GH–IGF-I axis and its components.

The effect of a single exercise

GROWTH HORMONE

One should differentiate between the acute effects of a single bout of exercise on the GH–IGF axis from neuroendocrinological adaptations that occur in physically active people or in response to long-term programs of endurance training.

The GH response to exercise is dependent on the duration and intensity of the exercise bout, the fitness level of the exercising subject, the timing of blood sampling, refractoriness of pituitary GH secretion to exercise stimuli and other environmental factors. Therefore, standardized exercise protocols should be used to evaluate the GH response to acute exercise.

Several previous studies reported that the GH response to exercise is greater in *less fit* subjects (Buckler 1972). However, in those earlier studies, subjects were asked to perform exercise tests at the same *absolute*, rather than *relative*, power. As a consequence, due to the great variability in fitness, some subjects exercise below, while others exercise above, their lactic/anaerobic threshold (LAT). This is important since several investigators (Felsing *et al*. 1992) have demonstrated that circulating GH levels increased only in response to above, but not below LAT, and that exercise loads of 75–90% of maximal aerobic power yielded a greater GH rise than milder

loads (Hartley *et al*. 1972; Sutton & Lazarus 1976). Therefore, results of studies in which the GH response to exercise was tested at an absolute work rate demonstrate simply that as individuals become fitter, the stress associated with exercise at an absolute work rate diminishes.

In contrast to the observation that the exercise input should be sufficient to cause a sizeable metabolic effect in order to stimulate GH secretion, we previously demonstrated a small but significant GH response to an exercise input that had no systemic effect on heart rate or circulating lactate levels (i.e. unilateral wrist flexion) (Eliakim *et al*. 2000). These data suggest that factors like perceived exertion and associated psychological stress may lead to activation of the hypothalamic–pituitary axis and GH release even in exercises involving small muscle groups.

The duration of exercise for stimulation of GH secretion should be at least 10 min (Bar-Or 1983), since exercise of shorter duration (e.g. 5 min at above LAT [Felsing *et al*. 1992]) was not accompanied by increases in circulating GH levels. Moreover, exercise-induced GH peak occurs 25–30 min after the start of the exercise, irrespective of the exercise duration (Schwarz *et al*. 1996; Eliakim *et al*. 1999), and occurs few minutes earlier in women (Wideman *et al*. 1999). Thus, when the exercise task is brief (e.g. 10 min) a peak may be reached after its cessation, while when the exercise task is long (e.g. 45 min) the peak may be reached while the individual is still exercising. Blood sampling, however, should be timed to the exercise-induced GH peak.

Pituitary refractoriness, a time in which the normal pituitary gland will not respond sufficiently to a stimulus for GH release could also influence the GH response to exercise. We previously demonstrated that the GH response to exercise was inhibited if a spontaneous, early morning, GH pulse had occurred within 1 h prior to the exercise test (Eliakim *et al*. 1999). This inhibition occurred probably due to the phenomenon of GH autoinhibition (Pontiroli *et al*. 1991) in which the elevated circulating GH from the previous spontaneous pulse attenuated the pituitary's response to exercise. Cappon *et al*. (1994) previously demonstrated a refractory period of at least 1 h following exercise induced GH secretion (i.e. the subsequent GH response to exercise was attenuated), and suggested that exercise-induced elevation in free fatty acids or alterations in parasympathetic–sympathetic tone might have been responsible (Casanueva *et al*. 1984, 1987; Klijman & Frohman 1991). Ronsen *et al*. (2001) showed a recovery from pituitary refractoriness to GH secretion if a second bout of high intensity endurance exercise was performed 3 h after the first session. Consistent with this report, integrated 1.5-h GH concentrations were significantly greater if differences between the exercise bouts (30 min, 70% $\dot{V}O_{2max}$) were 3.5 h and not 1 h (Kanaley *et al*. 1997).

Environmental factors as well as some pathological states may interfere with GH response to exercise. Administration of a high fat meal attenuated the magnitude of GH response to exercise (Cappon *et al*. 1993), and this inhibition was correlated with circulating levels of SS. High ambient temperature may in itself increase circulating GH level, while low temperature attenuates GH release (Buckler 1973). Obesity and polycystic ovarian syndrome (Wilkinson & Parkin 1974) are characterized by attenuated GH response to exercise. Exercise-associated GH release is reduced in amenorrheic athletes, reflecting possibly decreased GHRH response to exercise compared to eumenorrheic athletes (Waters *et al*. 2001). Finally, the age-related decline in GH secretion was associated also with reduced GH response to exercise (Zaccaria *et al*. 1999).

The increase in circulating GH levels following exercise may serve in the diagnosis of GH deficiency. Since GH is secreted in pulses, and during most of the day its levels are very low, a single random blood sample can not differentiate between the healthy and GH-deficient child. To overcome this, a number of provocative tests to stimulate pituitary GH release have been developed (Fraiser 1974). Most of these provocative tests use pharmacological agents (Cowell 1995) and present some risk (e.g. hypoglycemia) for the patient. Moreover, the interpretation of a normal GH response to *pharmacological* stimuli can be questioned because it does not necessarily give information about *physiological* GH secretion. These confounding factors have led a number of investigators to emphasize the role of *physiological* stimulation tests such as exercise, or

to focus on less variable circulating substances, like IGF-I and/or its BPs, in the diagnosis of GH-deficiency in children (Rosenfeld *et al*. 1995).

INSULIN-LIKE GROWTH FACTORS

It is now well established that acute exercise lead to an increase in circulating IGF-I levels. Interestingly, several studies demonstrated that the exercise-induced IGF-I increase occurred following very short high intensity exercise (i.e. 90 s) (Kraemer *et al*. 2000), occurred 10 min following the beginning of endurance exercise (Bang *et al*. 1990; Schwarz *et al*. 1996) and occurred in exercise of both below and above the LAT (Schwarz *et al*. 1996). Exercise was also associated with an increase in urinary IGF-I (De Palo *et al*. 2002).

The mechanism for the transient increase in circulating IGF-I in response to exercise is not readily apparent. One possibility would be the classic mechanism of increased hepatic IGF release due to exercise-induced secretion of GH. As noted earlier, GH increases significantly mainly in response to high intensity exercise, while IGF-I increases for both low and high intensity exercise. Moreover, circulating IGF-I reaches its peak *before* the GH peak (i.e. 10 vs. 30 min) (Fig. 13.1), while increases in serum IGF-I, due to *de novo* IGF-I synthesis in the liver and transport to the circulation, occurs several hours *after* the administration of endogenous GH (Marcus *et al*. 1990). In addition, earlier studies showed (Bang *et al*. 1990) that exercise led to increases in IGF even in subjects with pituitary insufficiency. These studies suggest that the exercise-associated increase in IGF-I is, in fact, not related to GH and must reflect rapid changes in IGF-I distribution in the circulation due to release from marginal pools or changes in IGF-I removal. In addition, the transient nature of the increases suggests that hemodynamic or metabolic effects of exercise might play a role. Exercise in humans is accompanied by the rapid autotransfusion of hemoconcentrated blood from the spleen into the cellular circulation (Flamm *et al*. 1990), by increased blood flow to the exercising muscle and by loss of plasma water (Convertino *et al*. 1981). Each of these phenomenona might explain, in part, an increased IGF concentration by changes in IGF flux and/or volume of distribution.

Another possible source for the increase in circulating IGF-I can be a release from the exercising muscle. To test this, we used a simple approach in which subjects performed a unilateral repeated flexion of the wrist against relatively high resistance, while during- and post-exercise blood samples were collected simultaneously from the basilic vein of both the exercising (representing local release) and resting arm (representing systemic response) (Eliakim *et al*. 2000). We found a bilateral, simultaneous increase in IGF-I suggesting that the local

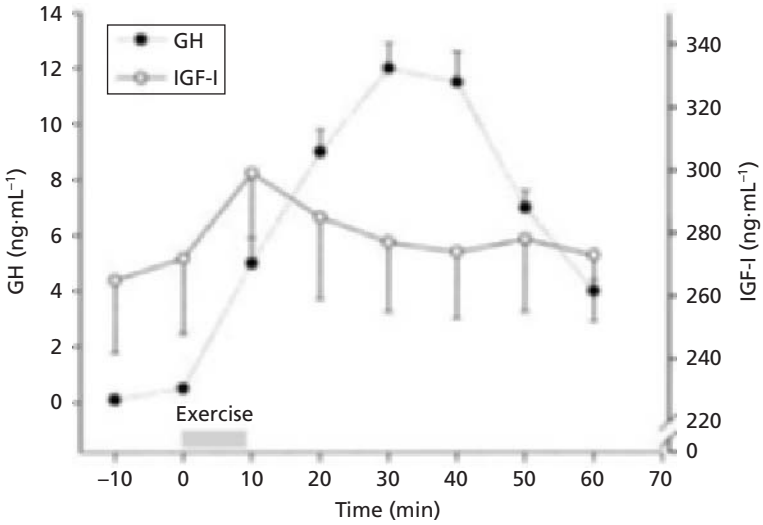

Fig. 13.1 Typical growth hormone (GH) and insulin-like growth factor I (IGF-I) response to intense endurance-type exercise bout. Peak IGF-I level occurs before the GH peak emphasizing that the exercise-induced increase in circulating IGF-I is GH-independent.

exercising muscle was not the source for of the IGF-I increase.

Interestingly, Elias *et al*. (2000) showed a transient rise in circulating IGF-I immediately after exercise test to exhaustion. IGF-I levels fell thereafter to reach nadir level 60–90 min following the exercise, and then returned gradually to baseline levels. Consistent with this observation prolonged and intense exercise sessions (1.5 h of intense soccer practice in children [Scheet *et al*. 1999] or 1.5 h of wrestling practice in adolescents [Nemet *et al*. 2002]) were associated with decreases in circulating IGF-I levels. Interestingly, these sessions were associated with increases in proinflammatory cytokines, and the authors suggested that the increase in these proinflammatory cytokines mediated the decrease in circulating IGF-I.

The effect of resistance exercise on circulating IGF-I is inconsistent. Several studies found an increase in circulating IGF-I and free IGF-I following strength exercise (Bermon *et al*. 1999). Moreover, eccentric exercise was associated with increases in muscle IGF-I mRNA suggesting that IGF-I may modulate tissue regeneration after mechanical damage (Bamman *et al*. 2001). However, other studies found no change in circulating IGF-I following heavy resistance exercise (Nindl *et al*. 2001), or even reduced IGF-I levels the morning after high and moderate intensity resistance workout (Raastad *et al*. 2000). These conflicting results reflect probably differences in exercise protocols, fitness level of the participants, timing of blood sampling, etc.

There have been far fewer investigations in humans regarding the physiological responses of IGF-II compared with IGF-I. Several studies (Bang *et al*. 1990; Schwartz *et al*. 1996) showed an acute, endurance exercise associated rise in IGF-II. Circulating IGF-II may play an important role in bone growth and development (Mohan & Baylink 1991); however, the biological importance of the exercise-induced increase in IGF-II has yet to be determined.

INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

Several studies found increases in IGFBP-3 following endurance exercise (Schwarz *et al*. 1996; Chadan *et al*. 1999). Interestingly, Schwarz and coworkers have demonstrated that IGFBP-3 levels measured by radioimmunoassay (RIA) increased with both low and high intensity exercise, and were greater during the above-LAT protocols (Schwarz *et al*. 1996). In contrast to their RIA measurements, IGFBP-3 measured by Western ligand blotting (WLB) did not change. As a potential explanation for the discrepancy between RIA and WLB data they suggested that the antibody used in the RIA recognizes both intact and fragment forms of IGFBP-3 while the WLB method measures only the intact form of IGFBP-3. They therefore measured the rate of IGFBP-3 proteolysis as a function of exercise and were intrigued to find that proteolysis did occur in the high intensity exercise, and that this increased proteolysis was associated with the peak increase in IGF-I and IGF-II serum concentrations. It is important, however, to note that increases in IGFBP-3 proteolytic activity occurred in this study only for high intensity exercise, while increases in IGFBP-3, IGF-I and IGF-II occurred for both low and high intensity exercise. Moreover, other studies did not find significant increase in IGF-I proteolytic activity following endurance exercise (Dall *et al*. 2001).

The mechanism for the increased IGFBP-3 proteolysis following high intensity exercise is not clearly understood. It was suggested that changes in total and free-ionized serum calcium concentrations (Lamson *et al*. 1993) and marked serum and muscle acid-base changes (Martin & Baxter 1986) play a role in exercise-induced IGFBP-3 proteolysis.

Few studies demonstrated changes in other IGFBPs following exercise, including increases in IGFBP-1 (Suikkary *et al*. 1989; Hopkins *et al*. 1994) and IGFBP-2 (Chadan *et al*. 1999; Nindl *et al*. 2001). These BPs exist in the circulation in much smaller quantities than IGFBP-3, and appear to play a lesser role in circulating IGF bioavailability. However, it is clear that the exercise-induced effect on circulating IGF-I is not only mediated by alteration of the amount of IGF-I but rather by the effect on its BPs.

The effect of exercise training on the GH–IGF-I axis

Several independent studies of healthy human beings have demonstrated significant correlation between physical fitness and circulating components of the

GH–IGF-I axis. Weltman *et al*. (1994) demonstrated significant positive correlation between 24-h integrated GH concentrations and peak $\dot{V}O_2$ in young healthy male, but not female, adults. They also demonstrated inverse correlation between 24-h integrated GH concentrations and body fat in both men and women. They suggested that hyperinsulinemia associated with excess body fat and decreases levels of physical activity may reduce GH release (Yamashita & Melmed 1986), and since men, in general, have more central fat than women (Bouchard & Despres 1989), there is a greater influence of adiposity on GH secretion in men. They further speculated that the higher estradiol levels in female adults, which stimulate GH release (Ho *et al*. 1987), may oppose the inhibitory effect of insulin and therefore explain the gender-related differences of the correlation between fitness and GH concentration. Consistent with this hypothesis, we recently demonstrated in healthy prepubertal girls a remarkable relationship between adiposity (determined by the body mass index [BMI] percentile), fitness and indirect indicators of GH responsiveness (i.e. GHBP) and insulin sensitivity (i.e. IGFBP-1) (Eliakim *et al*. 2001). It appears that above about the 70 percentile of BMI for age, fitness, GH levels and insulin sensitivity all begin to decrease even in healthy children (Fig. 13.2). Such information might prove to be clinically useful in identifying children who would benefit from programs of physical activity.

The positive correlation between fitness and GH levels is consistent with animal experiments of Borer *et al*. (1986) who noted increased GH pulse amplitude in physically active, rapidly growing hamsters compared with sedentary controls. They suggested that the fit state was associated with increased circulating endorphins and/or increased tissue sensitivity to endorphins. Endorphins inhibit SS and reduce the inhibition of pituitary GH secretion, and as a consequence pituitary GH secretion is increased.

We previously described that both functional (i.e. $\dot{V}O_{2max}$) and structural (i.e. thigh muscle volume determined by magnetic resonance images) indices of fitness were correlated with mean overnight GH levels, GHBP and serum IGF-I levels in pre- and late-pubertal girls (Eliakim *et al*. 1996, 2001). These cross-sectional data suggest that fitness in healthy, prepubertal and adolescent girls is associated with anabolic adaptations of the GH–IGF-I system.

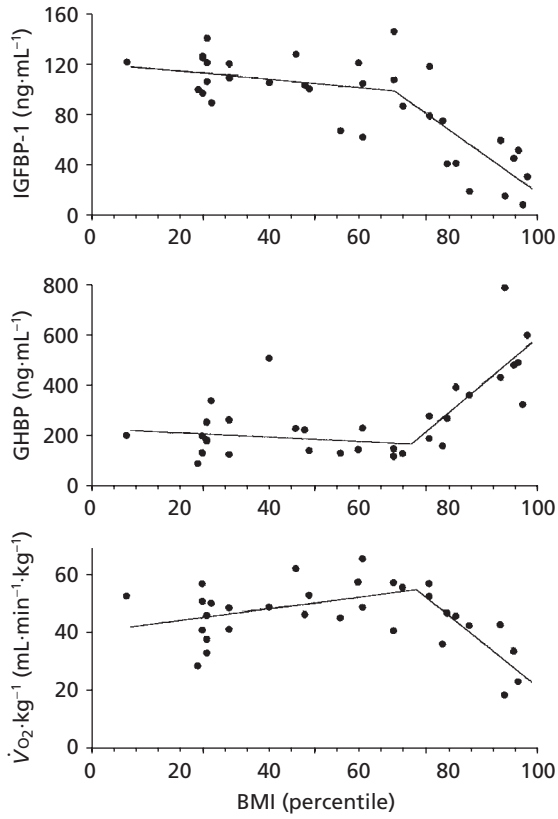

Fig. 13.2 Relationship between age-adjusted body mass index (BMI) percentiles and insulin-like growth factor binding protein-1 (IGFBP-1) (top panel), growth hormone binding protein (GHBP) (middle panel), and $\dot{V}O_{2peak}$·kg^{-1} (bottom panel). The data suggest that there exists a BMI percentile threshold (~ 70%) above which insulin sensitivity, GH activity and fitness all begin to decrease in otherwise healthy, prepubertal girls.

The significant correlation between fitness and mean overnight GH levels resulted probably from an increase in peak GH amplitude since only peak amplitude (and not peak frequency or width) correlated with mean GH.

The positive correlation between GHBP and fitness is unique. GHBP is the extracellular domain of the GH receptor (Rosenfeld 1994), and therefore reflect

tissue GH receptor capacity. Ligand-mediated receptor regulation appears to exist for GH and GHBP in a number of situations. GHBP decreases in acromegaly (Kratzsch *et al*. 1995) and during exogenous recombinant human growth hormone (rhGH) therapy (Legar *et al*. 1995), and is high in obesity despite low GH (Jorgensen *et al*. 1995). But ligand-mediated receptor down-regulation does not appear to operate during normal growth when *both* GH and GHBP increase in the prepubertal years (Baumman *et al*. 1989). Our data suggest that the relatively trained or fit state in prepubertal and adolescent girls is another example of simultaneous increases in both GH and GHBP. The mechanism of these responses is not known, but suggests anabolic adaptations of both the ligand and receptor.

Collectively, it seems that increasing levels of physical activity stimulate GH pulsatility and, as a consequence, circulating IGF-I. It is compelling to speculate that the stimulation of the GH–IGF-I axis by exercise contributes—along with genetic, nutritional and other environmental factors—to an increase in muscle mass and, ultimately, to improved cardiorespiratory responses to exercise (such as $\dot{V}o_{2peak}$). Our data suggest that this mechanism operate in prepubertal and adolescent girls even as spontaneous growth proceeds.

Significant positive correlation between $\dot{V}o_{2max}$ and both circulating GH and IGF-I levels were also found in healthy pre- and post-menopausal women (Kelley *et al*. 1990). Both $\dot{V}o_{2max}$ and IGF-I concentrations decline with age. However, when the influence of both age and fitness was analyzed using multiple regression, $\dot{V}o_{2max}$ remained the only independent predictor of circulating IGF-I. Therefore, it was concluded that the decrease in serum IGF-I was probably the result of age related decline in physical activity and fitness, and was not related to aging per se. Along with these observations, positive correlation between $\dot{V}o_{2max}$ and circulating IGF-I were reported in young and old male adults (Poehlman & Copeland 1990), and higher levels of IGF-I were found in trained middle-aged men (Manetta *et al*. 2002).

Virtually all of the major IGFBPs (1–6), each of which is known to influence IGF-I bioactivity in different ways, were also related to indexes of fitness, suggesting that the BPs may play a role in the interaction between growth and exercise. With the exception of IGFBP-3, the amount of IGFBPs in the circulation is low, and the exact role of these IGFBPs in the circulation has yet to be determined. Nonetheless, it is noteworthy that the cross-sectional relationships between fitness and IGFBPs were consistent with current understanding of the biological activity of IGF-I binding proteins based on tissue studies. IGFBP-1 and -2, known to inhibit IGF-I function, were found to be inversely correlated with muscle mass (Eliakim *et al*. 1996, 2001). IGFBP-4, a known inhibitor of the anabolic functions of IGF-I in bone tissue culture experiments (Mohan *et al*. 1995), was found to be inversely correlated with muscle mass (Eliakim *et al*. 1996) and $\dot{V}o_{2max}$ (Eliakim *et al*. 1998a). In contrast, the IGF-I potentiating binding protein, IGFBP-5, was positively correlated with muscle mass (Eliakim *et al*. 2001). Accordingly, these data suggest the possibility that the generally increased IGF-I bioactivity in fitter subjects might not be related only to changes in circulating IGF-I but also to changes in IGFBPs.

Very few studies examined the effect of endurance training on the GH–IGF-I axis longitudinally. Smith, A.T. *et al.* (1987) studied a group of healthy young adult men for 10 days and found that increased physical activity exacerbated the well-described reduction in IGF-I that accompanies caloric restriction. Reduced IGF-I associated with training has been observed in high school wrestlers and in highly trained young female gymnasts (Jahreis *et al*. 1991; Roemmich & Sinning 1997). In these studies the training program was accompanied by loss of body mass providing clear evidence for a negative energy balance and catabolic state. However, a recent report demonstrated that while inadequate caloric intake and negative energy balance is a major cause for the training-associated IGF-I decrease, IGF-I level may fall even when energy balance and weight stability are maintained (Nemet *et al*. 2004).

We recently reported the effect of a brief (5 weeks) randomized, prospective endurance-type training intervention on the GH–IGF-I axis in pre- and late-pubertal boys and girls. Based on the cross-sectional data, we hypothesized that training would lead

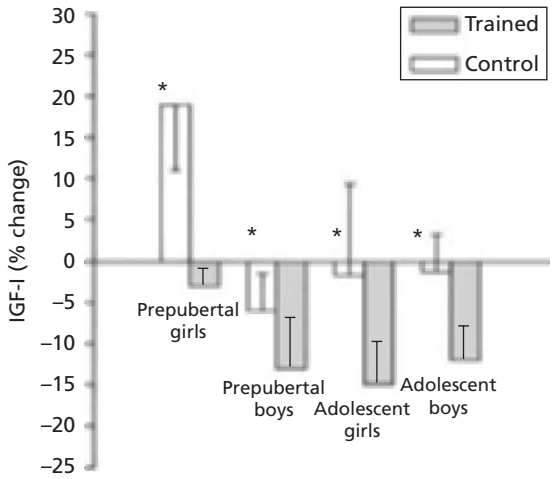

Fig. 13.3 Effect of a brief endurance-type exercise training on circulating insulin-like growth factor I (IGF-I) levels in prepubertal and adolescent boys and girls. Training was associated with a significant decrease or attenuated IGF-I response in all groups.

to increases in circulating GH and IGF-I levels. Training was accompanied by about 15% higher total energy expenditure (by the doubly labeled water technique), and resulted in significant increases in $\dot{V}o_{2max}$ and thigh muscle volume in the trained but not the control subjects (Eliakim *et al*. 1996, 1998, 2001; Scheet *et al*. 2002). In contrast to our hypotheses, there was a significant decrease in GHBP, IGF-I and IGFBP-3 in the trained prepubertal girls (Eliakim *et al*. 2001); a significant decrease in IGF-I and IGFBP-3, with a significant increase in IGFBP-2 in the trained prepubertal boys (Scheet *et al*. 2002); a significant decrease in IGF-I and IGFBP-5 in the trained late-pubertal girls (Eliakim *et al*. 1996); and a significant decrease in GHBP and IGF-I, with a significant increase in IGFBP-2 in the trained late-pubertal boys (Fig. 13.3) (Eliakim *et al*. 1998b). Interestingly, endurance training had no effect on GH pulsatility patterns in any of these groups.

These effects are commonly observed in energy-deficient states like food deprivation or disease-associated malnutrition (Smith, W.J. *et al*. 1995; Tonshoff *et al*. 1995), but 'catabolic' neuroendocrine adjustments occurred in the present studies even though training *increased* thigh muscle volume. Moreover, despite the significantly greater energy expenditure in the training group, the exercise intervention was not accompanied by weight *loss*.

A potential mechanism for this seemingly unexpected systemic 'catabolic-type' adaptation was the reduction in circulating GHBP in the trained subjects. As noted earlier, lower GHBP circulating levels may reflect fewer tissue receptors and reduced tissue responsiveness to GH (Rosenfeld 1994). As a consequence, since circulating IGF-I depend on GH-induced hepatic production (Lowe 1991), IGF-I levels in the training group decreased.

The exercise training effect on GHBP observed here appears to be unique. In several pathological states (malnutrition [Postel-Vinay *et al*. 1995], obesity [Jorgensen *et al*. 1995]) the relationship between GH and GHBP suggest the well-described phenomenon of ligand-induced receptor regulation. In studies (Eliakim *et al*. 1998b, 2001), training did lead to a reduction in GHBP, but *without* any change in mean GH or GH pulsatility. Thus, the possibility exists that lower GH receptor is the *initiating* mechanism in the growth axis response to training. The mechanism for such a direct, training effect on GH receptors or GHBPs is not known.

Interestingly, training was associated with increases in IGFBP-2, a known inhibitor of IGF-I action, and with reduction in IGFBP-5 in adolescent women. IGFBP-5 is one of the binding proteins that enhance some of the IGF-I mitogenic effects (i.e. in bones [Mohan *et al*. 1995]). These observations emphasize again that the exercise training induced decrease in IGF-I bioavailability is mediated not only by acting on IGF-I itself but by altering its binding proteins.

Recently, Scheet *et al*. (2002) intriguingly suggested the hypothesis that proinflammatory cytokines were involved in the training-induced decreases of IGF-I (Fig. 13.4) (Scheet *et al*. 2002). They demonstrated that in brief 5-week endurance training in prepubertal boys increases in $\dot{V}o_{2max}$ were positively correlated with changes in tumor necrosis factor-α (TNF-α). This suggested that children who trained the hardest and had the biggest increase in fitness also had the largest increase in circulating level of proinflammatory cytokines. In addition, changes in IGFBP-3 were inversely correlated with changes in TNF-α and interleukin-6 (IL-6), suggesting

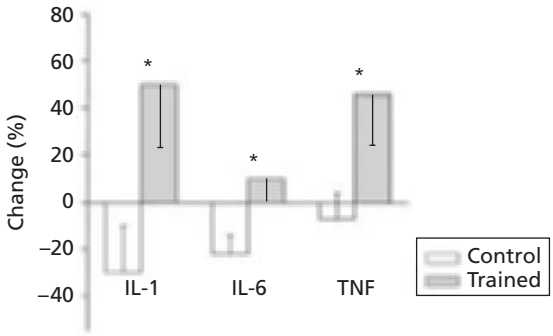

Fig. 13.4 The effect of 5-week aerobic-type exercise intervention on proinflammatory cytokines (interleukin-1β, interleukin-6 and tumor necrosis factor-α). The stress of training was associated with a significant increase of the proinflammatory cytokines. IL, interleukin; TNF, tumor necrosis factor.

that increase in the inflammatory response mediates the training-associated decrease of components of the GH–IGF-I axis.

These observations suggest the hypothesis that a sudden imposition of a training program which is associated with substantial increase in energy expenditure leads initially to an increase in pro-inflammatory cytokines and, as a consequence, to decreases in IGF-I levels. Further, if the training adaptation is successful, the proinflammatory cytokines fall, and with that decrease, the suppression of IGF-I diminishes, an anabolic 'rebound' in the GH–IGF-I axis may occur, and IGF-I level exceed the pretraining level. Exactly how and when this switch takes place, and whether the initial catabolic-type stage is necessary for the ultimate anabolic adaptation, remains unknown.

Adaptations to the training-induced increase in proinflammatory cytokines and decrease in circulating IGF-I start early with a training-associated increase in IGF-I receptor binding capacity (Lee *et al*. 2000). This increase in IGF-I affinity probably reflects the phenomenon of ligand-mediated receptor up-regulation.

Fitter subjects were found to have lower levels of IL-1 receptor antagonist (IL-1ra). This agent is stimulated by the inflammatory cytokines and act to block their biological activity at the receptor level. Therefore, training-associated reduced inflammatory response leads to lower level cytokines and to a lower steady state level of IL-1ra. Finally, and consistent with the two phases hypothesis, longer periods of training (5 months [Koziris *et al*. 1999] and 1 year [Weltman *et al*. 1992]) were indeed associated with increases in circulating GH and IGF-I levels.

Very few studies have examined the effect of resistance training on the GH–IGF-I axis. Raastad *et al*. (2001) demonstrated that despite an increase in muscle strength following 2 weeks of heavy strength training, IGF-I decreased in the 8th day and returned to baseline levels 4 days later. Kraemer *et al*. (1999) found no change in circulating IGF-I but an increase in resting IGFBP-3 following 10 weeks of resistance training program in young men. Twelve weeks of high-volume resistance training resulted in type I and type II muscle fiber hypertrophy in college men, and there was a significant correlation between the muscle hypertrophy and training-associated increase in GH level (McCall *et al*. 1999). Longer resistance training programs (25 weeks) were associated with increase in serum IGF-I after the 13th week of training in young adults (Borst *et al*. 2001). Since increase in muscle strength occurred also mainly during the first 13 weeks, the authors suggested that IGF-I mediated, at least partially, the resistance training-associated improvement in muscle strength. These results suggest that a biphasic change in circulating IGF-I occurs also in response to resistance training in young adults. In contrast, no changes in circulating IGF-I was found in old adults (Kraemer *et al*. 1999; Häkkinen *et al*. 2001), suggesting age-dependent differences in the IGF-I axis response to strength training.

The training-induced increase in muscle mass, despite the circulating decrease in IGF-I level, suggest that the local tissue effect of exercise on growth factors differ from systemic effects. Very few studies have examined the effect of brief exercise or training on skeletal muscle IGF-I levels. Yan *et al*. (1993) demonstrated that an acute bout of eccentric exercise led to an increase in IGF-I immunoreactivity in rat type II muscle 4 days post-exercise. Consistent with Yan's observation, we found that 5 days of treadmill training in young female rats resulted in a significant increase in muscle size and muscle IGF-I protein without changes in IGF-I mRNA and

circulating IGF-I (Eliakim *et al*. 1997). These results suggest that the early mechanisms of the training adaptation involve translational or post-translational increases in muscle tissue IGF-I, and that local muscle IGF-I regulation may be dissociated from central control mechanisms. Along these lines, Phillip *et al*. (1994) suggested that increases in IGF-I in the kidney was not due to an increase in local synthesis but rather to an increase in IGF-I uptake from the circulation by a non-membrane associated IGFBP-1. This observation emphasizes the possible role of local IGFBPs (which are strongly attached to the cell surface by a membrane integrin-binding domain) in the regulation of the early adaptation of local IGF-I to exercise training.

Nevertheless, compensatory hypertrophy of the plantaris and soleus muscle after unilateral excision of the gastrocnemius tendon was accompanied by a significant increase in IGF-I mRNA after only 2 days (levels of IGF-I protein were not measured in these studies) (De Vol *et al*. 1990). This suggests that different types and amount of muscular work could lead to a different time course and pattern of local IGF-I increase.

Longer periods of exercise training do, however, lead to stimulation of *IGF-I* gene expression. Zanconato *et al*. (1994) found an increase in *hepatic IGF-I* gene expression following 4 weeks of endurance training in young rats. In addition, the authors showed that the 4 weeks of endurance type training led to increases in the exercising *muscle IGF-I* gene expression and protein. Interestingly, inhibition of GH (by hypophysectomy [De Vol *et al*. 1990] or by GH releasing hormone antibody [Zanconato *et al*. 1994]) actually enhanced the local IGF-I response to increased muscular effort. It is clear from these observations that inhibition of GH alone cannot block the autocrine and paracrine effects of IGF-I, emphasizing the GH-independence of the 'local' IGF-I anabolic adaptations to physical activity.

Singh *et al*. (1999) studied the effect of progressive resistance training in frail elderly humans. They found that training was associated with increased muscle strength and with muscle fiber remodeling, and that higher baseline muscle IGF-I predicted the increase in muscle strength.

Recently, it was suggested that there are two isoforms of IGF-I in the muscle (McKoy *et al*. 1999; Goldspink & Yang 2001). One of the isoforms is detectable only after mechanical stimulation and therefore was named mechano growth factor (MGF). Its response to resistance training is higher in young adults, indicating age-related desensitivity to mechanical loading (Hameed *et al*. 2003). MGF is smaller and unglycosylated, and has a shorter half-life time and a different receptor binding affinity than the systemic liver IGF-I. The muscle has another isoform of IGF-I. This isoform is similar to the liver-type IGF-I, and is also up-regulated by exercise. Induction of MGF expression occurs mainly after stretch and electrical stimulation. MGF has an important role in local protein synthesis and in the prevention of apoptosis and, therefore, plays a pivotal role in local tissue repair and remodeling. This emphasizes again the significant importance of the local IGF-I response to exercise training.

What is the advantage to the organism of simultaneous central catabolism and local anabolism early in the adaptation to increased physical activity? We speculate that this adaptive mechanism might reduce global anabolic function, thereby conserving energy sources, but still allows for local tissue growth in response to environmental stresses like exercise training.

Consistent with this speculation is the phenomenon of attenuated somatic growth and reduced circulating IGF-I despite muscle adaptation to intense exercise training in nutritionally self-deprived young elite athletes (e.g. female gymnasts [Theintz *et al*. 1993]). The dissociation between target tissue and central neuroendocrine GH–IGF-I responses was also demonstrated in other environmental conditions (Moromisato *et al*. 1996). Rats exposed to hypoxia had a reduced growth rate and circulating IGF-I, but the relative size of their heart and lung was increased along with local *IGF-I* gene expression. This indicates that a local anabolic adjustment to reduced oxygen carrying capacity had occurred but that the central response was catabolic and overall growth was reduced.

In summary, there are differences between the local and systemic GH–IGF-I response to exercise training (Fig. 13.5). The local and more important skeletal muscle response is anabolic from very early

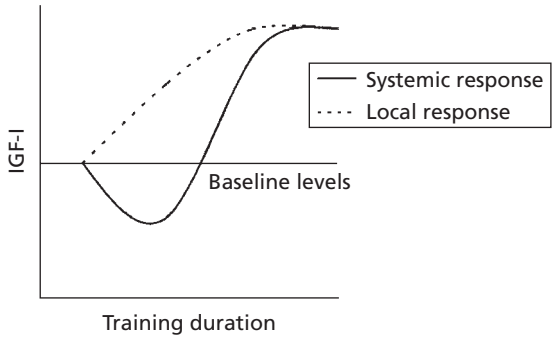

Fig. 13.5 Differences between local and systemic adaptations of insulin-like growth factor I (IGF-I) to exercise training. While muscle IGF-I increases from very early stages of training, there are at least two phases in the systemic IGF-I response. The first phase is an acute catabolic-type response characterized by decreases in circulating IGF-I, but at some point anabolic rebound occurs and IGF-I increases.

stages, and result mainly from GH-independent autocrine and paracrine IGF-I release. The systemic GH–IGF-I response to a training program has at least two phases: the first is an acute catabolic-type response with a decrease in circulating IGF-I. At some later point, depending probably also on the nutritional and energy balance of the individual, a chronic anabolic adjustment of the GH–IGF-I axis occurs. Whether the initial catabolic phase is necessary for the later anabolic adaptation, and whether this response is affected by the fitness level of the individual is still unknown.

Possible practical applications for the coach and athlete

The effectiveness of physical training depends essentially on the intensity, volume, duration and frequency of training, and on the individual ability to tolerate training. An imbalance between the training load and the individual's tolerance leads to under or overtraining. Therefore, many efforts have been made to find objective parameters to quantify the balance between training load and the athlete's tolerance, with limited success. The endocrine system, by modulation of anabolic and catabolic processes, plays a major role in the physiological adaptation to exercise training (Urhausen & Kindermann 2000). For example, the change in the testosterone/cortisol ratio, as an indicator of the anabolic–catabolic balance, has been used with limited success to determine the physiological strain of training (Kuoppasalmi & Adlercreutz 1984; Hoffman *et al*. 1997). The data presented here suggests that changes in circulating IGF-I may also serve as a possible marker of the training load, and that sustained reduced circulating IGF-I levels may indicate overtraining. This is particularly important in young competitive athletes, and particularly in sports that typically combine intensive training with caloric restriction (aesthetic sports [e.g. gymnastics] or weight-categories sports [e.g. wrestling]). Reduced circulating IGF-I level in these circumstances indicates negative energy balance, which may lead to attenuated somatic growth (Jahreis *et al*. 1991; Theintz *et al*. 1993; Roemmich & Sinning 1997). Therefore circulating IGF-I levels can serve as a very important alerting sign, and the athletes should be aware that even when they reduce the training intensity, they would not be always able to compensate for the growth loss during long periods of negative energy balance.

Measurements of IGF-I levels can also help the athlete and coach in the preparation for competition. We recently determined the effect of 4 weeks of training on fitness, self-assessment physical conditioning scores and circulating IGF-I in elite professional handball players during their preparation for the junior world championships (Eliakim *et al*. 2002). Training consisted of 2 weeks of intense training followed by 2 weeks of relative tapering. Both circulating IGF-I and physical conditioning scores decreased initially, and returned to baseline levels at the end of training. We found a significant positive correlation between the changes in circulating IGF-I and the physical conditioning scores.

Tapering down the training intensity prior to the competition is a well-known training methodology to help the athlete to achieve his best performance. The results of this study demonstrated that this strategy is indeed associated with parallel changes in both IGF-I (an objective measure) and in individual conditioning self-assessment (a subjective measure). Therefore, both measures may assist coaches and athletes in their training preparations.

However, as noted earlier (e.g. Eliakim *et al*. 1996, 2001), despite the decrease in circulating IGF-I, fitness improved. These results suggest that while changes in *circulating* IGF-I are good markers of the general condition and energy balance of the athlete, they are not necessarily good predictors of the athlete's performance.

Recently, we measured IGF-I level throughout the training season in elite judokas. IGF-I decreased significantly during periods of heavy training, but returned to baseline levels during tapering down, and reached a peak above baseline levels during the competition period (Fig. 13.6) (unpublished data). Nemet *et al*. (2004) showed similar patterns for free IGF-I during the training season in competitive wrestlers. It is still unknown what the permitted decrease of IGF-I during periods of heavy training is, or the optimal increase during periods of tapering down and reduced training intensity. However, we believe that the inability to increase circulating IGF-I levels before the target competition should be an alarming sign for both the athlete and his/her coach that the athlete's general condition is not optimal.

Finally, we demonstrated that local IGF-I changes are more important than systemic changes in the muscle adaptation to exercise training. However, if indeed GH changes following exercise play a key role in the anabolic effects of exercise, attention should be paid by athletes and trainers to factors that may inhibit the GH response to exercise and attenuate the training effect (e.g. low exercise intensity; short intervals between multiple daily exercise sessions; exercise following high fat meal; continuous training despite menstrual irregularities, etc.).

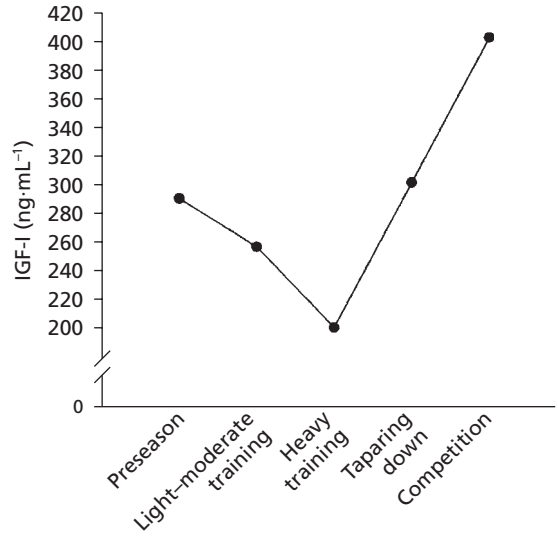

Fig. 13.6 A typical example of insulin-like growth factor I (IGF-I) levels in elite judoka during the training season. IGF-I level decreased significantly during heavy training, and increased above baseline levels before the target competition.

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Chapter 14

The Role of MGF and Other IGF-I Splice Variants in Muscle Maintenance and Hypertrophy

GEOFFREY GOLDSPINK, SHI YU YANG, MAHJABEEN HAMEED,

Introduction

Molecular biology methods have drastically changed the way we think about hormones. The classical definition of a hormone was 'a chemical substance produced in a specialized gland released into the blood stream and transported to different tissue to elicit a physiological purpose'. We are now aware that hormones, or factors, are produced by many different types of tissues, and that some have an autocrine or a paracrine effect. This is exemplified by the insulin-like growth factor I (IGF-I) system, a family of different forms of IGF-I, each of which has a different biological effect. These are derived from the splicing of the IGF-I gene in response to different signals. The human genome has now been sequenced and comprises about 40 000 different genes. However, we know there are many more different proteins, so it is therefore evident that the same gene must be spliced differentially to generate this phenotypic diversity. The IGF gene appears to have evolved from a single insulin-like gene that is expressed in vertebrates. A similar insulin-like gene is present in the *C. elegans*, the nematode, and in the chordate amphioxus, closely related to the common ancestor of the vertebrate. During vertebrate evolution however, the gene has been duplicated to give rise to insulin, IGF-I and IGF-II genes. Experiments on *C. elegans* have shown that the ancestral insulin-like gene prevents cell death and considerably extends the life of the worm. The system is even more versatile because the insulin-like growth genes can be spliced to produce different RNA transcripts and different polypeptides with different biological activity.

IGF-I, originally called 'somatomedin', was regarded as a general growth factor produced by the liver under the influence of growth hormone (GH). Later, it became apparent that it is expressed by most tissues and exists as different splice variants, each of which has a somewhat different action.

Growth hormone–IGF-I axis

The original somatomedin hypothesis originated in the 1950s following early efforts to understand the regulation of somatic growth by pituitary-derived GH. It was suggested that this did not act directly on its target tissues to promote growth, but there were intermediary substances involved (Daughaday & Reeder 1966). The term 'somatomedin' was later adopted (Daughaday *et al*. 1972) to reflect the growth promoting actions of these substances, which were subsequently characterized and later called insulin-like growth factors (Rinderknecht & Humbel 1978; Klapper *et al*. 1983). However, in 1985, Green *et al*. proposed the 'dual effector hypothesis' (Green *et al*. 1985), which suggested that GH had direct effects on peripheral tissues that were not mediated by IGF-I and that GH stimulated local IGF-I production. It is now clear that one of its main roles is stimulating the release of IGF-I from the liver and that, in addition, GH stimulates the formation of a ternary IGF binding complex, including insulin-like growth factor binding protein 3 (IGFBP-3) and the acid-labile subunit (ALS), which stabilizes IGF-I in the serum. GH is secreted from somatotroph cells located within the anterior pituitary gland in a pulsatile manner, and also has itself other specific functional

effects on local tissues. These include a protein anabolic effect (increased DNA, RNA and protein synthesis), stimulation of growth and calcification of cartilage, an increased mobilization of fats, and use of fats as an energy source. The exercise-induced increase in GH is thought to mediate, either directly or indirectly, many of the somatotrophic events surrounding tissue remodeling (reviewed by Nindl *et al*. 2003).

Unquestionably, the GH–IGF-I axis plays a role in postnatal growth and development, and these hormones reach their peak during adolescence. However, with increasing age, a further decline in the circulating levels of GH and IGF-I occurs, such that older people can be regarded as partially GH deficient (Rudman *et al*. 1981). The relationship between the age-related decline in the GH–IGF-I axis and loss of muscle mass and strength has been extensively studied. In young GH-deficient adults, administration of recombinant human growth hormone (rhGH) was shown to have positive effects on muscle mass and function (Cuneo *et al*. 1991). Another study where GH-deficient adults were treated with GH for an extended period of time concluded that there was not only increased muscle strength but also decreased body fat (Beshyah *et al*. 1995). This led to the belief that older individuals with decreased levels of circulating GH and IGF-I would also benefit from rhGH therapy. However, studies which have combined GH administration and resistance training in both young (Yarasheski *et al*. 1992) and older men (Yarasheski *et al*. 1995) have shown that the rates of protein synthesis are no greater when resistance training is combined with GH than when resistance training is performed alone. Furthermore, in older people the changes in muscle mass and function have also been reported to be similar between both groups (Lange *et al*. 2002). It should be noted that studies involving the use of rhGH, such as those listed above, are likely to have used the 22 kDa molecular isoform of GH. This is the predominant form of GH found in the plasma GH. The effects of the other molecular isoforms of GH, of which there are thought to be more than a hundred (Baumann, 1991), are yet to be determined. The roles of circulating GH and IGF-I, particularly with regard to muscle adaptation in later life, are still unclear. Systemic growth factors may be of relatively minor importance in muscle hypertrophy. For example, in one study the overloaded muscles of hypophysectomized rats were still able to hypertrophy despite significantly reduced systemic IGF-I levels (Adams & Haddad 1996). These findings, coupled with the simple observation that it is only challenged muscles that hypertrophy and not all the muscles of the body, highlights the importance of a 'local' system of muscle adaptation.

Expression of IGF-I splice variants in muscle

Muscles can be stimulated to grow rapidly if mechanically challenged; electrical stimulation of a muscle held in a stretched position also promotes both muscle lengthening (by the addition of new sarcomeres in series) and increases cross-sectional area, by adding sarcomeres in parallel (Goldspink *et al*. 1992). Using this approach in combination with specific primers and RT-PCR (reverse transcription-polymerase chain reaction) it was possible to detect two different RNA transcripts (Yang *et al*. 1996), which subsequent cloning and sequencing identified as being derived from the *IGF-I* gene by alternative splicing (Yang *et al*. 1996). The first, IGF-IEa was also detected in the resting muscle, and corresponded to the transcript commonly expressed in the liver. The second, not detected in resting muscle, corresponded to IGF-IEb.

The terminology of the IGF-I is a problem when attempting to apply it to non-hepatic tissues and therefore this splice variant in muscle was named mechano growth factor (MGF), due to its apparent regulation by mechanical signals (Yang *et al*. 1996); it has a different carboxy-peptide sequence to the liver type of IGF-I. An additional problem is that MGF would be classified as IGF-IEb in rat but IGF-IEc (as identified in hepatoma cells by Chew *et al*. 1995) in humans (Table 14.1). Also, in human muscle an additional transcript has been detected (Hameed *et al*. 2004), which confusingly has been termed IGF-IEb but which differs from the rat IGF-IEb. It is therefore apparent that the muscle IGF-I isoforms while related to the liver isoforms, need to be characterized separately (Table 14.1 and Fig. 14.1).

Table 14.1 The different names used to describe the insulin-like growth factor I (IGF-I) isoforms in the literature.

IGF-I isoform	Names used in the literature
IGF-IEa	L.IGF-I*, m-IGF-I†
IGF-IEb	
IGF-IEc	IGF-IEb (rat)‡, MGF*

*Yang *et al*. (1996); †Musaro *et al*. (2001); ‡Rotwein *et al*. (1986).
MGF, mechano growth factor.

Mechano growth factor

As well as MGF being expressed in response to mechanical activity, it was noted that its E domain had an insert that changes the open reading frame. Amino acids are encoded by nucleotide base triplets and any insert that is not a multiple of three therefore changes the downstream sequence. There is a 52 base pair insert in the rat and a 49 base pair insert in humans. This has important functional consequences, as the 3'RNA sequence codes for a different carboxy peptide sequence that is involved in the recognition of the binding proteins. Also, in the case of MGF, the carboxy peptide (encoded in exons 5 and 6) sequence acts as a different growth factor to the peptide that binds to the IGF-I receptor (encoded in exons 3 and 4). The E-domain peptide alone has been shown to induce division of mononucleated myoblasts and thus activate the muscle satellite (stem) cells required for muscle hypertrophy and repair (Yang & Goldspink 2002). MGF is apparently not glycosylated and there is evidence that it has a short half-life unless bound to the binding protein which may be intracellular. Therefore MGF can be regarded as an autocine/paracrine or local growth factor produced locally in response to mechanical stimuli and acts on those muscle fibers that produce it. Hence it is an important signaling molecule in the local regulation of muscle growth.

Systemic IGF-I is produced by active muscle

As mentioned above, IGF-IEa is also expressed in skeletal muscle as well as in several other non-hepatic tissues. It has a similar sequence to the main isoform produced by the liver and is therefore assumed to have a systemic action. However, muscle expresses at least two of the major binding proteins of the systemic form of IGF-I and their expression tends to be up-regulated as is that of IGF-IEa, by exercise. As the IGF-IEa produced by muscle will bind to these binding proteins in the extracellular matrix as well as in the serum, it is expected to have more effect on the muscles that produce it than on other muscles; its action can be regarded as autocrine and paracrine, as well as endocrine.

As well as having a different carboxy peptide sequence, the expression kinetics are different to those of MGF. It was shown by Haddad and Adams (2002), that in response to resistance-type exercise in rats, the mRNA of MGF was expressed earlier than IGF-IEa mRNA. In support of this, Hill and Goldspink (2003) showed that in rats that, following muscle damage, MGF is produced as a pulse lasting a few days, whereas the expression of IGF-IEa

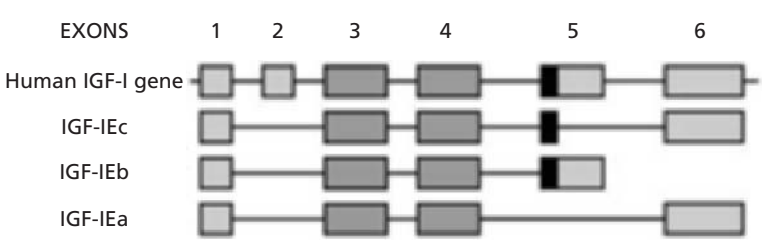

Fig. 14.1 Schematic representation of the human IGF-I gene comprising of six exons. The three splice variants, IGF-IEa, IGF-IEb and IGF-IEc (also known as mechano growth factor, MGF) expressed in muscle are shown with the relevant exons. The black box in exon 5 represents the first 49 base pairs (bp) (52 bp in the rat) which gives rise to the alternatively spliced, mechano-sensitive MGF isoform.

increases as MGF declines but stays elevated for much longer.

IGF-I and its splice variants in human muscle

In a study of young military recruits, Hellsten *et al*. (1996) reported an increase in IGF-I immunoreactivity in muscle after 7 days of strenuous exercise (which included terrain marching and warfare exercises). More, recently, in a study of elderly people, Singh *et al*. (1999) reported a 500% increase in IGF-I levels in the quadriceps muscles following a 12-week period of resistance training, as determined by immunohistochemistry. Resistance training consisted of three sets of eight repetitions at 80% of the most recently determined 1 repetition maximum (1-RM) for the hip and knee extensor muscles, 3 days per week for 10 weeks. It is clear that this type of exercise training, where the active muscles must overcome high loads, is the type of exercise that results in muscle hypertrophy. However, these studies failed to distinguish between the different IGF-I splice variants. Recently, the mRNA levels of MGF and IGF-IEa were measured using real-time quantitative PCR shortly (2.5 h) after a single bout of high intensity knee extensor exercise. Subjects in this study performed 10 sets of six repetitions of the knee extensor muscles at 80% of their 1-RM. (Hameed *et al*. 2003a). In young subjects it was observed that MGF mRNA levels were significantly increased as a result of weightlifting exercise, but no such change was observed in older subjects. Furthermore, at this short time point after exercise IGF-IEa mRNA levels were unchanged in both groups. These observations were interesting in that they were in general agreement with animal experiments in which MGF levels were shown to increase before those of IGF-IEa, suggesting that the two isoforms were differentially regulated. No relationship was observed with muscle myosin heavy chain isoform composition, but of note was the observation that the subject who showed the most dramatic increase in MGF was the subject whose muscles expressed the most MHC-IIX. Weightlifting exercise comprises both concentric and eccentric components and in a recent study, Bamman *et al*. (2001) reported a 62% increase in IGF-I mRNA concentration in the muscle, 48 h after an acute bout of eccentric, but not concentric contractions. Shortly (2.5 h) after a bout of eccentric cycling exercise, we again detected a significant increase in MGF, but not IGF-IEa. The eccentric exercise consisted of 60 min of reverse pedal cycling divided into six work intervals: 0–6 min at 50%, 6–12 min at 75%, 12–20 min at 100%, 20–25 min at 130%, 25–40 min at 100% and 40–60 min at 75% of the load corresponding to the previously determined concentric $\dot{V}O_{2max}$ (Hameed *et al*. 2003b). Thus, it is possible that Bamman *et al*. (2001) were measuring increases in IGF-IEa and not MGF at this latter time point.

Whilst it is clear that mechanical activity plays a pivotal role in regulating local IGF-I expression in muscle, it was unclear as to whether there may be further regulation, provided by other hormones, notably GH. Some insight into this possibility was gained from the results of a recent longitudinal study where the relationship between exogenous GH administration and strength training exercise was studied in older people. The subjects (age 74 ± 1 year) were assigned to either resistance training (consisting three different lower body exercises: leg press, seated knee extension and seated knee flexion, which were performed three times a week and consisted three to five sets of 8–12-RM per session) with placebo, resistance training combined with rGH administration alone. GH administration without training did not change MGF mRNA levels when measured at 5 weeks (Fig. 14.2), but significantly increased IGF-IEa levels (237%). In contrast, 5 weeks of resistance training significantly increased expression of MGF (163%) and to a lesser extent IGF-IEa (68%). However, when GH treatment was combined with exercise MGF levels were dramatically increased (456%). These data suggest that exogenous GH administration causes an overall up-regulation of transcription of the IGF-I gene prior to splicing, which results in more of the primary transcript of IGF-I. In the absence of strength training exercise, splicing is towards the IGF-IEa isoform, but when combined with the mechanical loading it splices towards the MGF isoform. Furthermore, in this study another splice variant, IGF-IEb was detected and cloned. The function of this third muscle isoform is not yet known.

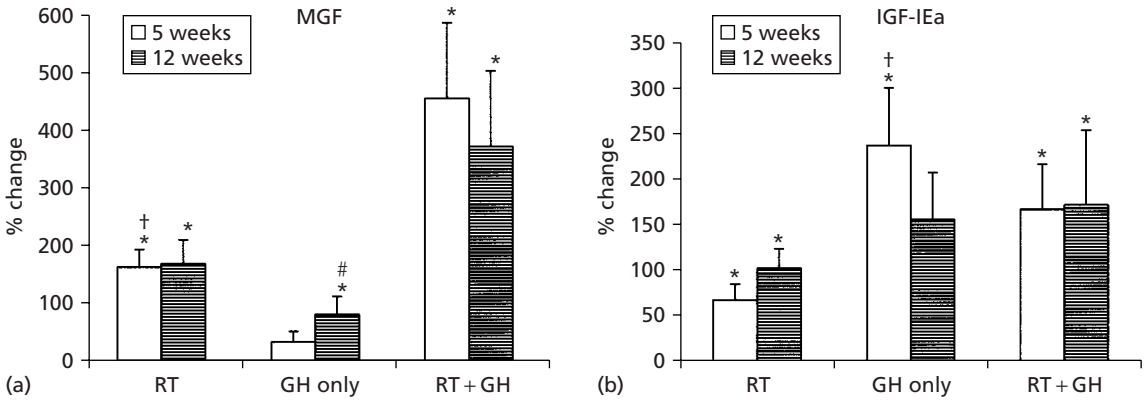

Fig. 14.2 Changes in the expression of mechano growth factor (MGF) (a) and IGF-IEa (b) mRNA after 5 (open bars) and 12 (lined bars) weeks in elderly men in the three intervention groups: Growth hormone only (GH), resistance training only (RT) and resistance training in combination with growth hormone (RT + GH). Values are expressed as a percentage change from baseline at 5 and 12 weeks. Bars and error bars represent mean values and SEM, respectively. *, Significant difference from baseline (#) from 5 weeks ($P < 0.05$). †, Significant difference in % change between isoforms ($P < 0.05$). (Data taken from Hameed *et al*. 2004.)

The structure of IGF-I

IGF-I peptides exist as single chain polypeptides consisting of about 70 amino acid residues. Not only are these derived by alternative splicing of the *IGF-I* gene but, like insulin, the initial peptide undergoes a process of post-translational alteration. The primary structure of IGF-I is similar to that of proinsulin (43% sequence homology) as it comprises an amino terminal B region and an A region that is separated by a short connecting C domain. Unlike proinsulin, however, it also has a D region extension peptide and an E peptide at its carboxyl terminus (Lowe, 1988), and is therefore longer than insulin.

The tertiary structure of IGF-I (Fig. 14.3) was initially predicted using computer graphics. This was based on the three-dimensional crystalline structure of insulin as determined by X-ray diffraction. As mentioned, IGF-I is somewhat longer than insulin although its receptor domain is very similar and acts as a marker for interactive molecular graphics for visualizing the conformation of IGF-I and IGF-II peptides. In predicting the tertiary structure of IGF-I, the conservation of the cysteine and glycine residues between IGF-I and proinsulin are particularly important. The hydrophobic core of insulin: A2 Ile, A16 Leu, B12 Val, B15 Leu and B24 Phe (insulin notation) is conserved as well. The most obvious differences between IGF-I and insulin are in the C domain. The extra carboxy terminal regions endow the IGF-I splice variant with special properties which determine their *modus operandi*. As well as a hydrophobic core similar to that of proinsulin, IGF-I has three S–S bridges which determine the three dimensional conformation of this polypeptide. However the presence of the disulphide links make it difficult to synthesise IGF-I chemically and native function structure and stability require the presence of all three S–S bridges (Narhi *et al*. 1993).

Receptors mediate the cellular effects of IGF-I

The biological activity of any hormone depends on the ability of the target cells to respond to the signal in the extracellular milieu. This is a function of the cell receptors as well as post-receptor mechanisms. IGF-I and IGF-II molecules interact with an array of cell surface receptors that may be present individually or in various combinations on target cells. Both IGF-I and IGF-II are believed to interact with the IGF-I receptor (IGF-IR), that is structurally and functionally related to the insulin receptor (IR) with which it shares > 50% amino acid identity. Despite

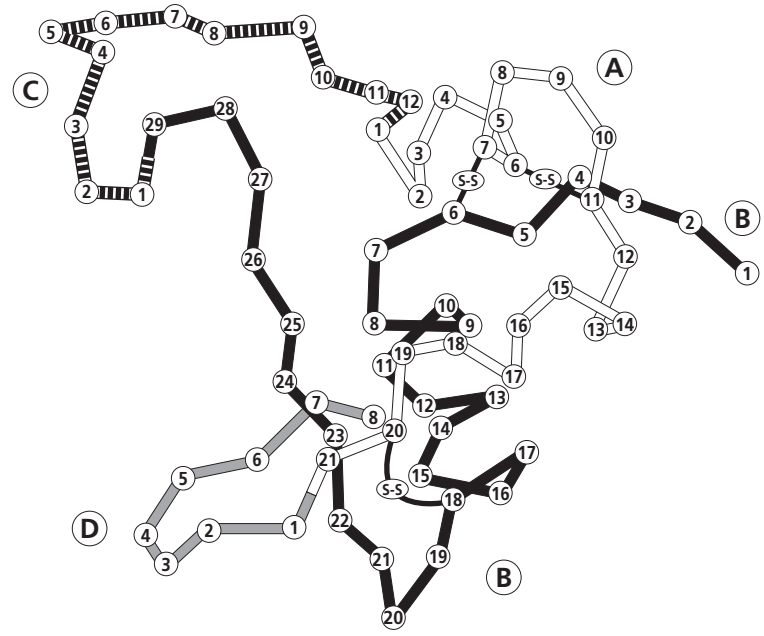

Fig. 14.3 Tertiary structure of insulin-like growth factor I (IGF-I) showing the A, B, C and D domains. Amino acid residues are numbered within each domain. The amino B and A domains are separated by a short interconnecting C domain. The D domain and E domain (not shown) form the carboxyl terminus of the protein. As well as the hydrophobic core similar to that of proinsulin the molecule has three disulfide bridges (shown as S–S), which determine its three-dimensional conformation. Two of these are located in the core of the molecule (B18–A20; A6–A11) and the third on the surface (B6–A7). (Adapted from Blundell *et al*. 1983.)

this similarity, IGF-I does not tend to bind with the IR, except at pharmacological doses. This is largely due to its affinity being approximately two orders of magnitude higher for the IGF-IR compared to the IR. As well as binding to the IGF-IR, IGF-II can also bind to a second receptor known as the IGF-IIR with high affinity.

The IGF-IR is a tyrosine specific protein kinase with an extracellular ligand-binding site. This receptor seems to mediate most actions of IGFs in skeletal muscle. For example, the IGF-IR appears to mediate amino acid and hexose uptake in rat soleus muscles (Yu & Czech 1984), BC3H1 muscle cells (De Vroede *et al*. 1984) and DNA synthesis in chick muscle satellite cells (Duclos *et al*. 1991). The IGF-I receptor is therefore believed to mediate several actions of IGF-I, such as stimulation of amino acid uptake, proliferation, differentiation and inhibition of protein degradation (Ewton *et al*. 1987).

The complexity of IGF-I signaling is increased by the formation of hybrid receptors that result from the dimerization of IGF-IR and IR hemireceptors. Each hybrid receptor consists of a single α and β subunit linked by disulphide bonds. In some circumstances, these hybrid receptors may outnumber homoreceptor molecules at the cell surface (reviewed by Le Roith & Roberts 2003). These IGF-IR/IR hybrid receptors bind IGF-I with high affinity, but have a reduced affinity for insulin. This can be attributed to the ability IGF-I to bind to either IGF-IR α subunit, whereas for insulin to bind effectively requires interaction with both the β subunits found in the IR.

It is interesting to note that among known muscle growth factors, IGF-I is unique in its ability to stimulate both proliferation and terminal differentiation of post-mitotic myotubes. This may be due to different IGF-I splice variants within muscle tissue. A recent study showed that different IGF-I splice variants have different roles in myoblast proliferation and differentiation (Yang & Goldspink 2002). It also showed that different actions of these different splice variants are mediated through a different receptor.

IGF-I binding proteins

The roles of the specific binding proteins in determining the autocrine or paracrine actions of the IGF system are becoming increasingly apparent (Florini *et al*. 1996; Damon *et al*. 1997). Amongst the seven

IGFBPs described so far, four (IGFBP-2, 4, 5 and 6) are produced by different myoblast cell lines whereas only IGFBP-4, 5 and 6 are expressed by adult skeletal muscle (Florini *et al*. 1996; Putzer *et al*. 1998). IGFBPs have been associated with the systemic IGF system, but their expression in skeletal muscle would have the effect of retaining IGF-I within the muscle tissue. In the unbound state the IGF-I peptides have a short half-life and the IGFBPs were previously thought of as carrier proteins in the serum. However, when expressed within the muscle they modulate the endocrine as well as the local influences of IGF-I (Mohan *et al*. 1996). The precise actions of IGFBPs at the tissue level are unknown, but it is apparent that they stabilize and augment local IGF-I bioavailability (Jones & Clemmons 1995; Mohan *et al*. 1996; Clemmons *et al*. 1998).

Both mRNA and protein levels of IGF-I and IGFBPs have been shown to be up-regulated during regeneration after ischaemic injury (Jennische & Hall 2000). *In situ* hybridization studies have shown IGFBP-5 to be restricted to regenerating muscle cells, whereas connective tissue cells expressed IGFBP-4 (Boes *et al*. 1992). Mechanically loading, or unloading the muscle, has been shown to regulate several of the binding proteins in muscle. For example, Awede *et al*. (1999) reported that overloading the muscles in mice increased the expression of IGFBP-4 mRNA, but decreased that of IGFBP-5. In contrast, unloading of mouse muscle resulted in reduced levels of IGFBP5 mRNA, but did not affect levels of IGFBP-4. Both of these binding proteins were assumed to mediate the effects of IGF-I via regulation of the free IGF-I concentration in muscle and possibly via competition with IGF receptors for IGF-I (Awede *et al*. 1999). Experiments are underway to characterize the specific binding protein for MGF, which differs from the other binding proteins for the other splice variants.

Biological action of the IGF-I splice variants

All the IGF-I splice variants have the same receptor-binding domain encoded by exons 3 and 4. This is apparently responsible for the anabolic effects of IGF-I. These have been clearly demonstrated by numerous *in vitro* studies, where it has been shown that IGF-I acts to increase the diameter of myotubes, suppress protein degradation, increase amino acid uptake and stimulate protein synthesis (Ewton *et al*. 1987; Vandenburgh *et al*. 1991; Florini *et al*. 1996; Semsarian *et al*. 1999; Bodine *et al*. 2001; Rommel *et al*. 2001). Its expression during muscle hypertrophy has been shown by using several animal models, including stretch-induced hypertrophy of the muscle. For example, Schlechter *et al*. (1986) and Czerwinski *et al*. (1994) reported an increased expression of muscle IGF-I mRNA, and DeVol *et al*. (1990) demonstrated that there was a threefold increase in IGF-I mRNA levels in the soleus and plantaris muscles in 11–12-week-old female rats following tenotomy-induced hypertrophy. This particular study employed hypophysectomized rats, which further suggests that the observed increase in IGF-I mRNA expression was GH independent. Later studies utilizing a similar model of functional overload in both normal and hypophysectomized rats found that both mRNA and protein levels of IGF-I were increased in muscle, prior to the attainment of significant hypertrophy, and remained elevated for up to 28 days during the hypertrophy process (Adams & Haddad 1996). In another study which utilized treadmill training of GH-suppressed rats, levels of IGF-I mRNA and protein increased by 55% and 250% respectively (Zanconato *et al*. 1994). Furthermore, overexpression (Coleman *et al*. 1995) or direct infusion (Adams & McCue 1998) of IGF-I in muscle results in hypertrophy, whereas inhibition of intracellular signaling components associated with IGF-IR activation can prevent this response (Bodine *et al*. 2001). Another study, examining the association between local IGF-I overexpression and atrophy induced by hind limb unloading, concluded that overexpression of IGF-I in the muscles of transgenic mice was not shown to prevent unloading-induced atrophy (Criswell *et al*. 1998).

Genetic manipulation of IGF-I in muscle

Transgenically modified mice that overexpress the IGF-I gene have been produced in several laboratories. Initial experiments of this kind by Mathews *et al*. (1988) in which the GH gene was overexpressed,

reported a 30% increase in muscle and an increase in bone density (Mathews *et al*. 1988). These experiments involved using a metallothionein promoter, which when activated increased the expression of IGF-I in most tissues. Later, experiments involved IGF-I transgenes that were under the control of muscle regulatory elements, such as the α-actin promoter (Coleman *et al*. 1995). Although serum levels of IGF-I were not significantly elevated in these mice, there was a considerable increase in muscle mass. More recently, work by the same group has shown that both muscle and motor neuron regeneration was enhanced in these mice when compared to wild type (Rabinovsky *et al*. 2003). It is difficult to determine whether the full length cDNA has been used in these gene transfer experiments and if this insert includes the sequence for a binding protein. This is important if we are to understand whether or not the expression of IGF-I is muscle specific; this is usually determined by whether the introduced DNA is under the control of a muscle specific regulatory sequence. IGF-I knock-out mice (lacking the entire IGF-I gene sequence) have also been produced. Therefore, none of the IGF-I peptide can be transcribed (Baker *et al*. 1993). However, these mice do not survive very long after birth, as their muscles appear to be dystrophic. Interestingly, the results of recent tissue specific gene deletion experiments using the Cre-loxP model of gene deletion have questioned the role of GH and liver IGF-I in controlling postnatal growth and development (Sjogren *et al*. 1999; Yakar *et al*. 1999). This homologous recombination system was used to create a liver specific deletion of the IGF-I gene but allowed normal expression of this gene in other non-hepatic tissues, such as heart, muscle, fat, spleen and kidney. The effect of this liver specific gene deletion of IGF-I on growth and development in these mice was to reduce circulating IGF-I levels at 6 weeks of age compared with wild type animals. Interestingly, measurements of body size and individual organ weights at 6 weeks showed no difference between the 'knockout' animals and their wild type littermates. Thus, postnatal growth and development proceeded without the contribution of liver derived IGF-I (Sjogren *et al*. 1999), emphasizing the role of the local IGF-I system in the process.

Gene transfer of the different IGF-I splice variants

The finding that muscle fibers can be transfected by a simple intramuscular injection of a plasmid vector containing the cDNA sequence of a gene of interest provides a method for treating medical conditions that are associated with marked muscle loss. More recently, experiments in which constructs containing the IGF-I splice variants, including MGF have been performed. For example, in one such experiment a plasmid construct containing MGF cDNA was injected into the muscles of normal mice to determine the role of the MGF splice variant in muscle maintenance. This study resulted in a 25% increase in the mean muscle fiber size in injected muscle within 2 weeks, and this was shown to be due to an increase in the size of the muscle fibers (Goldspink & Yang 2001). Similar experiments by other groups have also been carried out using a viral construct containing the liver type of IGF-I (IGF-IEa). This also resulted in a less than 20% increase in muscle mass, but this took over 4 months to develop (Barton-Davis *et al*. 1998). As well as treating certain medical conditions, gene transfer of this type may well be open to abuse. The use of an adenoviral vector for delivery would make detection relatively simple using PCR based methods, as this virus infects most cell types. Plasmid vectors are in contrast more difficult to detect, but new approaches that involve determining the different actions of the gene products from those of introduced gene are underway.

IGF-I signaling pathways in muscle hypertrophy

The signaling pathways by which IGF-I promotes skeletal muscle hypertrophy remain unclear, with roles suggested for both the calcineurin/NFAT (nuclear factor of activated T-cells) pathway (Musaro *et al*. 1999; Semsarian *et al*. 1999) and the P13-kinase/Akt pathway (Rommel *et al*. 2001). More recently, studies investigating the hypertrophic response both *in vitro* and *in vivo* have reported that it is the Akt/mTOR pathway and not the calcineurin pathway which is involved in promoting hypertrophy,

by activating downstream targets such as $P > 0.056$ kinase (Bodine *et al*. 2001; Rommel *et al*. 2001). In addition, it was also reported that IGF-I might in fact act via Akt to inhibit the calcineurin/NFAT signaling pathway during this process (Rommel *et al*. 2001). Unfortunately studies conducted to date have looked at the expression of total IGF-I in response to muscle overload.

IGF-I and sarcopenia

Increasing age is associated with a loss of muscle and mass and function, but the mechanisms underlying this process remain unclear. There is a reduction in circulating levels of GH and IGF-I (Rudman *et al*. 1981) and an actual loss of muscle fibers (Lexell *et al*. 1988). There is some emerging evidence that IGF-I has a role to play the maintenance of muscle in later life. Using a recombinant adeno-associated virus containing a myosin light chain (MLC1/3) promoter, Barton-Davis *et al*. (1998) injected the cDNA of an IGF-I construct into the extensor digitorum longus (EDL) muscles of young (6 months) and old (27 months) mice. This resulted in overexpression of IGF-I (Ea isoform) in this muscle, but did not give rise to elevated circulating levels of IGF-I in plasma. Four months post-injection, the injected muscle of the younger animals was on average 15% larger and stronger than the non-injected muscle. In the older animals, the injected muscle was 27% stronger when compared with the non-injected old animals, resulting in similar values for mass and function to the 6-month-old animals. An age-related improvement in muscle mass and function was also demonstrated in a very recent study using a mouse model in which transgenic animals were bred to overexpress the IGF-IEa gene (which they termed m.IGF-I) (Musaro *et al*. 2001). At 6 months of age, the fibers of the transgenic animals were 32 μm in diameter as compared to 18 μm in the wild type animals. There was, however, preferential hypertrophy of the faster fiber types (46 μm in the transgenic animal vs. 32 μm in the wild type) with little effect on the slow fibers (16 μm vs. 18 μm). This was attributed to the low expression of the MLC regulatory cassette (1/3 locus) in slow muscle fibers. In the older transgenic animals, muscle mass was maintained in animals 20 months of age, whilst it was significantly atrophied in the wild type animals. These genetic manipulation studies suggest that local IGF-I may be an important factor in maintaining muscle mass in old age. Recently, Owino *et al*. (2001) used tendon ablation to overload the soleus and plantaris muscles of rats to study any age-related differences in the response of the muscle IGF-I isoforms. Animals of different ages (4, 12, 24 months) were overloaded for 5 days. They reported that the MGF mRNA was up-regulated by ~ 1200% in the young animals when compared to the control leg, but was up-regulated to a significantly less extent (~ 500%) in the old animals. The Ea isoform was also up-regulated, but showed no clear age-related effect. In recent studies on humans, and as mentioned previously, Hameed *et al*. (2003a) reported that older men showed a reduced MGF response when measured shortly after a single bout of knee extensor weightlifting exercise compared to young subjects. However, Singh *et al*. (1999) showed that overall muscle IGF-I levels could be increased in older people as a result of a 10-week period (three times per week) of strength training exercise. As discussed previously, Hameed *et al*. (2004) showed that such a training regimen could cause an increase in the level of transcript expression all three of the IGF-I splice variants (IGF-IEa, IGF-IEb and MGF) in the muscles of older people. Interestingly, it was MGF that showed the most marked increase (see Figure 14.2). The ability to do this no doubt contributes to the retained capacity, of even very elderly muscle, to hypertrophy in response to strength training exercise.

Mechano growth factor, satellite cells and muscle damage

Satellite cells in skeletal muscle were first described by Mauro (1961). It is now realized that these cells provide the extra nuclei for postnatal growth (Moss & Leblond 1970; Schultz 1996) and that they are also involved in repair and regeneration following local injury of muscle fibers (Grounds 1998). In normal adult undamaged tissue, the satellite cells are quiescent and usually detected just beneath the basal lamina. They express M-cadherin (M-cad) (Bornemann & Schmalbruch 1994; Irintchev *et al*.

1994) when activated, and commence to coexpress myogenic factors including c-met, MyoD and myf5 and later myogenin (Cornelison & Wold 1997; Beauchamp *et al.* 2000; Qu-Petersen *et al*. 2002). The origin of satellite cells is still somewhat uncertain, as they were thought to be residual myoblasts (reviewed by Seale & Rudnicki 2000), but there is accumulating evidence that some may also originate from pluripotent stem cells derived from progenitor cells of the vasculature (Qu-Petersen *et al*. 2002). Pluripotent stem cells from bone marrow cells (Ferrari *et al*. 1998), as well as epidermal cells (Pye & Watt 2001), have also been shown to fuse and adopt the muscle phenotype when introduced into dystrophic muscle.

It has been established that even in normal muscle, local injury does occur from time to time (Wernig *et al*. 1990), but in certain diseases such as the muscular dystrophies, the muscle fibers are markedly more susceptible to damage, in particular to the membrane (Cohn & Campbell 2000). The contractile system of muscle fibers also sustains damage during eccentric contractions, that is to say when the muscle is activated whilst being stretched. It is interesting to note that the forces generated by activation combined with stretch exceed even those of a maximal isometric contraction. In the muscle fibers involved, the sarcomeres may be pulled out to such a degree that there is no longer any overlap of the actin and myosin filaments, thus causing damage (Lieber & Friden 1999).

During regeneration of skeletal muscle in young rats following ischaemia or myotoxin-induced damage, elevated expression of IGF-I has been reported (Jennische & Hansson 1987; Jennische *et al*. 1987; Edwall *et al.* 1989) which was diminished by the 15th day of recovery (Marsh *et al*. 1997). In a more recent study, the response of the different isoforms of IGF-I, IGF-IEa and MGF to such stimuli were studied for the first time and related to the activation of muscle satellite (stem) cells *in vivo* (Hill & Goldspink 2003). Results of the experiments, in which damage was induced by bupivacaine, demonstrated a surge of IGF-IEa mRNA expression that was maximal at 11 days and diminished thereafter to similar levels as those in the non-injected animals. On the other hand, MGF mRNA showed a much earlier transient response, which peaked at 4 days post-bupivacaine injection and decreased thereafter. Following mechanical damage, the peak in MGF expression occurred even earlier. It seems that in both myotoxin- and mechanical activity-induced damage models, the temporal expression pattern for each IGF-I splice variant showed similar differential gene splicing sequences, with MGF peaking before IGF-IEa. This temporal difference in expression of the two muscle IGF-I mRNA transcripts has also been described in the rat following commencement of resistance exercise (Haddad & Adams 2002). As M-cad expression peaked well before IGF-IEa, whether it was measured as mRNA or protein, it is unlikely that the systemic type of IGF-IEa is responsible for initial activation of satellite cells. However, it is not possible to say from these data whether this was due to an increase in number of satellite cells as it is known that quiescent satellite cells do stain to some extent for M-cad protein (Rosenblatt *et al*. 1994). Nevertheless, it represents a marked increase of M-cad whether it is in existing satellite cells or an increase in the number of these cells or both.

MGF and IGF-IEa splice variants apparently yield the same mature peptide, which is derived from the highly conserved exons 3 and 4 of the IGF-I gene. These exons, present in all known IGF splice variants are known to encode the IGF-I receptor ligand domain. A mechanism of extracellular endoproteolysis of the IGF-I prohormone results in the same mature peptide (Gilmour 1994), even though the splice variants of IGF-I may have different 3′ sequences including the E domain. It has been suggested that IGF-I precursors could be pluripotent, in a form analogous to that of the prohormone propiomelanocortin and proglucagon (Siegfried *et al*. 1992). The observation that a synthetic peptide derived from the rat Eb domain induces proliferation in epithelial cells is noteworthy (Siegfried *et al*. 1992). The growth promoting properties of the MGF E peptide and its role as an independent growth factor is supported by recent cell culture experiments where stable transfection with MGF was shown to stimulate myoblast proliferation but differentiation was suppressed. The addition of a synthetic MGF peptide or the medium from MGF-transfected cells

onto normal C2C12 cells also inhibited their differentiation. Yet this inhibition was reversed when the peptide or the medium were withdrawn. In contrast, cells of the liver type, systemic IGF-I (IGF-IEa) positive clone did form myotubes and the normal cell lines showed less cellular proliferation as well as forming myotubes. Of particular interest was the observation that when an IGF-I receptor antibody was added to the muscle cell cultures, cell proliferation induced by MGF was not inhibited, whereas their stimulation to increase in mass and to form myotubes by IGF-I was reduced. These data strongly suggest that MGF is involved in another signaling pathway in addition to that associated with the IGF-I receptor (Yang & Goldspink 2002).

The results of these studies provide additional insight into the complexity and implication of the IGF-I system in conditions of damage and subsequent regeneration. IGF-IEa and MGF are produced by the active muscle in rodents and humans and have been shown to be positive regulators of muscle hypertrophy (McKoy *et al*. 1999; Goldspink 2001; Owino *et al*. 2001; Hameed *et al*. 2003). However, as reported here, the MGF isoform is acutely induced, whereas IGF-IEa has a delayed effect that is sustained during the later phase of regeneration. When comparing mechanical damage with myotoxin damage it is apparent that both involve a relatively rapid expression of the MGF splice variant, although it may seem that this growth/repair factor has been misnamed 'mechano growth factor'. However, even in the case of myotoxin-induced damage it is likely that the damaged tissue mass is subjected to increased mechanical strain. Also, it is known that the cells swell following damage, thus resulting in the same cellular response. As the expression of the autocrine splice variant (MGF) precedes satellite cell activation it is likely that this form of IGF-I is associated with satellite cell activation, not the systemic IGF-IEa type. This is in accord with the finding that MGF is not appropriately expressed in dystrophic muscles (Goldspink 1996) and the decrease in MGF mRNA levels in response to mechanical overload in older muscles (Owino *et al*. 2001). There is a deficiency of active satellite cells in both these situations in which local tissue repair becomes increasingly impaired. Future experiments investigating the expression of the two transcripts and activation of satellite cells in young and old muscles after the therapeutic application of MGF and IGF-IEa to ameliorate muscle loss are in progress.

Summary

It is becoming increasingly clear that muscle adaptation to high resistance exercise as well as its response to contraction induced damage or otherwise, is mediated at the local level by local growth and repair factors. The IGF-I family comprises members that play different roles in the growth and repair processes. Alternative splicing is a subtle and sophisticated mechanism by which different IGF-I isoforms can be generated from the same gene to have different biological roles. Understanding these roles and how these IGF-I isoforms may interact with other control processes in muscle (e.g. testosterone, myostatin, ubiquitin, etc.) will help in optimizing training regimens for athletes. Furthermore, an understanding of the signaling processes triggered by exercise training will also provide us with the means to develop more effective methods for testing exogenous enhancing substances used in doping.

Acknowledgments

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Chapter 15

Adrenal Gland: Fight or Flight Implications for Exercise and Sports

MICHAEL KJÆR

Introduction

Fight and flight reaction has been important for humans throughout history. To be able to react rapidly and mobilize energy for fast movements away from danger, or to be able to exert high amounts of muscle force in confrontations, requires a system by which a co-ordinated series of events can be stimulated by a fast-releasing hormonal system. The adrenal gland, especially the adrenal medulla, is very well suited for this. This is because epinephrine has potent effects in this regards; the half-life of epinephrine is very short whereby mobilization is quick. Finally, several of the effects that epinephrine exerts can be initiated, even in the resting state, when adequate stimulus (mental stress) can elevate circulating levels of epinephrine to a level that will bring forward, for example, metabolic effects.

Energy turnover increases with onset of exercise, and autonomic/endocrine mechanisms—especially those involving hormones released from the adrenal gland—play an important role in this regulation. This results in increased mobilization of substrate: i.e. (a) glycogenolysis in skeletal muscle; (b) glucose release from the liver to support muscle glycolysis; (c) free fatty acid release from both adipose and muscle tissue for beta-oxidation in skeletal muscle; and (d) increase protein synthesis. In addition to playing an important role in substrate mobilization and combustion, adrenal gland related endocrine mechanisms together with autonomic mechanisms can also: (i) influence blood distribution to various organs; (ii) stimulate sweat glands and influence thermoregulation; (iii) increase contractility of skeletal muscle; and (iv) cause suppression/stimulation of components involved in the human immune system during exercise.

Adrenal gland neuroendocrine changes with exercise

A major component in the autonomic control during exercise is the adrenergic activity, which can be assessed in humans both by direct measurements of electrical activity in superficial sympathetic nerves and by determination of circulating norepinephrine and epinephrine in the blood. Direct recording of sympathetic activity in humans can, for practical reasons, be performed to resting muscle only (Searls *et al*. 1988), whereas direct recording of sympathetic activity to the adrenal medulla can be determined in animal models. Epinephrine is released from the adrenal medulla in response to sympathetic neural activity to the gland during exercise, whereas adrenal cortical hormones (i.e. cortisol) are released due to pituitary hormonal stimulation by adrenocorticotropic hormone (ACTH). Levels of norepinephrine and epinephrine in arterial blood increase with exercise intensity, expressed by the percentage of maximal individual performance (% $\dot{V}O_{2max}$), and, as clearance of these hormones only changes moderately with exercise, changes in plasma levels can be attributed to changes in secretion and release (Kjær *et al*. 1985). It has been shown that whole-body clearance of epinephrine increases by 15% at low-exercise intensities and decreases around 20% below basal levels after more intense exercise (Kjær *et al*.

1985). However, as the increase in plasma epinephrine seen during dynamic exercise in humans is five to 10-fold, these changes are caused by increases in secretion from the adrenal medulla rather than by changes in clearance. A major contributor to epinephrine clearance is the hepatosplancnic area as well as the kidneys.

Epinephrine and cortisol responses are influenced by glucose levels during exercise, and falling glucose markedly enhance the responses of these two glucocounteregulatory hormones during prolonged exercise (Galbo *et al*. 1977). In addition to influencing carbohydrate and fat metabolism, epinephrine per se has been shown to increase protein metabolism in isolated electrically stimulated muscle. Although epinephrine responses can be seen to acute mental stress, these changes are by far lower than the ones observed during physical exercise.

Adrenomedullary activity after physical training

Vigorous endurance training will reduce the catecholamine response to a given absolute workload, whereas neither sympathetic nerve activity nor norepinephrine levels at maximal workloads differ between individuals with different training status. This supports the view that physical training does not alter the capacity of the sympathetic nervous system—and thus most likely neither the sympathetic activity directed towards the adrenal medulla—but that responses to submaximal exercise are linked closely to the relative rather than to the absolute workload. Surprisingly, however, in a 24-h study it has been found that, if anything, highly trained individuals had a higher catecholamine release over the day compared with sedentary individuals. Epinephrine response in trained versus sedentary individuals has been shown to be enlarged when stimulated by a variety of stimuli such as hypoglycemia, caffeine, glucagon, hypoxia and hypercapnia (Kjær *et al*. 1986, 1988; Kjær & Galbo 1988). This indicates that the capacity to secrete epinephrine from the adrenal medulla improves with training, the development of a so-called 'sports adrenal medulla' reflecting a hypertrophy phenomenon similar to that seen in a heart adapting to physical training. This is supported by findings in trained men (sprint) who showed a higher epinephrine response to short-term exercise compared with that of untrained counterparts (Jacob 2004). Interestingly, no significant difference could be obtained in trained versus untrained women using this short-term sprint test. In rats who underwent 10 weeks of intense swim training, the adrenal medullary volume and the adrenal content of epinephrine was larger in trained rats (both male and female) compared with controls who were either weight matched, sham trained or cold stressed (Stallknecht *et al*. 1990). Interestingly, the training induced increase in adrenal medulla volume was paralleled by the increase in adrenal gland weight, indicating that the major stimulus was on the medulla rather than the adrenal cortex. Furthermore, no increase in total gland weight was seen after sham training or cold stress, indicating that these 'mental' types of stress did not result in any major gland adaptation. Although these findings indicate that the improved secretion capacity of epinephrine is a result of training, this will most likely require several years of training. In well-trained athletes who underwent hypoglycemia before and 4–5 weeks after an injury that resulted in inactivity, epinephrine responses did not change with this short-lasting alteration in activity level (Kjær *et al*. 1992). It could be speculated that an early influence upon the adrenal medullary volume is needed, for example during adolescence, and some reports seem to indicate this (Jacob 2004). However, even if this adaptation requires long-term stimulation, it is interesting that endocrine glands apparently can adapt to physical training and alter their secretion capacity, similar to other tissues like muscle and heart.

Motor control and neural reflex influence on adrenal gland responses

Trying to address the role of motor control for adrenal endocrine responses during exercise can basically be studied either by enhancing the voluntary effect to carry out a certain workload and thus exagerating the central effort, or it can be studied by decreasing the contribution of motor centers by, for

example, performing involuntary exercise and, so to speak, 'letting the work being done to you'. Attempts to increase motor center activity for a given force output has been adressed in humans using partial neuromuscular blockade to weaken the maximal force by 30–70% (Kjær *et al*. 1987). It was found that an exercise-induced increase in levels of circulating catecholamines (and pituitary hormones [growth hormone and ACTH]) was augmented compared to control experiments with saline infusion. These findings are supported by experiments in paralyzed cats, where direct stimulation of the subthalamic locomotor areas in the brain resulted in adrenergic hormonal responses similar to the one seen during voluntary exercise (Vissing *et al*. 1989). Together, these experiments support the view that motor center activity can directly stimulate sympatho-adrenergic activity during exercise both directly and independently of feedback from contracting muscle. That these central factors coupled to exercise intensity are not sufficient to elicit a maximal adrenergic and pituitary hormonal responses can be demonstrated in several ways. When exercising with a small muscle group, for example one-legged knee extension even at maximal intensity, only a small catecholamine response can be observed (Savard *et al*. 1989). Furthermore, when maximal work output was reduced by more than 60% with neuromuscular blockade (tubocurarine), despite subjects working at the highest possible effort, adrenergic responses were far from maximal. Mechanisms other than central motor control therefore need to be active during exercise, and one such mechanism is neural feedback from contracting the skeletal muscle. This can be investigated by using lumbar epidural anesthesia in doses sufficiently high enough to block impulses in thinner afferent nerves (type III and IV or type C) but still preserving motor nerves and the ability to perform exercise to the highest possible degree. Such an approach evidently has weaknesses, and negative findings of a blockade does not definitely exclude a role of afferent nerves since a perfect distinction between afferent and efferent nerves cannot fully be obtained by this approach. Nevertheless, during static exercise, but not during dynamic exercise, catecholamine responses were inhibited when afferent responses were absent. Interestingly, both ACTH and β-endorphin responses during submaximal exercise were abolished during epidural anesthesia (Kjær *et al*. 1989). In support of a role of afferent nerves in adrenergic and pituitary responses, hormonal levels in the blood increased in response to direct stimulation of these nerve fibers in cats (Vissing *et al*. 1994).

Hepatosplanchnic glucose production and adrenal gland responses to exercise

During intense exercise the rise in hepatic glucose production parallels a rise in plasma epinephrine levels. In swimming rats, the removal of the adrenal medulla reduced the hepatic glycogenolysis. Furthermore, exercise-induced increase in hepatic glucose production was diminished by adrenodemedullation in running rats (Sonne *et al*. 1985). However, most studies have been unable to demonstrate any direct effect of epinephrine on liver glycogen breakdown during exercise. In running dogs, evidence has been provided that epinephrine may play a minor role in liver glucose output late during exercise, probably due to an increased gluconeogenic precursor level (Moates *et al*. 1988). Furthermore, adrenalectomized individuals maintain a normal rise in hepatic glucose production during exercise, and only when epinephrine is infused in these patients was hepatic glucose production augmented during the early stages of exercise (Howlett *et al*. 1999). In humans the role of liver nerves and epinephrine have been studied with application of local anesthesia around the sympathetic coeliac ganglion innervating liver, pancreas and adrenal medulla (Kjær *et al*. 1993). Pancreatic hormones were standardized by the infusion of somatostatin, glucagon and insulin. During blockade, the exercise-induced epinephrine response was inhibited by up to 90%, and presumably liver nerves were also blocked, but this did not diminish the glucose production response to exercise. This indicates that sympatho-adrenergic activity is not responsible for the exercise-induced rise in splanchnic glucose output. In further support of this hypothesis, the exercise-induced increase in liver glucose production was identical in liver-transplanted patients compared to healthy

control subjects, as well as kidney-transplanted patients who received a similar hormonal and immunosuppressive drug treatment as the liver-transplanted patients (Kjær *et al*. 1996a). Finally, in experiments in exercising dogs that underwent a selective blockade of hepatic α- and β-receptors, it was demonstrated that circulating norepinephrine and epinephrine do not participate in the stimulation of glucose production during intense exercise (Coker *et al*. 1997). Taken together, sympathetic liver nerves or circulating norepinephrine play no role in glucose mobilization from the liver during exercise, and circulating epinephrine only plays a minor role during intense exercise and late, prolonged exercise. Cortisol has been shown only to play a minor role in hepatic glucose production during exercise, and only seems to be of importance if inadequate secretion of other hormones is present.

Epinephrine effect on muscle carbohydrate metabolism

Epinephrine has been demonstrated to stimulate glycogen breakdown in skeletal muscle during contraction both in exercising animals and humans when supra-physiological doses were used (Richter 1996). Later studies using more physiological epinephrine doses have not been able to demonstrate any increased glycogen breakdown despite higher activation of phosphorylase compared with control studies). In line with this, in adrenalectomized individuals no impairment in glycogen degradation was found during exercise, neither was muscle glycogenolysis increased by substituting epinephrine during exercise (Kjær *et al*. 2000). It was furthermore shown that activation of glycogen phosphorylase and hormone-sensitive lipase is only present if these individuals receive infusion with epinephrine to mimic changes in epineprine occuring in healthy individuals during exercise. This indicates the importance of epinephrine in the activation of glycogenolytic and lipolytic pathways. It also indicates that such activation occurs in parallel for intramuscular triglyceride and glycogen by adrenergic activity, and that the choice of substrate for energy production takes place at another level in the muscle (Kjær *et al*. 2000).

Spinal-cord-injured individuals are characterized by a lack of voluntary control over their lower limbs, and are absent of neural feedback from muscles to higher brain centers. Development of equipment has allows for functionally electrical-stimulated exercise on an ergometer, causing oxygen uptake rates to rise to around 1.0–1.5 $L \cdot min^{-1}$. This allows for the study of carbohydrate and fat metabolism and metabolic changes during exercise. Using involuntary exercise in spinal-cord-injured individuals as an intervention showed that, in the absence of motor control and neural feedback from muscle, mobilization of glucose from the liver was impaired, resulting in a gradual drop in plasma glucose during exercise (Kjær *et al*. 1996b). In line with this, healthy individuals who were paralyzed by an epidural blockade also had impaired glucose mobilization response (Kjær *et al*. 1998). Furthermore, in spinal-cord-injured patients during voluntary arm cranking, euglycemia was maintained during exercise. These findings indicate that neural mechanisms are crucial for the matching of glucose mobilization to peripheral glucose uptake during exercise, and that blood-borne mechanisms are not sufficient to accomplish this. During electrical exercise by spinal-cord-injured individuals, the primary energy source is glycogenolysis, and higher levels of lactate have been found both in muscle and blood. Furthermore, the glucose uptake is several-fold higher in spinal-cord-injured patients compared with healthy controls working at the same oxygen uptake rate.

Sympathoadrenergic activity and fat metabolism

Intravenous infusion of epinephrine in resting humans caused an increase in lipolytic activity as determined by microdialysis of subcutaneous adipose tissue, an effect that was desensitized by repeated epinephrine infusions (Stallknecht 2003). Spinal-cord-injured patients were investigated during arm cranking, and lipolysis was determined by microdialysis in subcutaneous adipose tissue both above and below the cutaneous border separating the sympathetic innervated region (proximal–clavicular region) from the desympathectomized areas (distal–umbilical region) (Stallknecht *et al*.

2001). In both regions lipolysis increased with exercise, evidence that direct sympathetic innervation is not of major importance for the lipolysis during muscular work. In contrast, circulating epinephrine may be a likely candidate for lipolysis activation. Training causes a decreased adipose tissue mass and adipocyte size, but the sympathoadrenergic system does not seem to be crucial for this adaptation.

Not only adipose tissue but also intramuscular fat can be stimulated by epinephrine, and both lipoprotein lipase (LPL) and hormone sensitive lipase (HSL) play important roles in this regulation. HSL might be under control by both contractions and epinephrine (Donsmark 2002), and it has recently been shown that activation of HSL and glycogen phosphorylase occurs in parallel in adrenalectomized individuals who receive an infusion with epinephrine during exercise (Kjær *et al*. 2000). This could indicate that the mobilization of intramuscular triglyceride and glycogen occurs when simultaneously stimulated by adrenergic activity, and that the choice of substrate for energy production takes place at another level.

Summary

The adrenal gland plays an important role in releasing hormones that are crucial for carbohydrate, fat and protein metabolism, as well as for regulating organ blood distribution, thermoregulation, immunmodulation and skeletal muscle contractility during and after exercise. Epinephrine secretion increases with the relative work intensity, and adrenal medulla hypertrophy can occur in response to prolonged intense training ('sports adrenal medulla'). Both direct motor center activity and afferent neural feedback signaling from contracting muscle play a role in the regulation of hormonal release from the adrenal gland during exercise. Adrenal gland hormones (epinephrine and cortisol) participate in the redundant regulation of hepatic glucose release during physical activity.

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Chapter 16

The Adrenal Medulla: Proenkephalins and Exercise Stress

JILL A. BUSH AND N. TRAVIS TRIPLETT

The adrenal gland

The two endocrine adrenal glands are relatively small glands that are situated superior to the kidney. These glands are essential for life; removal of them would result in death. Diseases of the adrenal glands occur but are generally treated with synthetic derivatives of the hormones these glands produce. The main function of the adrenal glands is to respond to stressed of the body by producing hormones that regulate blood glucose, increase heart rate and ventilation, and maintain the fluid homeostasis in the body. The adrenal gland has the highest rate of blood flow in the body per gram of tissue, and is comprised of two main types of tissue; the cortex and medulla (Kaplan 1988). The adrenal medulla is the innermost part of the adrenal gland and comprises only about 10% of the total gland tissue. The adrenal medulla is a sympathetic ganglion in which postganglionic fibers have ended in secretory vesicles. Upon nervous system stimulation, via preganglionic nerves reaching the adrenal medulla via the splanchnic nerve, hormones and neurohormones (i.e. epinephrine and peptide F) are secreted from chromaffin cells. Adrenal medullary hormones are not essential for life but are produced and secreted during stressful-like events. The adrenal cortex, on the other hand, is essential for life. This outer cortex layer secretes glucocorticoids, involved in carbohydrate and protein metabolism (i.e. cortisol); mineralcorticoids, involved in maintenance of extracellular fluid volume (i.e. aldosterone); and minor-effecting sex hormones involved in reproduction. The adrenal cortex is stimulated primarily by the anterior pituitary release of adrenocorticotropic hormone.

Adrenal medullary chromaffin system

The adrenal medulla contains cells known as chromaffin cells which constitute the majority of space within the medulla. The chromaffin system serves as the method of storage and secretion for catecholamines and proenkephalin peptide fragments, including peptide F, E, and B (Lewis *et al*. 1979; Viveros *et al*. 1979). They resemble storage and secretory granules of other endocrine or exocrine glands found within the body. These chromaffin granules are highly specialized, electron-dense and osmiophilic organelles smaller than mitochondria. Communication between the sympathetic chromaffin system and the body is rapid, where the neural response is instantaneous and the hormonal response occurs within minutes or hours. Stimulation of chromaffin cells by sympathetic preganglionic neurons of the splanchnic nerve (Coupland 1965, 1972) occurs in response to the neurotransmitter acetylcholine secreted from preganglionic neurons arising from the thoracolumbar region of the spinal cord (Fig. 16.1).

Besides enkephalins and catecholamines, some other constituents of the chromaffin granules exist including chromogranin A, nucleotides and ascorbic acid. Extra-adrenal chromaffin cells are located adjacent to the aorta as paraganglia, in carotid bodies, in viscera, and in sympathetic ganglia. To date, there is only indirect evidence for selective discharge of granular contents upon neural stimulation

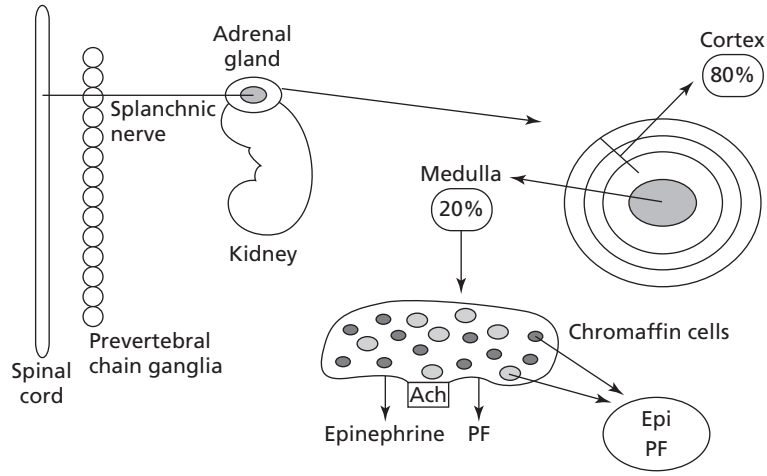

Fig. 16.1 Neural innervation and chromaffin granules of the adrenal gland. Ach, acetylcholine; Epi, epinephrine; PF, peptide F.

of the adrenal medulla. However, exocytosis of the chromaffin granule is far from simple, and this process could provide a potential mechanism for the differential release pattern with exercise stress. Unfortunately, there is no data available to support exocytosis and hence the differential release pattern of adrenal medullary neurohormone (i.e. peptide F and epinephrine) upon stimulation. Stimulation of nicotinic and muscarinic receptors is involved in acetylcholine-related exocytosis of the chromaffin cells. Several endogenous substances (e.g. substance P, neurotensin, vasoactive intestinal peptide) within the adrenal medulla may competitively bind to nicotinic receptors, thus inhibiting the release of epinephrine (Livett, B.G. 1984). High concentrations of nicotine demonstrated a proportional release of enkephalin-containing peptides and total catecholamine concentration (Wilson *et al*. 1982; Livett, B.G. 1984). If exocytosis of chromaffin cells containing proenkephalin fragment peptide F and epinephrine can be altered by such endogenously produced substances, then this would provide a plausible explanation for a differential release pattern of peptide F and epinephrine.

Chromaffin granules contain membrane-bound proteins and glycoproteins. Such soluble factors include a proton pump adenosine triphosphatase (ATPase), catecholamine and nucleotide carrier proteins, cytochrome b_{561}, actin, glycoproteins, and dopamine β-hydroxylase pump ATPase is a necessary constituent of the chromaffin granule (Fig. 16.2). Via hydrolysis of ATP on the exterior membrane surface, protons (H^+) are injected into the granule, providing a more acidic, positively charged environment versus the cytosol. An electrochemical H^+ gradient is created in the interior of the granule. Due to the biosynthetic pathways of epinephrine and dopamine, the energy supplied by the electrochemical proton gradient is necessary for the uptake of these two substances into the cell. The enzyme necessary to breakdown norepinephrine into epinephrine is located in the cytosol of the cell. Thus norepinephrine must be exported from the cell, exposed to the enzyme, and epinephrine recaptured for storage inside the granule. Other factors may also be involved in the exocytotic process of the granules. A Ca^{2+} influx is a primary elicitor of exocytosis. Stimulation via acetylcholine or high extracellular K^+ increases the intracellular Ca^{2+} concentration of the chromaffin cells. As the granules move toward the plasma membrane, it may encounter the cytoskeletal structure actin. The increase in Ca^{2+} may decrease the viscosity of this interaction permitting easier granular movement. Calmodulin selectively phosphorylates membrane proteins, allowing for the fusion of plasma and granular membranes. Synexin, in the presence of Ca^{2+}, can polymerize into large aggregates which bind to phospholipids within the membrane. Also, recycling of the granular membrane to and from the plasma membrane is

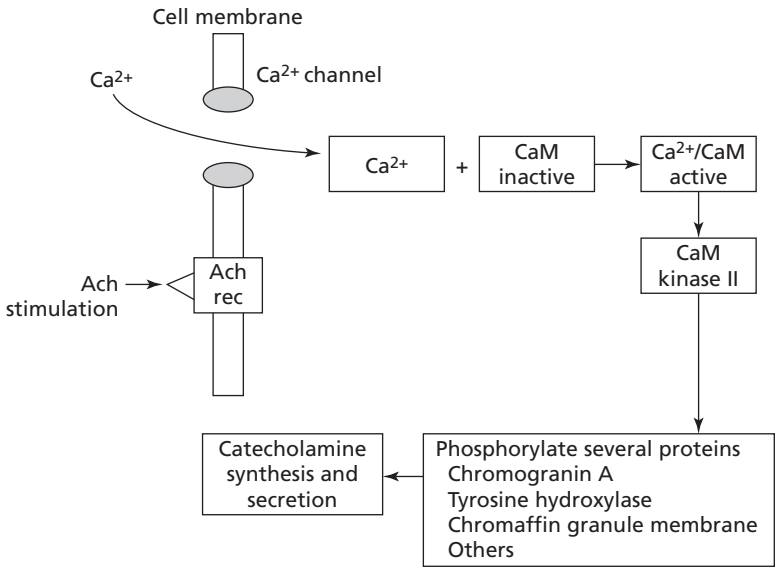

Fig. 16.2 Signal transduction pathway in acetylcholine-stimulated adrenal medullary cells. Ach, acetylcholine; CaM, calmodulin. (Modified from Yanagihara *et al*. 1996.)

continuously occurring. All of these aforementioned processes are important in the role of exocytosis of the chromaffin granules containing epinephrine and peptide F in the differential release of either neurohormone.

The chromaffin cells also secrete enkephalins, a group of substances that possess morphine-like analgesic activity and are also produced by the brain (Undenfriend & Kilpatrick 1984). The enkephalins and some larger peptide fragments which contain enkephalin sequences, known as the enkephalin-containing peptides (ECPs), are derived from the large molecular weight preproenkephalin. These ECPs include peptides in the 3–5 kDa range, which would include peptides E, F and B (Undenfriend & Kilpatrick 1984; Hiddinga *et al*. 1990). Studies with these peptides have found parts of their amino acid sequences to be highly conserved among different species (Lewis & Stern 1983) and are found in the blood in physiologically relevant concentrations with long half-lives (≥ 15 min), indicating their biological significance (Katzenstein *et al*. 1987). However, many of the functions of these substances are as yet unknown.

Proenkephalin peptide F

It is important to understand some of the fundamental aspects of the proenkephalin polypeptide precursor and the processing of this peptide precursor to peptide F in order to appreciate the potential physiological roles of peptide F. The preproenkephalin polypeptide (30 kDa) is the precursor of the enkephalins ([Met]- and [Leu]-enkephalin) and ECPs and is found in various regions of the body: brain, adrenal medulla and activated T-lymphocytes, and hemoglobin molecules (Hughes *et al*. 1975; Kimura *et al*. 1980; Brantl *et al*. 1986; Zurawski *et al*. 1986; Martin *et al*. 1987; Ivanov *et al*. 1997; Zhao *et al*. 1997).

Peptide F, a 3.8 kDa ECP, is post-translationally processed from preproenkephalin (Fig. 16.3) in a series of cleavages by trypsin (amino end) and carboxypeptidase B (carboxyl end) which cleave at the enkephalin sequences (Undenfriend & Kilpatrick 1984). Peptide F, the more common peptide product of proenkephalin precursor measured in circulation (Kilpatrick *et al*. 1980, 1981; Wasserman *et al*. 1986), consists of 34 amino acids (amino acid sequence 107–140) and contains two [Met]-enkephalin sequences (Lewis 1982; Lewis & Stern 1983), expressing structural similarities to classical opiates (Hansen & Morgan 1984). Opioid peptides typically play an essential role in the brain as neurotransmitters where [Met]- and [Leu]-enkephalin are the most prominent end-products.

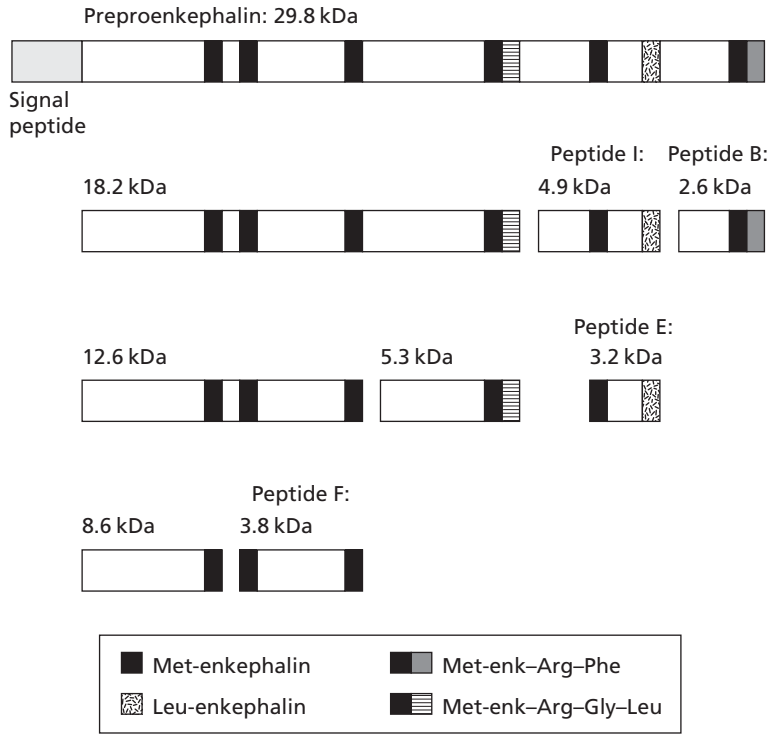

Fig. 16.3 Post-translational processing of enkephalin-containing peptides from preproenkephalin. (Modified from Lewis & Stern 1983, Undenfriend & Kilpatrick 1984 and Katzenstein *et al*. 1987.)

Interestingly, a breakthrough in the discovery of the preproenkephalin precursor molecule became apparent when Kimura *et al*. (1980) reported information pertaining to possible precursors for the enkephalins, [Met]- and [Leu]-enkephalin, secreted from the adrenal medulla. With the discovery of few fragments in the brain, the determination of the structure of the precursor became difficult. ECPs derived from the biosynthetic processing of proenkephalin within the central nervous system cannot fully account for the total amount of these peptides found in the circulatory pool (Lewis & Stern 1983). However, a higher concentration of [Met]- and [Leu]-enkephalin is derived through central nervous system processing (Lewis 1982; Lewis & Stern 1983). Discovery of more proenkephalin fragments in the adrenal medulla gave rise to better biochemical determination of sequences of the preproenkephalin precursor and to potential physiological stress responses. It was also thought at the time that the more labile enkephalins were transported via the higher molecular weight fragments (e.g. to peptide F) since ECPs have a greater circulating period (i.e. 15–60 min) than the smaller [Met]- and [Leu]-enkephalins (i.e. 1–2 min) (Kilpatrick *et al*. 1980, 1981; Kimura *et al*. 1980; Boarder & McArdle 1986).

Transportation of 3–8 kDa peptides through the circulation could be enhanced by the non-enkephalin regions within the peptide structure (Boarder & McArdle 1986), thus adding to the prolonged plasma half-life of these peptide fragments. The roles of the [Met]- and [Leu]-enkephalin in the periphery became speculative at best due to a high potential for protease degradation, within 1–2 min (Kraemer *et al*. 1987). Thus, the larger structure of peptide F with a longer prolonged circulating half-life (15–60 min) provided the potential for physiological roles in the peripheral circulation (e.g. communication between different biocompartments).

Concomittant release of peptide F and epinephrine from adrenal medulla

Peptide F is co-stored with epinephrine within chromaffin cells of the adrenal medulla (Viveros *et al*.

1979; Lewis & Stern 1983). However, the biochemical and molar-equivalent relationship of epinephrine and peptide F within these chromaffin cells is less understood. The relationship of the co-storage of peptide F and epinephrine may provide a potential mechanism for differential release of these neurohormones from the adrenal gland in response to stress. It is known that trypsin and carboxypeptidase β digestion of preproenkephalin within the adrenal medulla occurs within 2 h of proenkephalin precursor synthesis and that the peptide F fragment is found in the chromaffin granules within 2 h of the precursor synthesis (Wilson 1991). Tetrabenazine, which has been shown to inhibit catecholamine uptake into the storage vesicles, did not inhibit uptake of proenkephalin fragments into vesicles. In fact, it enhanced the peptide's assimilation (Wilson 1991). This research would suggest that, not only is peptide F a final processing product assimilated into the chromaffin vesicles, but also that termination of the process may depend upon the co-storage of epinephrine (Wilson 1991).

Studies have shown that epinephrine and enkephalin-containing fragments are co-released from cultured adrenal chromaffin granules (Schultzberg *et al*. 1978; Livett, A.R. *et al*. 1981). This co-secretion, under similar physiological stimulation, also underscores the importance of a biological role for peptide F during stress. It is also possible that peptide F and epinephrine can have a complementary action on various biological target tissues. For example, both epinephrine (McCarthy & Dale 1988) and peptide F (Hiddinga *et al*. 1994) have modulating effects on the immune system. This may be similar to a model used by other neurohormones and peptides which are co-stored and co-secreted and possess dissimilar/similar biological activities (e.g. insulin and serotonin within pancreatic β-cells in humans [Richter *et al*. 1986] and neuropeptide Y and norepinephrine within bovine sympathetic nerves [Bastiaensen *et al*. 1988; De Potter *et al*. 1988]).

Epinephrine is the primary stress hormone in the body. Its biosynthesis and release may influence peptide F release and appearance in the blood (Kraemer *et al*. 1985b, 1991). While it is important to understand the processing and storage of peptide F, it is important to understand the processing of epinephrine as well, as a close relationship exists between these neurohormones. The enzymatic processing of newly synthesized preproenkephalin may be affected by epinephrine and thus may affect the co-storage of peptide F (Wilson 1991). Wilson and colleagues (Wilson *et al*. 1982; Wilson 1991) found that pharmacological agents and neurotransmitters activated proenkephalin synthesis in the chromaffin cells. They found that inhibitors of vesicular catecholamine uptake (e.g. tetrabenazine and resperine) enhanced the processing of proenkephalin and thus the content of ECPs in the chromaffin cells. This data suggested that epinephrine or possibly other constituents of the chromaffin cells (e.g. adenosine triphosphate [ATP]) may inhibit proenkephalin processing. Thus, this mechanism of action can alter the molar concentration of peptide F and epinephrine within different chromaffin cells.

It is important to understand the basic processing of epinephrine. Briefly, amines containing a 3,4-dihyroxyphenyl nucleus are referred to as catecholamines and are derived from the amino acid tyrosine. Tyrosine can also be derived from the conversion of phenylalanine in the liver via phenylalanine hydroxylase. Tyrosine and phenylalanine can be found in the diet of foods high in protein. Epinephrine is mainly derived from the adrenal medulla; however, the necessary enzymes involved in biosynthesis are also present within neurons of the central nervous system in minute amounts. Norepinephrine is primarily found within the central nervous system as a neurotransmitter for sympathetic neurons, either inhibitory or excitatory. Approximately 80% of total stimulated adrenal medullary catecholamine secretion is epinephrine, while the remaining 20% is norepinephrine.

The initial step in epinephrine synthesis involves conversion of tyrosine to 3,4-dihydroxyphenylalanine (dopa) via the enzyme tyrosine hydroxylase, which is phosphorylated and activated when stimulated by acetylcholine (Fig. 16.4). Dopa, in the presence of an aromatic L-amino acid decarboxylase, is decarboxylated to form dopamine. The two aforementioned enzymes are located in the cytosol of the medulla where such enzymatic reactions occur. Dopamine enters the granule and is hydroxylated by dopamine β-hydroxylase from the granular mem-

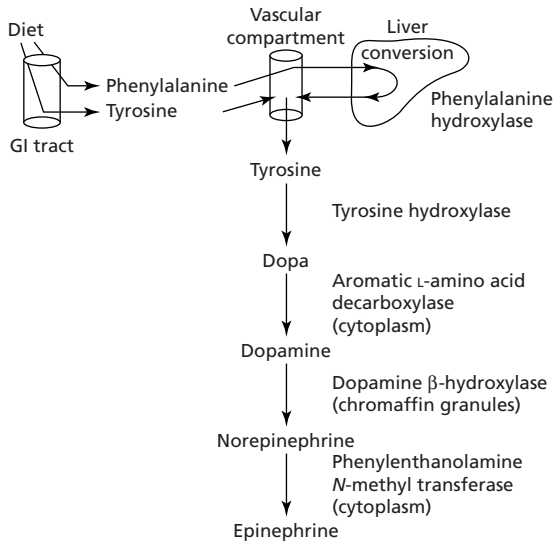

Fig. 16.4 Biosynthetic pathway of adrenal medulla-derived epinephrine. (Modified from Genuth 1988.)

brane to form norepinephrine. Norepinephrine returns to the cytosol for methylation into epinephrine via phenylethanolamine *N*-methyl transferase (PNMT), using *S*-adenosyl-L-methionine as a donor. Epinephrine is reincorporated into the granule for storage and secretory preparation. This process is dependent on the energy from the proton pump ATPase. Catecholamines within the granule are bound to ATP and chromogranin A, preventing egress of the stored hormones.

In the periphery (i.e. adrenal gland), epinephrine, norepinephrine and dopamine serve as neurohormones, while in the central nervous system, they can serve as neurotransmitters. The plasma half-life of epinephrine is about 1–2 min. The plasma concentration of epinephrine at rest is ~ 0.05 $ng \cdot mL^{-1}$ and can elevate from 0.27 $ng \cdot mL^{-1}$ to 4.1 $ng \cdot mL^{-1}$ during exercise. Epinephrine affects the state of arousal, the 'fight or flight' response, and the contractility of heart and skeletal muscle. Epinephrine elevates heart rate, increases blood flow to skeletal muscle, increases the metabolic rate, and increases substrate utilization during exercise via release of glucose and free fatty acids into the blood. Epinephrine binds to receptors on the cell surface of the plasma membrane of target cells, interacting with both α- (α_1, α_2) and β- (β_1, β_2, β_3) adrenergic receptors. Epinephrine has a high affinity for β_2-receptors which are located extrajunctionally on non-innervated target cells. Such receptors mediate lactate production and vasodilation in skeletal muscle. The role of epinephrine release during acute exercise stress may obviate the role played by peptide F, as the pattern of response between the two neurohormones sometimes differs (Kraemer *et al*. 1985b, 1991). This is evident when data are expressed relative to the molar ratio of peptide F to epinephrine. A decrease in the molar ratio (usually observed during exercise) would indicate the predominance of epinephrine secretion into the circulation, whereas as increase in the molar ratio (usually observed during recovery) would indicate the predominance of peptide F secretion. However, it cannot be discounted that different chromaffin cells contain varying concentrations of each neurohormone (Wilson *et al*. 1982; Livett, B.G. 1984), or that chromaffin cells are selectively released upon stimulation. Epinephrine has been shown to play a greater role during exercise (Kjær *et al*. 1985; Brooks *et al*. 1988; Kjær & Galbo 1988), while peptide F may play a greater role during recovery from exercise (Kraemer *et al*. 1985b, 1991). The difference in release patterns may explain important biological functions. The possibility exists that, even though epinephrine and peptide F are co-stored and co-secreted by similar stimuli, they may have different physiological roles within the same biocompartment.

Physiological role of peptide F

Although peptide F contains two [Met]-enkephalin sequences, it responds only weakly to classical opiate action tests (Lewis & Stern 1983). Therefore, the [Met]-enkephalin sequence contained in peptide F is not necessarily indicative of enkephalin-like functions. The non-enkephalin segments of the peptide F molecule have an important role in their function, as they may be the primary binding sequences for the molecule. Figure 16.5 outlines potential physiological roles for peptide F that will be discussed in the following section.

To date, one of the most important evidences supporting the study of the response pattern of

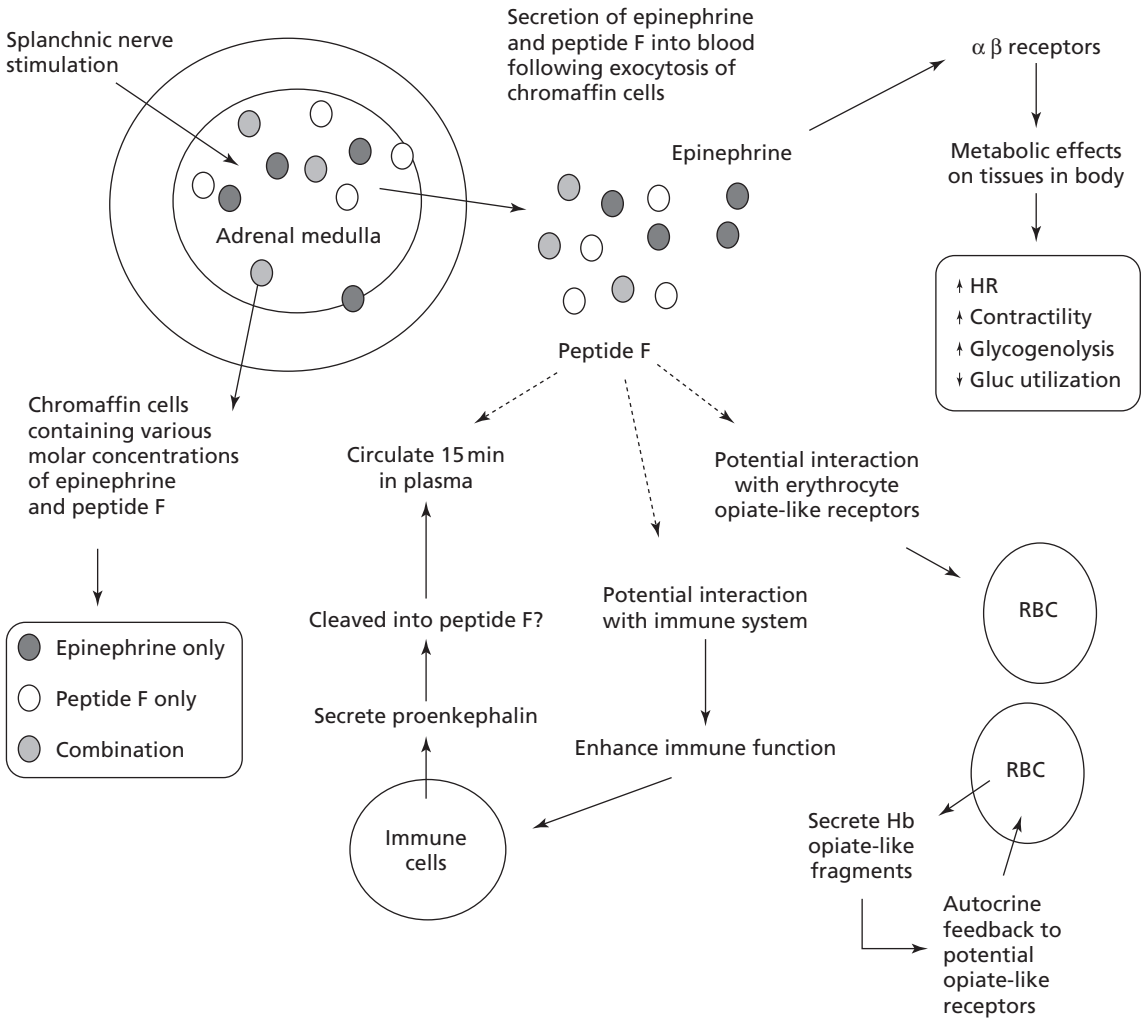

Fig. 16.5 Potential interaction of peptide F within the three biocompartments of blood. HR, heart rate; RBC, red blood cell. (From Bush *et al*. under review.)

peptide F is the interaction between peptide F and the immune system (Hiddinga *et al*. 1994; Triplett-McBride *et al*. 1998). In addition, it is possible that these interactions can take place in the blood biocompartments. Peptide F has been shown to have a relationship with T- and B-cells *in vitro* (Hiddinga *et al*. 1994) and to be related to B-cell activation and individual fitness level (Triplett-McBride *et al*. 1998).

The longer half-life of peptide F prolongs its biological viability to interact with the different blood biocompartments and the immune system. However, the immune system may not be the only target site for this proenkephalin peptide F fragment. Other target tissues and sites may exist as this is an evolving form of study.

Hiddinga *et al*. (1994) reported a possible role for peptide F as a regulatory neurohormone within the immune system. Unlike the suppressive effects of [Met]-enkephalin on the immune system (Johnson *et al*. 1982; Marotti *et al*. 1993), peptide F seems to enhance immune function (Hiddinga *et al*. 1994). *In vitro* research using purified peptide F at physiolo-

gical concentrations demonstrated that, following 15 min of peptide F incubation with T-cells, there was an increase in antibody-forming B-cells in culture, significantly enhancing the antigen-specific antibody-forming cell response of lymphocytes to an antigenic challenge of trinitrophenyl-Ficoll (TNP-Ficoll) (51 ng·mL^{-1}). Studies in athymic nude mice (e.g. mice expressing delayed and defective T-cell development) demonstrated that peptide F was directly involved in an alteration of the cell-mediated immune response (i.e. T-cell activation) rather than directly involved in the humoral-mediated immune response (i.e. antibody production from B-cells). This data has shown that lymphocytes could be a possible target site for peptide F, and that subsequent interaction causes an enhancement in immune function.

Methods of identifying the peptide F receptor

Competitive binding with naloxone. Hiddinga *et al*. (1994) also attempted to indirectly identify a possible receptor mechanism for peptide F through naloxone competition. Naloxone functions as an antagonist for opiate receptor binding (e.g. [Met]-enkephalin) (Simonds 1988). Murine splenocytes were either treated with peptide F or [Met]-enkephalin to determine which molecule had the greatest effect on the antibody-forming cell response of B-cells. Naloxone was added at final concentrations of 0.1, 1.0 and 10.0 µmol (i.e. 10–1000 times greater than the concentration of either plasma peptide F or plasma [Met]-enkephalin). At a 10 nmol concentration, purified peptide F and [Met]-enkephalin, respectively, yielded enhancement and suppression on the antibody-forming response of B-cells. To activate the lymphocytes, antigens (1% sheep red blood cells [SRBC] or TNP-Ficoll) were added to the culture at both an optimal (51 ng·mL^{-1}) and suboptimal (5 ng·mL^{-1}) concentration following 15 min of incubation with naloxone (10 µmol) and/or peptide F (10 nmol). Naloxone was unable to block the antibody-forming response of B-cells elicited by peptide F. This indicated that peptide F may interact with a yet-to-be-determined receptor other than an opioid receptor (i.e. a peptide receptor) on lymphocytes. [Met]-enkephalin-induced suppression of the antibody-forming response of B-cells, and this action was blocked by the addition of naloxone. This suggested that this low molecular weight enkephalin was bound to opiate receptors found on lymphocytes (Sibinga & Goldstein 1988). To test if concentration of either molecule was an important factor in modulating immune function, peptide F and [Met]-enkephalin were added simultaneously to cultures in an equimolar concentration (10 nmol) in the presence of naloxone. [Met]-enkephalin again induced suppression of the antibody-forming response of B-cells, and this action was blocked by naloxone. Peptide F, on the other hand, induced an increase in antibody production, and this increase was not blocked by naloxone. This again suggested the possibility of different mechanisms of receptor binding on the same immune cells. It may be that naloxone inhibited the immunosuppressive effects of [Met]-enkephalin, thereby potentiating the immunoenhancing effects of peptide F. These data also implied that even though the peptide F molecule contained [Met]-enkephalin sequences, peptide F may not have interacted with opioid receptors found on lymphocytes. It may be that the non-enkephalin (peptide) sequences of peptide F play a greater role in receptor binding (Roth *et al*. 1989). This line of research has proven to be very beneficial in determining and locating a possible receptor and/or target site for peptide F, which to date are not fully defined. Such data and the relationship of immune cells and peptide F supported the need for research since peptide F was found in quantifiable concentrations in the white blood cell biocompartment of the blood.

Immunochemical approach via flow cytometry. Since these studies in the 1980s, Bush *et al*. (under review) have performed other methodological techniques to determine the receptor for peptide F on immune cells. It was reasoned that an immune cell responsive to peptide F would bear peptide F receptors. An immunochemical approach was used to concurrently identify cells displaying a peptide F receptor and to identify the leukocyte subtype. Monoclonal antibodies to be used for identification of subclasses of human leukocytes were commercially available. No antibodies were available to identify peptide F

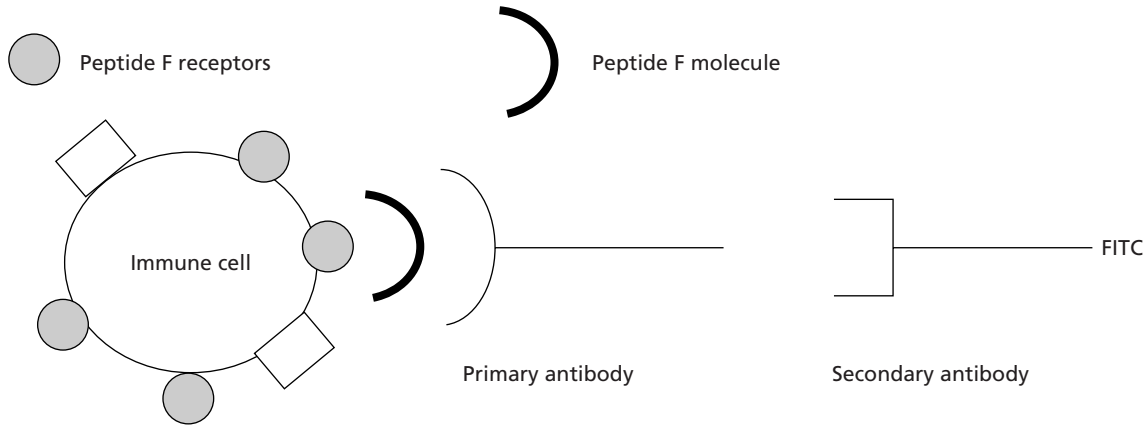

Fig. 16.6 Indirect immunophenotyping technique for detection of a peptide F receptor. Primary antibody = rabbit anti-peptide F. Secondary antibody = goat anti-rabbit and fluorescein isothiocyanate (FITC) labeled. (From Bush *et al*. under review.)

receptors. In fact, peptide F receptors have not been characterized. Therefore, an indirect approach and flow cytometry was employed (Fig. 16.6).

An antibody to the ligand (i.e. peptide F) was used to determine a receptor identified by ligand binding. A series of experiments including numerous controls to optimize the system and to verify specificity were performed. Such experiments included variation of the dose of peptide F and anti-peptide F antibody; utilization of several blocking buffers to eliminate background staining; and testing in the absence of exogenous peptide F, primary antibody, and secondary antibody. Normal rabbit serum (several different animals) was used as a negative control. Dual labeling techniques were used to try to localize the labeling to a subclass of leukocytes.

Using the indirect approach (Fig. 16.6), it was found that between 20–30% of the white blood cells stained positively for peptide F (Bush *et al*. under review). The secondary antibody showed essentially no binding in the absence of the primary antibody, suggesting that antibody binding was specific. Addition of peptide F to the cells did not greatly change the percentage of cells that bound the antibody, suggesting near endogenous saturation of receptors. However, normal rabbit serum used in place of the primary antibody also produced similar results. It was unlikely that binding to F_c-receptors occurred because cells were preincubated with goat serum, human serum and calf serum as F_c-blocking agents. We were unable to reduce the binding of normal rabbit serum to levels acceptable to determine specific binding to peptide F (Bush *et al*. under review). Further research on determining the receptor for peptide F needs to be done.

Effect of exercise on adrenal medullary proenkephalins

Peptide F has been shown to elevate during exercise (Kraemer *et al*. 1985a, 1985b, 1990a, 1991; Bush *et al*. 1998, under review; Triplett-McBride *et al*. 1998). Three of the studies by Kraemer *et al*. (1985a, 1985b, 1992) compared peptide F responses in endurance-trained and untrained men. The pattern of release of peptide F in untrained men in response to cycle ergometer exercise (8-min stages) at increasing intensities to $\dot{V}o_{2peak}$ (Kraemer *et al*. 1985a, 1985b) was marked by increases in peptide F concentrations with increasing exercise intensity, with a surge approximately 5 min after the cessation of exercise. Concentrations of peptide F were reduced, though not back to resting levels, by 15 min after exercise. With trained men, peptide F elevated at approximately 50% $\dot{V}o_{2peak}$, dropped as exercise intensity approached 100% $\dot{V}o_{2peak}$, and then surged again at 5 min after the cessation of exercise. By 15 min after the cessation of exercise peptide F concentrations

had dropped, though not to resting concentrations (Kraemer *et al*. 1985a, 1985b). The Triplett-McBride *et al*. (1998) investigation was performed in women and the results were in contrast to prior studies in men. For example, although the exercise mode was the same (cycle ergometer), only the trained group showed any peptide F response to the exercise, with a peak at the highest exercise intensity of 80%. The untrained group showed no exercise response in peptide F.

Peptide F is also not under conscious control as demonstrated by Kraemer *et al*. (1992) in an investigation utilizing hypnosis and the responses of peptide F to exercise. This study also compared trained to untrained men performing cycle ergometer exercise at 25% and 50% $\dot{V}O_{2peak}$. The hypnosis condition involved the suggestion of performing cycle ergometer exercise at 50% and 75% $\dot{V}O_{2peak}$ while subjects were actually cycling at 25% and 50% $\dot{V}O_{2peak}$. The investigators found no significant differences in peptide F concentrations between conditions (control versus hypnosis) or between rest and exercise, suggesting that the actual intensity of exercise was still too low to elicit a peptide F response, despite the suggestion of higher intensity. Other studies by Kraemer *et al*. (1987, 1988, 1990a, 1991) examined peptide F responses to exercise in healthy men, and demonstrated increases with exercise of long enough duration and high enough intensity.

Two studies (Kraemer *et al*. 1988, 1991) also utilized cycle ergometer exercise, although varying exercise protocols were employed. The 1988 study examined peptide F responses to steady state (80–85% $\dot{V}O_{2peak}$) exercise to exhaustion with and without caffeine and at varying altitudes and altitude exposures (sea level, acute altitude and chronic altitude). The investigators found that peptide F concentrations were higher at sea level (mid- and post-exercise) and lower at chronic altitude (post-exercise) after caffeine ingestion. The results also indicated that post-exercise peptide F concentrations were lower than sea level concentrations at both acute and chronic altitude after caffeine ingestion. The exercise responses of peptide F were mixed. At sea level, only the caffeine ingestion trial produced significant differences from pre-exercise concentrations for the mid- and post-exercise measurements. At acute altitude, the only significant difference from pre-exercise peptide F concentrations occurred at the mid-exercise measurement in the caffeine ingestion trial. For the chronic altitude condition, the only significant increase in peptide F concentrations occurred with no caffeine ingestion at the mid-exercise measurement. The authors attributed most of these differences to the time of altitude exposure (17 days).

A study (Kraemer *et al*. 1991) examined peptide F and catecholamine responses to exhaustive exercise on a computerized cycle ergometer at various percentages (36%, 55%, 73% and 100%) of maximal leg power. The focus of the study was to compare patterns of response of peptide F, epinephrine, lactate and norepinephrine. There was a significant increase in peptide F concentration immediately post-exercise at the 36% exercise intensity level, which was the longest in duration (3.5 min). There was also a significant increase in epinephrine immediate post-exercise at both the 36% and 55% exercise intensity levels and at 15 min following the 100% exercise intensity level. The results indicated that these peptide F and epinephrine frequently have an inverse relationship at the higher intensity levels. There were also significant increases in norepinephrine immediately after each exercise intensity, 5 min after the low to moderate exercise intensities, and 15 min at the lowest exercise intensity level, suggesting a differential exercise response pattern than the encephalin. Similar exercise response patterns were observed with whole blood lactate following all the exercise intensity levels. Although these relationships were not significant, the authors point out that these relationships do not support the theory of co-secretion of these substances from the chromaffin cells of the adrenal medulla as differential response patterns to exercise may exist.

Another study (Kraemer *et al*. 1987) examined peptide F responses to steady state (70% $\dot{V}O_{2max}$) treadmill exercise of long duration (100 min) before, during and after heat acclimation. The investigators found that higher concentrations of peptide F in the heat were due to a reduction in degradation to [Met]-enkephalin. The investigators also found no significant differences pre- to post-exercise or between test days (before, during and after acclimation), and

proposed that these findings may be a result of degradation processes in the longer duration exercise. In another investigation, Kraemer *et al*. (1990a) found that healthy men who performed treadmill exercise of increasing intensity (7-min stages) exhibited a peptide F response similar to that of the untrained men in the 1985 investigations (Kraemer *et al*. 1985a, 1985b). The main difference between these two groups of men was that the peak in peptide F concentration was at the maximal exercise intensity for the healthy men, not 5 min into recovery as was found in the 1985 investigations using untrained men. This may be explained by the difference in fitness level and in exercise mode; cycling (non-weight-bearing) versus treadmill (weight-bearing).

The modality of exercise (aerobic versus anaerobic) utilized can change the response of peptide F. Kraemer *et al*. (1992) studied the response of plasma peptide F to a high intensity cycle ergometer test in 10 healthy, active men. Four different intensities were utilized: 100% maximal leg power (equivalent to 318% $\dot{V}O_{2max}$ for 6 s), 73% maximal leg power (equivalent to 230% $\dot{V}O_{2max}$ for 15 s), 55% maximal leg power (equivalent to 175% $\dot{V}O_{2max}$ for 45 s) and 36% maximal leg power (equivalent to 115% $\dot{V}O_{2max}$ for 180 s). This study showed the differential pattern of epinephrine and peptide F in response to the exercise stress (i.e. epinephrine increased while peptide F decreased). With the longer duration of exercise, peptide F was elevated (i.e. 115% $\dot{V}O_{2max}$ for 180 s) during exercise and returned to the resting level by 5–15 min post-exercise. This study gave initial insight into the difference in peptide F concentration in response to an anaerobic-type exercise, such as high-intense short-duration cycling or resistance exercise. Such data demonstrated that the duration and/or volume of exercise may influence the concentration of peptide F in the plasma.

Resistance exercise is anaerobic in nature. Two studies have examined the responses of peptide F concentrations to resistance exercise (Bush *et al*. 1998; Fry *et al*. 1998). Fry *et al*. (1998) examined the peptide F response to an overtraining protocol. Subjects were tested following a high intensity resistance exercise training protocol (overtrained) versus a low intensity resistance exercise training protocol (control). A high intensity training protocol was performed for 2 weeks (i.e. 10 repetitions of 1-repetition maximum [1-RM] squat exercise every day for 2 weeks) and a low intensity training protocol (i.e. 50% 1-RM squats 1 day per week for 2 weeks and 1-RM testing 1 day per week for 2 weeks). Acute testing was administered at the beginning, middle and end of the 2-week training period. Subjects performed continuous repetitions at 70% 1-RM until exhaustion. No change in plasma peptide F concentration was observed in response to acute exercise stress and/or the overtraining protocol. The overtraining protocol may have produced adaptations in the adrenal medullary chromaffin system. The lack of peptide F response to this type of training stimulus may have been due to inhibitory factors negatively affecting the proenkephalin biosynthesis into the chromaffin cells (Wilson *et al*. 1982; Livett, B.G. 1984; Wilson 1991) or exocytotic suppression of existing peptide F from the chromaffin cells.

Reductions in the immune function with overtraining have been observed (Mackinnon 1992). Such types of high intense training had negative effects on health and the immune system, since hormones such as cortisol and epinephrine, exhibiting immunosuppressive effects, were elevated during this type of training (Kuipers & Keizer 1988; Fry *et al*. 1991, 1994). The lack of change in plasma peptide F concentration in response to overtraining may have negatively impacted the immune system; and under such conditions plasma peptide F would not be available to reverse any negative effects on immune function produced by either cortisol or epinephrine (Kuipers & Keizer 1988; Fry *et al*. 1991, 1994).

A second study (Bush *et al*. 1998) utilized a 16-set resistance exercise protocol. Four sets each of 10-RM and 15-RM bench press, bent over row, military press and squat exercise were performed by resistance trained men. The 10- and 15-RM protocols both produced a decrease in plasma peptide F concentration immediately following the acute resistance exercise stress. At 15 min post-exercise, an increase in plasma peptide F was observed following only the 10-RM protocol. The higher forces involved in performing the 10-RM protocol may account for the differential response in plasma peptide F. An 80%

increase in peptide F concentration was observed 4 h post-exercise following both protocols. This study suggested that circulating peptide F may play a role during recovery periods from moderate resistance exercise to potentially increase immune function during repair of disrupted muscle (Fridèn *et al*. 1983; McCully & Faulkner 1985; Round *et al*. 1987; Fielding *et al*. 1991; Nieman *et al*. 1995; McBride *et al*. 1998). The increase in peptide F 4 h after resistance exercise was not observed in the control group. This finding was quite surprising. It may be speculated that there was an increase in production and/or secretion of peptide F from the adrenal medulla, or that peptide F bound to receptors on target tissues were released.

Studies during heat acclimation in adults have shown a reduction in plasma [Met]-enkephalin (Kraemer *et al*. 1987), potentially due to the increased degradation of the peptide in the circulatory system of a decrease in the processing from the large precursor unit. Circulating encephalin and opiates released during stress (Viveros & Wilson 1983) and exercise (Howlett *et al*. 1984; Farrell *et al*. 1987) could mediate the effects of stress reduction. There appears to be a significant difference in the enzyme concentrations responsible for hydrolyzing enkephalins such that the enkephalin concentrations were higher in a trained individual (Jaskowski *et al*. 1989).

Exercise studies performed to exhaustion indicate an increase in plasma levels of [Met]-enkephalin and that this is observed in an exercise-intensity dependent manner (Sommers *et al*. 1990). It is known that [Met]-enkephalin is co-released with epinephrine from the adrenal gland (Viveros *et al*. 1979; Wilson *et al*. 1982; Wilson 1991). In an exercise study of moderate (70% $\dot{V}o_{2max}$) to exhaustive exercise (120% $\dot{V}o_{2max}$) focusing on the co-release patterns of epinephrine and [Met]-enkephalin, peak plasma epinephrine levels were observed 1 min post-exercise, whereas peak plasma [Met]-enkephalin levels were observed during the moderate exercise intensity, declined during the high exercise intensity and returned to baseline within 1 min post-exercise (Boone *et al*. 1992). Similar to the peptide F fragment, a differential exercise response pattern exists for epinephrine and [Met]-enkephalin.

Overtraining is defined as an increase in training volume and/or intensity resulting in a decrease in physical performance (Fry *et al*. 1991). An overtraining study done by Fry *et al*. (1998) examined resistance-trained adult men in which they performed 100% 1-RM barbell squats for a 2-week period, resulting in a decrease in their 1-RM strength in the overtrained state. Plasma testosterone and cortisol were decreased while in the overtrained state. Growth hormone, however, was not influenced by the high intensity resistance overtraining protocol. Epinephrine is elevated in response to high intensity resistance overtraining protocols (Fry *et al*. 1994) with no effect of overtraining response to circulating levels of peptide F (Fry *et al*. 1998). This indicates the high intensity resistance overtraining protocol appeared to overwhelm the capacity of the adrenal medullary chromaffin cells to secrete peptide F.

Effect of exercise on peptide F in other blood biocompartments

It has been reported that upon activation of lymphocytes, a secretagogue was the proenkephalin precursor (Roth *et al*. 1989). Lymphocytes differ in their origination and maturation. The lymphocytes are a subclass of leukocytes, consisting of T- and B-cells, ranging in size from 6–10 μm and comprising 20–25% of circulating leukocytes. Their functions are crucial during an immune response and include recognition of antigens, production of antibodies against invading antigens, production of lymphokines, action of cytotoxicity and memorization of previous antigen encounters. T-lymphocytes are generated within the bone marrow and mature in the thymus, while B-lymphocytes are generated and mature within the bone marrow and fetal liver. Proliferation of mature lymphocytes occurs while they are surveying the system in the secondary lymphoid organs.

Approximately 1–2% of total lymphocytes recirculate hourly, providing frequent communication with any foreign cells present in the system. During this time of circulation, proenkephalin fragments (e.g. peptide F) may interact with these immune cells. T-lymphocytes are categorized by their functional ability: T-helper and T-cytotoxic cells. T- and

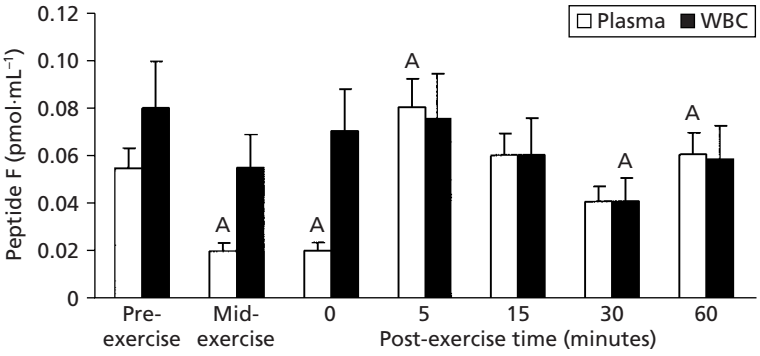

Fig. 16.7 Peptide F concentrations in plasma and white blood cell biocompartments at rest, mid-exercise and for 60 min into recovery following 30 min of cycling at 80% $\dot{V}o_{2max}$. A = $p \leq 0.05$ difference from corresponding pre-exercise, mean ± SE. WBC, white blood cell compartment.

B-lymphocytes are further differentiated by their function during humoral-mediated or cellular-mediated immunity. Cell-mediated immunity deals with cell-to-cell interactions and involves primarily the attacking of foreign substances or antigens. Humoral-mediated immunity functions to supply the system with antibodies (i.e. immunoglobulins) which have the ability to recognize the antigens (Sigal & Ron 1994). It is important to understand the difference between the two immune responses as Hiddinga *et al*. (1994) reported. Specifically, a significant cell-mediated response was produced between proenkephalin peptide fragments and T-cells, which then caused a humoral-mediated response (e.g. increased number of antibody-forming B-cells).

Based on the known interaction between peptide F and immune cells (Hiddinga *et al*. 1994), a study was designed to examine the presence of peptide F within the white blood cell biocompartment of blood. A progressive exercise stress protocol had been shown to cause an increase in plasma peptide F concentration (Kraemer *et al*. 1985b, 1991). In a progressive endurance exercise protocol, eight healthy men (21.0 ± 1.0 years) performed a 30-min cycle exercise at 80% $\dot{V}o_{2max}$ and returned for a second session under quiet control conditions. Blood samples were taken pre-exercise, mid-exercise, immediately post-exercise, 5 min, 15 min, 30 min and 60 min post-exercise. Similar to previous research, there was an exercise-induced response of peptide F in the plasma biocompartment at 5 min post-exercise (Kraemer *et al*. 1985a, 1985b). Unique to this pilot study was the presence of peptide F observed within the white blood cell biocompartment both during rest and following exercise. Subsequently, there was a decrease ($p \leq 0.05$) in white blood cell biocompartment peptide F concentration at 30 min post-exercise (Fig. 16.7). It might be speculated that during early recovery periods from exercise (i.e. 5–15 min post-exercise), peptide F was bound to immune cell receptors and then internalized, and thus a response of peptide F to exercise may not have been readily apparent until 30 min post-exercise. The presence of peptide F 30 min post-exercise in the white blood cell biocompartment was observed and may be attributed to a saturation of peptide F immune receptors.

The peptide F concentration observed in the plasma biocompartment following exercise was not similar to the concentration of peptide F observed in the white blood cell biocompartment. It may be that there was a shift of peptide F concentration from the white blood cell biocompartment to the plasma biocompartment, or that immune cell receptors for peptide F were fully saturated following exercise (i.e. immediate, 5 min and 15 min post-exercise). These results indicated that a possible interaction between peptide F and cells of the immune system existed, and that exercise modulated the concentration of peptide F within this biocompartment. This initial pilot data was studied within the context of utilizing a progressive endurance exercise model. Examining the effect of acute resistance exercise on the peptide F concentration has been a topic of interest. Such data in this pilot study

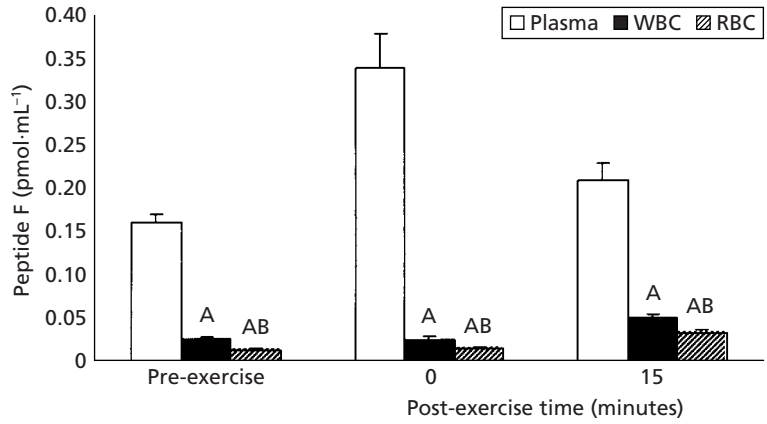

Fig. 16.8 Comparison of peptide F concentration in three biocompartments following resistance exercise. A = $p \leq 0.05$ difference from corresponding plasma biocompartment; B = $p \leq 0.05$ difference from corresponding white blood cell biocompartment, mean ± SE. RBC, red blood cell biocompartment; WBC, white blood cell biocompartment.

supported the examination of peptide F within the white blood cell biocompartment, and the response of peptide F in this biocompartment to resistance exercise to secrete cytokines which produce not only paracrine effects but also autocrine effects on the same activated immune cells to up-regulate receptor expression for further stimulation (Sigal & Ron 1994). Although the receptor for peptide F has not been characterized, it may potentially bind to cells within the blood biocompartments (i.e. immune cells). Erythrocytes possess peptide receptors for other hormones, such as insulin and insulin-like growth factor I (Horuk *et al*. 1993; Hagino *et al*. 1994). There is also the possibility of peptide F in the red blood cell biocompartment interacting with similar peptide receptors or a yet to be determined opioid.

In another study (Bush *et al*. under review) men aged 22 ± 0.9 years performed an acute resistance exercise protocol where they completed six sets of 10-RM squats on the Smith-squat machine. Blood samples for analysis of peptide F concentrations were obtained at baseline, immediate post- and 15 min post-exercise. There was a hierarchy of peptide F concentrations where the plasma biocompartment showed the highest ($p < 0.05$) concentration of peptide F versus the white blood cell and red blood cell biocompartments at baseline and during recovery (Fig. 16.8). Furthermore, the concentration in the white blood cell biocompartment was higher than that of the red blood cell biocompartment (Fig. 16.8). This hierarchy of concentration was consistent both under resting conditions and in response to the resistance exercise.

The increase in peptide F concentration in the white blood cell biocompartment occurred with a significant increase in the total number of white blood cells measured in that biocompartment. During the recovery period, the number of peptide F molecules per white blood cell was significantly increased by nearly 50%, indicating the potential significance of the interaction of peptide F within the white blood cell biocompartment. The interaction with the white blood cell biocompartment can be supported by the fact that peptide F may be binding to immune cell surface recpetors (Sibinga & Goldstein 1988), subsequently becoming internalized, and resulting in less measurable peptide F in either the plasma or white blood cell biocompartment. The increase in the number of peptide F molecules associated with immune cells during recovery can be potentially explained by: (i) a down-regulation of immune cell surface receptors; or (ii) an increase in production and secretion of peotide F from the adnreal medulla or immune cells, as observed by an increase in peptide F concentrations with the white blood cell biocompartment. The measurable amount of peptide F in the red blood cell biocompartment may be reflective of a shift in the peptide F among the three different blood biocompartments. However, the physiological significance of the observance of peptide F in the white and red blood cell biocompartments remains to be elucidated.

Summary

Neural stimulation is the primary mechanism involved with the activation and release of compounds in the adrenal medulla. Peptide F is an ECP secreted from the chromaffin cells of the adrenal medulla. Peptide F has been shown to be secreted along with epinephrine during exercise (Kraemer *et al*. 1985a, 1985b, 1987, 1988, 1990a, 1991, 1992; Bush *et al*. 1998, under review; Triplett-McBride 1998). However, the pattern of release of these two substances during and after exercise may differ (Kraemer *et al*. 1985b, 1990a, 1991). Interestingly, the pattern of release of peptide F during and after an acute bout of exercise also differs between highly trained male endurance athletes and untrained men (Kraemer *et al*. 1985a, 1985b). Many of the biological functions of peptide F are unknown, but it has been demonstrated that peptide F may improve the activation and function of the T-cells of the immune system (Hiddinga *et al*. 1994). Peptide F has been shown to enhance helper T-cell activation *in vitro* and subsequently cause increased production of antibody by B-cells (Hiddinga *et al*. 1994). However, this relationship has not yet been studied *in vivo*. The complexity and degree of interactions between the endocrine and immune systems are just beginning to be understood. Some investigators have examined the possibility of bi-directional regulation between these systems (Stein *et al*. 1985; Bateman *et al*. 1989). It has been shown that T-cells have receptors for enkephalins (Wybran *et al*. 1979) and appear to have receptors specific for peptide F (Hiddinga *et al*. 1994). It has also been demonstrated that T-cells can synthesize and release the small ECPs for autoregulation of function (Blalock 1992, 1994). Therefore, it is possible that one of the main roles of peptide F is to enhance immune cell function to counteract and balance the suppressive effects of other endocrine hormones such as cortisol and epinephrine. While the modulation of endogenous peptide F may promote movement to various target tissues, it appears that the mechanisms are related to adrenal medullary function, primarily regulation of peptide F concentrations related to the response of stress (i.e. exercise).

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Chapter 17

Exercise and the Hypothalamic–Pituitary–Adrenal Axis

WARRICK J. INDER AND GARY A. WITTERT

The hypothalamic–pituitary–adrenal (HPA) axis constitutes a hormonal network which is activated during stress. 'Stress' may take many forms, including acute episodes such as life-threatening illness, surgery or hemorrhage, or more chronic stressors such as depression or eating disorders. All of these forms of stress have been extensively studied, and the extent of the activation of the HPA axis quantified. Exercise, whether it is an acute episode of strenuous exercise or chronic endurance training is also known to activate the HPA axis. This chapter will briefly outline the physiological regulation of the HPA axis, followed by a comprehensive review of the specific effects of exercise and exercise training on the axis in human subjects.

Overview of the regulation of cortisol production by the adrenal cortex

The target end-organ of the HPA axis, the adrenal cortex, secretes cortisol during basal conditions in a circadian pattern, with higher levels in the early morning hours which fall over the course of the day, reaching low levels by midnight (Krieger *et al*. 1971). Cortisol has a number of physiological roles. These include sodium and water balance and blood pressure control, maintenance of glucose homeostasis, adipogenesis, inhibition of osteoblast function and anti-inflammatory actions including suppression of the immune response (Stewart 2003). Cortisol is under the influence of adrenocorticotropic hormone (ACTH) secreted by the anterior pituitary (Stewart 2003). ACTH binds to corticotropin receptors in the adrenal cortex, which leads to cortisol production and release (Catalano *et al*. 1986). Cortisol inhibits its own secretagogues, with a negative feedback loop operating to inhibit ACTH from the pituitary as well as acting at the hypothalamic level on both corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (Keller-Wood & Dallman 1984; Stewart 2003). This feedback tends to avoid prolonged and inappropriate periods of hypercortisolism. CRH and AVP, present in the parvicellular region of the paraventricular nucleus in the hypothalamus (Pelletier *et al*. 1983), are the primary regulators of ACTH secretion (Orth 1992; Kjær, A. 1993). CRH is a 41-amino-acid peptide first isolated in the sheep in 1981 (Vale *et al*. 1981). It is a potent stimulus of both ACTH production and secretion (Orth 1992). CRH binds to specific high affinity CRH receptors on the corticotrope (Chen *et al*. 1993). Intracellular signaling is via the protein kinase A/cyclic AMP second messenger system (Aguilera *et al*. 1983). Increased secretion of CRH is thought to play a major role to increase ACTH and cortisol levels during many forms of acute stress (Chrousos 1992; Orth 1992). AVP, a 9-amino-acid peptide, binds to a specific receptors on corticotropes known as V_3-(sometimes designated V_{1b}) receptors (Sugimoto *et al*. 1994). In turn, the protein kinase C second messenger system is activated, leading to ACTH release (Liu *et al*. 1990). CRH and AVP are co-localized in the median eminence of the hypothalamus (Whitnall *et al*. 1987). AVP acts synergistically with CRH to further amplify ACTH secretion during stress (Gillies *et al*. 1982, Rivier & Vale 1983). AVP is also a critical regulator of sodium and water balance and a potent vasoconstrictor (Jard 1988).

The presence of additional stimulatory and inhibitory factors which may affect ACTH secretion has been proposed (Grossman & Tsagarakis 1989; Alexander *et al*. 1996). A number of other hormones, cytokines and neurotransmitters interact with the HPA axis mainly through effects on CRH and less commonly AVP. There is evidence that leukemia inhibitory factor (LIF) is a stimulus to pituitary ACTH secretion (Auernhammer & Melmed 2000), but the physiological role of other factors interacting directly at the level of the pituitary or adrenal glands remains unclear. The regulation of the HPA axis is summarized in Fig. 17.1.

The effect of acute exercise stress on the HPA axis and factors modulating the response

Mechanism of the cortisol response to exercise

Like other acute stressors, acute intense exercise is a potent activator of the HPA axis (Luger *et al*. 1987). The increase in plasma cortisol occurs in spite of an increase in clearance from the circulation (Few 1974). Even anticipation of competition may result in a cortisol increase (Suay *et al*. 1999) and psychological stress occurring prior to exercise increases the cortisol response (Kaciuba-Uscilko *et al*. 1994).

Studies in humans and large mammals have indicated an important role for both CRH and AVP in the exercise-induced stimulation of ACTH secretion which then causes the cortisol rise at the adrenal level. Noting the limitations of peripheral CRH measurement, some (Elias *et al*. 1991; Harte *et al*. 1995; Inder *et al*. 1998a) but not all (Luger *et al*. 1987; Wittert *et al*. 1991) studies have found an increase in plasma CRH after exercise (Fig. 17.2). Duration of the exercise may be important in explaining the differences, along with the sensitivity of the CRH assay. When exercise is performed under conditions of a continuous CRH infusion, at a level where the corticotrope CRH receptors would be fully saturated, a robust rise in ACTH and cortisol above the elevated baseline is noted (Smoak *et al*. 1991). This implies a factor additional to CRH being crucial in the ACTH response. In the pituitary venous effluent of the exercising horse, plasma AVP levels are markedly increased (Alexander *et al*. 1991). In humans, both acute high intensity exercise (Wittert *et al*. 1991) and prolonged submaximal exercise (Inder *et al*. 1998a) are associated with increases in plasma AVP that parallel ACTH and cortisol levels. The extent of the rise in AVP also appears to determine the degree to which the exercise-induced activation of the HPA axis is inhibited by exogenous glucocorticoids (Petrides *et al*. 1994). In a group of exercising men pretreated with 4 mg dexamethasone, four out of 11 were noted to exhibit a significant increment in ACTH and cortisol following exercise. In this group, the plasma AVP response was six times that of the group where dexamethasone abolished the ACTH/cortisol rise (Petrides *et al*. 1997). Subsequent studies comparing the group without dexamethasone suppressibility (designated high responders) have also shown a greater integrated cortisol response to psychological stress (Singh *et al*. 1999). This may identify a group of individuals who have a greater HPA axis response to different stressors mediated via AVP. Changes in plasma AVP are also correlated with alterations in plasma osmolality, but the increase observed during intense exercise is greater than expected due to osmolality changes alone (Wade & Claybaugh 1980). In particular, the increase in plasma AVP correlates with changes in plasma osmolality during prolonged submaximal exercise, but this relationship is lost during subsequent incremental exercise to exhaustion (Inder *et al*. 1998a). The reduction in plasma volume which occurs during exercise may also contribute to the AVP response (Robertson & Athar 1976).

β-Endorphin is an opioid peptide derived from proopiomelanocortin (POMC) (Morley 1981). Previously it was thought to be secreted in equimolar amounts to ACTH, and a number of studies have examined the plasma β-endorphin response to exercise (Carr *et al*. 1981; Rahkila *et al*. 1987; Petraglia *et al*. 1988; Schwarz & Kindermann 1989). A major problem with much of this research is the finding of 100% cross-reactivity with β-lipotropin and acetylated forms of β-endorphin in most radioimmunoassays. Therefore, much of the β-endorphin-like immunoreactivity may not be opioid active. Using more specific immunoradiometric assays, it is now clear that in the basal state, β-endorphin is

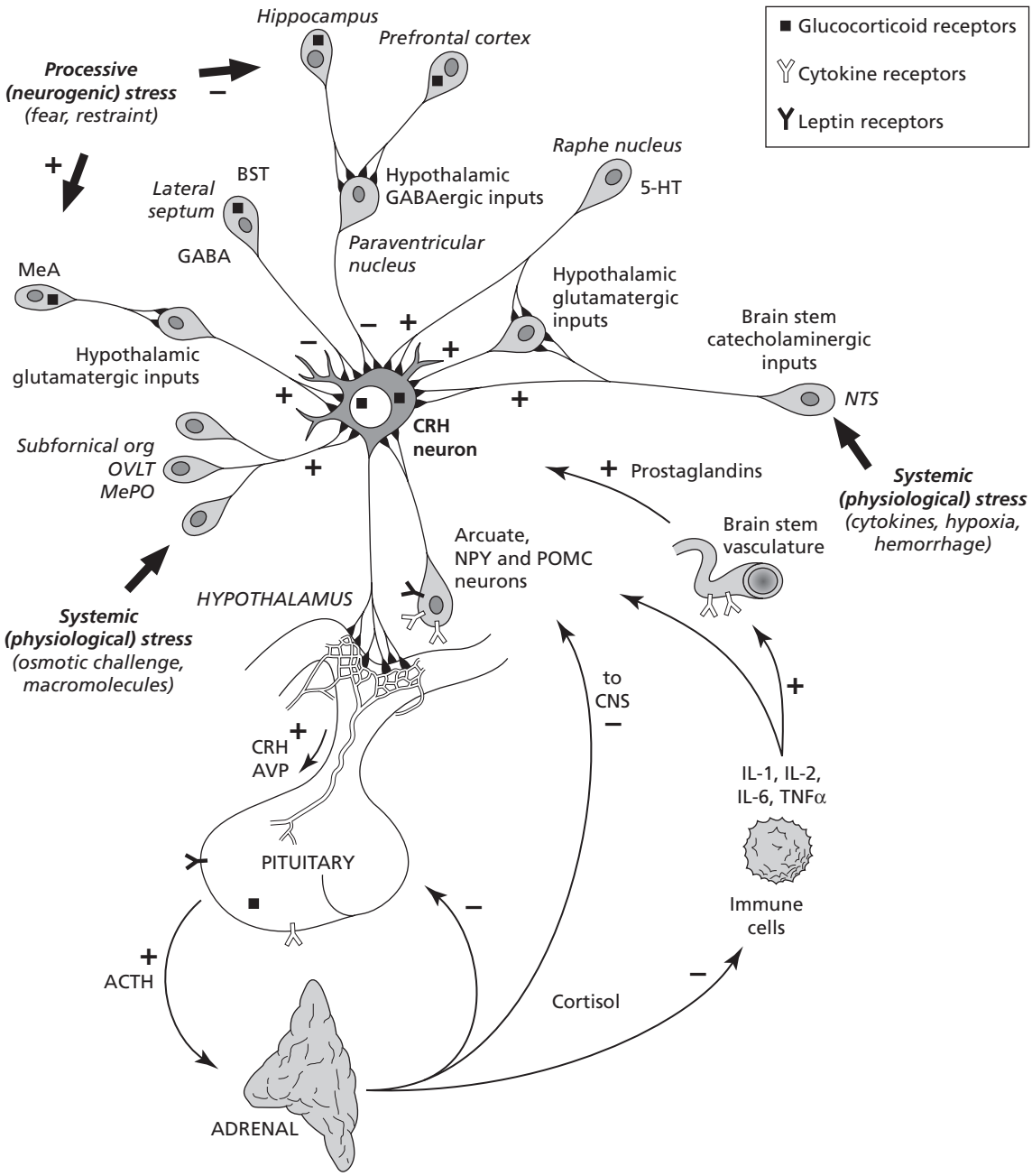

Fig. 17.1 Regulation of the hypothalamic–pituitary–adrenal (HPA) axis. Neuronal and neurotransmitter input to hypothalamic corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) -containing neurons result in release of these hormones into the hypothalamic–hypophysial portal system. This causes an increase in secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn stimulates cortisol release from the adrenal cortex. Cortisol acts at both pituitary and hypothalamic level via negative feedback to inhibit ACTH, CRH and AVP.

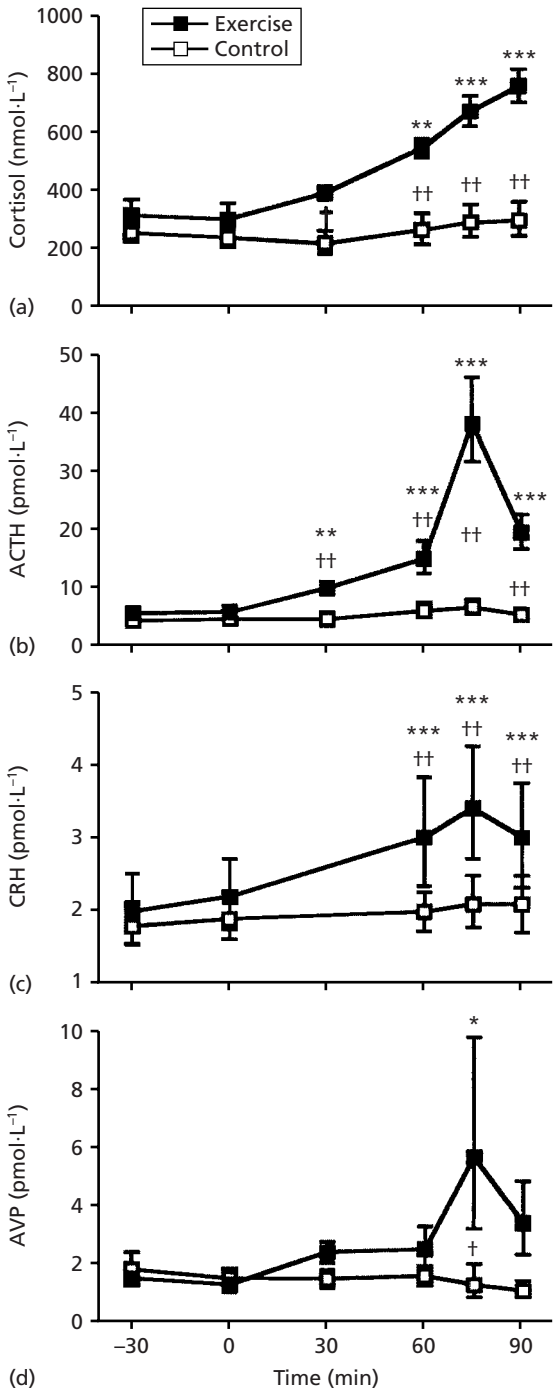

Fig. 17.2 (*left*) Changes in plasma levels (geometric mean ± SEM) of (a) cortisol, (b) adrenocorticotropic hormone (ACTH), (c) corticotropin-releasing hormone (CRH) and (d) arginine vasopressin (AVP) in six male athletes undergoing a cycle ergometer-exercise protocol aiming to maintain each athlete at 70% $\dot{V}O_{2max}$ for 1 h between 0 and 60 min, followed by an incremental increase in absolute intensity of 25 W every 2 min until exhaustion. Significant difference from baseline (–30 min) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significant difference between exercise and control time-matched data $^{\dagger}P < 0.01$, $^{\dagger\dagger}P < 0.001$.

undetectable in most normal subjects (Gibson *et al*. 1993). True β-endorphin$_{1–31}$ is released during exercise into peripheral blood in only about 50% of subjects, and represents only a small proportion of β-endorphin immunoreactivity (Harbach *et al*. 2000). It appears, however, that exercise does increase central levels of endogenous opioid peptides (Thoren *et al*. 1990). An infusion of naloxone, an opioid receptor antagonist, increases the perceived effort of exercise (Grossman *et al*. 1984; Sgherza *et al*. 2002). Highly trained athletes have evidence for increased central opioid tone, probably induced by training (Inder *et al*. 1995). Basal plasma levels of β-endorphin do correlate with the subsequent ACTH response to naloxone, a marker of central opioid tone (Inder *et al*. 1998b). Activation of endogenous opioid peptides has been implicated in the positive mood effects of exercise. They have also been hypothesized in a causative role in exercise induced hypothalamic amenorrhea (Laatikainen 1991).

Effect of the intensity and duration of exercise

Short duration exercise to > 60% $\dot{V}O_{2max}$ results in ACTH and cortisol release proportional to the intensity of the exercise (Davies & Few 1973; Howlett 1987; Luger *et al*. 1987; Kjær, M. *et al*. 1988; Deuster *et al*. 1989; Wittert *et al*. 1991). Even high intensity exercise for as short as 1 min activates ACTH and cortisol secretion (Buono *et al*. 1986). Short-term submaximal exercise does not result in activation of the HPA axis even in conditions of extreme heat (Kenefick *et al*. 1998). Exercising at 50% $\dot{V}O_{2max}$ for 20 min does not cause an increase in cortisol levels, while at 70% $\dot{V}O_{2max}$, ACTH and cortisol increase (Luger *et al*. 1987). When subjects have been

subjected to a progressive increase in exercise intensity in 10-min blocks, commencing at 40% $\dot{V}O_{2max}$, increased ACTH was only seen after 80% $\dot{V}O_{2max}$ was reached (de Vries *et al*. 2000). Following 1 h of cycle ergometer exercise at 70% $\dot{V}O_{2max}$, cortisol levels increased from baseline; yet a further significant increase in AVP, CRH, ACTH and cortisol was observed when the intensity was then progressively increased over a 10-min period until exhaustion (Inder *et al*. 1998a). Using salivary as opposed to plasma cortisol, a cortisol rise following 1 h of exercise was observed only at 76% $\dot{V}O_{2peak}$, but not at 45% or 62%, while 40 min of exercise failed to increase salivary cortisol at any of the three intensities (Jacks *et al*. 2002).

These observations are best explained by the exercise being performed at or exceeding the anaerobic threshold. Previous studies where the participants have exercised at the individualized anaerobic threshold have demonstrated that exercise below this point does not result in activation of the HPA axis (Kindermann *et al*. 1982; Gabriel *et al*. 1992). During incremental exercise to exhaustion, plasma ACTH and β-endorphin rise only once the individual anaerobic threshold is reached (Schwarz & Kindermann 1990).

Although some studies have failed to demonstrate a cortisol rise in response to prolonged low intensity exercise (Hoffman *et al*. 1994), ultra-long distance running is associated with similar cortisol rises to repeated bursts of shorter, higher intensity exercise (Nagel *et al*. 1992). Cortisol levels at the completion of a 100-km ultramarathon were significantly elevated over baseline (Pestell *et al*. 1989). A 75-km cross-country ski race significantly elevated plasma cortisol levels (Vasankari *et al*. 1993). It has been suggested that the activation of the HPA axis seen during prolonged low intensity exercise is dependent on the development of hypoglycemia (Tabata *et al*. 1991). In six subjects who exercised for 14 h at 50% $\dot{V}O_{2max}$, the cortisol, ACTH and CRH responses were totally abolished when plasma glucose concentrations were maintained at pre-exercise levels. The authors suggested a threshold plasma glucose concentration of $< 3.3\ mmol \cdot L^{-1}$ (Tabata *et al*. 1991). In an earlier study from the same group, cycling at 50% $\dot{V}O_{2max}$ for up to 3 h or exhaustion elicited an increase in ACTH and cortisol only in the latter part of the exercise when blood glucose decreased (Tabata *et al*. 1990).

Timing of exercise

The HPA axis response to some stimuli may be influenced by the ambient basal cortisol level. For example, the cortisol increment may be less in the morning when basal levels are higher (DeCherney *et al*. 1985). This is thought to be due negative feedback inhibition. Kanaley *et al*. (2001) demonstrated that although basal and peak cortisol levels in response to exercise were highest at 0700 h, the incremental response was greater compared to control day when the exercise occurred at 2400 h. In contrast, when comparing area under the curve and the circadian baseline, no difference was found in women exercising at different times of the day (Thuma *et al*. 1995). When bouts of equivalent exercise are repeated later in the day, the ACTH and cortisol response to the second bout are greater than the first, or a single bout performed on another day (Ronsen *et al*. 2001a).

Type of exercise

Squats and intermittent higher intensity cycling result in a cortisol response in contrast to cycling beneath the anaerobic threshold which does not (Vanhelder *et al*. 1985). Estimation of the cortisol response to rowing has lead to conflicting results. Although a significant increases in plasma cortisol in response to maximal (7 min) and submaximal (40 min) rowing on rowing apparatus and 8×2000 m on the water (Snegovskaya & Viru 1993) has been shown to occur in one study, a subsequent study was unable to demonstrate an increase in plasma cortisol in response to maximal rowing to exhaustion on a rowing ergometer (Jurimae & Jurimae 2001). Similar results were found when the rowing was performed at a lower intensity for 2 h, with no change in plasma cortisol seen (Jurimae *et al*. 2001). Cortisol levels were noted to increase following kayak races of 19 and 42 km, with the extent of the rise being more pronounced in the longer race (Lutoslawska *et al*. 1991). Swimming for 30 min

caused an increase in plasma cortisol at higher ambient water temperatures but not at 20°C (Deligiannis *et al*. 1993). Activation of the HPA axis can be induced by resistance exercise as well as through endurance exercise. During a high (100% of subject's maximum) and moderate (70% of subject's maximum) intensity strength workout, plasma cortisol increased more during the high intensity protocol (Raastad *et al*. 2000). Three sets versus one set of resistance exercises resulted in a greater cortisol increase (Gotshalk *et al*. 1997).

Age

In middle-aged men, resistance exercise consisting of sets of bench press, sit-ups and leg press, increased plasma cortisol concentrations (Häkkinen & Pakarinen 1995). This effect was not seen however in women or elderly men. When comparing young, middle-aged and elderly men, Silverman and Mazzeo (1996) found lower basal cortisol in the elderly sedentary subjects, but elevated basal levels in the trained subjects of all ages. Peak cortisol response to maximal exercise showed an age-related decline, independent of training status. There was no difference between men of different ages in the cortisol response to a 45-min submaximal exercise (Silverman & Mazzeo 1996).

Gender

Using 90 min of cycling at 80% of the anaerobic threshold (approximately 50% $\dot{V}O_{2max}$), there was no difference in cortisol response between men and women, matched for body mass index (BMI) and physical fitness (Davis *et al*. 2000). Similar results have been obtained when 30 min of treadmill running was employed (Kraemer, R.R. *et al*. 1989). Rahkila *et al*. (1987) found no difference between men and women in cortisol and ACTH response either during a graded treadmill exercise to exhaustion or anaerobic treadmill exercise. Therefore, both sexes appear to have a similar response to aerobic endurance exercise. Following dexamethasone, however, the plasma cortisol and AVP response to high intensity (90–100% $\dot{V}O_{2max}$) was greater in women than men, possibly indicating a greater AVP response in women or a reduced sensitivity to glucocorticoid negative feedback (Deuster *et al*. 1998). African-American women may have an increased ACTH response to exercise, but cortisol responses are similar to Caucasian women (Yanovski *et al*. 2000).

Altitude

When comparative exercise has been done at low and moderate altitude, cortisol levels were demonstrated to increase during exercise under both conditions; however, ACTH did not show an increase above baseline at moderate altitude (el-Migdadi *et al*. 1996). Comparison of athletes undergoing interval training at sea level and 1800 m did not reveal any statistically significant difference in cortisol response, although the sympathetic nervous system response was greater at higher altitude (Niess *et al*. 2003). Marathon runners competing at high altitude develop higher basal cortisol levels on acclimatization which increase further on completion of the race (Marinelli *et al*. 1994). Similar data were observed in a group of healthy volunteers undertaking a rigorous trekking expedition in the Himalayas, where cortisol levels increased after 2 weeks (Martignoni *et al*. 1997). While circadian rhythms were maintained, 30% of the subjects demonstrated failure of dexamethasone suppression (Martignoni *et al*. 1997). Overall, it appears that acclimatization to altitude results in an increase in basal cortisol levels compared to sea level, while the cortisol response to exercise is preserved.

Nutrition

Several studies have examined the effects of dietary changes and supplement use, before, during and after exercise, on the plasma cortisol response. Ingestion of carbohydrates during prolonged (2.5 h) cycling or running at approximately 70% $\dot{V}O_{2max}$ resulted in a lower cortisol response to the exercise and a lower rating of perceived exertion (Utter *et al*. 1999). Similar results were obtained by Deuster *et al*. (1992) who observed that ingestion of a 7% glucose polymer/fructose/electrolyte solution at a rate of

200 mL every 30 min abolished the exercise-induced cortisol rise during a 2-h run at 60–65% $\dot{V}O_{2max}$ compared to exercise ingesting an equal volume of water. Murray *et al*. (1991) using a carbohydrate solution compared to a water placebo, also noted an improvement in a 4.8-km performance test after 2 h of prolonged cycling exercise at 65–75% $\dot{V}O_{2max}$, in addition to a reduction in the ACTH and cortisol response.

Three days on a ketogenic diet of equal energy content resulted in higher cortisol levels both before and after exercise in comparison to a control mixed diet (Langfort *et al*. 1996). Glycerol, which has been suggested as an aid to maintain hydration during exercise, did not result in any difference in plasma cortisol response to 1 h of cycle ergometer exercise at 70% $\dot{V}O_{2max}$ followed by an incremental increase in intensity to exhaustion (Inder *et al*. 1998c).

Creatine is a popular nutritional supplement among athletes. Short-term creatine supplementation for 5 days did not alter the cortisol response to a 1 h session of heavy resistance training, although levels tended to be higher during recovery following the creatine (Op't Eijnde & Hespel 2001). Post-exercise feeding with whole food, supplemental drink, carbohydrate beverage or placebo made no difference to cortisol levels measured over 24 h following exercise (Bloomer *et al*. 2000).

The effect of exercise training on the HPA axis

The objective of training is to optimize human performance; training produces a number of neuroendocrine adaptations, which result in alterations of the activity of the HPA axis. This response is determined by the training volume, intensity, type of exercise, optimum periods of rest (regeneration) and poorly defined psychological factors.

Effect of training on basal activity of the HPA axis

The nature of the effect of training on basal activity of the HPA axis remains unresolved. Normalization of plasma cortisol levels after prolonged endurance exercise may take up to 18–24 h (Lutoslawska *et al*. 1991; Heitkamp *et al*. 1996). The recovery from intense, prolonged exercise in trained athletes is associated with greater plasma ACTH levels, but similar plasma cortisol levels as compared to control subjects (Duclos *et al*. 1997). Intensive training for an ultramarathon (Tharp & Buuck 1974; Pestell *et al*. 1989; Wittert *et al*. 1996), or experimentally in recreational athletes (Lehmann *et al*. 1993), has been shown to increase pituitary ACTH secretion without affecting plasma cortisol levels. A number of other studies are consistent with this. In male and female joggers, a season of training was without affect on basal plasma cortisol levels (Ronkainen *et al*. 1986). In middle- and long-distance runners, neither an increase in training intensity nor 12 weeks of intense training in competitive swimmers (Mujika *et al*. 1996) affected basal plasma cortisol levels (Lehmann *et al*. 1992; Flynn 1997). While this suggests reduced adrenal responsiveness to ACTH, Duclos *et al*. (1998) showed that that responsiveness to ACTH did not appear to be decreased after endurance training (Duclos *et al*. 1998) but the sensitivity of the HPA axis to glucocorticoid feedback, at least at the level of the pituitary, was decreased (Duclos *et al*. 2001). In trained men, there is a decreased sensitivity of monocytes to cortisol 24 h after the last bout of exercise. This may be related to a process of desensitization that may act to protect the body from prolonged, exercise-induced cortisol secretion (Duclos *et al*. 2003).

In contrast to these studies, it has been shown that intensive training on a treadmill increased resting plasma cortisol levels without affecting basal ACTH (Kraemer, W.J. *et al*. 1989). In competitive swimmers, a short-term increase in training distance may increase basal plasma cortisol levels (without necessarily affecting performance) (Kirwan *et al*. 1988), and trained cyclists have been reported to have higher basal cortisol levels than untrained controls (Silverman & Mazzeo 1996).

Age, gender, nutrition, mood, type and duration of activity and training status may all contribute to the effects of physical training on the HPA axis. There is no difference in the response of male and female athletes to a sudden increase in training (O'Connor *et al*. 1991). In prepubescent gymnasts, 5

consecutive days of training (3 h each day at moderate intensity), had no significant effect on basal plasma cortisol levels (Rich *et al*. 1992). In contrast, in a similar group of gymnasts 7–16 weeks of training resulted in increased basal cortisol levels, but energy intake was 31% below recommended levels (Filaire *et al*. 2003). Accordingly, the increased cortisol levels were likely the consequence of an energy deficit rather than training per se. In well-nourished young male gymnasts training had no significant effect in basal cortisol levels (Daly *et al*. 1998).

The response of plasma cortisol may be determined by both the duration and the nature of the training program, because sprint interval (associated with a substantial anaerobic component to training) but not endurance training has been observed to increase basal plasma cortisol (Kraemer, W.J. *et al*. 1989; Wittert 1996). An increase in training volume as opposed to intensity has been observed to induce a decrease in both resting and exercise-induced cortisol levels (Lehmann *et al*. 1992), which is characteristic of an overtraining syndrome. In contrast a twofold increase in training volume has been shown not to affect plasma cortisol levels, and there was no difference in the endocrine responses to an increase in training volume with cross-training as compared to mode-specific training (Flynn *et al*. 1997). The hormonal response to 5 weeks of aerobic training is similar regardless of whether training was carried out under conditions to simulate increased altitude (2500 m) or at sea level (Engred *et al*. 1994). Similarly, season appears to have no effect on the response to exercise training (Ronsen *et al*. 2001b). In elderly men there is substantial individual variability in the effect of endurance training on the HPA axis, although in general the changes appear to be similar to those that occur in younger men (Struder *et al*. 1999).

The effect of resistance exercise training on basal plasma cortisol levels is variable, and either no change (Häkkinen *et al*. 1988a, 1988b; Fry *et al*. 1994) or a decrease has been reported (Alen *et al*. 1988); the difference probably related to lower training volumes. Increasing resistance, training volume, or intensity may result in an increase in resting cortisol levels (Fry & Kraemer 1997). A doubling of training volume has also been observed to result in a decrease in intensity-dependent, exercise induced, increases in cortisol levels (Fry & Kraemer 1997). Although 2 years of intensive weight training was without affect on basal cortisol levels in adolescent males (Fry *et al*. 1994), 1 week of an overreaching stimulus induced an increase in early morning cortisol levels in this group of subjects (Fry *et al*. 1994). In young men, high-intensity weight training sufficient to induce an overtrained state resulted in a slightly increased exercise-induced testosterone/cortisol ratio but decreased exercise-induced cortisol. This hormonal profile is distinctly different from what has been previously reported for other types of overtraining, indicating that high relative intensity resistance exercise overtraining may not be successfully monitored via circulating testosterone and cortisol (Fry *et al*. 1998).

Effect of training on the response to subsequent acute exercise

The cortisol response to exercise of the same relative intensity (Rolandi *et al*. 1985; Deuster *et al*. 1989) is not affected by training, although the response to the same absolute intensity may decrease (Buono *et al*. 1987; Deuster *et al*. 1989; Botticelli *et al*. 1992; Hickson *et al*. 1994). In other words, some degree of adaptation occurs. In contrast, activation of the HPA axis in response to supramaximal exercise is greater in endurance-trained subjects than in untrained subjects (Furell *et al*. 1987; Snegovskaya & Viru 1993). The nature of the exercise training may to some extent determine the response of the HPA axis to subsequent acute exercise; when a substantial anaerobic component is present, the cortisol response to subsequent exercise may be increased (Fry *et al*. 1997).

Exercise and abnormalities of the HPA axis

Overreaching and overtraining

If adaptation to the increased training regimen does not occur, or if insufficient recovery time is included in training regimens, overreaching and subsequently overtraining may occur. Overreaching may be con-

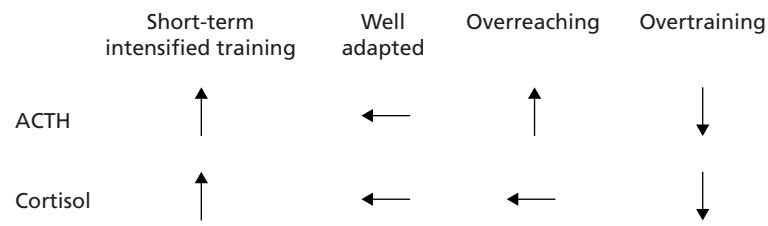

Fig. 17.3 Effect of exercise training on plasma adrenocorticotropic hormone (ACTH) and cortisol levels.

sidered as short-term overtraining and may be a normal part of athletic training, or may be seen as a short-term consequence of ultra-endurance events. By contrast overtraining results in a state described as burnout or staleness, which is characterized by increased fatigue, altered mood state, increased infections and suppressed reproductive function. Overtraining may be, at least in part, the consequence of inadequate periods of 'regeneration' (Lehmann *et al*. 1997).

From the HPA axis standpoint, it has been suggested that the earliest stage of overreaching (or very early overtraining) may be reflected by a reduced responsiveness to ACTH, which is initially compensated for by an increased pituitary ACTH response but a decreased adrenal cortisol response (Wittert *et al*. 1996; Lehmann *et al*. 1997). When fully evolved, the overtraining syndrome is characterized by both increased basal and 24-h urinary cortisol levels and a reduced ACTH and cortisol response to physical activity (Barron *et al*. 1985; Lehmann *et al*. 1992). In highly trained distance runners who undertook a 38% increase in training intensity over 3 weeks, six of the subjects developed sustained fatigue and the increase in serum cortisol, normally induced by 30 min of submaximal exercise, was lost (Verde *et al*. 1992). The severest and most fully evolved form of the overtraining syndrome is characterized by underactivity of both the HPA axis and sympathoadrenal system. This complete pattern is only observed subsequent to high-volume endurance overtraining at high caloric demands (Figs 17.3 and 17.4) (Lehmann *et al*. 1998).

Whether overtraining induced by high volume resistance exercise produces similar effects to highly aerobic activities is not entirely clear. After maximal-intensity-resistance exercise training, basal plasma cortisol or ACTH levels appear unaffected, but the responsiveness to exercise is decreased (Fry & Kraemer 1997). Some data suggests that hormone responses to exercise load are superior in indicating heavy training-induced stress when compared with resting hormone levels. These responses indicated decreased adrenocortical activity. However, since marked individual differences were found in training- and overtraining-induced hormonal changes, individual hormonal profiles are needed to follow-up training effects (Uusitalo *et al*. 1998).

Athletic amenorrhea

Exercise-associated disorders of the reproductive axis in women are associated with abnormalities of the HPA axis; the response of plasma cortisol to both maximal and submaximal acute exercise is reduced in amenorrheic compared to eumenorrheic athletes (Loucks & Horvath 1984; De Souza 1991). Amenorrheic athletes have been found to have higher mean basal (Ding *et al*. 1988; Loucks *et al*. 1989) early morning (Loucks *et al*. 1989) or mid-afternoon- (De Souza *et al*. 1991) blood and 24-h urine (Loucks *et al*. 1989) cortisol levels. There is also evidence that basal CRH stimulation is increased (Loucks *et al*. 1989; Hohtari *et al*. 1991) and that adrenal sensitivity to ACTH is reduced (De Souza *et al*. 1991, 1994).

Summary

Trained and untrained individuals have a robust increase in ACTH and cortisol in response to acute high intensity exercise. This is mediated via the hypothalamus by both CRH and AVP. The extent of the cortisol rise is proportional to the relative

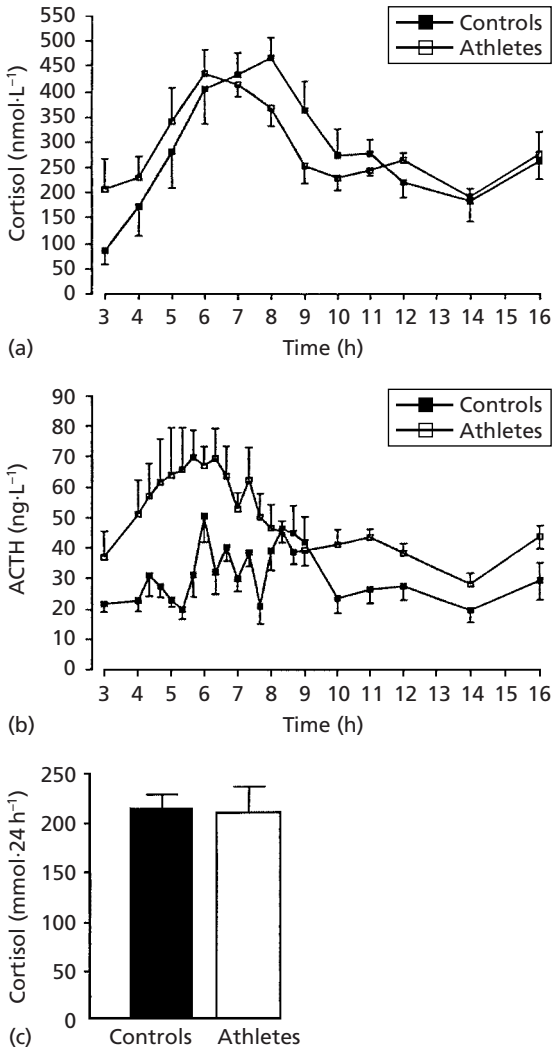

Fig. 17.4 Adaptation of the hypothalamic–pituitary–adrenal (HPA) axis to chronic exercise stress in humans. Mean (± SEM) plasma concentrations of (a) cortisol, (b) adrenocorticotropic hormone (ACTH) and (c) a 24-h urinary free cortisol excretion in ultramarathon athletes and control subjects.

intensity of the exercise as expressed as a percentage of $\dot{V}o_{2max}$. Older people may have a blunted response, while no difference exists between male and female subjects. At exercise levels below the anaerobic threshold, the duration of exercise has to be more prolonged to produce a significant increase in cortisol levels. The effect of resistance exercise on the HPA axis appears to be most variable, and there are both gender and age-specific affects. Variable responses have been observed during other forms of exercise, such as swimming and rowing. Carbohydrate supplementation during more prolonged exercise can attenuate or abolish the cortisol rise, which may implicate relative hypoglycemia as a potential factor inducing the activation of the HPA axis in this setting. At altitude, exercise still results in a significant rise in plasma cortisol, although basal cortisol levels are higher following acclimatization.

Although a great deal has been learned about the effects of physical activity on the HPA axis, there is still much conflicting information and many areas of confusion. The response of the HPA axis to any stress is dependent not only on the nature of the stress, but also on the environment in which the stress is imposed as well the inherent characteristics of the individual concerned (genetic factors, gender, personality, prior experience), concomitant stressors and nutritional state. In addition, the timing and manner in which samples are collected will also influence the results obtained.

In general, it appears that the response to short-term intensified training is an increased plasma cortisol level, particularly if an anaerobic element is involved. Progressively adaptation occurs, with a decreasing adrenal response to ACTH to exercise at the same relative intensity. With overreaching, the absolute cortisol response is decreased, and when overtraining occurs, HPA axis activity is decreased as a whole (Barron *et al*. 1985). The precise factors that may modify an appropriate adaptive response and lead to overreaching or overtraining remain to be determined.

The extent to which abnormalities of the HPA axis are simply consequences of, or are inherent to the pathophysiology of, a variety of conditions related to exercise training are not entirely clear. Whether activity of the HPA axis can be used as a marker of training stress and adaptation, by establishing the activity of and following longitudinally the HPA axis of individual athletes remains to be determined.

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Chapter 18

Influence of Energy Availability on Luteinizing Hormone Pulsatility and Menstrual Cyclicity

ANNE B. LOUCKS

Introduction

Over the past 30 years three types of scientific studies have been conducted to investigate the high prevalence of menstrual disorders in athletes. Surveys have found the highest prevalences in competitive endurance, aesthetic and weight-class sports and have led to several hypotheses about the cause of these disorders. Subsequent observational studies comparing hormone measurements in amenorrheic and eumenorrheic athletes contradicted most of those hypotheses. Then prospective experiments controlling the administration of the two remaining hypothetical causal factors, exercise stress and energy availability, have shown that low energy availability (defined as dietary energy intake minus exercise energy expenditure) disrupts reproductive function in physically active women and that exercise has no suppressive effect on reproductive function beyond the impact of its energy cost on energy availability.

In this research, most observational studies of menstrual disorders in athletes have focused on participants in aerobic sports, and most prospective experiments have employed aerobic exercise training to investigate the mechanism of these disorders. Therefore, very little information is available concerning whatever differences there may be between the influences of aerobic and anaerobic exercise on the prevalence and mechanism of menstrual disorders. However, since the prevalence of menstrual disorders in female bodybuilders is high, and since dietary restriction is common practice in bodybuilding, low energy availability probably accounts for most cases of menstrual disorders in anaerobic sports, too.

Menstrual disorders caused by factors other than athletic training, including pregnancy, lactation, eating disorders, pituitary tumors, hyperandrogenism, polycystic ovary syndrome, depression and various organic diseases, are beyond the scope of this review, even though some (such as polycystic ovary syndrome and anorexia nervosa) may be disproportionately represented amongst athletes due to the self-selection of affected women into activities in which hyperandrogenism or low body weight, respectively, offer a competitive advantage. The subject of this review is the disruption of reproductive function by athletic training itself, the mechanism of which has been traced to the failure of female athletes to sufficiently increase their dietary intake in compensation for their exercise energy expenditure, and which may be prevented by dietary reform without any moderation of their exercise regimen.

Regulation of the reproductive system

The length and regularity of menstrual cycles vary considerably across the general population of women as well as during an individual woman's reproductive years (Treloar *et al*. 1967). The median length of the menstrual cycle amongst North American adolescents is 33 days, and the median standard deviation of variations in the length from month to month is 7 days. By the age of 20 years, the median length and standard deviation have decreased to 29 and 4 days, respectively. These

numbers continue to decline slowly until the age of 40 years when both numbers begin to increase greatly during the years preceding menopause, when menstruation ceases permanently.

Moreover, the average age of menarche (i.e. the first menstrual cycle) has declined dramatically over the past 150 years in all Western societies—from an average of 17 years to 12.4 years (Styne & Grumbach 1991; Anderson, S.E. *et al*. 2003). What reactivates the reproductive system at puberty is unknown, just as what deactivates it in infancy and again at menopause are also unknown.

The regulation of the menstrual cycle by the hypothalamic–pituitary–ovarian (HPO) axis includes both negative and positive feedback mechanisms, as well as inputs from the central nervous system and other systems at various levels within the axis. The glands of the HPO axis secrete their hormones rhythmically. Indeed, the secretion of gonadotropin-releasing hormone (GnRH) pulses into the pituitary portal blood by neurons within the hypothalamus of the brain must occur at an optimal frequency if the axis as a whole is to function normally. These GnRH pulses stimulate the pulsatile secretion of luteinizing hormone (LH) from the pituitary gland into the blood. By sampling blood at 10 min intervals for 24 h, the frequency and amplitude of LH pulses can be studied.

In response to the proper pulsatile and monthly rhythmic stimulation by LH and pituitary follicle-stimulating hormone (FSH), clusters of ovarian cells ('ovarian follicles') grow and secrete increasing amounts of estrogen. Gradually, too, one of the follicles becomes dominant and eventually the rising concentration of estrogen in the blood exerts a positive feedback on LH secretion. In response, the pituitary gland secretes a surge of LH into the blood, causing the dominant follicle to rupture, thereby releasing an egg cell for fertilization. The remaining cells of the dominant follicle then undergo rapid chemical and morphological changes to form the corpus luteum, a body that begins secreting both progesterone and estrogen into the blood. The interval during which a dominant follicle develops, from menses until ovulation, is known as the follicular phase of the menstrual cycle. The interval during which the corpus luteum is active from ovulation until the next menses is known as the luteal phase of the cycle.

Estrogen and progesterone have profound influences on the uterine endometrium as well as many other tissues in the body. Estrogen stimulates endometrial proliferation and progesterone causes it to become highly vascularized. These are necessary, hospitable conditions for the successful implantation of a fertilized egg. If no fertilization occurs after several days, the ability of the corpus luteum to secrete progesterone becomes exhausted, the structural integrity of the endometrium collapses, and menstruation ensues. Under normal circumstances, the secretory capacity of the corpus luteum is sustained long enough for the rapidly dividing cells derived from a fertilized egg to become implanted in the endometrium 6 or 7 days after fertilization. If the secretory capacity of the corpus luteum is exhausted too soon, the endometrium sloughs off before implantation can occur. The likelihood of this increases when the luteal phase is shorter than 10 days. Thus, infertility can result from either the failure of the ovary to release an egg for fertilization or the failure of a fertilized egg to become properly implanted into the endometrium.

Characterization of female athletes

The ovarian axis

Amenorrheic athletes produce low levels of estrogen and progesterone every day indicating a complete absence of follicular development, ovulation, and luteal function (Fig. 18.1, AA) (Loucks *et al*. 1989). By contrast, even the most eumenorrheic competitive athletes display extended follicular phases and abbreviated luteal phases with blunted progesterone concentrations (Fig. 18.1, CA) compared to eumenorrheic sedentary women (Fig. 18.1, CS). Similar observations have been made in eumenorrheic women running recreationally as little as 12 miles (20 km) per week (Ellison & Lager 1986; Broocks *et al*. 1990; Pirke *et al*. 1990; De Souza *et al*. 1998).

The proximal cause of ovarian dysfunction in amenorrheic and eumenorrheic athletes is a disruption of the pulsatile rhythm of LH concentrations in the blood, upon which ovarian function critically

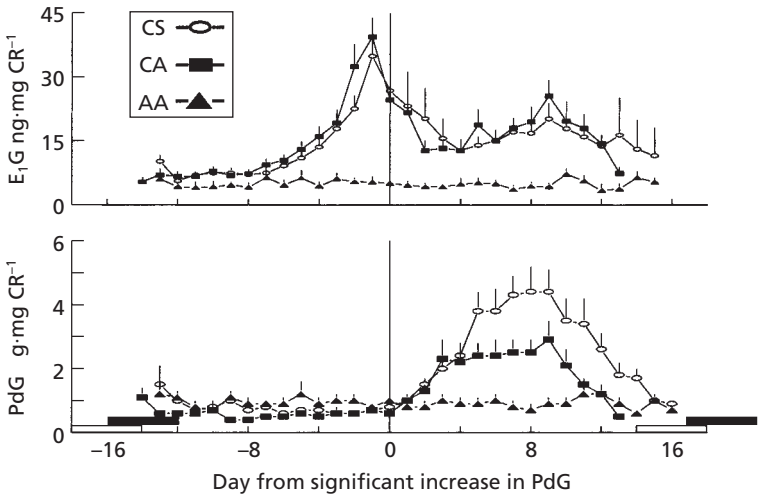

Fig. 18.1 Urinary estrone-glucuronide (E_1G), an estradiol metabolite, and pregnanediol-glucuronide (PdG), a progesterone metabolite, over an entire menstrual cycle in cyclic sedentary women (CS) and cyclic athletes (CA), and over an entire month in amenorrheic athletes (AA). The mass of each metabolite (ngE_1 and μPdG) excreted in overnight urine samples was normalized to the mass (mg) of creatinine (CR) excreted in the same samples. The black and open bars at the bottom of the figure indicate the days of menses in the CS and and CA women, respectively, at the beginning and the end of the cycle of observation. (From Loucks *et al*. 1989.)

depends (Veldhuis *et al*. 1985; Yahiro *et al*. 1987; Loucks *et al*. 1989; Laughlin & Yen 1996). In a eumenorrheic sedentary young woman, the 24-h LH profile in the early follicular phase is characterized by regular, high frequency pulses of low amplitude (Fig. 18.2, CS) (Loucks *et al*. 1989). During sleep, the frequency slows and the amplitude increases. Eumenorrheic athletes display a slower, but still regular rhythm of larger pulses (Fig. 18.2, CA). Amenorrheic athletes display even fewer pulses, at irregular intervals (Fig. 18.2, AA).

Experimental administration of GnRH has demonstrated that the disruption of LH pulsatility in amenorrheic and eumenorrheic athletes is caused by a disruption of GnRH pulsatility and not by a pituitary disorder (Veldhuis *et al*. 1985; Loucks *et al*. 1989). Therefore, the neuroendocrine mechanisms by which the GnRH pulse generator can be disrupted are the focus of much research.

The prevalence of amenorrhea in endurance, aesthetic and weight-class sports can be as much as 10 times higher than in the general population (Otis *et al*. 1997). The less severe disorders of ovarian function (follicular and luteal suppression and anovulation) may display no menstrual symptoms at all so that affected women are entirely unaware of their condition until they undergo an endocrine workup. Amongst eumenorrheic athletes, the incidence of follicular and luteal suppression and anovulation appears to be extremely high. Repeated endocrine workups have found that 79% of eumenorrheic female runners were luteally suppressed or anovulatory in at least 1 month out of 3 (De Souza *et al*. 1998).

Other endocrine axes

Mildly elevated resting serum cortisol levels in amenorrheic and eumenorrheic athletes (Ding *et al*. 1988; Loucks *et al*. 1989; De Souza *et al*. 1991, 1994; Laughlin & Yen 1996) encouraged the hypothesis that their reproductive disorders might be due to the stress of exercise. Since cortisol is a glucoregulatory hormone activated by low blood glucose levels, however, these elevated cortisol levels might also be explained as part of the physiological response to chronic energy deficiency.

Indeed, more extensive endocrine observations found that amenorrheic athletes also display low levels of plasma glucose (Laughlin & Yen 1996), insulin (Laughlin & Yen 1996), insulin-like growth factor I (IGF-I) (Zanker & Swaine 1998a), insulin-like growth factor I/insulin-like growth factor binding protein-1 (IGF-I/IGFBP-1) (an index of IGF-I bioavailability) (Laughlin & Yen 1996), leptin (Laughlin & Yen 1997; Thong *et al*. 2000) and triiodothyronine

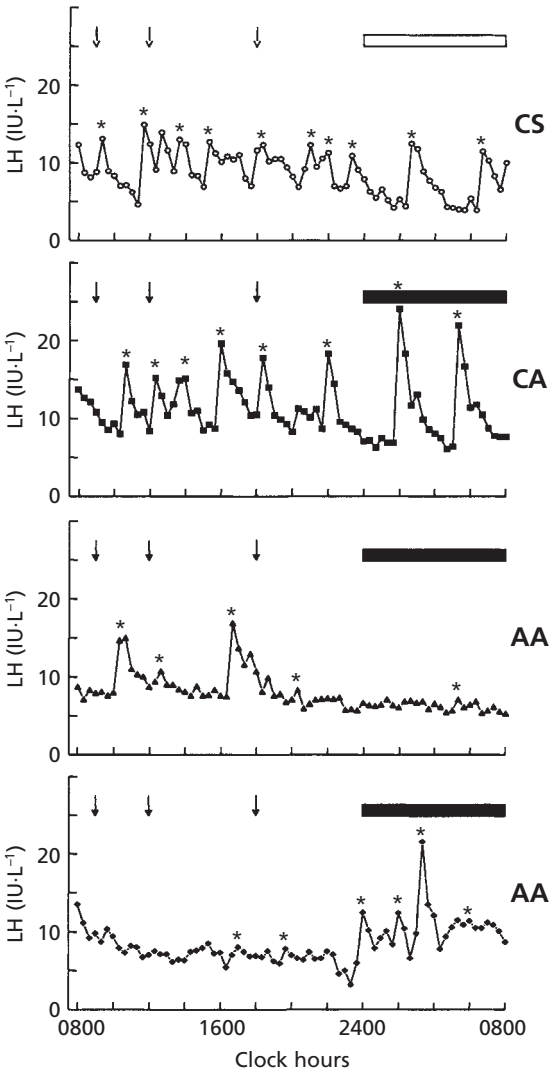

Fig. 18.2 The 24-h pulsatile rhythms of luteinizing hormone (LH), expressed as international units per liter ($IU \cdot L^{-1}$), for a cyclic (regularly menstruating) sedentary woman (CS), a cyclic athlete (CA) and two amenorrheic athletes (AA). Among amenorrheic athletes, the extensively quiescent, irregular pulsatile rhythms differed markedly. Pulsatile rhythms were considerably less variable among cyclic sedentary women and athletes. (From Loucks *et al*. 1989.)

(T_3) (Myerson *et al*. 1991; Loucks *et al*. 1992; Zanker & Swaine 1998a, 1998b), as well as low resting metabolic rates (Myerson *et al*. 1991). They also display elevated growth hormone (GH) (Laughlin & Yen 1996) in addition to the mildly elevated cortisol levels (Loucks *et al*. 1989; De Souza *et al*. 1991; Laughlin & Yen 1996). All these abnormalities are signs of chronic energy deficiency.

Compared to eumenorrheic sedentary women, luteally suppressed eumenorrheic athletes also display low levels of insulin (Laughlin & Yen 1996), leptin (Laughlin & Yen 1997) and T_3 (De Souza *et al*. 2003), and elevated levels of GH (Laughlin & Yen 1996) and cortisol (Loucks *et al*. 1989; Laughlin & Yen 1996), but the magnitudes of these signs of energy deficiency are less extreme than in amenorrheic athletes.

Energy intake and expenditure

Because of large variances between individuals, we were unable to distinguish between the dietary and exercise habits and histories of the amenorrheic and eumenorrheic athletes that we studied (Loucks *et al*. 1989, 1992). At that time, it did not occur to us to estimate each individual's energy availability (dietary energy intake minus exercise energy expenditure). Both groups of athletes reported their similar body weights to be stable, despite dietary energy intakes indistinguishable from those of sedentary women (Loucks *et al*. 1989). That is, their dietary energy intakes were much less than would be expected for their level of physical activity, a feature commonly observed amongst athletic women (Drinkwater *et al*. 1984; Marcus *et al*. 1985; Nelson *et al*. 1986; Kaiserauer *et al*. 1989; Myerson *et al*. 1991; Laughlin & Yen 1996).

Recently, extensive observational data on the energy and carbohydrate intakes of athletes in many sports have been compiled (Burke *et al*. 2001). If these data are to be believed, one observation is particularly noteworthy: with the notable exception of cross-country skiers, female athletes consume ~ 30% less energy and carbohydrates—normalized for body weight—than do male athletes.

The repeatedly reported combination of a stable body mass and an unexpectedly low dietary energy intake in female athletes is very controversial. Many investigators have been skeptical of the dietary records of female athletes, because studies comparing such data to estimations or measurements

of their energy expenditure have repeatedly found apparently huge negative energy balances, some exceeding 4 $MJ \cdot day^{-1}$ in athletes with stable body weights (Mulligan & Butterfield 1990; Wilmore *et al*. 1992; Edwards *et al*. 1993; Beidleman *et al*. 1995; Trappe *et al*. 1997; Hill & Davies 2002). Such large discrepancies have been interpreted as indicating not that female athletes are undernourished but rather that they grossly underreport their actual dietary intake. In support of this allegation, investigators have cited certain other special subpopulations that have been found to underreport (Wilmore *et al*. 1992; Edwards *et al*. 1993). Actually, underreporting of dietary intake is common in all populations (Mertz *et al*. 1991) and a meta-analysis of studies comparing dietary assessments to measurements of energy expenditure by doubly labeled water found that women do not underreport more than men (Trabulsi & Schoeller 2001).

Other investigators have questioned the methods used to measure energy intake and expenditure, and, indeed, the study that found virtually identical energy intakes in female and male cross-country skiers took extraordinary pains to achieve accurate measurements of energy intake (Sjodin *et al*. 1994). As a result of such concerns, quantitative criteria have been developed to assess whether reported energy intakes in studies of various numbers of subjects over various lengths of time pass what might be called the laugh test (Goldberg *et al*. 1991).

On the other hand, a stable body weight is *not* necessarily proof of energy sufficiency, because behavior modification and endocrine-mediated alterations in resting metabolic rate can counteract the potential influences of dietary energy excess or deficiency on body mass (Leibel *et al*. 1995). Energy intake and energy expenditure are also very difficult to measure reliably, even with doubly labeled water. Considering the lack of confidence in studies of energy balance in athletes, therefore, it is surprising that investigations of energy intake and expenditure in female athletes have not included biochemical measurements, because underreporting does not account for biochemical evidence of energy deficiency. As described above, metabolic substrates and hormones measured in amenorrheic and eumenorrheic athletes tell a consistent story of chronic energy and carbohydrate deficiency resulting in the mobilization of fat stores, the slowing of metabolic rate and a decline in glucose utilization, with more extreme abnormalities in amenorrheic athletes and less extreme abnormalities in eumenorrheic athletes (Myerson *et al*. 1991; Loucks *et al*. 1992; Jenkins, P.J. *et al*. 1993; Laughlin & Yen 1996, 1997; De Souza *et al*. 2003). So, while some might suggest that lower energy and carbohydrate intakes would be appropriate for women if their energy and carbohydrate expenditures were less than those of men, biochemical data demonstrate that female athletes are, indeed, chronically energy deficient.

Clinical concerns

The stability of body weight in athletes whose energy intake is estimated to be much less than their energy expenditure has been attributed to an increase in metabolic 'efficiency' (Westerterp & Saris 1991; Westerterp *et al*. 1992), but 'efficiency' is not the appropriate concept to apply to pathological adjustments to chronic energy deficiency. The oxidation of scarce metabolic fuels in the muscular work of locomotion makes these fuels unavailable for immune function, growth, tissue turnover, reproductive development and function, and other important physiological functions. For example, 50% of peak bone mass is deposited during adolescence, but the impairment of this process has led to some young amenorrheic athletes having the bone densities of 60-year-old women. Recently, a case study has been published documenting the 20-year history of clinical osteoporosis in an amenorrheic athlete (Zanker *et al*. 2004).

Bone is continuously remodeled at millions of local sites by coupled processes of bone resorption by osteoclast cells followed by bone formation by osteoblast cells. Normally, an increase in osteoclast activity stimulates an increase in osteoblast activity and bone resorption and formation are said to be coupled, but under pathological circumstances they become uncoupled. When the activity of osteoclasts exceeds that of osteoblasts, the resulting net bone loss is irreversible, because once they have left a bone remodeling site osteoblasts do not return to finish the job of refilling it.

At menopause, bone density declines because the normal suppression of osteoclast activity by estrogen is released, thereby increasing the rate of bone resorption while bone formation is unaffected (Marcus *et al*. 1996) or increased (Nielsen *et al*. 2004). In younger women by contrast, energy deficiency reduces the rate of bone formation by suppressing bone growth factors that stimulate osteoblast activity, and when this energy deficiency is severe enough to induce amenorrhea, the suppression of osteoclast activity by estrogen is also released, thereby increasing the rate of bone resorption. Thus, most studies of anorexia nervosa patients have found both a reduction in bone formation as well as an increase in bone resorption (Stefanis *et al*. 1998; Caillot-Augusseau *et al*. 2000; Hotta *et al*. 2000; Audi *et al*. 2002; Gordon *et al*. 2002), although a few have found only reduced formation (Soyka *et al*. 1999) or increased resorption (Lennkh *et al*. 1999). Refeeding anorexia nervosa patients has been found either to increase bone formation alone (Stefanis *et al*. 1998; Hotta *et al*. 2000) or to increase bone formation while also reducing resorption (Caillot-Augusseau *et al*. 2000; Heer *et al*. 2002; Soyka *et al*. 2002).

Fewer studies of bone turnover have been conducted in amenorrheic athletes. Unfortunately, the results of these studies have been less consistent than those of anorexia nervosa patients, with results showing no difference in either formation or resorption (Hetland *et al*. 1993; Stacey *et al*. 1998), reduced formation (Okano *et al*. 1995), and reduced formation and resorption but with resorption outweighing formation (Zanker & Swaine 1998a). Therefore, more studies of bone turnover in amenorrheic athletes need to be conducted before we can speak confidently about whether the mechanism of bone loss in amenorrheic athletes differs from that in anorexia nervosa patients. Such studies will need to be interpreted with care, however, since markers of bone turnover are systemic in nature and may not reveal local changes in bone turnover in the lumbar vertebrae and other trabecular sites where bone loss commonly occurs in amenorrheic athletes, especially if bone density is simultaneously increasing elsewhere, such as in the heel.

In young athletic women, bone mineral density declines by as much as 20% in proportion to the number of menses that they have missed (Drinkwater *et al*. 1990), resulting in an increased rate of stress fractures (Warren *et al*. 2003). Oral contraceptives have been recommended to restore bone loss in amenorrheic athletes (Otis *et al*. 1997; Anderson, S.J. *et al*. 2000), but this treatment has failed to increase bone density in young amenorrheic women by more than a few percent (Gulekli *et al*. 1994; Keen & Drinkwater 1997; Zanker *et al*. 2004). Restoring menses has been more successful for increasing bone density in some women, but recovered amenorrheic athletes have not kept pace with the bone growth of their eumenorrheic peers (Warren *et al*. 2003). In recovered anorexia nervosa patients who had been clinically well for 14–23 years, bone density in the femur remained 14% lower than in control subjects (Hartman *et al*. 2000). The inability of estrogen to restore lost bone in amenorrheic athletes and anorexia nervosa patients may indicate a potentially reversible suppression of bone formation caused by a continuing deficiency of nutritional growth factors such as IGF-I, T_3 and insulin, or it may indicate that the bone loss is irreversible due to the uncoupling of bone resorption and formation.

IGF-I and IGF-I/IGFBP-3 (another index of IGF-I bioavailability) have been found to predict spinal and femoral neck bone mass in gymnasts and runners (Maddalozzo & Snow 2000). When anorexia nervosa patients with osteopenia were treated with a combination of oral contraceptives and recombinant IGF-I their bone density did increase more than in a similar group who were administered oral contraceptives alone (Grinspoon *et al*. 2000, 2002). The benefit was only a few percent, however, and it may have been illusory because the latter group inexplicably reduced their dietary intake by 40% during the study resulting in a 15% decline in their endogenous IGF-1 production. Therefore, the superiority of the combined treatment has not been established. This treatment has not yet been tested in amenorrheic athletes.

Skeletal demineralization and osteoporotic fractures are the most alarming clinical consequence of athletic amenorrhea. Osteoporotic spinal fractures result in permanent disabilities and chronic pain. Osteoporosis in a 20-year-old athlete is a disaster. Osteopenia is a disaster waiting to happen.

Therefore, early detection and intervention are critical for minimizing permanent skeletal damage. Physicians should not defer treatment until after osteopenia and osteoporosis are manifest. The American College of Sports Medicine has published a position stand on the female athlete triad as a syndrome requiring prompt intervention to prevent chronic undernutrition from inducing reproductive disorders and skeletal demineralization (Otis *et al*. 1997). The American Academy of Pediatrics has published a similar warning (Anderson, S.J. *et al*. 2000). Since skeletal demineralization must proceed for many months before a reduction in bone density is measurable, repeated measures of biochemical markers of bone turnover, which respond immediately to undernutrition and hypoestrogenism should be considered as early indicators of skeletal demineralization.

Of course, long before reductions in bone density are measurable, the first clinical consequence of amenorrhea is infertility, since amenorrheic women are not developing egg cells that can be fertilized. Eumenorrheic physically active women with short luteal phases and low progesterone levels may also be at risk for infertility due to failures of implantation. Paradoxically, irregularly cycling and oligomenorrheic athletes may be at increased risk for unintended pregnancies if they do not use contraceptives, because their day of ovulation is less predictable than that of eumenorrheic women.

Another consequence of the hypoestrogenism in amenorrheic athletes is impaired endothelium-dependent arterial vasodilation (Hoch *et al*. 2003a, 2003b), which reduces perfusion of working muscle and increases the risk of developing cardiovascular disease, and which is restored by estrogen replacement therapy and by the return of regular menstrual cycles (Hoch *et al*. 2003b). Impaired skeletal muscle oxidative metabolism has also been reported in amenorrheic athletes, suggesting that they may be at a physiological disadvantage in their ability to perform repeated exercise bouts compared to their eumenorrheic competitors (Harber *et al*. 1998). In another report of estrogen-deficiency symptoms, 75% of amenorrheic athletes reported vaginal dryness compared to only 7% of eumenorrheic athletes (Hammar *et al*. 2000).

Proposed mechanisms of reproductive disturbances

As mentioned above, many competing hypotheses about the cause and mechanism of reproductive disorders in athletes have been offered over the years. The three most prominent hypotheses are discussed below.

Body composition

The body composition hypothesis held that the ovarian axis is disrupted when the amount of energy stored in the body as fat declines below a critical level (Frisch & McArthur 1974). Outside the research community this has been the most widely publicized explanation for menstrual disorders in athletes, but it has been the least widely believed explanation within the research community. Despite early associations of menarche and amenorrhea with body composition (Frisch & McArthur 1974), later observations of athletes did not consistently verify this association (e.g. Laughlin & Yen 1996), nor did they find the appropriate temporal relationship between changes in body composition and menstrual function (for reviews see Scott & Johnston 1982; Loucks & Horvath 1985; Sinning & Little 1987; Bronson & Manning 1991). Eumenorrheic and amenorrheic athletes span a common range of body composition (Loucks *et al*. 1984; Sanborn *et al*. 1987; Laughlin & Yen 1997) leaner than most eumenorrheic sedentary women. Among women distance runners, energy balance is a better predictor of estradiol levels ($r = 0.88$) than are body mass index (BMI) ($r = 0.42$) or percent body fat ($r = 0.48$) (Zanker & Swaine 1998c). Furthermore, if the growth and sexual development of young animals are blocked by dietary restriction, normal LH pulsatility resumes only a few hours after *ad libitum* feeding is permitted, long before any change in body mass or composition (Bronson 1986). Further evidence that body composition does not play a causal role in the mechanism of menstrual disorders was reported in an experiment on severely obese women (body weight ~ 130 kg; BMI ~ 47) (Di Carlo *et al*. 1999). Surgical reduction of their stomach volume reduced the amount of food that these patients could eat, re-

sulting in rapid weight loss and amenorrhea while the patients were still overweight (body weight ~ 97 kg; BMI ~ 35). Thus, the body composition hypothesis confused causation with correlation, which occurs in athletes because a lean body composition and menstrual disorders are both consequences of low energy availability.

Nevertheless, interest in the body composition hypothesis was rejuvenated several years ago with the discovery of the hormone leptin. Synthesized and secreted by adipose tissue, leptin was originally thought to communicate information about fat stores (Maffei *et al*. 1995). Later reports of leptin varying profoundly before any changes in adiposity in response to fasting (Kolaczynski *et al*. 1996a; Weigle *et al*. 1997), dietary restriction (Weigle *et al*. 1997), refeeding after dietary restriction (Kolaczynski *et al*. 1996a; Jenkins, A.B. *et al*. 1997) and overfeeding (Kolaczynski *et al*. 1996b) led to the hypothesis that leptin signals information about dietary intake, particularly carbohydrate intake (Jenkins, A.B. *et al*. 1997). Then leptin was found to be regulated by the tiny flux of glucose through the hexosamine biosynthesis pathway in muscle and adipose tissue (Wang *et al*. 1998; Rossetti 2000; Obici *et al*. 2002; Ravussin 2002). Since then, we have shown that the diurnal rhythm of leptin depends actually on energy *availability* or more specifically on carbohydrate availability (Hilton & Loucks 2000).

Exercise stress

The stress hypothesis held that the stress of exercise activates the adrenal axis, which disrupts the GnRH pulse generator by various mechanisms. To be meaningfully distinct from the energy availability hypothesis, the stress hypothesis further implied that the adrenal axis is activated by some aspect of exercise other than its energy cost.

Certainly, there are central and peripheral mechanisms by which the adrenal axis can disrupt the ovarian axis. Considerable animal research has shown that GnRH neurons are disturbed by activation of the hypothalamic–pituitary–adrenal axis via pathways involving corticotropin-releasing hormone (CRH) and endogenous opioid and proopiomelanocortin-derived peptides, or by increased cortisol negative feedback (Rivier & Rivest 1991; Chrousos & Gold 1992). Furthermore, early experiments by Selye (1939) induced anestrus and ovarian atrophy in rats by abruptly forcing them to run strenuously for prolonged periods. Others induced anestrus in rats by forced swimming (Asahina *et al*. 1959; Axelson 1987), by forced running (Chatterton *et al*. 1990), and by requiring animals to run farther and farther for smaller and smaller food rewards (Manning & Bronson 1989, 1991). Elevated cortisol levels in such studies were interpreted as signs of stress, and the induced reproductive disorders were widely interpreted as evidence that 'exercise stress' had a counter-regulatory influence on the female reproductive system. These experiments induced extreme activations of the adrenal axis, however, raising cortisol concentrations by several hundred percent, in contrast to the mild 10–30% elevations seen in amenorrheic athletes (Loucks *et al*. 1989; Laughlin & Yen 1996), hypothalamic amenorrhea patients (Berga *et al*. 1989) and anorexia nervosa patients (Gold *et al*. 1986). Whether such mild elevations in cortisol influence the GnRH pulse generator was entirely speculative.

Only one experiment had successfully employed exercise to induce menstrual disorders in eumenorrheic women. That experiment (Bullen *et al*. 1985) imposed a high volume of aerobic exercise abruptly, in imitation of Selye (1939). It caused a large proportion of menstrual disorders in the first month, and an even larger proportion in the second. The disorders were more prevalent in a subgroup fed a controlled weight-loss diet than in another subgroup fed for weight maintenance, but even the weight maintenance subgroup may have been underfed, since body mass is an unreliable indicator of energy balance (Leibel *et al*. 1995).

The first cracks in the stress hypothesis appeared when glucose administration during exercise was found to prevent the cortisol response to exercise in both rats (Slentz *et al*. 1990) and in men (Tabata *et al*. 1991) in the laboratory. This finding was later confirmed in a field experiment when the cortisol response to a strenuous, prolonged hill walk was prevented by eating larger meals (Ainslie *et al*. 2003). Then LH pulsatility was disrupted in habitually sedentary, eumenorrheic women through a

combination of dietary restriction and exercise (Williams *et al*. 1995), but this did not resolve the ambiguity about whether exercise stress or energy availability disrupts the reproductive system in exercising women.

Since all previous animal and human investigations of the influence of the 'activity stress paradigm' on reproductive function had confounded the stress of exercise with the stress of forcing animals to exercise and/or with energy deficiency, there was, in fact, no unconfounded experimental evidence that the stress of exercise, independent of its energy cost, disrupts reproductive function in voluntarily exercising women. Therefore, we conducted an experiment to determine the independent effects of exercise stress and energy availability on LH pulsatility (Loucks *et al*. 1998). We defined, measured and controlled energy availability operationally as dietary energy intake minus exercise energy expenditure. Needing an operational definition of exercise stress, however, we were confronted by a fundamental problem. Despite 60 years of research on responses to exercise stress, we could find no objective definition of exercise stress itself. So, we defined exercise stress as *everything* associated with exercise, except its energy cost.

Figure 18.3 shows the experimental design in which we assigned habitually sedentary women of normal body composition to sedentary or exercising groups and then administered balanced (45 kcal [188 kJ]·kg fat free mass [FFM]$^{-1}$·day^{-1}) and restricted 10 kcal [42 kJ]·kg FFM^{-1}·day^{-1}) energy availability treatments to them in random order under controlled conditions in the laboratory. The energy availability of the balanced sedentary group was achieved by feeding them 45 kcal [188 kJ]·kg FFM^{-1}·day^{-1} of energy in the form of a clinical dietary product. In the other trial, their energy availability was reduced by dietary restriction alone. The exercising group expended 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1} of energy in supervised exercise on a treadmill in the laboratory. Their 10 kcal [42 kJ]·kg FFM^{-1}·day^{-1} restricted energy availability was achieved by feeding them a dietary energy intake of 40 kcal [167 kJ]·kg FFM^{-1}·day^{-1} similar to the sedentary women in their balanced treatment. Their balanced energy availability was achieved by

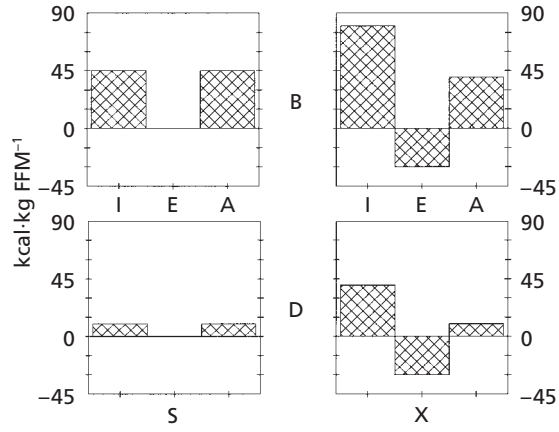

Fig. 18.3 Experimental design. Dietary energy intake (I) and exercise energy expenditure (E) were controlled to achieve balanced (B = 45 kcal·kg FFM^{-1}·day^{-1}) and deprived (D = 10 kcal·kg FFM^{-1}·day^{-1}) energy availability (A = I – E) treatments. Deprived energy availability was achieved by dietary restriction alone in sedentary women (S) and by exercise energy expenditure alone in exercising women (X) (1 kcal = 4.18 kJ). (From Loucks *et al.* 1998.)

increasing their dietary energy intake in compensation for their exercise energy expenditure. After 4 days of these treatments, we drew blood samples from the subjects at 10-min intervals for 24 h to assess LH pulsatility.

The results in Fig. 18.4 show that exercise stress had no suppressive effect on LH pulse frequency, whereas low energy availability suppressed LH pulse frequency, regardless of whether the low energy availability was caused by dietary energy restriction alone or by exercise energy expenditure alone. We also obtained similar results (not shown) when half of the reduction in energy availability was caused by dietary energy restriction and half by exercise energy expenditure. Low energy availability also suppressed T_3, insulin, IGF-I and leptin (Hilton & Loucks 2000) while increasing GH and cortisol in a pattern very reminiscent of amenorrheic and luteally suppressed eumenorrheic athletes.

Unexpectedly, the effects of low energy availability on LH pulse frequency and on the metabolic hormones were smaller in the exercising women than in the dietarily restricted women, even though their balanced and low energy availabilities were exactly

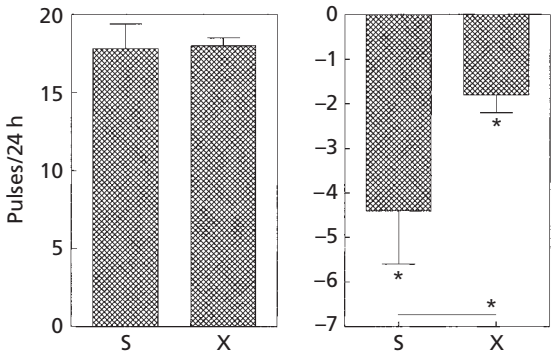

Fig. 18.4 Left: luteinizing hormone (LH) pulse frequency in sedentary (S) and exercising (X) women with the same energy availability. Right: reduction in LH pulse frequency caused by low energy availability in sedentary (S) and exercising (X) women. * = $p < 0.01$. (From Loucks 2004; adapted from Loucks *et al*. 1998.)

matched. This surprised us, because no-one had ever hypothesized that exercise would be *protective* of reproductive function. Further investigation revealed that the exercising women had a higher carbohydrate availability (defined observationally as dietary carbohydrate intake minus carbohydrate oxidation during exercise), due to a glucose-sparing alteration in skeletal muscle fuel selection during energy deprivation. As a result, the carbohydrate availabilities of the exercising and sedentary women were above and below the brain's daily glucose requirement, respectively.

Since this experiment, amenorrhea has been induced in monkeys by training them to run voluntarily on a motorized treadmill for longer and longer periods while their food intake remained constant (Williams *et al*. 2001a). The monkeys became amenorrheic abruptly in 7–24 months, after one or two cycles of luteal suppression. When the diet of half of the monkeys was then supplemented without any moderation of their exercise regimen, their menstrual cycles were restored (Williams *et al*. 2001b). The rapidity of recovery was directly related to the number of calories consumed.

Further data undermining the stress hypothesis have been reported in a study of young male soldiers participating in the 8-week US Army Ranger training course (Friedl *et al*. 2000). This course is divided into four 2-week phases in forest, desert, mountain and swamp environments during which trainees undergo daily military skill training, 8–12 km patrols carrying 32 kg rucksacks, sleep deprivation (~ 3.6 h of sleep per night) and dietary intakes during alternate weeks of ~ 8.4 and ~ 21 MJ per day. During the course, trainees lost ~ 12 kg of body weight. Blood sampling at the end of each week revealed that T_3, IGF-I and testosterone levels fell ~ 20%, ~ 50% and ~ 70%, respectively, during weeks on diets of 8.4 MJ·day^{-1} and returned to normal initial levels during alternate weeks on diets of 21 MJ·day^{-1}, despite continued exposure to all other training stresses. Thus, exercise appears to have no deleterious effect on reproductive function in either men or women apart from the impact of its energy cost on energy availability. If the adrenal axis disrupts the GnRH pulse generator in athletes, it probably does so by mediating the influence of energy availability.

Energy availability

The energy availability hypothesis recognizes that mammals partition energy amongst six major metabolic activities: cellular maintenance, thermoregulation, immunity, locomotion, growth and reproduction (Wade & Schneider 1992). The expenditure of energy in one function, such as locomotion, makes it unavailable for others, such as reproductive development and function. Specifically, this hypothesis holds that failure to provide sufficient metabolic fuels to meet the energy requirements of the brain causes an alteration in brain function that disrupts the GnRH pulse generator. In this regard, it is important to remember that because fatty acids do not cross the blood–brain barrier, the brain relies on glucose for energy. Furthermore, the brain has no glucose storage capacity, and in humans the brain is so large and so metabolically active that its daily energy requirement exceeds the entire liver glycogen storage capacity (Bursztein *et al*. 1989). In addition, because skeletal muscle lacks the enzyme to return glucose derived from muscle glycogen to the bloodstream, muscle glycogen stores are not available to the brain. By contrast, skeletal muscle does have access to liver glycogen stores. Therefore,

working skeletal muscle competes directly against the brain for all available carbohydrate stores in the body. In a marathon race, working muscle oxidizes as much glucose in 2 h as the brain needs in a week.

Considerable data from biological field trials support the hypothesis that mammalian reproductive function depends on energy availability, particularly in women (for reviews see Bronson & Manning 1989; Wade & Schneider 1992; Bronson & Heideman 1994; Wade *et al*. 1996; Schneider & Wade 2000). Anestrus has been induced in Syrian hamsters by food restriction, the administration of pharmacological blockers of carbohydrate and fat metabolism, insulin administration (which shunts metabolic fuels into storage) and cold exposure (which consumes metabolic fuels in thermogenesis) (Wade & Schneider 1992). Disruptive effects on the reproductive system were independent of body size and composition.

Considerable laboratory research suggests that GnRH neuron activity and LH pulsatility are regulated by brain glucose availability via two separate mechanisms involving the area postrema (AP) in the caudal brain stem and the vagus nerve (Knobil 1990; Minami *et al*. 1995; Levin *et al*. 1999; Muroya *et al*. 1999; Wade & Jones 2003). Glucose-sensing neurons in the AP of the hindbrain appear to transmit information to the GnRH pulse generator in the arcuate nucleus of the hypothalamus in the forebrain via neurons containing catecholamines, neuropeptide Y and CRH. These glucose-sensing neurons are activated by fasting (Mizuno *et al*. 1999), due in part to reductions in the inhibitory influences of insulin (Schwartz *et al*. 1992) and leptin (Mizuno *et al*. 1998) as well as glucose.

Having demonstrated that low energy availability, not exercise stress, disrupts LH pulsatility in exercising women, we investigated the dose–response relationship between energy availability and LH pulsatility and between energy availability and metabolic substrates and hormones in exercising women (Loucks & Thuma 2003). Energy availability was set at 10, 20, 30 and 45 kcal [42, 84, 125 and 188 kJ]·kg FFM^{-1}·day^{-1} by having all subjects perform 16 kcal [63 kJ]·kg FFM^{-1}·day^{-1} of exercise at 70% $\dot{V}o_{2max}$ while consuming 25, 35, 45 or 60 kcal [104, 146, 188, or 251 kJ]·kg FFM^{-1}·day^{-1} of dietary energy. All subjects were administered the balanced energy availability treatment (45 kcal [188 kJ]·kg FFM^{-1}·day^{-1}) and one of the restricted energy availability treatments in random order.

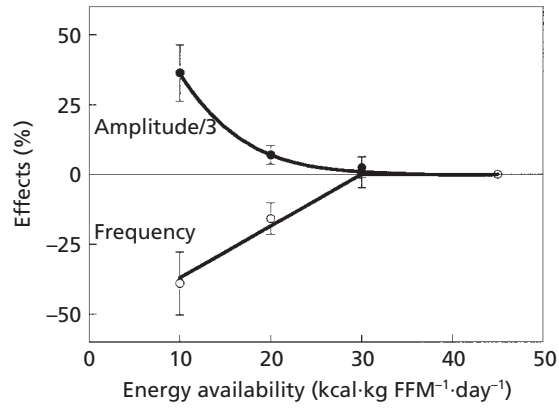

Fig. 18.5 Dose-dependent effects of restricted energy availability on luteinizing hormone (LH) pulse amplitude (● top) and frequency (○ bottom). Effects are expressed relative to values at 45 kcal·kg FFM^{-1}·day^{-1}. Effects on LH pulse amplitude are divided by three for graphical symmetry. As energy availability declines from 45 kcal·kg FFM^{-1}·day^{-1}, effects occur below 30 kcal·kg FFM^{-1}·day^{-1} and become more extreme as energy availability is further reduced (1 kcal = 4.18 kJ). (From Loucks & Thuma 2003.)

Figure 18.5 shows the dose-dependent effects of energy availability on LH pulsatility. LH pulse frequency was suppressed and pulse amplitude was increased below a threshold of energy availability at ~ 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1}, suggesting that athletes may be able to prevent menstrual disorders by maintaining energy availabilities above 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1}.

We also found that the disruption of LH pulsatility is substantially more extreme in women with short luteal phases (Fig. 18.6). If the latter finding is confirmed through further investigations, the screening of women for luteal length may be a convenient way to identify those who need to take extra care to avoid falling below the threshold of energy availability needed to maintain normal LH pulsatility.

Statistical analysis showed that the dose-dependent effects on LH pulsatility were similar to those on the metabolic substrates glucose and

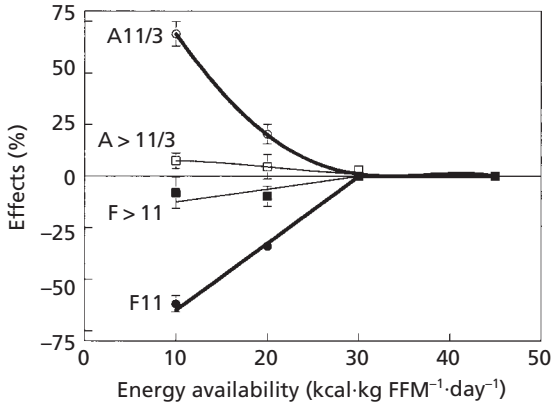

Fig. 18.6 Dose-dependent effects of restricted energy availability on luteinizing hormone (LH) pulse amplitude (□, ○ top) and frequency (■,● bottom) in subgroups of women with luteal phases of 11 days (○, ●) and > 11 days (□, ■). Women with shorter luteal phases (< 11 days) had been excluded from participation in the experiment. Effects are relative to values at 45 kcal·kg FFM^{-1}·day^{-1}. Effects on LH pulse amplitude are divided by three for graphical symmetry. Women with luteal phases of 11 days displayed substantially more extreme disruption of LH pulsatility (1 kcal = 4.18 kJ). (From Loucks & Thuma 2003.)

β-hydroxybutyrate and to the metabolic hormones GH and cortisol, and unlike those of the other metabolic hormones including insulin, IGF-I, T_3 and leptin. These results support the hypothesis that reproductive function reflects the availability of metabolic fuels, especially glucose, which may be signaled in part by activation of the adrenal axis. The role of leptin in mediating the influence of energy metabolism on the reproductive axis remains controversial with both proponents (Hileman *et al*. 2000) and skeptics (Schneider & Wade 2000). The central melanocortin mechanism by which leptin inhibits food intake does not appear to mediate leptin's stimulatory effect on the GnRH pulse generator (Hohmann *et al*. 2000).

The maintenance of normal LH pulsatility in this experiment despite a 33% restriction of energy availability to 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1} demonstrated for the first time that LH pulsatility is not simply proportional to energy availability. Rather, the regulation of the reproductive system in women seems to be robust against reductions in energy availability as large as 33%. Since the exercise energy expenditure in this experiment was ~ 836 kcal [3.5 MJ]·day^{-1}, these results suggest that many women may be able to maintain normal LH pulsatility while running up to 8 miles (13 km) a day as long as they do not simultaneously reduce their dietary energy intake below 45 kcal [188 kJ]·kg FFM^{-1}·day^{-1}. If they do reduce their dietary energy intake, as many exercising women do, then they risk falling below the threshold of energy availability needed to maintain normal LH pulsatility.

We also determined the dose-dependent effects of energy availability on biochemical markers of bone turnover in this experiment (Ihle & Loucks 2004). We found that bone resorption increased and became uncoupled from a suppression of bone formation only when energy availability was restricted severely enough to suppress estradiol (i.e. to 10 kcal [42 kJ]·kg FFM^{-1}·day^{-1}). If left to continue, such uncoupling may cause irreversible reductions in bone density. By contrast, bone formation was impaired by much less severe restrictions of energy availability (i.e. as high as 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1}), a level not at all unusual in weight control programs, in close association with the effects of low energy availability on insulin, IGF-I and T_3. Such reductions in the rate of bone formation may prevent even regularly menstruating, physically active women from achieving their genetic potential for peak bone mass.

Recently, a group of physically active university women displayed high scores on a test of dietary restraint without any of them reporting irregular menstrual cycles (McLean *et al*. 2001). From the data provided, we estimate that their energy availabilities were reduced only ~ 22% compared to similar women with low restraint scores. Similarly, monkeys maintained on a 30% restricted diet for 6 years showed no disruption of menstrual cycling or reproductive hormones and no reduction in bone mineral density, despite a fat mass 46% lower than that in monkeys fed a control diet (Lane *et al*. 2001). Since their body mass declined during the study, their dietary intake normalized to their body mass had, in fact, declined by only ~ 20%. Recent cross-sectional comparisons of estimated energy availability in athletes also support the notion that menstrual function is disrupted at the threshold of

energy availability needed to maintain normal LH pulsatility (Thong *et al*. 2000). Amenorrheic athletes were estimated to habitually self-administer an energy availability of ~ 16 kcal [67 kJ]·kg FFM^{-1}·day^{-1}, while eumenorrheic athletes habitually self-administered ~ 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1}.

Implications for athletic training

Research done to date suggests that athletes may be able to prevent or reverse menstrual disorders by increasing their dietary energy intake without any modification of their exercise regimen. In short-term experiments, an energy availability of 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1} appears to be sufficient to preserve normal LH pulsatility. More short-term experiments are needed to determine the independent effects of carbohydrate and energy availability on LH pulsatility, to identify the exact physiological mechanism by which low energy or carbohydrate availability disrupts LH pulsatility, and to investigate further whether low energy availability causes more extreme disruptions of reproductive function in certain identifiable women. More prolonged experiments are needed to verify that short-term effects on LH pulsatility are predictive of chronic effects on ovarian function, and to identify practical, effective and acceptable interventions for preventing and reversing menstrual disorders in athletes. Research is also needed to investigate how body weight is maintained in chronically energy-deficient athletes.

Part of the nutritional challenge for athletes is that 'there is no strong biological imperative to match energy intake to activity-induced energy expenditure' (Blundell & King 1999, p. 5581). Experimental food deprivation increases hunger, but the same energy deficit produced by exercise energy expenditure does not (Hubert *et al*. 1998). Studies have shown that hunger is suppressed briefly by a single bout of intense exercise (Blundell & King 1998), and two bouts of intense exercise in a single day induces no increase in *ad libitum* food intake on that or the following 2 days (King *et al*. 1997). Furthermore, large shifts in carbohydrate and fat oxidation (Stubbs *et al*. 1995a, 1995b) and in glycogen stores (Snitker *et al*. 1997) produce no changes in *ad libitum* macronutrient intake. Even a 20% increase in energy expenditure during 40 weeks of marathon training induced no increase in energy intake (Westerterp *et al*. 1992). In our own laboratory, women say that they have to force themselves to eat far beyond their appetites to consume the amount of food that compensates their dietary energy intake for their exercise energy expenditure and to prevent the disruption of LH pulsatility. Other investigators have had to offer exercising amenorrheic monkeys special treats to induce them to increase their energy intake enough to restore their menstrual cycles (Williams *et al*. 2001b). Consequently, to improve their performance while protecting their health, athletes must learn to eat by discipline instead of appetite.

Complicating that discipline, another part of the nutritional challenge for athletes is that energy balance is not the objective of athletic training. Athletic performance is maximized, in part, by a sport-specific (and in team sports, position-specific) optimum body size, body composition and mix of stored metabolic fuels. Therefore, much of an athlete's training aims to modify her body to achieve these objectives. It is also important to know that macronutrients are metabolized differently and stored separately so that the conversion of one macronutrient into another for storage does not represent important metabolic pathways (Flatt 1988). Therefore, an athlete needs to manage fat, protein and carbohydrate balances separately to optimize their pursuit of sport-specific body size, body composition and energy store objectives.

As part of this discipline, athletes would benefit from monitoring biomarkers of their progress and pitfalls on the path toward their particular body size, body composition and energy store objectives. Much applied research is needed to validate the utility of such biomarkers. Ideally, a single measurement of a biomarker would provide the desired information accurately, unambiguously, inexpensively, conveniently, non-invasively, safely, privately and quickly without intentional or unintentional confounding by other factors. Few, if any, biomarkers fulfill all these criteria. An obvious candidate for monitoring progress in reducing fat mass is skinfold thickness, which is simple, direct,

immediate and inexpensive, and perhaps good enough. We are unaware of any inexpensive, non-invasive method for assessing muscle and liver glycogen stores, which are essential for both athletic performance and reproductive health, but a convenient method for assessing carbohydrate deficiency is readily available. In our experience, urinary ketones identify carbohydrate-deficient individuals almost as reliably at 30 kcal [125 kJ]·kg FFM^{-1}·day^{-1} as they do at 10 kcal [42 kJ]·kg FFM^{-1}·day^{-1}. A single prolonged exercise bout on an energy-restricted diet is sufficient to elevate plasma ketones (Ainslie *et al*. 2003). Athletes can purchase 'keto-sticks' inexpensively in most pharmacies to monitor their urinary ketones in the privacy of their own homes.

The health of female athletes has always been a high priority of women's sports, but undernutrition has become standard practice, especially in endurance, aesthetic and weight-class sports. If equally pervasive voluntary reforms by athletes, coaches and judges are unlikely, then mandatory institutional reforms like Rule 3 in National Collegiate Athletic Association (NCAA) men's wrestling (NCAA 2002) may be necessary to protect the health of female athletes. Rule 3 restricts weight loss objectives and sets individualized minimum weights for competition. In so doing, it prevents widespread weight loss practices that had previously placed the health of participants at risk.

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Chapter 19

Oral Contraceptive Use and Physical Performance

JACI L. VANHEEST, CARRIE E. MAHONEY AND CAROL D. RODGERS

Oral contraceptive use is common in both recreational and elite female athletes. Oral contraceptives are generally comprised of a combination of estrogen- and progesterone-like compounds. The composition is dependant upon the varying levels of the two steroid derivatives produced by the various manufacturers. In addition to their use for contraception, women may also be prescribed oral contraceptives for cycle regulation and to manage various cycle dysfunctions (i.e. amenorrhea or dysmenorrhea). Side effects such as weight gain, fluid retention and nausea initially caused many athletic women to avoid the use of oral contraceptives. However, with the introduction of lower dose formulations the negative side effects have been reduced and the numbers of women using these drugs has increased. The impact of the oral administration of ovarian steroids on physical performance has been evaluated over the past several decades. Research, however, remains incomplete as to the potential positive and/or negative effects of these compounds on sport performance. The following chapter will address the endogenous hormonal control of the menstrual cycle, the various types and mechanisms of action of exogenous hormones and the influence of these drugs on physical performance.

Overview of normal menstrual cycle

The menstrual cycle is regulated primarily by a group of five hormones; gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen and progesterone. These hormones are released as part of a classical endocrine axis known as the hypothalamic–pituitary–gonadal (HPG) axis (Fig. 19.1). Regulation of the HPG axis occurs through both short- and long-loop feedback systems.

GnRH is secreted from the hypothalamus in a pulsatile manner throughout the menstrual cycle. Hypothalamic GnRH pulsatility is essential for regular menstrual cyclicity. On average, the frequency of GnRH secretion is once per 90 min during the early follicular phase and once per 60–70 min during the luteal phase. The release of both FSH and LH is induced by GnRH with LH being the more sensitive of the two hormones with respect to changes in GnRH levels (Larsen *et al*. 2003).

FSH is secreted by the anterior pituitary gland and is essential for follicular growth. Its secretion is highest and most critical during the 1st week of the follicular stage. At the level of the ovary, FSH induces estrogen and progesterone secretion by activating aromatase and p450 enzymes. FSH also induces the proliferation of granulosa cells and expression of LH receptors on granulosa cells.

LH is secreted by the anterior pituitary gland and is required for both growth of preovulatory follicles and luteinization and ovulation of the dominant follicle. During the follicular phase of the menstrual cycle, LH induces androgen synthesis by theca cells; stimulates proliferation, differentiation, and secretion of follicular thecal cells; and increases LH receptors on granulosa cells. The preovulatory LH surge drives the oocyte into the first meiotic division and initiates luteinization of thecal and granulosa cells. The resulting corpus luteum produces high levels of progesterone and some estrogen.

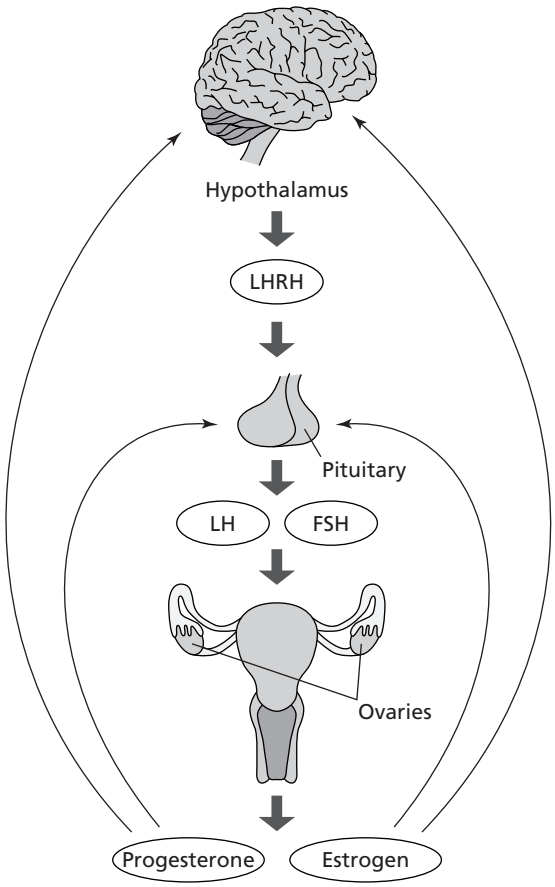

Fig. 19.1 The hypothalamic–pituitary–gonadal (HPG) axis. FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone.

Estrogen is produced at the level of the ovary and is crucial for the development of the antrum and maturation of the Graafian follicle. Estrogen is predominant at the end of the follicular phase, directly preceding ovulation. Estradiol is primarily derived from androgens produced by thecal cells. The androgens migrate from the thecal cells to the granulosa cells, where they are converted into estradiol by aromatase enzyme. The actions of estradiol include induction of FSH receptors on granulosa cells, proliferation and secretion of follicular thecal cells, induction of LH receptors on granulosa cells, and proliferation of endometrial stromal and epithelial cells. At low circulating levels, estrogens exert negative feedback on LH and FSH secretion; however, at very high levels estrogens exert positive feedback on LH and FSH secretion. Estrogen further induces proliferation of granulosa cells and synthesis of estrogen receptors, establishing a positive feedback loop on itself. In the uterine endometrial cycle, estrogen induces proliferation of the endometrial glands (Knobil 1999).

In the presence of a GnRH pulse, the pituitary and ovarian hormones exert mutual control over the circulating levels of each other. The complex interactions between pituitary and ovarian hormones involve forward control, positive feedback, and negative feedback mechanisms. They also serve to sustain a self-perpetuating monthly endocrine cycle. Figure 19.2 illustrates the relationships between the relative amounts of key hormones of the menstrual cycle. Day one of the menstrual cycle begins with menstruation, which occurs at the beginning of the follicular phase.

The follicular phase of the menstrual cycle spans the first day of menstruation until ovulation. The primary purpose of the follicular phase is to develop a viable follicle capable of undergoing ovulation. The early events of the follicular phase are initiated by a rise in FSH levels that occurs on the 1st day of the cycle. This rise in FSH levels can be attributed to a decrease in progesterone and estrogen levels at the end of the previous cycle and the subsequent removal of their inhibitory effect on FSH. FSH stimulates the development of 15–20 follicles each month and stimulates follicular secretion of estradiol by up-regulating secretion of androgens by the theca externa, and by inducing the aromatase enzyme receptor on granulosa cells (Yen 1999). FSH further induces expression of FSH receptors by follicles. As estradiol levels increase under the influence of FSH, estradiol inhibits the secretion of FSH and FSH levels decrease.

Under normal circumstances, one follicle evolves into the dominant follicle, destined for ovulation, while the remaining follicles undergo atresia. It is currently not known how the dominant follicle is selected; yet it has been observed that the dominant follicle always expresses an abundance of FSH receptors. As FSH levels decrease towards the end of the follicular phase, the developing follicles must

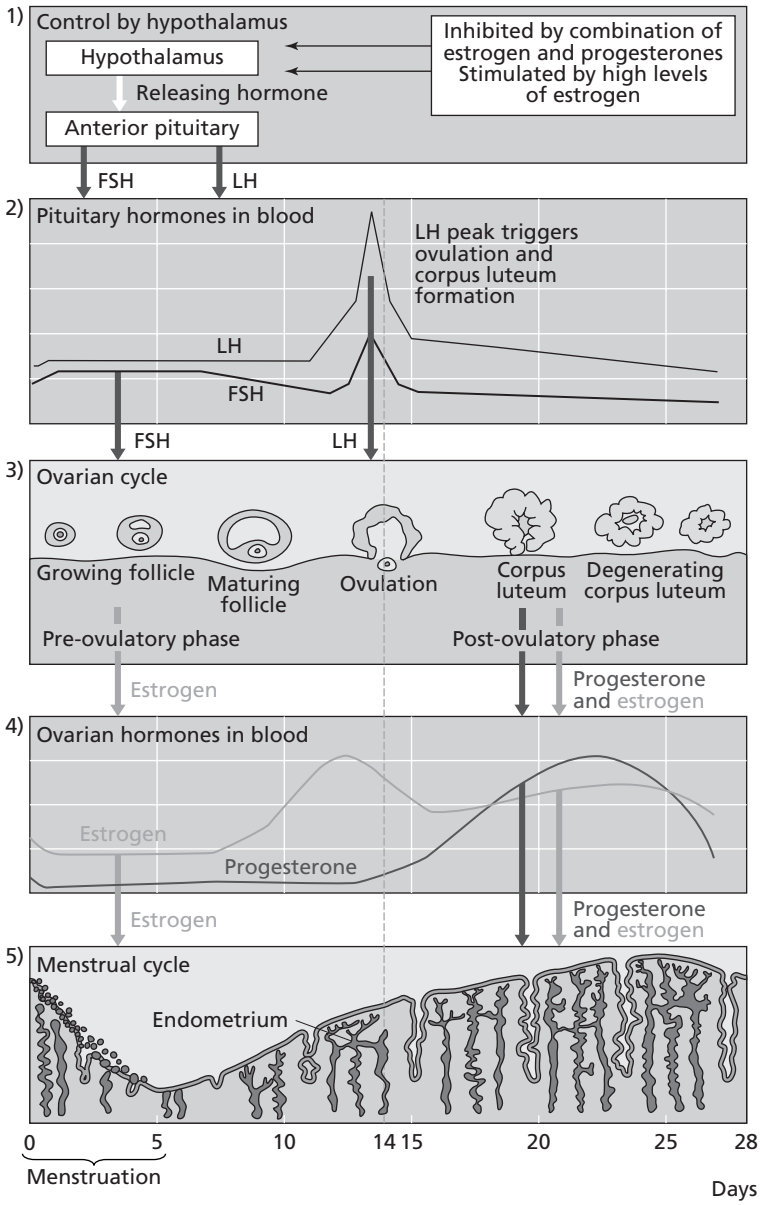

Fig. 19.2 The relationships between the relative amounts of key hormones of the menstrual cycle. FSH, follicle-stimulating hormone; LH, luteinizing hormone.

compete for relatively small amounts of FSH. The dominant follicle, with its high concentration of FSH receptors is able to acquire more FSH even as FSH levels decrease. This enables the dominant follicle to continue to synthesize estradiol which is essential for its complete maturation. Since the remaining follicles can no longer produce the needed amount of estradiol due to the decreasing FSH level they cease to develop and ultimately undergo atresia. The dominant follicle may also release paracrine factors that stimulate apoptosis in the other follicles. As the dominant follicle continues to mature it secretes increasing amounts of estrogen. Estrogen levels peak towards the end of the follicular phase of the menstrual cycle. At this critical point, estrogen exerts positive feedback on

LH (positive feedback is usually associated with having estradiol present in excess of 1100 pmol·L^{-1} for > 24 h), generating a dramatic preovulatory LH surge. It is important to note that estrogen can only exert a positive feedback on LH at this precise stage in the menstrual cycle; if estrogen is artificially provided earlier in the cycle, ovulation will not be induced (Yen 1999; Larsen *et al*. 2003).

The luteal phase is characterized by the luteinization of those components of the follicle that were not ovulated. It is initiated by the LH surge. The granulosa cells, theca cells and some surrounding connective tissue are all converted into the corpus luteum, which eventually undergoes atresia. The major effects of the LH surge are the conversion of granulosa cells from predominantly androgen-converting cells to predominantly progesterone-synthesizing cells by the expression of new LH receptors. This fosters increased progesterone synthesis, and a reduced affinity of granulose cells for estrogen and FSH in these cells. Combined, these changes promote increased progesterone secretion with some estrogen secretion. Progesterone secretion by the corpus luteum peaks between 5 and 7 days post-ovulation. High progesterone levels exert negative feedback on GnRH and subsequently GnRH pulse frequency decreases. As GnRH pulse frequency decreases, FSH and LH secretion also decreases. The corpus luteum further loses its FSH and LH receptors. The lack of FSH and LH stimulation precipitates atresia of the corpus luteum and its subsequent evolution into the corpus albicans. With the decline of both estrogen and progesterone levels, an important negative feedback control on FSH is removed and FSH levels rise once again to initiate the next menstrual cycle (Yen 1999).

The LH surge is required for ovulation. Under the influence of LH, the primary oocyte enters the final stage of the first meiotic division and divides into a secondary oocyte and the first Barr body. The LH surge induces release of proteolytic enzymes, which degrade the cells at the surface of the follicle, and stimulates angiogenesis in the follicular wall and prostaglandin secretion. These effects of LH cause the follicle to swell and rupture. At ovulation, the oocyte is expelled into the peritoneal cavity. The oocyte adheres to the ovary and muscular contractions of the fallopian tube bring the oocyte into contact with the tubal epithelium to initiate migration through the oviduct.

Generally, estrogen (estradiol) is produced continuously during the cycle. It is very low during the early follicular phase, rising to a peak during the late follicular phase and triggering ovulation. Progesterone and estrogen increase during the luteal phase, and the increased estrogen causes the endometrium, the inner lining of the uterus, to thicken and mature. Progesterone helps to mature and stabilize the endometrium. It also prevents further endometrial proliferation and mitosis and changes the endometrium to a secretory structure that is ready for implantation of the fertilized ovum. At the end of the luteal phase, concentrations of estrogen and progesterone decrease, which triggers menstruation and the subsequent breakdown of the endometrium. Breakdown of the endometrium occurs assuming no implantation of an ovum has occurred during this period.

Inhibin is a glycoprotein that has been isolated as a heterodimer with two subunits linked by disulphide bonds. The two isoforms of inhibin are termed inhibin A and inhibin B. Inhibin is produced by the granulosa cells and the corpus luteum in females (deKrester & Robertson 1989; Groome *et al*. 1996). Control of inhibin synthesis and release is via the hormones FSH (granulosa cells) and LH (corpus luteum). Level of inhibin (serum) peak mid-cycle and in the mid-luteal phase, with a decline in concentrations prior to menstruation (Sehested *et al*. 2000). Inhibin has FSH-suppressing properties, as well as, more recently discovered immune, nervous system and hemopoietic properties (deKrester & Robertson 1989; Sehested *et al*. 2000). In addition, inhibin may have paracrine actions related to growth and maturation of the ovary and classical endocrine actions in the maturation of the HPG axis (Groome *et al*. 1996; Sehsted *et al*. 2000). Additional research is necessary to fully elucidate the role of inhibin in human physiology.

Overview of oral contraceptives

Oral contraceptive pills are available in three major formulations—fixed-dose, combination phasic and

daily progestin. Monophasic pill formulations contain a fixed dose of estrogen and progestin throughout the cycle. Biphasic and triphasic (multiphasic) pills reduce the total amount of progestin throughout the cycle as to mimic normal physiologic levels. Ethinyl estradiol and mestranol are typical synthetic estrogens used in oral contraceptive formulas. Older formulations contained mestranol in high doses (50 μg) compared to the newer low dose formulations (< 50 μg of estrogen compounds ethinyl estradiol or mestranol). First generation progestins include norethindrone and norethindrone acetate or ethynodiol diacetate and norethynodrel. Second generation progestins are typically norgesterel and levonorgestrel (Larsen *et al*. 2003). Finally, desogestrel, norestimate and gestrodene are third generation progestins. The third generation progestins are designed to be selective for progestin receptors, thus reducing the atherogenic properties of these compounds (Godsland *et al*. 1992). Table 19.1 lists oral contraceptives that are currently available in the USA. It is important to note that the new generation progestins are assumed to be less androgenic compared to the early formulations. However, all combination oral contraceptives are beneficial in the clinical treatment of various conditions including hirsuitism (Thorneycroft 1999). Low dose oral contraceptives are considered to have a lipid neutral affect resulting in a reduced atherosclerosis rates in humans.

First generation oral contraceptives were associated with many negative side effects such as weight gain, fatigue, headaches and nausea (Lebrun *et al*. 2003). These side effects have been minimized by the second and third generation lower dose drugs. These formulations have 30–40% lower levels of hormones resulting in significantly reduced side effects (Greenblatt 1985). Studies evaluating changes in body weight and body composition in both nonactive and athletic women using oral contraceptives support these findings. Female athletes (Lebrun *et al*. 2003) and non-athletes (Rosenberg 1998) report non-significant weight gains (~ 1 kg over 6 weeks) or no change in body weight and body composition, respectively. However, the impact of small body weight and body composition changes associated with oral contraceptive use in female athletes cannot be overlooked when discussing elite sport performance.

Mechanism of action of oral contraceptives

The mechanism by which estrogen and progesterone prevent ovulation is through the suppression of the FSH and LH mid-cycle surge. Combination formulations are highly effective at inhibiting the secretion of gonadotrophic hormone, and through this mechanism prevent ovulation. Conversely, progestin-only products are inconsistent in the suppression of ovulation and operate through the inhibition of the release of GnRH from the hypothalamus. In this way, progestin only products attenuate the levels of FSH, LH, estradial and progesterone (Speroff *et al*. 1993).

These induced changes in circulating hormone levels precipitate changes in endometrial functioning. Changes in ovum transport and implantation, coupled with alterations in the composition of the cervical mucus (increase viscosity and reduce volume) are also evident (Larsen *et al*. 2003). Together, the hormonal and endometrial alterations result in a negative environment for success of ovulation, implantation and support of the ovum.

Oral contraceptives also impact carbohydrate, protein and fat metabolism (Table 19.2). Carbohydrate metabolism is influenced by the dose, potency and chemical structure of the progestin in the drug. The role of the synthetic estrogens on glucose metabolism is unclear, however they may act synergistically with the progestins to cause impaired glucose tolerance. Epidemiological studies indicate low risk or no risk for developing diabetes in oral contraceptive users (new generation). However, it is prudent to consider the glucose handling status of each woman when prescribing oral contraceptives. Low dose norethindrone-type progestins are preferable in these women.

Estrogens used in oral contraceptives increase the hepatic production of various globulins such as Factor V, VII, X and fibrinogen. These globulins enhance thrombosis. In addition, synthetic estrogens also increase the synthesis of angiotensinogen (which is converted to angiotensin) resulting in elev-

Table 19.1 Oral contraceptive formulations. (Data from Mishell 1999 and Larsen *et al*. 2003.)

Brand name	Product type	Progestagen	Estrogen
Levlen	C	0.15 mg levonorgestrel	30 μg ethinylestradiol
Tri-Levlen	C, T	0.5 or 0.075 or 0.125 mg levonorgestrel	30 μg/40 μg /30 μg ethinylestradiol
Ovcon-35	C	0.4 mg norethindrone	35 μg ethinylestradiol
Ovcon-50	C	1.0 mg levonorgestrel	50 μg ethinylestradiol
Desogen	C	0.15 mg desogestrel	35 μg ethinylestradiol
Mircette	B, C	0.15 mg/0 mg desogestrel	30 μg/10 μg ethinylestradiol
Micronor	P	0.35 mg norethindrone	
Modicon	C	0.5 mg norethindrone	35 μg ethinylestradiol
Ortho-Cept	C	0.15 mg desogestrel	30 μg ethinylestradiol
Ortho-Cyclen	C	0.25 mg norgestimate	35 μg ethinylestradiol
Ortho-Novum 1/35	C	1.0 mg norethindrone	35 μg ethinylestradiol
Ortho-Novum 1/50	C	1.0 mg norethindrone	50 μg mestranol
Ortho-Novum	C, T	0.5 mg/0.75 mg/L mg norethindrone	35 μg ethinylestradiol
Ortho-Novum	C, B	0.5 mg/1.0 mg norethindrone	35 μg ethinylestradiol
Ortho-tricyclin	C, T	0.18 mg/0.215 mg/0.25 mg norgestimate	35 μg ethinylestradiol
Estrostep	C, T	1.0 mg norethindrone	20 μg/30 μg/35 μg ethinylestradiol
Loestrin 1/20	C	1.0 mg norethindrone	20 μg ethinylestradiol
Loestrin 1.5/30	C	1.5 mg norethindrone	30 μg ethinylestradiol
Norlestrin 1/50	C	1.0 mg norethindrone	50 μg ethinylestradiol
Norlestrin 2.5/50	C	2.5 mg norethindrone	50 μg ethinylestradiol
Brevicon	C	0.5 mg norethindrone	35 μg ethinylestradiol
Norinyl 1 + 35	C	1.0 mg norethindrone	35 μg ethinylestradiol
Norinyl 1 + 50	C	1.0 mg norethindrone	50 μg ethinylestradiol
Nor-Q.D.	P	0.35 mg norethindrone	
Tri-Norinyl	C, T	0.5 mg/1 mg/0.5 mg norethindrone	35 μg ethinylestradiol
Demulen 1/35	C	1.0 mg ethynodiol diacetate	35 μg ethinylestradiol
Demulen 1/50	C	1.0 mg ethynodiol diacetate	50 μg ethinylestradiol
Alesse	C	0.1 mg levonorgestrel	20 μg ethinylestradiol
Lo/Ovral	C	0.3 mg levonorgestrel	30 μg ethinylestradiol
Nordette	C	0.15 mg levonorgestrel	30 μg ethinylestradiol
Ovral	C	0.5 mg norgestrel	50 μg ethinylestradiol
Ovrette	P	75 mg norgestrel	30 μg ethinylestradiol
Triphasil	C, T	50 mg/75 mg/125 mg levonorgestrel	40 μg /40 μg /30 μg ethinylestradiol

B, biphasic; C, combination; P, progestagen only; T, triphasic.
Androgenic activity (relative to 1 mg of norethindrone: norethindrone (1 mg) = 1; levonorgestrel (1 mg) = 8.3; drospirenone (1 mg) = 0; desogestrel (1 mg) = 3.4; norgestimate (1 mg) = 1.9; ethynodiol diacetate (1 mg) = 0.6).

ated blood pressure in some women (Larsen 2003). The androgenic progestins increase the synthesis of sex hormone binding globulin (SHBG). SHBG binds the 19-nortestosterone compounds used in oral contraceptives. Overall, the incidence of venous and arterial thrombosis is directly related to the dose of the estrogen, which is relatively low in the new generation formulations.

Synthetic estrogens used in oral contraceptives cause an increase in high-density lipoproteins (HDL), total cholesterol and triglycerides, and a decrease in low-density lipoproteins (LDL) (Larsen 2003). Conversely, progestin elements cause a decrease in HDL, total cholesterol and triglycerides, and an increase in LDL. The negative impact of the progestins in the early oral contraceptives was related to the high androgenic activity of these compounds. Today's low dose oral contraceptives are less androgenic and appear to have a more lipid neutral response (Thorneycroft 1999). When evaluating the

Table 19.2 Metabolic effects of oral contraceptives. (Data from Dorflinger 1985 and Godsland *et al*. 1992.)

Steroid derivative	Substrate pathway	Metabolic effect
Ethinyl estradiol	Protein	Decrease amino acids
Ethinyl estradiol	Carbohydrate	No change in plasma insulin or glucose tolerance
Ethinyl estradiol	Lipid	Increase cholesterol, HDL and triglyceride Decrease LDL cholesterol
19-Nortestosterone derivatives	Proteins	None
19-Nortestosterone derivatives	Carbohydrate	Increase plasma insulin Decrease glucose tolerance
19-Nortestosterone derivatives	Lipid	Decrease cholesterol, HDL and triglyceride Increase LDL cholesterol

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

metabolic response to various oral contraceptives, it is critical that the type, dose and combination of each synthetic hormone be assessed.

Impact of oral contraceptive use on physical performance

The literature regarding the impact of oral contraceptive use on performance has grown substantially over the past several decades. Early studies exploring the effect of first generation, high dose drugs are often distinctly different from the more recent work evaluating the low dose multiphasic compounds. While the focus of this review will be on the second and third generation drugs that are currently being used by women participating in sport, earlier work will be discussed when appropriate.

Aerobic performance

Maximal oxygen consumption ($\dot{V}o_{2max}$) is an often used measure when assessing aerobic ability. Normal ovarian hormone cyclicity does not appear to affect $\dot{V}o_{2max}$ (De Souza *et al*. 1990; Bemben *et al*. 1992, 1995; Lebrun *et al*. 1995; Lynch & Nimmo 1998); however, data examining the impact of hormonal alterations as induced by changes in exogenous hormones, such as would occur with oral contraceptive use, is less conclusive.

Studies evaluating the administration of exogenous ovarian hormones have varied in the dose and type of oral contraceptives utilized. Research in the early 1980s failed to demonstrate any significant difference in performance between oral contraceptive users and non-users (McNeill & Mozingo 1981; Huisveld *et al*. 1983). A subsequent short duration (21 day) evaluation of 1 mg norethindrone also did not demonstrate a significant difference in $\dot{V}o_{2max}$ between oral contraceptive users and non-users (Bryner *et al*. 1996). Interestingly, however, endurance trained women who used monophasic oral contraceptives for a 6-month period experienced a significant decrease in $\dot{V}o_{2peak}$ (Notelovitz *et al*. 1987). Further evaluation of a triphasic preparation performed by Casazza *et al*. (2002) with moderately active women also showed a significant reduction (–11%) in $\dot{V}o_{2peak}$ following 4 months of oral contraceptive use. Peak heart rate and minute ventilation, however, were not different These results were supported by the work of Lebrun *et al*. (2003) who showed a 4.7% decrease in $\dot{V}o_{2max}$ following a 2-month triphasic oral contraceptive intervention protocol.

Potential factors that could impact maximal aerobic capacity include stroke volume, oxygen transport capacity, oxygen extraction capacity and muscle blood flow. The reductions in $\dot{V}o_{2max}$ may logically be associated with a reduction in stroke volume and/or oxygen transport capacity. Stroke volume has been shown to increase in perimenopausal women following hormone replacement therapy (Kamali *et al*. 2000). However, resting

ferritin, hemoglobin concentration and serum iron concentration remain unchanged in women using oral contraceptives (Mooij *et al*. 1992) which would suggest that these primary factors are not the mechanism by which aerobic potential is limited.

Blood flow regulation via mechanisms such as sympathetic nervous system (SNS) activation has also been cited as a factor that may be responsible for the observed reductions in maximal aerobic capacity. The evaluation of various conditions where ovarian hormones are elevated provides experimental evidence for this mechanism. In pregnancy where there is a period of high estrogen and progesterone concentrations SNS activation is suppressed, in conjunction with a decreased level of circulating catecholamines (McMurray *et al*. 1993). In this way, a reduction in SNS activation with oral contraceptive administration might also be responsible for the decreases in aerobic capacity shown in these studies.

Anaerobic and strength performance and muscle damage

The current literature on the relationship of exogenous administration of ovarian hormones and anaerobic metabolism and/or muscular strength is extremely limited. Early work by Petrofsky *et al*. (1976) identified a reduction in muscle strength during the luteal phase of normally cycling females. The potential for oral contraceptive use to blunt this strength decline was subsequently evaluated in a group of athletic women by Lebrun *et al*. (2003). Anaerobic performance on the anaerobic speed test and isokinetic strength measured on the Cybex dynamometer was determined prior to and following administration of a triphasic formulation for 2 months. Although this study failed to find a significant difference in either anaerobic performance or isokinetic strength, the relationship of high levels of ovarian hormones to anaerobic and/or strength performance capacity is significantly underexamined and must be further researched before any definitive conclusions can be established.

The influence of endogenous and/or exogenous estrogens on exercise induced muscle damage has been evaluated over the past decade. Ultrastructural muscle damage is a typical consequence of strenuous exercise such as resistance training. The role of estradiol on membrane structure and permeability has been evaluated in both animal and human models.

Women have responded to strenuous exercise with lower serum creatine kinase (CK) concentrations compared to men, which has been associated with lower total muscle mass in women (Shumate *et al*. 1979; Rogers *et al*. 1985). However, animal studies support the role of estradiol in reducing membrane permeability in response to exercise (Amelink *et al*. 1988, 1990; Bar & Amelink 1997).

Research evaluating the role of estradiol on muscle damage following exercise in women using oral contraceptives is both limited and equivocal. Miles and Schneider (1993) reported no influence of estradiol on CK activity following exercise. The relationship between delayed onset muscle soreness and oral contraceptive use was reported following a 50-min stepping routine in women using oral contraceptives compared to controls. Oral contraceptives were associated with a reduction in self-reported soreness scores; however, the mechanism for these findings remains unclear (Thompson *et al*. 1997).

Recent studies have utilized eccentric muscle damage protocols. Downhill running (30 min) was used by Carter *et al*. (2001) to elicit damage in oral contraceptive users and non-users. Both groups experienced significantly elevated CK and muscle soreness scores compared to baseline measures. Differences were evident between the groups at 72 h following damage with the oral contraceptive users exhibiting a reduction in CK compared to the control group. These data support a protective role of estrogen following muscle damage. In contrast, Savage and Clarkson (2002), using a 50 repetition eccentric muscle contraction protocol of the elbow flexors, showed no difference in measures of soreness, serum CK or range of motion of the elbow joint. However, oral contraceptive users had delayed force recovery (maximal isometric strength) at 2 days following muscle damage.

The role of exogenous estrogens on muscle damage following exercise remains unclear. Animal

studies support the protective role (anti-oxidant characteristics) of estradiol on membrane permeability. Human studies vary in methodology and outcome measures. Further research is necessary to clarify the potential benefits of estrogen on muscle stability following strenuous physical activity.

Heat tolerance

Menstrual cycle phase is associated with varied responses to thermal stressors. Exercise during the luteal phase is associated with an elevated core temperature (0.4°C) compared to the follicular phase (Stachenfeld *et al*. 2000). In contrast, oral contraceptive users have less variability in core temperature between phases than non-users. The primary factor for these phasic alterations in core temperature is the oscillations in progesterone across the menstrual cycle (elevation during the luteal phase). Use of oral contraceptives containing progestins would cause elevations in core temperature during exercise similar to women during the luteal phase. The influence of progesterone on core temperature is further supported by the response of women using the long-term contraceptive method of injectable progesterone (i.e. Depot-Provera [Cheung *et al*. 2000]). These women experience an elevated core temperature during the 24–36 h following administration of the drug. Martin and Buono (1997) reported elevated core temperature (0.3°C) and heart rate (8 b·min^{-1}) in women using oral contraceptives containing synthetic progestins while exercising in the heat (30°C, 50% relative humidity). These changes in core temperature and heart rate are similar to those seen in women exercising in the heat during the luteal phase. It appears that second and third generation oral contraceptives mimic the thermoregulatory responses of women exercising in a hot environment during the luteal phase. Further research is necessary to more clearly understand the impact of low dose contraceptive use on performance in hot and humid environments.

Substrate utilization and metabolic flux

The metabolic effects of contraceptive steroids varied depending on the formulation. Table 19.2 illustrates the influence of estrogen ethinyl estradiol and the 19-nortestosterone derivative progrestins on metabolism. The influence of the oral contraceptives is directly related to dosage and potency of the steroid derivative in the formulation. The metabolic effects at rest have been evaluated in relation to side effects of the drugs. Only recently have scientists begun to understand the role of oral contraceptive use in female athletes during physical exercise.

Current research on the metabolic alterations associated with oral contraceptive use during exercise is limited. Cross-sectional studies have compared oral contraceptive users with non-users. Early work by Bonen *et al*. (1991) reported significantly elevated free fatty acid concentrations in oral contraceptive users when compared with non-users during basal or resting conditions. This tendency also appears to occur during exercise with reductions in respiratory exchange ratio (RER) and a potential enhancement in lipid oxidation also evident in oral contraceptive users (Bemben *et al*. 1995).

More recently, studies using stable isotope technology to evaluate both glucose and lipid metabolism during exercise and at rest have demonstrated a decrease in glucose rate of appearance (Ra), disappearance rate (Rd) and metabolic clearance rate in women using oral contraceptive during exercise on a cycle ergometer (45% or 65% $\dot{V}o_{2peak}$) (Suh *et al*. 2003). Under similar exercise conditions, triglyceride mobilization was measured using glycerol rate of appearance (infusion of [1,1,2,3,3-^{2}H]glycerol). Glycerol Ra increased significantly at both exercise intensities in the oral contraceptive condition. The elevated Ra was associated with a significantly increased plasma cortisol concentration but no difference in RER (Casazza *et al*. 2004). These data would suggest that endogenous ovarian hormones do play a role in affecting glucose and lipid metabolism during exercise. The exercise induced hormonal perturbations clearly dictate the metabolic flux in normal cycling women. Oral contraceptives (triphasic) provide a greater stimulus to lipid and glucose flux during exercise conditions. The level of circulating ovarian hormones appears to be critical in these metabolic alterations.

Summary

Oral contraceptive use has grown in females participating in sport and physical activity. The role that these exogenous ovarian hormones play in physical performance remains to be fully elucidated. It is clear that third generation lower dose formulations result in reduced side effects for women. These changes have made the use of oral contraceptives more appealing to a broad group of female athletes including elite caliber performers.

The reductions seen in aerobic capacity appear to be associated with potential alterations in blood flow due to suppression of SNS activation coupled with a reduction in circulating catecholamines. These findings are similar to those seen during pregnancy. Anaerobic and/or strength performance appear to be unchanged during oral contraceptive use. Potential thermoregulatory issues exist for women using oral contraceptives; however, the responses mimic women exercising during the luteal phase of the menstrual cycle. The most prominent finding regarding oral contraceptive use during exercise is on substrate metabolism. Studies using stable isotope methodologies have provided significant insight into the interplay between lipid and glucose metabolism during exercise in women using exogenous hormones.

The literature examining oral contraceptive use during exercise is relatively limited. It is important to consider the potential negative or ergogenic effects of exogenous ovarian steroid use in elite female athletes. Longitudinal studies of longer durations are necessary to provide practical recommendations to female athletes.

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Chapter 20

Energy Balance and Exercise-Associated Menstrual Cycle Disturbances: Practical and Clinical Considerations

NANCY I. WILLIAMS AND MARY JANE DE SOUZA

Introduction

Historically, opportunities for women to engage in physical activity increased dramatically with the passing of Title IX in the USA. To date, over three decades of subsequent research examining the impact of exercise on women's bodies and behavioral aspects of the athletic lifestyle have brought attention to gender-specific issues such as musculoskeletal health, weight and diet concerns, and the impact of exercise on the menstrual cycle. Looking back, it appears that research on the musculoskeletal effects of exercise and weight and diet concerns developed soon after observations of disturbances in reproductive function associated with exercise. After an early report documenting a higher prevalence of menstrual abnormalities in athletes than non-athletes (Erdelyi 1962), numerous other studies confirmed this finding in the late 1970s and early 1980s (Feicht *et al*. 1978; Dale *et al*. 1979; Baker *et al*. 1981), focusing mostly on amenorrhea and delayed menarche in long-distance runners (Feicht *et al*. 1978) and ballet dancers (Frisch *et al*. 1980; Warren 1980). Interestingly, while Frisch and Revelle (1971) had put forth the hypothesis that menarche depended on the achievement of a critical body weight level of body fat, Warren (1980) observed that changes in body weight and body composition did not correlate with the onset of menarche in dancers who acutely decreased their exercise due to injury. Warren thus popularized the concept that the 'energy drain' or metabolic cost of exercise might be a contributing factor to alterations in menstrual cyclicity in female athletes. The first links between exercise-associated amenorrhea and compromised bone were made by Drinkwater *et al*. (1984) and Cann *et al*. (1984) who observed significantly lower bone mineral content in amenorrheic runners. Drinkwater *et al*. (1990) then documented a significant relationship between lumbar spine bone mineral density (BMD) and menstrual history in female athletes. An authoritative review by Loucks and Horvath in 1985 (Loucks & Horvath 1985) helped highlight the importance of exercise-associated menstrual disturbances (EAMD) as a research area and synthesized the available information concerning plausible mechanisms of the interactions between exercise and reproductive function. Researchers' attention was focused on the potential contributions of body composition, training habits, diet, physical stress and psychological stress in the etiology of EAMD. In 1987, a link between 'eating problems' and amenorrhea in ballet dancers was reported by Brooks-Gunn *et al*. (1987), and in 1991, Wilmore (1991) put forth the idea that athletes in both endurance or appearance sports are at an increased risk for disordered eating, secondary amenorrhea and bone mineral disorders. Shortly after, the female athlete triad (Fig. 20.1), i.e. a condition comprised of restrictive eating, menstrual disorders and skeletal demineralization was recognized (Yeager *et al*. 1993), and subsequently described in a Position Stand published by the American College of Sports Medicine (ACSM) in 1997 (Otis

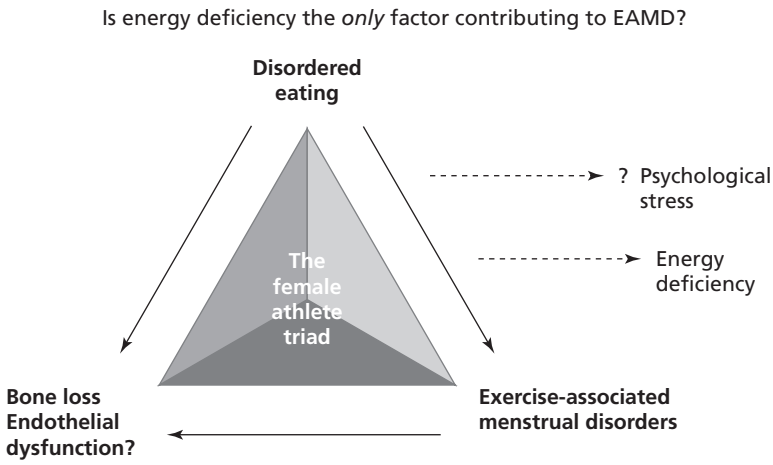

Fig. 20.1 The relationship between components of the female athlete triad. Disordered eating and exercise combined to produce low energy availability, causing exercise-associated menstrual disturbances (EAMD). Psychosocial stress is theoretically depicted as a factor that may exacerbate the effects of low energy availability on EAMD. (Modified and reprinted with permission from Williams 2003.)

et al. 1997). Since that time, recognition of the importance of the triad and the need to more specifically define the components, establish the prevalence, physiological underpinnings and the clinical consequences has driven the research in the area of exercise and the menstrual cycle, and stimulated a revision of the 1997 ASCM Position Stand. A current understanding of the practical and clinical considerations of the effects of exercise on the menstrual cycle in today's active woman must include the broader scope of the female athlete triad, and consequently recognize the complexity of interrelationships among its components. A multidisciplinary approach to this issue involving behavioral, physiological, sociocultural and medical perspectives is warranted. This chapter will provide current information on the practical issues relating to the impact of exercise on menstrual function, such as the identification, prevention and treatment of menstrual disorders, an exploration of factors that predispose certain individuals to menstrual disorders and considerations for the restoration of normal menstrual function. Clinical issues arising from the association of physical activity and disturbances in reproductive function will also be examined. These include the impact of endocrine changes associated with menstrual disturbances on fertility, bone health and possibly the cardiovascular system, and the association of menstrual disturbances with restrictive eating.

Exercise-associated menstrual disturbances: definitions and prevalence

Overview

Important aspects of the relationship between exercise and the menstrual cycle have been revealed through descriptive, observational, cross-sectional and prospective studies utilizing both survey methods and physiological approaches. Our current understanding is that EAMD exist on a spectrum of severity, i.e. luteal phase defects (LPD), anovulation and amenorrhea. Prior to discussing the definitions and prevalence of the different EAMD, attention is warranted toward the appropriate use of terms. The term 'exercise-induced menstrual disturbances' implies that menstrual disturbances are directly related to participation in or the stress of physical exercise per se. Contrary to this idea, a variety of experimental approaches have yielded evidence that inadequate caloric intake is a causal factor in menstrual disturbances with exercise (Laughlin & Yen 1996; Loucks *et al*. 1998; Williams 1998; Williams *et al*. 2001b; De Souza *et al*. 2003) such that a state of low energy availability created by an imbalance of energy consumed as food and expended through exercise is the key factor in the onset of reproductive suppression. The use of a more fitting term is thus warranted, i.e. 'exercise-associated' or 'exercise-related' menstrual disturbances. Since estimates of

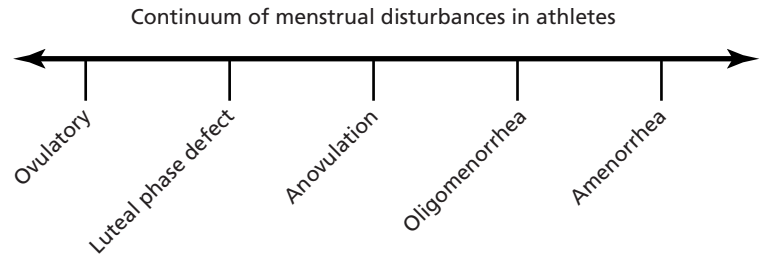

Fig. 20.2 Continuum of reproductive disturbances, ranging from ovulatory cycles, subtle presentations of luteal phase defects (LPD) and anovulatory cycles to the most severe menstrual disturbance, amenorrhea. Physically active women and athletes fluctuate between ovulatory cycles, LPD and anovulatory disturbances frequently. It also seems probable that amenorrheic athletes may experience LPD during recovery from amenorrhea. (Reprinted with permission from De Souza 2003.)

the prevalence of secondary amenorrhea are higher in athletes than in sedentary populations (Drew 1961), there is a *correlation* between athletic amenorrhea and physical exercise, but there is not a *causal* relationship.

Amenorrhea

Figure 20.2 depicts this continuum of menstrual disturbances ranging from subtle perturbations like LPD and anovulatory cycles to the most severe presentation, amenorrhea (De Souza 2003). In athletes, the amenorrhea is hypothalamic in origin and is manifested by suppressed levels of gonadotropins and ovarian steroids (Loucks & Horvath 1985; Velduis *et al*. 1985). The definition of amenorrhea in the literature has varied, but should be conservatively defined as no menses for a minimum of 3 months due to the clinical sequelae associated with chronic estrogen deficiency (Loucks & Horvath 1985). The prevalence of amenorrhea in athletes ranges from 1% to 66% (Feicht *et al*. 1978; Dale *et al*. 1979; Schwartz *et al*. 1981; Sanborn *et al*. 1982, 2000; Loucks & Horvath 1985), is highest in those sports with an aesthetic component like gymnastics and figure skating, and grossly exceeds estimates of this condition in sedentary women (2–5%) (Drew 1961; Petterson *et al*. 1973).

Delayed menarche

Delayed menarche, or primary amenorrhea, defined as failure to achieve menarche by the age of 16 years (Loucks & Horvath 1985), has been repeatedly reported in athletes participating in many sports, but again particularly in the aesthetic sports. The later ages at menarche in adolescent athletes are often attributed to regular exercise training without considering other factors known to influence pubertal growth and maturation (Malina 1994). Malina (1994) states that in adequately nourished adolescents, the timing of menarche is very dependent on hereditary factors; but menarche is also influenced by a number of social or biocultural variables, including the self-selective nature of participation in some sports, like gymnastics, figure skating and ballet, where selection occurs for specific factors associated with a delayed or later maturation (Malina 1994). The mechanisms that link these factors to a later age of maturation remain undefined. Following the review of several studies that examined menstrual disturbances in athletes, the average age of menarche was about 1 year later (13 vs. 12 years old) in the groups of amenorrheic athletes, but these differences were not statistically significant in the individual studies. In both human (Warren 1980) and animal studies (Cheung *et al*. 1997), however, the timing of puberty has been related to nutritional status (Wade *et al*. 1996), and specifically, to increased leptin concentrations providing a permissive effect on growth (Cheung *et al*. 1997). Since leptin concentrations can discriminate athletes of differing menstrual status (Laughlin & Yen 1997; De Souza *et al*. 2003), and evidence exists for a causal

role of energy availability in the development of menstrual disturbances (Williams *et al*. 2001a), it is reasonable to speculate that energy status may play a role in the common observance of a later age of menarche in amenorrheic athletes, if participation in exercise began prior to puberty.

Oligomenorrhea

Oligomenorrhea is defined by irregular and inconsistent menstrual cycles lasting from 36–90 days in length (Loucks & Horvath 1985). The key parameter of interest in the definition of oligomenorrhea is the unpredictable nature of the intervals between menstrual cycles. Given these inconsistent characteristics, it is a menstrual presentation that is difficult to study. As such, no definitive data exist on the prevalence of oligomenorrhea in athletes, except to note that cycles of irregular length are often reported in female athletes (Loucks & Horvath 1985) and oligomenorrhea as a menstrual category is frequently grouped together with amenorrhea in many studies (Gremion *et al*. 2001; Csermely *et al*. 2002; Cobb *et al*. 2003). When daily measurement of hormones has not been feasible or affordable, investigators have also used definitions that have included 3–4 or fewer menstrual cycles per year to define oligomenorrhea (Cobb *et al*. 2003). The ovarian profile of an oligomenorrheic athlete displays erratic, unpredictable and presumably inadequate estradiol (E_2) production as a given follicle struggles to achieve dominance, but certainly may result in an ovulatory cycle in an unpredictable manner.

Anovulation

Anovulation is defined as the absence of ovulation of an oocyte in the face of inadequate luteinizing hormone (LH) secretion secondary to inadequate estrogen priming in the follicular phase and the obvious absence of luteinization (Hamilton-Fairly & Taylor 2003). Anovulatory cycles are characterized by low E_2 and progesterone (P_4) levels throughout the cycle; however, much debate in the clinical forum continues regarding the specific criterion for confirming anovulation (Malcolm & Cumming 2003). Because serial ultrasound measurements to document ovulation are not always feasible, many researchers have relied on daily assessment of urinary ovarian steroid metabolites and urinary LH to confirm ovulation (or anovulation). Using this approach, De Souza *et al*. (1998a) have reported a 16% prevalence of anovulatory cycles in women that exercise at recreational levels despite having characteristic regular menstrual intervals of 26–32 days. Williams *et al*. (2000) have reported that in 32% of Division 1 athletes from a wide variety of sports who self-reported regular menstrual bleeding of 26–32 days, ovulation could not be detected. In most cases, a higher degree of estrogen exposure is present in anovulatory cycles compared to amenorrheic cycles; that is an anovulatory cycle is likely to have greater E_2 production in a 30-day period than an equivalent period in an amenorrheic athlete. In both of the latter conditions, the absence of progesterone from luteinized cells renders estrogen actions on some tissues unopposed.

LPD have been reported in women engaged in all levels of physical activity, from strenuous to recreational exercise, (Shangold *et al*. 1979; Ellison & Lager 1986; Broocks *et al*. 1990; Beitins *et al*. 1991; Winters *et al*. 1996; De Souza *et al*. 1998a; De Souza 2003). In women with LPD, the ovarian system functions at a level adequate for ovulation, but inadequate to support successful implantation, since the latter is dependent on adequate exposure of the endometrium to P_4 (Balash & Vanrell 1987). A reduction in luteal phase P_4 production and abbreviated luteal phases are the key characteristics of LPD. Reduced P_4 production during the luteal phase is also referred to as luteal phase inadequacy or insufficiency to describe the poor quality of the endometrium secondary to the low P_4 levels. The low P_4 levels that occur are either low in volume or low in duration of output, since they occur in the face of normal menstrual cycle lengths of 26–32 days (Jones 1976; Balash & Vanrell 1987). The other presentation of LPD in athletes is the shortening of the luteal phase, referring to luteal phases of 10 days or less (Sherman & Korenman 1974; Jones 1976; De Souza 2003). Clinically, LPD-associated P_4 inadequacy causes asynchronous follicular growth in the subsequent menstrual cycle, compromised oocyte maturation and differentiated (out-of-phase) func-

tion of the endometrium. All of the latter factors are associated with low rates of cycle fecundity and high rates of embryonic loss, i.e. infertility and spontaneous abortion (Jones 1976; Balash & Vanrell 1987). It is important to understand that women with exercise-associated LPD continue to ovulate, although some women do so as late as day 20 (De Souza 2003), reflective of the short luteal phases that are apparent in association with some presentations of LPD. The prevalence of LPD in non-active (i.e. sedentary) women is controversial, but estimates vary from 2% to 5%, and from 3% to 20% in women with infertility (Balash & Vanrell 1987; McNeely & Soules 1988). LPD occur in athletes at a much greater prevalence of approximately 79% than is reported in non-active women, representing the most common menstrual cycle abnormality associated with exercise (De Souza 1998a). It is important to note that EAMD occurs outside the competitive athletic arena as even recreationally active women have been recognized to exhibit a high prevalence of LPD (De Souza *et al*. 1998a). For a thorough review of LPD in athletes, see a recent review by De Souza (2003).

Exercise-associated menstrual disturbances: clinical considerations

Infertility

A clinical consequence of exercise-associated amenorrhea and anovulatory cycles is temporary infertility. This represents an obvious problem for women attempting to conceive. With amenorrhea, the absence of menses is noticeable and thus the individual is aware that conception is unlikely. However, if there is a spontaneous resumption of ovulation, pregnancy is possible because ovulation may not be preceded by a return of menses. Anovulatory cycles can be accompanied with regular and consistent menstrual bleeding intervals and thus the individual is not aware that conception is unlikely. This is in sharp contrast to irregular cycle intervals indicative of oligomenorrheic cycles where ovulation is unpredictable. Although difficult to diagnose, LPD may be the most common abnormality observed in athletes and contribute to 10% of infertility and 25% of habitual abortion; however, the infertility is related to poor P_4 production rather than ovulatory problems (Soules 1988). In women desiring to become pregnant, decreases in the duration or amount of P_4 secreted during the luteal phase has been correlated with the low cycle fecundity (Jones & Madigal-Castro 1970; Strott *et al*. 1970). Blacker *et al*. (1997) found that only very small differences in luteal phase P_4 accounted for unexplained infertility in a group of women compared to aged-matched controls. No differences in existed in the number of preovulatory follicles, the rate of follicular growth, or the mean diameter before follicle rupture and timed endometrial biopsy (Blacker *et al*. 1997). Alternatively, no differences were found in salivary P_4 obtained during the days prior to implantation in cycles that resulted in conception and the levels on the same days that did not result in conception. The major determinant of cycle fecundity was the robustness of the follicular phase, as the probability of conception was significantly related to the value of the mid-follicular E_2 concentration (Lipson & Ellison 1996). The previous studies suggest that subtle changes in luteal function and/or follicular E_2 levels may have implications for fertility. To date, no studies have been performed in female athletes with LPD to assess fertility but clearly the high prevalence of LPD in recreationally active women and athletes warrants more work in this area.

Effects on the cardiovascular system

Due to an observed cardioprotective effect of estrogens, persistently low E_2 levels in amenorrheic athletes may also have adverse effects on cardiovascular health. Clinically, one of the earliest signs of cardiovascular disease is a decrease in endothelial function, and is evident decades before overt coronary artery disease is present (Celermajer 1997; Luscher & Barton 1997; Schachinger *et al*. 2000). Atherosclerotic disease progression and adverse cardiovascular events have both been shown to be associated with a decrease in endothelial function (Celermajer 1997; Luscher & Barton 1997; Schachinger *et al*. 2000). In addition, physical changes to the endothelium and the availability of

nitric oxide—an important endothelial-derived relaxing factor—are also decreased in association with atherosclerotic disease (Luscher & Barton 1997).

It is well-established that estrogen plays a significant role in endothelial-dependent blood flow via nitric oxide and has effects on cholesterol and lipoproteins (Steinberg 1987; Celermajer 1997; Luscher & Barton 1997; Mendelsohn & Karas 1999; Schachinger *et al*. 2000). As such, hypoestrogenism has been associated with endothelium-dependent dysfunction (Celermajer 1997; Luscher & Barton 1997; Schachinger *et al*. 2000). The mechanism of action of estrogen on endothelial function is likely through the nitric oxide pathway, which is known to be critical in vascular control and during reactive hyperemia (Armour & Ralston 1998; Arora *et al*. 1998; Ayres *et al*. 1998). Genomic and non-genomic effects of estrogen have been shown to play a significant role in the up-regulation of endothelial nitric oxide synthase (eNOS) and the subsequent production and increased half-life of nitric oxide (Armour & Ralston 1998; Simoncini *et al*. 2002). Some studies (Armour & Ralston 1998; Simoncini *et al*. 2002) have suggested that estrogen affects vascular endothelial release of nitric oxide through actions that enhance the bioavailability of nitric oxide (production) by up-regulating the constitutive nitric oxide synthase and, conversely, by inhibiting superoxide anion production.

Coincident with declining estrogen levels, a study by Celermajer *et al*. (1994) has shown a reduction in endothelial-dependent vasodilation as soon as 3 months after natural menopause. Impaired endothelial function has also been demonstrated 1 week after surgical menopause (Ohmichi *et al*. 2003). Given these rapid reductions in endothelial-dependent vasodilation following both surgical and natural menopause, it is logical to question the impact of clinical and subclinical levels of hypoestrogenism in physically active women.

Recent data from our laboratory has identified reduced peripheral blood flow, and in another laboratory impaired endothelial cell dysfunction in amenorrheic athletes has been reported (Zeni-Hoch *et al*. 2003). These cardiovascular findings are most likely the result of chronic hypoestrogenism. Thus, these results suggest that chronic hypoestrogenism may predispose young physically active and athletic women to early or premature cardiovascular disease. To date, only two studies have examined the impact of athletic amenorrhea on endothelial function.

Zeni-Hoch *et al*. (2003) examined brachial artery flow-mediated dilation (endothelium-dependent) in amenorrheic athletes and compared them to women with oligomenorrhea and age-matched controls. Zeni-Hoch *et al*. (2003) reported that endothelial function was 80% lower in athletes with amenorrhea, compared to athletes with either normal menstrual cycles or oligomenorrhea. Disturbingly, the magnitude of impaired endothelium-dependent vasodilation in amenorrheic athletes was comparable to data previously reported in otherwise healthy post-menopausal women (Blumel *et al*. 2003) and older (60 + 2 years) coronary-artery-disease patients (Celermajer *et al*. 1992) after a similar flow-mediated stimulus. In contrast, endothelium-independent dilation to nitroglycerine was not different amongst the groups. Recent data from our laboratory demonstrate that amenorrheic athletes had lower resting (2.2 + 0.1 vs. 4.8 + 0.4; $p < 0.001$) and peak ischemic (42.8 + 2.1 vs. 52.9 + 2.0; $p = 0.004$) blood flow responses ($mL \cdot 100\ mL^{-1} \cdot min^{-1}$) using lower limb strain-gauge plethysmography (O'Donnell *et al*. 2004). The amenorrheic athletes also had lower resting supine heart rate (50.5 + 4.8 vs. 58.8 + 1.9; $p = 0.07$) and supine resting systolic blood pressure (90.4 + 5.7 vs. 106.8 + 2.0 mmHg; $p = 0.004$), compared to their eumenorrheic counterparts. In light of the known link between estrogen and vascular function, the observed attenuated blood flow response is likely a consequence of the chronic hypoestrogenism in the amenorrehic athletes. These findings confirm altered flow-mediated endothelial-dependent vasodilation. Lower resting heart rate and systolic blood pressure may be indicative of altered autonomic regulation, similar to that seen in anorexia nervosa patients. These findings represent an additional health paradigm associated with the female athlete triad and suggests that hypoestrogenism in amenorrheic athletes may lead to deleterious cardiovascular outcomes. Thus, the clinical sequelae of hypoestrogenism in young amenorrheic women

must be extended to include detrimental effects on cardiovascular function that could have serious consequences later in life. Since cardiovascular health is of concern in this young cohort, more evaluation is warranted.

Bone health

Chronic hypoestrogenism is a major cause of bone loss in women, regardless of age (Ott 1990; Matkovic *et al*. 1994). It is well-established that the chronic hypoestrogenism coincident with menopause is a major cause of osteoporosis in women (Matkovic *et al*. 1994; Kanis 2002). Like the anorexic patient, female athletes who experience amenorrhea also experience chronic hypoestrogenism (Zipfel *et al*. 2001).

Hypoestrogenism associated with secondary amenorrhea in athletes contributes greatly to bone loss (Cann *et al*. 1984; Drinkwater *et al*. 1984, 1990; Marcus *et al*. 1985; Matkovic *et al*. 1994). Since menstrual status is a reflection of E_2 production, the most severe menstrual perturbations exhibit the greatest reductions in E_2 production, and are associated with the most severe consequences to bone. The failure to achieve peak bone mass or premature bone loss, and appropriate clinical diagnostic criteria as measured by dual energy X-ray absorptiometry (DXA), i.e. the use of *t*-scores, should be encouraged (Ott 1990; Kanis 2002; Khan *et al*. 2002). Reduced BMD in amenorrheic athletes has been repeatedly reported by many investigators (Cann *et al*. 1984; Drinkwater *et al*. 1984, 1990; Marcus *et al*. 1985; Keen & Drinkwater 1997; Tomten *et al*. 1998; Gremion *et al*. 2001; Csermely *et al*. 2002; Cobb *et al*. 2003). Most often a *t*-score of –1.0 to –2.5 is observed in athletes with amenorrhea, and is referred to as osteopenia (Kanis 2002; Khan *et al*. 2002). The clinical criterion of osteopenia is associated with a 100% increase in the risk of fracture and is notably of long-term concern for the management of bone health in these individuals. The clinical sequelae of a low peak bone mass or premature bone loss includes an increased risk of stress fractures and fractures of the hip and spine (Carbon *et al*. 1990; Otis *et al*. 1997; Korpelainen 2001). Moreover, if the clinical criterion of osteoporosis is achieved in an athlete, an even greater risk of fracture is apparent (Kanis 2002). Similar data are available in women with E_2 deficiency associated with anorexia nervosa. Since exposure to E_2, along with genetic and nutritional factors, determines peak bone mass (Matkovic *et al*. 1994; Kanis 2002), a delay in menses related to exercise training may result in a lower than normal peak value. Peak bone mass appears to be a very good predictor of the rate of post-menopausal bone loss, and higher levels will delay the risk of fracture (Ott 1990; Matkovic *et al*. 1994; Kanis 2002).

It is well-documented that amenorrheic athletes unequivocally suffer from reductions in BMD, particularly in the lumbar spine (Cann *et al*. 1984; Drinkwater *et al*. 1984, 1990; Marcus *et al*. 1985; Keen & Drinkwater 1997; Tomten *et al*. 1998; Gremion *et al*. 2001; Csermely *et al*. 2002; Cobb *et al*. 2003). Even in oligomenorrheic athletes, very low BMD scores have been repeatedly reported (Tomten *et al*. 1998; Gremion *et al*. 2001; Csermely *et al*. 2002; Cobb *et al*. 2003). In one study of oligomenorrheic athletes, lumbar BMD was only 69% of that observed in an aged-matched menstruating group of women (Cobb *et al*. 2003). The low BMD experienced by amenorrheic athletes is both severe and likely irreversible, since resumption of menses offers minimal improvements in BMD, permanently compromising the attainment of peak BMD (Drinkwater *et al*. 1986; Jonnavithula *et al*. 1993; Keen & Drinkwater 1997). Even the administration of oral contraceptives and other hormonal replacement strategies fail to significantly improve BMD in amenorrheic and oligomenorrheic athletes (Cumming 1996; Fagan 1998). Dugowson *et al*. (1991) contend that the bone loss observed in amenorrheic athletes may be serious enough to result in osteoporotic fractures well before menopause.

Based on available data, the prevalence of osteopenia in amenorrheic athletes is estimated to range from 1.4% to 50% in athletes and exercising women (Drinkwater *et al*. 1984, 1990; Dugowson *et al*. 1991; Rutherford 1993; Young *et al*. 1994; Lauder *et al*. 1999; Pettersson *et al*. 1999; Khan *et al*. 2002; Cobb *et al*. 2003). It is important to recognize that the clinical endpoint of osteopenia is not necessarily accompanied by the other two components of

the triad (disordered eating and amenorrhea). That is, the existence of even one of these problems has been associated with a decrease in BMD, presumably because both disorders are associated with varying degrees of hypoestrogenism (Cobb *et al*. 2003). Six percent of the oligomenorrheic and/or amenorrheic runners in the study by Cobb *et al*. (2003) had BMD *t*-scores that were osteoporotic, whereas 48% had BMD *t*-scores that were osteopenic. Cobb *et al*. (2003) also demonstrated that 26% of the women who were menstruating and had some evidence of disordered eating had BMD *t*-scores that were osteopenic. Sports medicine professionals should aggressively move toward the understanding that the existence of even one subclinical entity associated with the female athlete triad may have a negative clinical impact on the long-term health of these athletes and physically active women.

Although physical activity has generally been considered to be protective of BMD, some research suggests that a proportion of physically active women, even in the face of apparently normal menstrual cycles, have reduced BMD (Prior *et al*. 1990). Data reported by Prior *et al*. (1990) suggested that LPD and anovulatory cycles resulted in progressively more spinal bone loss over a 1-year period in moderate- and long-distance runners, and they attributed this loss to lower P_4 levels. Methodological problems, however, are associated with the Prior *et al*. (Prior *et al*. 1990; De Souza *et al*. 1997, 2003) data set that limit the usefulness of the information. These limitations include: (i) the inferior methods used to assess LPD, which included the use of basal body temperatures and the pooling of single blood samples from the follicular and luteal phases as indicators of ovulatory status; and (ii) the confusing effect of combining E_2-deficient anovulatory cycles with LPD cycles, since it is likely that anovulatory cycles have very different E_2 production patterns compared to ovulatory LPD cycles, and thus have the potential to impact bone health. Since then, many researchers have been unable to reproduce the results of Prior *et al*. (1990) (Lindsay *et al*. 1978; Winters *et al*. 1996; De Souza *et al*. 1997; Waller *et al*. 1998; Lindsay 1999). Moreover, in postmenopausal women, progestins, whether prescribed alone or in combination with E_2, provides neither an independent nor an additive effect on bone (Lindsay *et al*. 1978; Lindsay 1999). Simply put, the data available *do not* support any significant effect of P_4 or LPD on bone. If there is a decrease in bone mass in association with EAMD, such as LPD, and anovulation, it is likely a product of decreased exposure to E_2.

De Souza *et al*. (1997) have reported that regardless of significantly lower P_4 levels in exercising women with LPD (not combined with women with anovulation), BMD at the lumbar spine and femoral hip were comparable to that observed in ovulatory sedentary women, provided the E_2 status was adequately maintained during the cycle. In the Women's Reproductive Health Study (Waller *et al*. 1998), sedentary women with LPD failed to present with reduced BMD as well. One concern is that in the study by De Souza *et al*. (1997), significantly lower E_2 levels were found in the follicular phase in the LPD runners. Even in the ovulatory exercising women in that study, E_2 levels were lower during the early follicular phase (days 2–5). Winters *et al*. (1996) have also reported reduced E_2 levels in the early follicular phase in trained runners. Importantly, they also found that the lumbar spine BMD was lower in their runners with the reductions in follicular phase E_2, compared to the active controls. Although this finding was not statistically significant at $p = 0.074$, it was likely a product of inadequate statistical power, and the potential physiological importance of the finding should not be dismissed. A limitation of the aforementioned studies is that they are cross-sectional and limited by small sample sizes. In the Michigan Bone Health Study, Sowers *et al*. (1998a, 1998b) found that 31 sedentary women who were slightly older than the subjects in the previous studies (mean age of 37 years) and who had reduced bone mass, also had the lowest E_2 levels during the luteal phase of two comprehensively monitored menstrual cycles.

Thus, it may be necessary to follow exercising women for several years while simultaneously documenting menstrual status and E_2 exposure to definitively answer the important question of long-term effects of LPD and anovulation on BMD. It is likely that women with anovulatory cycles may certainly experience more serious consequences

due to the greater severity of estrogen depletion association with this menstrual anomaly compared to ovulatory LPD cycles.

Energetics and exercise-associated menstrual disturbances: practical considerations

Demographics

With respect to the demographics of EAMD, the highest prevalence of amenorrhea in athletes is found in sports that emphasize a low body weight such as figure skating, ballet, long-distance running and gymnastics, but more recent studies have documented menstrual abnormalities in a wide variety of sports (Erdelyi 1962; Feicht *et al*. 1978; Dale *et al*. 1979; Schwartz *et al*. 1981; Sanborn *et al*. 1982; Loucks & Horvath 1985). Amenorrhea associated with exercise is a typically a diagnosis of exclusion; other causes of reproductive disturbances, i.e. pregnancy, androgen excess, gonadal dysgenesis, uterine or premature ovarian failure, or pituitary dysfunction, must be ruled out (Warren 1996). In physically active girls who have not experienced menarche, primary amenorrhea may ensue despite these individuals exhibiting normal growth (Warren 1996). While no studies have examined the prevalence of EAMD with increasing age, increased gynecological maturity may afford some protection from disruption of menstrual cycles (Rogol *et al*. 1992). The majority of studies examining EAMD have been performed in adolescents or women in their early 20s. No studies have examined racial or ethnic differences in the prevalence or endocrine presentation of EAMD. Characterizing effects of race is important because studies in premenopausal and perimenopasual women show differences in some reproductive hormone concentrations when Caucasian and African-American women are compared (Manson *et al*. 2001; Reutman *et al*. 2002), and a high degree of variability when multiple races are studied (Randolph *et al*. 2003). Additionally, because reproductive hormones change in varying directions with age and increasing body mass index (Randolph *et al*. 2003), studies examining racial influences must adjust for these and other (smoking, alcohol use and physical activity) factors. Another factor pertinent to the assessment of racial differences in EAMD is that racial and ethnic differences in body image and dieting behavior exist (White *et al*. 2003). African-American girls may be at a lessened risk for developing eating disorders because they do not display the same degree of negative beliefs about body size and shape when compared to Caucasian girls (White *et al*. 2003).

Low energy availability in practical terms

CALORIC RESTRICTION

The evidence for modulation of reproductive function by energy availability in exercising women comes from short- and long-term prospective studies in humans and animals and from observational and cross-sectional studies (Bullen *et al*. 1985; Loucks 1989; Williams *et al*. 1995; Laughlin & Yen 1996; Wade *et al*. 1996; De Souza *et al*. 1998a; Loucks *et al*. 1998; Loucks & Thuma 2003). The suppression of reproductive function during conditions of low fuel availability is well-recognized in the animal literature, and is thought to be an adaptive response to conserve fuel for more vital bodily processes, such as cellular maintenance and locomotion, and prevent the energetically costly investment of gestation and lactation (Wade *et al*. 1996). A key difference is that a state of low energy availability in today's active women may represent a choice to consciously restrict food intake to a level below that required to match the calories expended through exercise. Numerous studies have linked EAMD, particularly amenorrhea, with significantly higher scores on psychometric inventories of eating attitudes, indicating a conscious restriction of calories associated with a drive for thinness and low body fat (Brooks-Gunn *et al*. 1987; Myerson *et al*. 1991; Wilmore *et al*. 1992; Laughlin & Yen 1996; Lebenstedt *et al*. 1999; Warren *et al*. 1999). Dietary fat is often restricted as well (Laughlin & Yen 1996; Loosli & Ruud 1998). When less severe forms of menstrual disturbances are examined, i.e. anovulation and LPD, scores on these eating inventories are still higher than those in subjects who menstruate normally (Lebenstedt *et al*. 1999).

PHYSIOLOGICAL CUES

While significant associations between weight and diet concerns and restrictive eating have been related to menstrual disturbances in female athletes, it should not be assumed that *every* athlete that has EAMD is consciously restricting her food intake. Many studies describing the endocrine aspects of EAMD included only subjects that had no history of disordered eating (Loucks *et al*. 1989, 1992). If an athlete is not consciously restricting her food but is still energy restricted, it is logical to question whether the physiological cues to eat are not well-matched to energy needs. Perhaps strenuous exercise in some individuals alters the levels of, and/or sensitivity to, physiological cues for hunger or satiety in female athletes, or whether it influences cognitive cues for, or the response to, food intake. In an effort to address a plausible mechanism for insufficient dietary intake in female athletes, one report by Hirschberg *et al*. (1994) found that the response of gastrointestinal hormone cholecystokinin (CCK), a satiety peptide produced in the gut, was reduced in female long-distance runners compared to non-athletes when a 500 kcal (2093 kJ) meal was provided. Increased hunger was also reported by the athletes (Hirschberg *et al*. 1994). In response to acute exercise, Hilton and Loucks (2000) showed that leptin, a signal for satiety, decreases with exercise only when energy availability is also decreased. When the calories expended as exercise are compensated for with increased calorie intake, leptin levels did not change significantly. In amenorrheic athletes, leptin levels are lower than would be expected for their level of body fat (Laughlin & Yen 1997). Recently, the current authors have investigated a link between EAMD and ghrelin, a recently discovered growth hormone secretagogue released from the stomach that stimulates hunger and food intake in humans (Date *et al*. 2000). Ghrelin has been directly linked to the regulation of energy homeostasis, and is one of the most powerful known orexigens (Nakazato 2001; Wren *et al*. 2001). Interestingly, one report thus far has linked intracerebroventricular ghrelin administration to a suppression of LH pulsatility in ovariectomized rats, therefore establishing a plausible mechanism linking low energy availability to a suppression of menstrual cyclicity in female athletes (Furuta *et al*. 2003). This is not surprising, as other gut peptides, such as CCK, galanin and peptide YY (PYY) (3–36), have been discovered to exist in the brain and have neuroendocrine functions (Lopez & Negro-Vilar 1990; Challis *et al*. 2003; Moran & Kinzig 2004). We recently reported that weight loss resulting from a diet and exercise intervention in young women leads to an increase in resting levels of ghrelin, and the change in ghrelin is significantly correlated with the change in body weight during the intervention (Leidy *et al*. 2004). In another study we compared concentrations of ghrelin between women grouped based on their exercise and menstrual status. Fasting ghrelin levels were assessed in the following groups of women: sedentary and ovulating normally; athletes who are ovulating normally; athletes who are anovulatory or exhibit LPD; and athletes who are amenorrheic. Fasting ghrelin concentrations were found to be significantly elevated in the amenorrheic athletes compared to all other groups (De Souza *et al*. 2004). Because the amenorrheic athletes in the study exhibited other endocrine signs of energy deficiency—i.e. low levels of triiodothyronine, insulin, leptin and other alterations in metabolics hormones (Fig. 20.3)—but had been weight stable, it appears that physiological cues to stimulate food intake are responding appropriately to a state of low energy availability, whereas other cues, perhaps cognitive in nature, are preventing a return to energy homeostasis. This would agree with the findings of elevated concentrations of ghrelin in anorexics (Otto *et al*. 2001). With respect to *how* low energy availability develops in an active woman, much still needs to be learned in order to identify the predisposing factors that predict which athletes will consciously restrict their calories in addition to their training, and which athletes may simply not be able to, or physiologically stimulated to, increase their food intake to meet their energy needs.

PREDICTION OF EXERCISE-ASSOCIATED MENSTRUAL DISTURBANCES BASED ON ENERGY INTAKE AND EXPENDITURE

Despite advances in our understanding of the

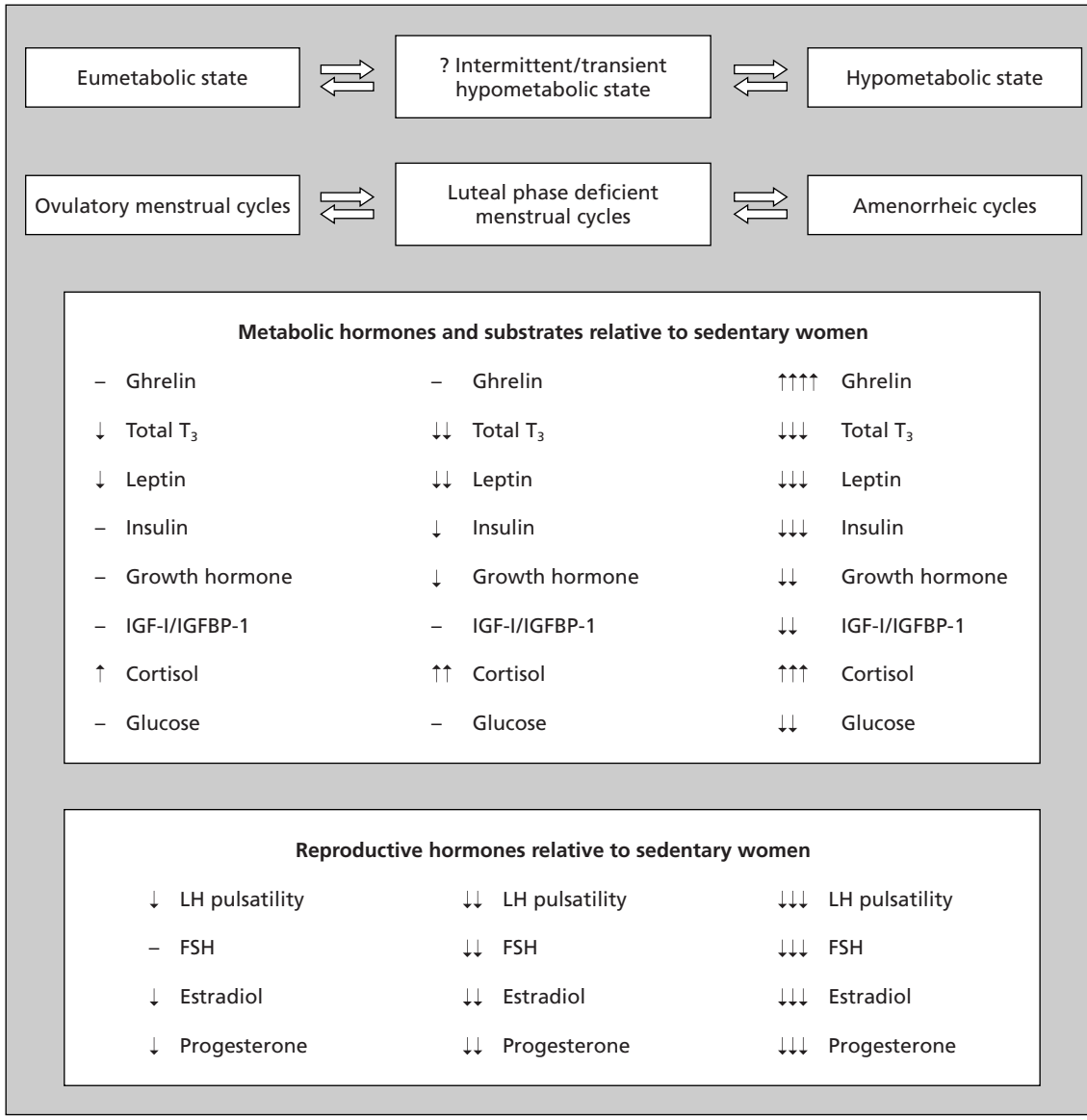

Fig. 20.3 The metabolic and reproductive hormone perturbations that have been identified to date and associated with exercise training and menstrual status, including eumenorrheic ovulatory cycles, luteal phase defect (LPD) cycles and amenorrhea. All values are depicted by arrows signifying the magnitude of the alteration reported. The proposed relationship to menstrual status is also shown. The repeated transitions from ovulatory cycles and LPD cycles are shown. Although not documented in the literature, it is likely that during recovery from amenorrhea, an individual is likely to experience LPD cycles. FSH, follicle-stimulating hormone; LH, luteinizing hormone; IGF-I, insulin-like growth factor I; IGFBP-1, insulin-like growth factor binding protein 1; T_3 triiodothyronine. (Modified and reprinted with permission from De Souza 2003.)

causal role that low energy availability plays in the etiology of EAMD, we have not yet developed practical guidelines regarding the optimal combination(s) of dietary intake and exercise energy expenditure to maintain ovulation in active women. Although it is believed that female athletes may not have to reduce their training to avoid EAMD, avoiding disruptions in menstrual function would conceivably necessitate the quantification of food intake *and* energy expenditure in order to determine how much food is required to match the level of calories expended. Because efforts to optimize performance may predispose an athlete to take in the minimum amount of calories required to prevent fatigue but maintain ovulation, it would be helpful to know 'how low you could go' without compromising reproductive function. Many (Drinkwater *et al*. 1984; Marcus 1985; Kaiserauer *et al*. 1989) but not all (Loucks 1989; Wilmore *et al*. 1992) studies of self-reported calorie intake in amenorrheic athletes report that calorie intake is lower than expected for the level of estimated energy expenditure, and lower when compared to eumenorrheic athletes with similar training regimens. It is difficult to translate the findings of these studies into advice for practitioners, because there is a tendency for most subjects to under-report food intake. Regarding the suppression of reproductive function associated with calorie restriction, neither the magnitude of the reduction in the calories consumed, nor the absolute amount of calories consumed appears to be what is sensed by gonadotropin-releasing hormone (GnRH) neurons. Support for this comes from Loucks' studies where short-term energy deficits are created by varying combinations of food intake and exercise and the resulting suppression of LH pulsatility is dependent on the magnitude of the energy deficit, regardless of if it is created through exercise or diet, or a combination (Loucks & Thuma 2003). Additionally, in studies in exercising monkeys, food intake remained constant as amenorrhea developed due solely to increases in the energy cost of daily exercise (Williams *et al*. 2001a). This dependence of EAMD on energy availability per se, and not a particular level of calorie intake or amount of exercise, presents a problem with regard to the detection and prevention of EAMD. It means that a wide range exists in the volume of exercise and or daily calorie intake that is associated with EAMD, and prevents either from being used as a benchmark. Perhaps the most progress in actually quantifying changes in energy intake and energy expenditure associated with changes in the reproductive axis in exercising women has been made by Loucks *et al*. (Loucks & Thuma 2003). A threshold of energy availability between 20 and 30 $kcal \cdot kg^{-1}$ (84 and 126 $kJ \cdot kg^{-1}$) lean body mass was identified such that no effect on the reproductive axis was observed until this threshold was achieved. When energy availability of exercising subjects was held between 20 and 30 $kcal \cdot kg^{-1}$ (84 and 126 $kJ \cdot kg^{-1}$) lean body mass, LH pulsatility, a proxy indicator of the GnRH pulse generator, was decreased. Importantly, because the initial energy availability of the subjects was 45 $kcal \cdot kg^{-1}$ (188 $kJ \cdot kg^{-1}$) lean body mass, these data also show that a decrease of energy availability by 33% had no effect on LH pulsatility, suggesting that the female reproductive axis can withstand substantial reductions in energy availability before the reproductive axis responds. Additionally, the data suggest that no change in LH pulsatility occurred even though exercise calories were as high as 840 kcal (3516 kJ), which might represent an 8-mile (13-km) run. However, whether a similar threshold to that identified by Loucks' short-term studies holds true for the effects of prolonged training on ovulation and menstrual cyclicity remains to be demonstrated. Further, whether the concept of a 'threshold' holds true for the development of less severe menstrual disturbances, such as LPD, is unknown. Ongoing studies aimed at inducing an quantifiable energy deficit with exercise combined with calorie restriction and perturbing the menstrual cycle are underway and may, in the future, provide practical guidelines for athletes and active women.

PREDICTION OF EXERCISE-ASSOCIATED MENSTRUAL DISTURBANCES BASED ON BODY WEIGHT AND BODY FAT

Predicting EAMD would be much easier if changes in reproductive function occurred at a predictable level of body weight and or body fat, as originally

proposed by Frisch and Revelle (1971). Bullen *et al*. (1985), in a prospective exercise training study over two menstrual cycles, showed that the incidence of EAMD increases with weight loss that averaged –0.45 kcal·wk^{-1} (–1.88 kJ·wk^{-1}) and –4.0 + 0.3 kg over the course of the study. Whether this rate and magnitude of weight loss represent potential guidelines for women just beginning an exercise program is questionable, since no individual correlations were reported between the magnitude of weight loss and the severity of menstrual disturbances, and menstrual cyclicity was disrupted in the group that maintained body weight. Other studies have shown that EAMD exist in women who represent a range of body weights and levels of percentage body fat (Sanborn *et al*. 1987), and the induction of amenorrhea can occur with no change in body weight (Williams *et al*. 2001a). Further, changes in reproductive hormone secretion resulting from short-term changes in nutritional intake occur without significant weight loss or gain (Bronson 1986; Cameron & Nosbisch 1991). Lastly, the effect of weight loss or the loss of body fat on reproductive function may or may not be dependent on initial body fat stores (Alvero *et al*. 1998), thereby imposing an additional interaction to consider. Therefore, advising female athletes not to lose a certain amount of weight to prevent EAMD, or to gain a certain amount of weight to restore menstrual cyclicity, is not reliable. Guidelines regarding the prevention of EAMD based on body weight are further complicated because of the large individual variability in the amount of weight lost in response to specific energy deficits (Ravussin *et al*. 2001).

REVERSING EXERCISE-ASSOCIATED MENSTRUAL DISTURBANCES BY INCREASING ENERGY AVAILABILITY

To date, the only non-medical advice provided for prevention and treatment of amenorrhea is that athletes reduce their training and increase their caloric intake. No specific dietary guidelines are available, and the rationale for reducing training may be an overly conservative approach, since exercise per se does not appear to play a role in exercise-associated amenorrhea. Quantification of the magnitude of change in energy availability associated with the induction of EAMD may be more difficult to achieve than quantification of the magnitude of change in energy availability required to restore menstrual cyclicity. One reason is that researchers have been more successful at documenting the reversal of amenorrhea (Drinkwater *et al*. 1986; Dueck *et al*. 1996; Kopp-Woodroffe *et al*. 1999; Perkins *et al*. 2001; Zeni-Hoch *et al*. 2003) in exercising women than inducing amenorrhea with a diet and/or exercise intervention (Williams *et al*. 2001a). In exercising monkeys who had developed amenorrhea, Williams *et al*. (2001b) showed that, unlike the induction of amenorrhea, the reversal (as noted by ovulation) exhibited a linear dose–response relationship with increased energy availability, such that the two monkeys that consumed 163% and 181% of their baseline intake recovered in 16 and 12 days, respectively, and two monkeys that consumed 138% and 141% recovered in 57 and 50 days, respectively. The time in days required for recovery was inversely related to the amount of extra calories consumed by each monkey ($r = -0.97$; $P < 0.02$). In contrast to the lack of change in body weight during the induction of EAMD, body weight increased significantly with the restoration of ovulation and subsequent menses. There was a significant correlation between body weight during the amenorrheic period and the time to recovery, i.e. the monkeys that weighed less recovered more quickly than the heavier monkeys, but there was no correlation between the magnitude of change in weight and the rapidity of the restoration. Overall, body weight increased 3–11%. Notably, the time to recovery for these monkeys was much shorter than the time it took to become amenorrheic.

Although the reversal of exercise-associated amenorrhea has been documented by several studies in humans, there have only been two small studies published on the reversal of amenorrhea attempted by a supervised intervention that modified diet and exercise (Dueck *et al*. 1996; Kopp-Woodroffe *et al*. 1999). Dueck *et al*. (1996) were the first to publish the results of a reversal of amenorrhea intervention in an athlete consisting of a 15-week diet and exercise intervention. The intervention consisted of increasing daily energy intake by 360 kcal (1507 kJ) and

decreasing exercise training by 1 day per week. The increase in energy intake was accomplished with a liquid supplement. At the end of the 15-week intervention, the subject remained amenorrheic, despite a 2.7 kg increase in body weight and increased body fat from 8% to 14%, likely a product of improved and positive energy balance from –155 kcal·day^{-1} (–648 kJ·day^{-1}) at baseline to +683 kcal·day^{-1} (+2855 kJ·day^{-1}) accomplished by the end of the 15-week intervention. The intervention also resulted in a 148% increase in serum LH levels. Interestingly, menstruation did resume in this amenorrheic athlete after an additional self-initiated 3 months of additional compliance to the protocol. A second study (Kopp-Woodroffe *et al*. 1999) attempted the reversal of amenorrhea with a 20-week diet and intervention in four amenorrheic athletes. The women were again provided with a 360 kcal·day^{-1} (1507 kJ·day^{-1}) liquid supplement and asked to restrict exercise training for 1 day per week. Three of the four subjects resumed normal menstrual function during or shortly following the intervention period, again likely a result of the improved energy balance of +164–292 kcal·day^{-1} (+686–1222 kJ·day^{-1}). The fourth subject withdrew from the intervention to begin hormone therapy for poor bone density. As reported in the study results, time to menstruation subsequent to ovulation in these three subjects ranged from 13 to 24 weeks. Using data reported for calorie intake and expenditure, the estimated changes in energy balance ranged from +8% to +16%.

The data from the previous studies provide the most useful information to date on how to achieve the restoration of menstrual cyclicity in amenorrheic athletes. An important consideration in attempting to reverse amenorrhea with an 'energy intervention' is that the alteration in diet, exercise, or both, be tailored to the individual's own preference. For some individuals, a reduction in training may not be as well-tolerated psychologically as an increase in calories, but for others a combination or only a change in training may be preferred. Careful monitoring of both exercise and diet is important since there may be a tendency for an athlete to unknowingly compensate for an increase in food intake with an increase in training frequency or duration (Kopp-Woodroffe *et al*. 1999). In the latter study, two out of four subjects could not completely adhere to the reduction in training called for by the intervention.

Summary and conclusions

EAMD can be associated with significant clinical outcomes, and thus continued efforts aimed at identification and prevention is necessary. Bone loss and endothelial dysfunction has been linked to hypoestrogenism secondary to amenorrhea in female athletes. The extent to which less severe EAMD are associated with changes in bone and cardiovascular function remain unclear, but is a fertile area for future research. The cause of EAMD is not completely understood but a causal relationship has been established between low energy availability and the induction of menstrual disturbances. Recovery of normal menstrual function appears to depend directly on the magnitude of change in energy availability, accomplished through an increase in food intake that may or may not be combined with decreased exercise training.

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Chapter 21

Regulation of Testicular Function: Changes in Reproductive Hormones During Exercise, Recovery, Nutritional Deprivation and Illness

SHALENDER BHASIN

Introduction

The mammalian testis is the site of germ cell development and androgen production (Rommerts 2004). Testosterone, a 19-carbon steroid secreted by the testis, is the predominant androgen in most mammals. Testosterone plays a critical role in mammalian reproduction; it is essential for maintaining sexual function, germ cell development and accessory sex organs. In the adult animal, testosterone has additional effects on the muscle, bone, hematopoeisis, coagulation, plasma lipids, protein and carbohydrate metabolism, psychosexual and cognitive function. During sexual differentiation of the mammalian fetus, testosterone masculinizes the Wolffian structures and causes the external genitalia to form a scrotum and penis. In addition, increasing testosterone levels during pubertal development promote somatic growth and virilization of boys.

Androgen production by the testes is regulated primarily by pituitary luteinizing hormone (LH), while germ cell development requires the co-ordinated action of follicle-stimulating hormone (FSH) and high intratesticular testosterone concentrations generated by the Leydig cells under the influence of LH (Rommerts 2004). Paracrine interactions between Sertoli and germ cells are also important in the regulation of spermatogenesis, although the precise role of Sertoli cell in regulation of germ cell development is not fully understood.

Testicular function is regulated by a series of feedback and feedforward mechanisms that operate at the level of the hypothalamus, the pituitary, and the testes (Fig. 21.1). Thus, LH and FSH secretion by the pituitary is stimulated by pulsatile gonadotropin-releasing hormone (GnRH) secretion, and regulated by negative feedbacks from gonadal hormones, including gonadal steroids, and inhibin and activin.

Gonadotropin-releasing hormone secretion by hypothalamic neurons

Developmental migration of GNRH neurons. The neurons that secrete GnRH originate in the region of the olfactory apparatus (Schwanzel-Fukuda & Pfaff 1989) and migrate, along the olfactory and vomeronasal nerves, into the forebrain and then into their final location in the hypothalamus (Fig. 21.2). This orderly migration of GnRH neurons requires the co-ordinated action of direction-finding molecules, adhesion proteins such as the KALIG-1 gene product and fibroblast growth receptor, and enzymes that help the neuronal cells burrow their way through intercellular matrix. Mutations of any of these proteins could arrest the migratory process and result in GnRH deficiency. In a subset of patients, failure of this developmental migration of GnRH neurons into their final location in the hypothalamus results in a clinical disorder called idiopathic hypogonadotropic hypogonadism (IHH) that is characterized by GnRH deficiency and impaired gonadotropin secretion by the pituitary (Legouis *et al*. 1991).

Hypothalamus as the integrating center for the male reproductive axis. The hypothalamus serves as the integrating center for the reproductive system and co-ordinates the regulatory signals from the higher

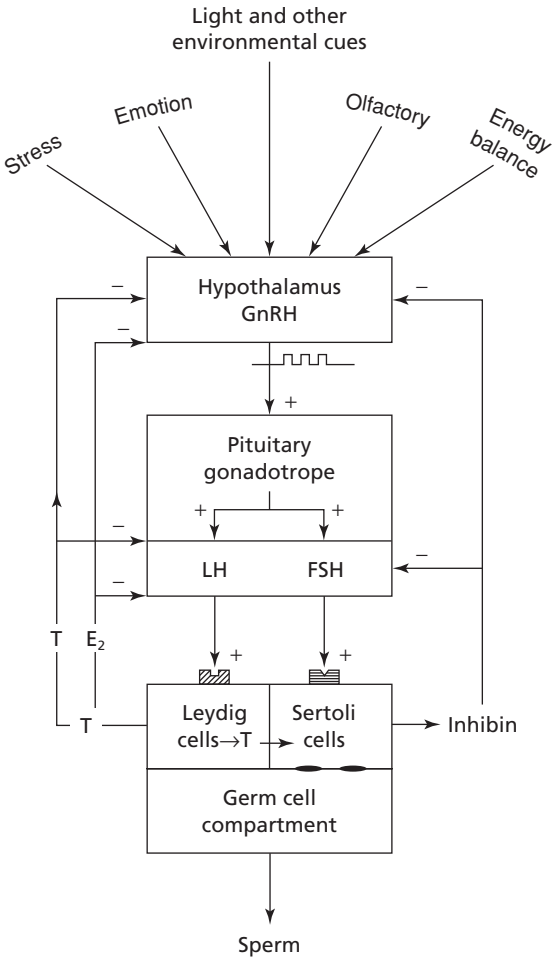


Fig. 21.1 A schematic diagram of the hypothalamic–pituitary–testicular axis. The hypothalamus serves as the integrating center for the male reproductive axis. Emotion, olfaction, energy balance, light and stress exert their effects on human reproduction through circuits that impinge on hypothalamic gonadotropin-releasing hormone (GnRH) secreting neurons. Pulsatile GnRH secretion by hypothalamic neurons stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion by the pituitary. LH binds to G-protein-coupled receptors on the Leydig cells in the testis and stimulates the production of testosterone; high intratesticular testosterone concentrations along with FSH are essential for initiating and maintaining spermatogenesis. Testosterone has a negative feedback effect on pituitary LH secretion and hypothalamic GnRH secretion directly and indirectly through its conversion to estradiol. Circulating glycoproteins secreted by the Sertoli cells, known collectively as inhibins, also regulate FSH secretion. As receptors for FSH and androgens are located on the Sertoli cells, it is generally believed that the hormonal influences on germ cells are mediated via the Sertoli cells.

centers and the feedback from the gonads (Knobil 1980; Crowley *et al*. 1991). The hypothalamus receives neural input from the central nervous system that reflects the effects of emotion, stress, light, olfactory stimuli, temperature and other environmental stimuli. The feedback signals from the gonads include steroid hormones (testosterone and estradiol) and protein hormones (inhibins and actvins).

Regulation of LH and FSH by pulsatile GnRH secretion. GnRH is a major regulator of gonadotropin secretion, and increases LH and FSH secretion from pituitary cells both *in vitro* and *in vivo*. Pulsatile secretion of GnRH is essential for maintaining normal LH and FSH secretion from the pituitary (Belchetz *et al*. 1978; Knobil 1980; Shupnik 1990; Crowley *et al*. 1991; Weiss *et al*. 1992). Continuous GnRH infusion or administration of a long-acting GnRH agonist leads to decreased LH and FSH secretion, a phenomenon referred to as down-regulation (Fig. 21.3) (Belchetz *et al*. 1978; Knobil 1980). The pattern of GnRH signal (amplitude and frequency) is important in determining the quantity and quality of gonadotropins secreted (Belchetz *et al*. 1978; Haisenleder *et al*. 1988, 1991; Kim *et al*. 1988a, 1988b; Yuan *et al*. 1988; Shupnik 1990; Weiss *et al*. 1992). Marked increase in GnRH pulse frequency also desensitizes the gonadotrope, resulting in decreased LH and FSH secretion (Belchetz *et al*. 1978; Mercer *et al*. 1988; Shupnik 1990). The electrophysiologic activity of hypothalamic GnRH neurons is linked to episodic GnRH secretion.

The transcription of *LH*-β gene is induced by pulsatile GnRH administration *in vitro* (Wierman *et al*. 1989; Shupnik 1990; Weiss *et al*. 1992). Continuous infusion of GnRH up-regulates only the α-gene transcription, but not LH or FSH β-subunit gene transcription (Haisenleder *et al*. 1988). Pulsatile GnRH administration also modifies polyadenylation of LH subunit mRNA (Weiss *et al*. 1992). The frequency of GnRH stimulus is important in differential regulation of *LH*-β and *FSH*-β genes (Haisenleder *et al*. 1988). Faster frequencies increase α and LH-β, and slower frequencies FSH-β, leading to speculation that alterations in GnRH pulse frequency may be one mechanism by which two functionally distinct gonadotropins can be regulated by a single hypothalamic-releasing hormone

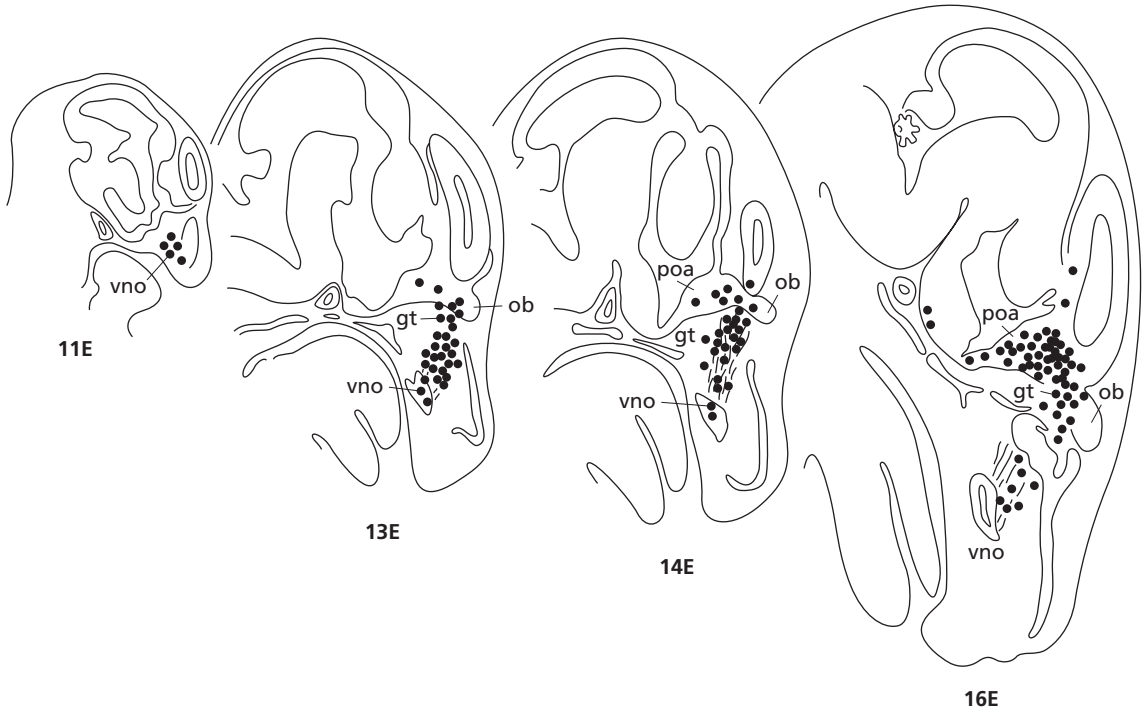


Fig. 21.2 Developmental origin and migration of the gonadotropin-releasing hormone (GnRH) secreting neurons. The neurons that secrete GnRH originate in the region of the olfactory apparatus. This figure shows the migratory route of GnRH secreting neurons in sagittal sections of the mouse embryo as determined by GnRH immunohistochemistry. On day 11, GnRH neurons are seen in proximity to the vomeronasal organ (vno) and the olfactory placode. By day 13, the migration of these neurons into the nasal septum and along the path of vomeronasal nerves and nervous terminalis is evident. By day 16, most of the GnRH neurons have entered the forebrain and some have entered into the preoptic area of the hypothalamus. gt, ganglion terminale; ob, olfactory bone; poa, preoptic area; vno, vomeronasal organ. (Reproduced from Schwanzel-Fukuda & Pfaff 1989.)

(Haisenleder *et al*. 1988). Continuous infusion of GnRH or administration of a GnRH agonist leads to a decrease in LH-β mRNA levels but α mRNA levels remain elevated (Haisenleder *et al*. 1988; Kim *et al*. 1988a, 1988b; Yuan *et al*. 1988).

A great deal of information about the physiology of GnRH secretion has emerged from examination of the LH and FSH pulse patterns in normal men and women, and from GnRH replacement studies in patients with idiopathic hypogonadotropic hypogonadism (IHH) (Urban *et al*. 1988; Crowley *et al*. 1991). Studies in such patients with hypothalamic GnRH deficiency have indicated that GnRH pulses at a dose of 25 $ng \cdot kg^{-1}$, given intravenously to men, can replicate normal pulsatile LH secretion in all its characteristics (Crowley *et al*. 1991). The peak GnRH levels achieved after intravenous administration of this dose (500–1000 $pg \cdot mL^{-1}$) are similar to those obtained in the primates by direct sampling of the hypophyseal-portal blood (Crowley *et al*. 1991). In men with IHH, an interpulse interval of 2 h appears optimum (Crowley *et al*. 1991). Increasing the frequency of GnRH pulses leads to progressive decrease in LH responsiveness to GnRH (Rebar *et al*. 1976). Decreasing the pulse frequency or increasing the interpulse interval increases the amplitude of the subsequent LH pulse. There is a linear relationship between the log of the dose of GnRH pulse and the amount of LH, FSH and free α subunit secreted (Spratt *et al*. 1986; Whitcomb *et al*. 1990). In the adult man, the magnitude of the LH response to GnRH is considerably greater than that of FSH.

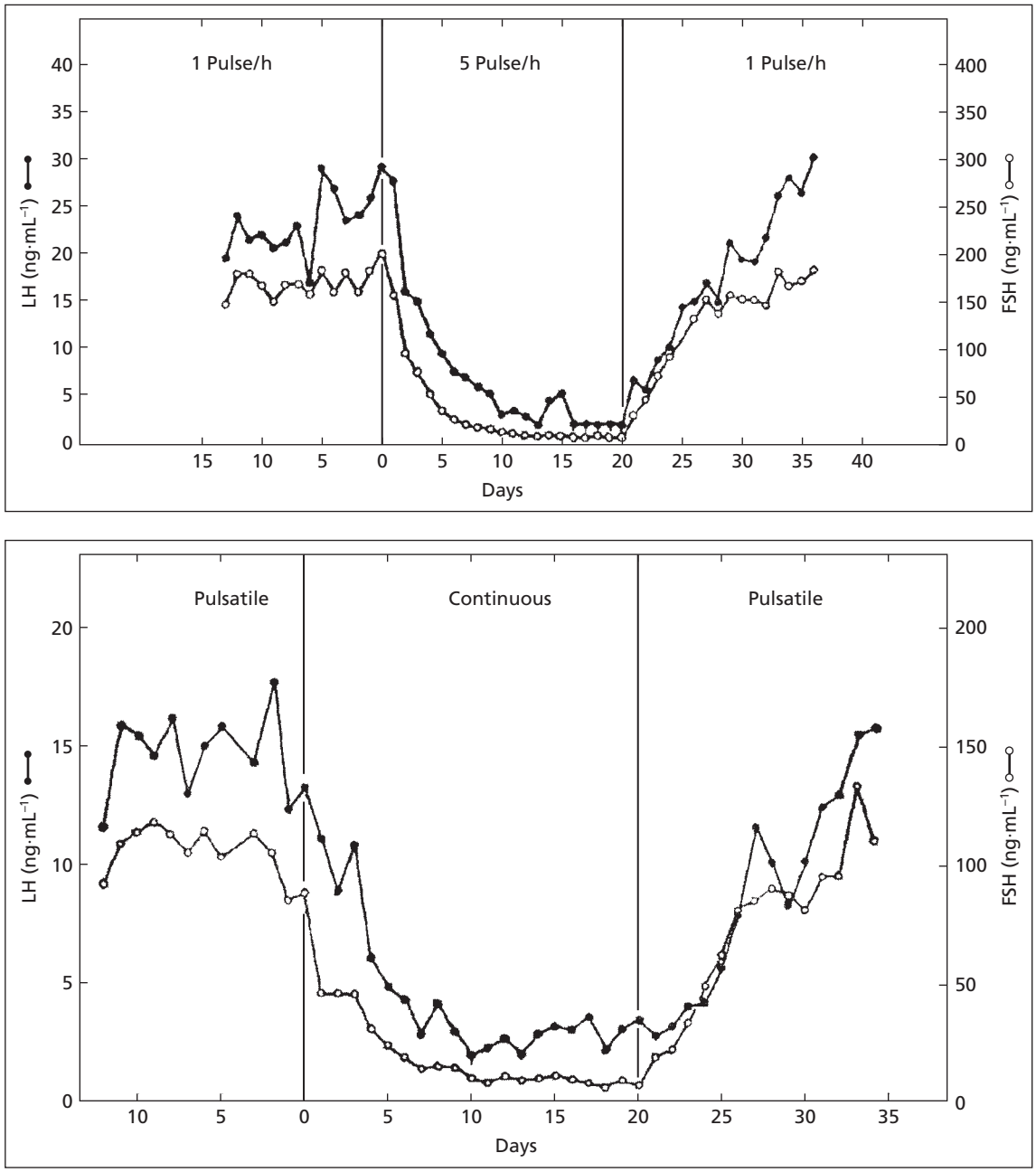


Fig. 21.3 Knobil's pioneering experiments demonstrated that the pulsatile nature of gonadotropin-releasing hormone (GnRH) signal is essential for maintaining normal luteinizing hormone (LH) and follicle-stimulating hormone (FSH) output. Knobil *et al*. (1980) ablated GnRH secreting neurons in the hypothalamus to create a monkey model that was deficient in GnRH. These monkeys also underwent gonadectomy in order to remove the feedback influences of the gonads. In this gonadectomized, GnRH deficient monkey model, pituitary LH and FSH secretion was normalized by pulsatile administration of GnRH at a frequency of one pulse per hour. Continuous infusion of GnRH decreased LH and FSH secretion, a phenomenon referred to as down-regulation. Similarly, administration of GnRH at an increased frequency of five pulses per hour resulted in decreased LH and FSH secretion. Restoration of pulse frequency to one pulse per hour restored normal LH and FSH output. (Reproduced with permission from Knobil *et al*. 1980 and Belchetz *et al*. 1978.)

Intensive sampling in normal adult men and women reveals a wide spectrum of LH pulse characteristics (Urban *et al*. 1988). The median values for LH pulse parameters in men, reported in one such recent study (Urban *et al*. 1988) were as follows: interpulse interval 55 min; LH peak duration 40 min; LH pulse amplitude 37% of basal (1.8 $mLU \cdot mL^{-1}$ incremental). Wide variations in LH pulse parameters in apparently healthy normal men and women dictate a need for caution in interpretation of mild deviations in LH pulse frequency and amplitude parameters. The sampling frequency and the paradigm used to quantitate pulse characteristics can have significant impact on the false-negative and false-positive rates and on the observed pulse parameters (Urban *et al*. 1988).

GnRH action on the gonadotrope is mediated via its binding to specific membrane receptors leading to aggregation of receptors and calcium-dependent LH release (Conn *et al*. 1981, 1982).

Gonadotropin secretion by pituitary

Functional anatomy and development of the pituitary gland. The bulk of immunocytologic evidence suggests that a single cell type within the pituitary secretes both LH and FSH (Moriarty 1973; Kovacs *et al*. 1985). Gonadotropes, the cells that secrete LH and FSH, constitute about 10–15% of anterior pituitary cells (Moriarty 1973; Kovacs *et al*. 1985) and are dispersed throughout the anterior pituitary close to the capillaries. Gonadotropes are easily demonstrable in the fetal and prepubertal pituitary gland (Childs *et al*. 1981); however, their number is low before sexual maturation. Castration leads to an increase in the size as well as the number of gonadotropes. Adenohypophyseal cells are derived from a common multipotential stem or progenitor cell. Genetic analyses of mutations associated with developmental disorders of the pituitary have revealed the molecular mechanisms of pituitary development and cell lineage determination (Ingraham *et al*. 1988; Scully & Rosenfield 2002). Co-ordinated, temporal expression of a number of homeodomain transcription factors directs the embroyological development of the pituitary and its differentiated cell types. Three homebox genes *Lbx3*, *Lbx4* and *Titf1* are essential for early organogenesis (Scully & Rosenfield 2002). Cell specialization and proliferation of differentiated cell types requires the expression of transcription factors, Pit1 and Prop1. Pit-1 has a POU-specific and a POU-homeo DNA-binding domain (Scully & Rosenfield 2002). The *Prop1* gene encodes a transcription factor with a single DNA-binding domain. While Pit-1 mutations are associated with deficiencies of growth hormone (GH), thyroid-stimulating hormone (TSH) and prolactin, mutations in Prop1 are associated with deficiencies of LH and FSH in addition to deficiencies of GH, prolactin and TSH. Expression of the *HESX1* gene precedes expression of Prop-1 and Pit-1; mutations in this gene are associated with septo-optic dysplasia and panhypopituitarism (Parks *et al*. 1999).

Biochemical structure and molecular biology of LH and FSH. The family of pituitary glycoprotein hormones includes LH, FSH, TSH and chorionic gonadotropin (CG) (Sairam 1983; Ryan *et al*. 1987; Gharib *et al*. 1990). Each of these hormones is heterodimeric, consisting of an α and a β subunit. The primary structures of the α subunits of LH, FSH, TSH and CG are nearly identical within a species; the biologic specificity is conferred by the dissimilar β subunit. Significant homology between the two subunits suggests that these subunits arose from a common ancestral gene. Individual subunits are not biologically active; formation of the heterodimer is essential for biologic activity. The subunits are linked internally by disulfide bonds; the location of the cysteines is important in determining the folding and the three-dimensional structure of the glycoprotein (Sairam 1983; Ryan *et al*. 1987; Gharib *et al*. 1990). The α subunit of LH contains two asparagine-linked carbohydrate chains, while the β-subunit chain contains one or two (Table 21.1) (Baenziger 1990). The CG β subunit also contains O-linked oligosaccharides not found on the LH dimer (Cole *et al*. 1984). Although free uncombined α subunit is secreted by the pituitary into the circulation, it is generally believed that the free β subunit is not secreted to any significant degree via this route.

Emergence of CG as a separate gonadotropin occurred relatively recently during evolution (Kornfeld & Kornfeld 1976; Fiddes *et al*. 1984). Unlike LH, which is found in the pituitaries of a

Table 21.1 General structure of the pituitary glycoprotein hormones. There is considerable homology among the glycoprotein α, LH-β, FSH-β, TSH-β and hCG-β genes and proteins. (Reproduced from Grossman *et al*. 1997.)

Subunit	Locus	Gene length (Kb)	No. of exons (introns)	mRNA length (Kb)	No. of amino acids	No. of glycosylation sites (location)*
Common α	6p21.1-23	9.4	4 (3)	0.8	92	2 (N: 52,78)†
TSH-β	1p22	4.9	4 (3)	0.7	118‡	1 (N: 23)
LH-β	19q13.3	1.5	4 (3)	0.7	121	1(N: 30)
CG-β	19q13.3	1.9§	4 (3)	1.0	145	6 (N: 13,30; S: 121,127,132,138)
FSH-β	11p13	3.9	4 (3)	1.8	111	2 (N: 7,24)

*Oligosaccharide chains are attached either to asparagine (N) (N-linked) or to serine (S) (O-linked). N or S residues are numbered according to their position in the respective sequence.
†Free subunit may also contain an additional site of O-glycosylation at threonine (T) 39.
‡118-Amino acid coding region; six amino acids can be cleaved at the C-terminal end.
§In contrast to all other glycoprotein hormone subunit genes which exist as a single copy, hCG is encoded by a cluster of six genes which vary in length.

large number of species, CG is found only in the placenta of certain mammalian species such as horses, baboons and humans (Fiddes *et al*. 1984). The α and β subunits of LH and FSH are encoded by separate genes (Fiddes *et al*. 1984). The LH–CG-β gene cluster in the human incorporates seven CG-like genes, including one which codes for the *hLH*-β gene (Fiddes *et al*. 1984). The general organization of the *LH*-β gene four exons and three introns is similar to other glycoprotein hormone β genes (Table 21.1).

Regulatory role of LH. Testosterone secretion by Leydig cells is under the control of LH. LH binds to G-protein-coupled receptors on Leydig cells and activates the cyclic adenosine monophosphate (cAMP) pathway. The luteinizing hormone–chorinonic gonadotropin (LH–hCG) receptor shares homology with other G-protein-coupled receptors such as rhodopsin, adrenergic, TSH and FSH receptors (Fig. 21.4) (McFarland *et al*. 1989; Sprengel *et al*. 1990). These G-protein-coupled receptors are transmembrane proteins that share a structural motif of seven membrane-spanning domains. The amino terminus of the receptor protein constitutes the extracellular domain. The carboxy terminus consists of the seven membrane-spanning segments and a small cytoplasmic tail, and has several serines and threonines that may serve as phosphorylation sites (McFarland *et al*. 1989; Sprengel *et al*. 1990).

LH increases the activity of the side-chain cleavage enzyme (Simpson 1979; Mori & Marsh 1982), a cytochrome P450-linked enzyme that converts cholesterol to pregnenolone, the rate-limiting step in testosterone biosynthesis. LH increases the delivery of cholesterol to the side-chain cleavage enzyme, thus increasing its capacity to convert cholesterol to pregnenolone (Simpson 1979; Mori & Marsh 1982). A steroidogenesis acute regulatory protein (STAR) makes cholesterol available to the cholesterol side-chain complex and regulates the rate of testosterone biosynthesis (Clark & Stocco 1996). Peripheral benzodiazepine receptor, a mitochondrial cholesterol-binding protein involved in cholesterol transport, is present in high concentration in outer mitochondrial membrane, and has also been proposed as an acute regulator of Leydig cell steroidogenesis. The long-term effects of LH include stimulation of gene expression and synthesis of a number of key enzymes in the steroid biosynthetic pathway, including the side-chain cleavage enzyme, 3-β hydroxysteroid dehydrogenase, 17-α hydroxylase and 17,20-lyase (Simpson 1979; Mori & Marsh 1982). Although LH also activates phopholipase C pathway, it is unclear if this pathway is essential for LH-mediated stimulation of testosterone production. In addition, insulin-like growth factor-I, insulin-like growth factor binding proteins, inhibins, activins, transforming growth factor-β, epidermal growth factor, interleukin-1, basic fibroblast growth factor, gonadotropin-releasing hormone, vasopressin and another group of poorly characterized mitochondrial

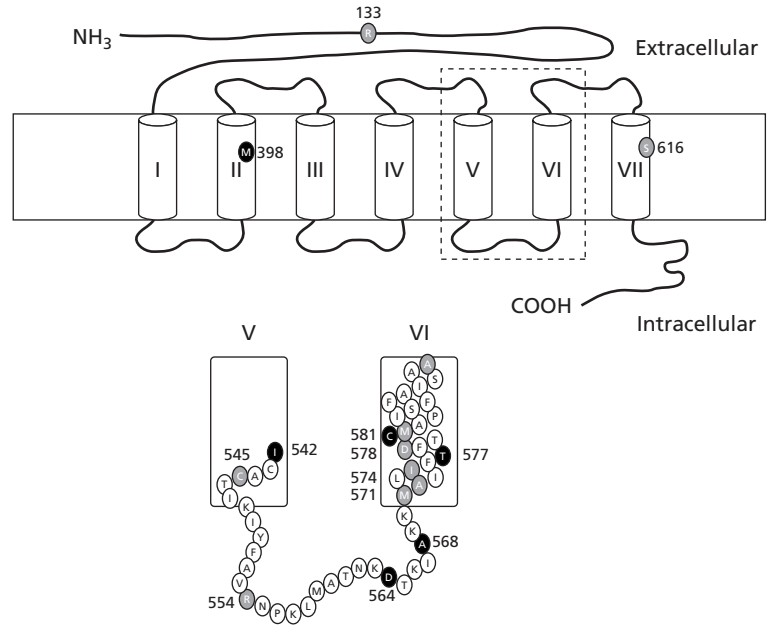


Fig. 21.4 General structure of the luteinizing hormone–chorinonic gonadotropin (LH–hCG) receptor. The normal amino acids are shown, but the activating mutations are indicated by black circles with light letters, whereas the inactivating mutations are indicated by gray circles with white letters. The corresponding codon numbers also are shown. Most activating mutations occur in the sixth transmembrane domain and third intracellular loop, whereas inactivating mutations occur in the extracellular domain, transmembrane domains V–VII and the third intracellular loop. Activating mutations include Asp578Gly, Ile542Leu, Asp564Gly, Asp578Tyr, Cys581Arg, Met571Ile, Thr577Ile, Ala568Val, Ala572Val and Met398Thr. Inactivating mutations are Cys545X, Ala593Pro, Arg554X, Ser616Tyr and Arg133Cys. An X indicates a stop codon, which is TAA, TAG or TGA. (Reproduced from Layman 1999.)

proteins have been implicated in control of steroidogenesis in Leydig cells.

The regulatory role of FSH in the male mammal. FSH binds to specific receptors on Sertoli cells and stimulates the production of a number of proteins including inhibin-related peptides, transferrin, androgen-binding protein, androgen receptor and 7-glutamyl transpeptidase. However, the role of FSH in the regulation of the spermatogenic process remains poorly understood. The prevalent dogma is that LH acts on Leydig cells to stimulate the production of high intratesticular levels of testosterone (Boccabella 1963; Steinberger 1971; Sharpe 1987). Testosterone then acts on spermatogonia and primary spermatocytes leading the germ cells through the meiotic division. FSH is felt to be essential for spermiogenesis, i.e. the maturation process by which spermatids develop into mature spermatozoa. However, evidence from experimental animals and patients with IHH treated with gonadotropins suggest that FSH plays a more complex role in maintaining quantitatively normal spermatogenesis.

In the rat and non-human primate, testosterone alone can maintain spermatogenesis when administered shortly after hypophysectomy or stalk resection (Marshall *et al*. 1983; Sharpe *et al*. 1988; Stager *et al*. 2004). However, if testosterone is given after a lapse of several weeks to months, it is much less effective in reinitiating spermatogenesis. Spermatogenesis maintained by treatment of hypophysectomized male rodent or non-human primate by testosterone is qualitatively, but not quantitatively, normal (Marshall *et al*. 1983; Sharpe *et al*. 1988; Stager *et al*. 2004). Combination of testosterone and FSH is more effective than testosterone alone in reinitiating spermatogenesis (Stager *et al*. 2004). Thus, although LH alone can maintain or reinitiate spermatogenesis, FSH is required for quantitatively normal sperm counts.

In men with prepubertal onset of both LH and FSH deficiency, LH or hCG alone is unable to initiate spermatogenesis (Bardin *et al*. 1969; Matsumoto *et al*. 1983, 1984; Finkel *et al*. 1985). On the other hand, if gonadotropin deficiency is acquired after the patient has completed pubertal development,

LH or hCG alone can reinitiate qualitatively normal spermatogenesis (Finkel *et al*. 1985). Thus, FSH is required for initiating the spermatogenic process but, once this has occurred, testosterone in high doses can maintain spermatogenesis. These observations suggest that FSH may be involved in some sort of 'programming' in the peripubertal period, after which LH alone can maintain germ cell development and maturation.

Androgen concentrations in the testis are much higher than those in serum. However, considerable confusion exists with regard to the significance of high intratesticular testosterone levels (Sharpe 1987; Sharpe *et al*. 1988; Stager *et al*. 2004). For example, stimulatory effects of exogenous testosterone on spermatogenesis in the rat are not associated with proportionate elevations of intratesticular testosterone levels. In the adult hypophysectorized or GnRH antagonist-treated monkey that is supplemented with testosterone, a direct relationship between testicular testosterone levels and spermatogenesis has not been found (Stager *et al*. 2004). The method of post-mortem collection of testicular tissue affects estimation of intratesticular testosterone concentrations (Stager *et al*. 2004). Thus, the relationship between intratesticular testosterone concentrations, FSH and spermatogenesis remains poorly understood. Androgen receptors are present on Sertoli and peritubular cells, some Leydig cells and endothelial cells of the small arterioles. However, we do not know whether androgen receptors are also present on germ cells. It is generally believed that androgen effects on spermatogenesis are mediated indirectly through Sertoli cells, although it is possible that testosterone might also directly affect germ cell development. Testosterone affects protein secretion by both round spermatids and Sertoli cells. The expression of androgen receptors is maximal in Stages VI–VII of the seminiferous epithelium; testosterone regulates germ cell apoptosis in a stage-specific manner.

The transduction of the FSH signals to the germ cells requires mediation of Sertoli cells, as FSH receptors are present on the Sertoli cells but are lacking on the germ cells. FSH receptor is also a G-protein-linked polypeptide consisting of a glycosylated extramembranous domain which is connected to its C-terminus containing seven transmembrane segments (Sprengel *et al*. 1990).

Feedback regulation of luteinizing hormone and follicle-stimulating hormone secretion

Feedback regulation by testosterone. Testosterone plays an important role in feedback regulation of gonadotropins in the male. Serum LH levels rise promptly and serum FSH gradually after castration in a number of experimental animals (Yamamoto *et al*. 1970; Badger *et al*. 1978). The mRNAs for α, LH-β (Gharib *et al*. 1986) and FSH-β (Gharib *et al*. 1987) increase after castration, although the changes in FSH-α mRNA are more modest. The postcastration rise in serum LH and LH-β mRNA levels is brought about both by an increase in the gonadotrope number and hypertrophy of individual gonadotropes (Childs *et al*. 1987). Testosterone replacement, started at the time of, or soon after, castration can attenuate the postcastration rise in α and LH-β mRNAs and serum LH levels, but has little effect on FSH-β mRNA levels (Gharib *et al*. 1986, 1987).

The effects of testosterone on FSH secretion and synthesis are complex. The net *in vivo* effect of testosterone administration to normal men is inhibition of serum FSH levels (Swerdloff *et al*. 1979; Winters *et al*. 1979). However, the direct effects of testosterone on FSH output at the pituitary level are stimulatory (Steinberger & Chowdhury 1977; Bhasin *et al*. 1987; Gharib *et al*. 1987). In isolated pituitary cell cultures, testosterone increases FSH release into the media (Steinberger & Chowdhury 1977). This is attended by a three to fourfold increase in FSH-β mRNA levels (Gharib *et al*. 1990). In intact male rats, in whom GnRH actions are blocked by administration of a GnRH antagonist, testosterone increases serum FSH levels in a dose-dependent manner (Bhasin *et al*. 1987). Bhasin *et al*. (1987) demonstrated that in castrated animals treated with a GnRH antagonist, graded doses of testosterone increase serum FSH levels. These data indicate that the stimulatory effects of testosterone on serum FSH levels are not mediated through effects on a gonadal inhibitor of FSH, but rather

directly at the pituitary level. Testosterone increases FSH-β but not LH-β mRNA levels. However in the intact male animal, testosterone inhibits GnRH-stimulated FSH secretion, accounting for the net inhibition of serum FSH levels.

Testosterone inhibits LH secretion when given to normal men and rats (Santen 1975; Matsumoto *et al*. 1984; Veldhuis *et al*. 1984). These inhibitory effects are largely exerted at the hypothalamic level, a conclusion supported by observations that testosterone decreases the frequency of LH pulses in eugonadal men (Matsumoto & Bremner 1984; Scheckter *et al*. 1989; Finkelstein *et al*. 1991a). Androgens have no direct effects on LH-β mRNA levels in rat pituitary monolayer cultures. Similarly, in GnRH antagonist-treated male rats, graded doses of testosterone lead only to an increase in FSH-β but not LH-β mRNA levels (Bhasin *et al*. 1987). In contrast to rats, in men with IHH, the amplitude of LH pulses, initiated and maintained by pulsatile GnRH therapy, is reduced by testosterone administration, indicating that in humans, testosterone has additional actions at the pituitary level in attenuating pituitary LH response to GnRH (Matsumoto *et al*. 1984; Scheckter *et al*. 1989; Finkelstein *et al*. 1991a). These studies demonstrate that testosterone or one of its metabolites inhibits gonadotropin secretion at both the pituitary and hypothalamic levels in men.

The inhibitory effects of testosterone are mediated directly by testosterone itself and indirectly through its metabolites, estradiol and dihydrotestosterone (DHT). Administration of inhibitors of aromatase or 5-α reductase is associated with increases in LH concentrations consistent with the role of estradiol and DHT in augmenting the inhibitory feedback effects of testosterone (Santen 1975; Finkelstein *et al*. 1991b; Gormley & Rittmaster 1992). However, administration of a non-aromatizable androgen, DHT, also inhibits LH secretion consistent with the proposal that aromatization of testosterone is not obligatory for mediating its inhibitory effects on LH secretion (Santen 1975). Similarly, 5-α reduction of testosterone is not essential for the LH-inhibitory effects of testosterone (Gormley & Rittmaster 1992). The hypothalamic effects of testosterone are mediated via opiatergic pathways (Veldhuis *et al*. 1984).

Feedback inhibition by estrogens. Estrogens can exert both stimulatory and inhibitory effects on gonadotropin synthesis and secretion depending on the dose, duration, the presence or absence of GnRH and other physiologic factors. Data from experimental animals suggest that the stimulatory effects of estrogens on gonadotropin synthesis and secretion *in vivo* are exerted directly at the pituitary level, while the inhibitory effects are mediated at the hypothalamic level (Neill *et al*. 1977; Clarke & Cummins 1982; Zmeili *et al*. 1986; Saade *et al*. 1989; Shupnik *et al*. 1989; Yamaji *et al*. 1992). Estrogen administration leads to a decrease in LH pulse frequency suggesting a hypothalamic site of action (Shupnik *et al*. 1989). Estradiol treatment of hypothalamic slices decreases GnRH mRNA expression (Hall & Miller 1986). Finally, transcription of all three gonadotropin subunits is negatively regulated by estradiol *in vivo*, even though the direct *in vitro* effects on pituitary are stimulatory (Neill *et al*. 1977; Clarke & Cummins 1982; Saade *et al*. 1989). Estradiol inhibits LH pulse amplitude in normal men and in GnRH-deficient men maintained on GnRH (Finkelstein *et al*. 1991b). These studies provide evidence that in men, estradiol inhibits LH by an action predominantly at the pituitary site.

Inhibins, activins and follistatins. The hypothesis that a peptide of gonadal origin selectively regulates FSH secretion was postulated over 70 years ago (McCullagh 1932); however, it wasn't until 1985 that the structure of inhibin-related peptides was finally characterized (Ling *et al*. 1985; Burger & Igarashi 1988; Vale *et al*. 1988; Ying 1988). Inhibins are dimeric proteins consisting of a common α subunit and one of two β subunits, β_A or β_B (Fig. 21.5). The heterodimers of $\alpha{:}\beta_A$ form inhibin A and $a{:}\alpha{:}\beta_B$ heterodimers form inhibin B (Vale *et al*. 1988). Inhibins A and B are equipotent in their FSH-inhibiting potency, although inhibin B appears to be the dominant circulating form of inhibin in men. In addition, β_A subunits can form homodimers called activin A or heterodimers with the β_B subunits called activin AB (Mason *et al*. 1985). Both activins A and B stimulate FSH secretion *in vitro*.

Inhibin-related peptides are widely distributed in organ systems and have significant homology with

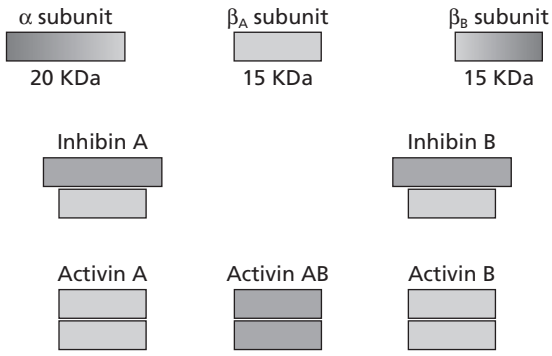


Fig. 21.5 General structure of the inhibin/activin family of gonadal hormones.

members of a family of proteins that includes Mullerian inhibiting substance, transforming growth factor-β, bone matrix proteins and the decapentaplegic gene complex of *Drosophila* (Vale *et al*. 1988; Ying 1988), which play an important role as regulators of growth and differentiation in diverse tissues. Thus, activin interacts with erythropoietin to stimulate erythropoiesis. Activin is also an important regulator of homeobox genes. In the testis, activin has been shown to regulate spermatogonial multiplication (Mather *et al*. 1990).

The role of inhibins in the adult male animal remains unclear. Immunoneutralization studies in rats reveal that infusion of anti-inhibin sera leads to an increase in serum FSH levels only in the female and prepubertal male animal, but not in the adult male animal (Rivier *et al*. 1986). These studies questioned the *in vivo* role of inhibin as an FSH regulator in the adult male. Subsequently, Culler & Negro-Vilar (1990) demonstrated that when Leydig cells in adult male rats are destroyed by a Leydig cell-specific toxin, ethane dimethane sulfonate (EDS), administration of anti-inhibin sera leads to an increase in serum FSH. These data suggest that under basal conditions in the adult male, testosterone plays a more important role in FSH regulation and that inhibin effects are unmasked only when testosterone levels are lowered. Indeed, using specific, two-site directed assays, an inverse relationship has been demonstrated between circulating inhibin B levels and FSH levels in healthy men and women, and in men with disorders of germ cell development.

Although the original inhibin hypothesis postulated inhibin as a selective regulator of FSH, under some conditions inhibins also regulate LH output (Vale *et al*. 1988). Conversely, both FSH and LH regulate inhibin production by Sertoli cells in the rat and in the human male (McLachlan *et al*. 1988; Keman *et al*. 1989; Krummen *et al*. 1989). FSH and LH both increase inhibin-α mRNA (Krummen *et al*. 1989, 1990). FSH effects on inhibin subunits are mediated via cAMP (Najmabadi *et al*. 1993).

Follistatins are another class of FSH inhibitors (Ueno *et al*. 1987) that are made up of glycosylated single chain polypeptides with homology to pancreatic secretory inhibitory protein and human epidermal growth factor. The mature follistatin protein contains four repeating domains; three are highly similar among themselves and to hEGF and PSTI. The physiologic role of follistatins is not known; emerging data suggest that they may act primarily to suppress FSH release. Follistatins are potent inhibitors of estrogen production in granulosa cells and can bind activin. Follistatins also act as binding proteins for other growth regulating proteins such as myostatin.

Activins regulate intragonadal function in both the male and the female (Vale *et al*. 1988). In the testis, activins suppress LH-stimulated testosterone production while inhibins suppress it. In granulosa cells, activins increase aromatase activity but inhibit progesterone synthesis (Vale *et al*. 1988; Ying 1988). Activin B may also act as an autocrine/paracrine mediator in the pituitary and modulate *FSH*-α gene expression.

Ontogenesis of luteinizing hormone and follicle-stimulating hormone secretion during different phases of human life

Fetal life. GnRH is demonstrable in the fetal hypothalamus at 6 weeks of gestation (Lee, P.A. 1988). Fetal pituitary contains measurable amounts of LH and FSH by 10 weeks, and by 11–12 weeks LH response to GnRH can be shown. Serum LH and FSH levels rise to a peak at about 20 weeks (Clements *et al*. 1980; Lee, P.A. 1988). In the second half of pregnancy, serum LH and FSH levels in the

fetus decline gradually. The mechanism of this decline is not known but several factors operative in the second half of pregnancy may be responsible. The rise in sex steroid secretion by the fetal gonad, the rising maternal estrogen levels and the development of negative feedback mechanisms may all contribute to tonic inhibition of the hypothalamus and the pituitary gland by the ambient sex steroid concentrations (Winter *et al*. 1975; Sizonenko 1979).

Placental hCG plays a significant role in stimulating androgen production by the fetal testis in early pregnancy (Lee, P.A. 1988). High androgen levels are required for differentiation of Wolffian structures in the male. In addition, FSH stimulates differentiation and development of seminiferous tubules. These data are consistent with observations that patients with IHH have normal differentiation of Wolffian structures and external genitalia because the placental hCG drives the fetal testis to produce sufficient androgen, even in the absence of pituitary LH and FSH. However, because of FSH deficiency, these patients have arrested or retarded development of seminiferous tubules. Testicular descent is partly dependent, during the later part of pregnancy, on androgen levels (Husmann 1991) which, during this period of fetal life, are maintained by pituitary LH.

Postnatal life and childhood years. After birth, serum LH and FSH levels rise again, albeit transiently (Winter *et al*. 1975; Sizonenko 1979). In the first 6 months of postnatal life, LH and FSH levels are measurable in blood (Winter *et al*. 1975). In fact, the pulsatile pattern of LH and FSH secretion is easily discernible during this brief period of reactivation of the hypothalamic–pituitary axis (Winter *et al*. 1976; Jakachi *et al*. 1982). Serum LH and FSH levels peak around 2–3 months of age and then decline to undetectable levels by 9–12 months; serum testosterone levels undergo similar changes. This brief period of postnatal life thus provides a window in which the normality of the hypothalamic–pituitary–gonadal axis can be assessed before gonadotropin and sex steroid levels fall back to the low range.

During childhood years, the hypothalamic–pituitary–gonadal unit is kept in abeyance until the onset of puberty (Lee, P.A. *et al*. 1976; Apter *et al*. 1978). However, the pituitary and the testis retain the ability to respond to GnRH and to hCG, respectively. The response of prepubertal pituitary to GnRH stimulus is relatively damped. In addition, the GnRH-induced rise in serum FSH in prepubertal humans is greater than that in LH. This is in contrast to an adult individual in which a single dose of GnRH causes a greater rise in LH. During pubertal maturation, serum LH and FSH levels rise (Sizonenko 1979). The activation of FSH secretion precedes that of LH. Sleep-entrained pulsatile secretion of LH is highly characteristic of early stages of puberty (Jakachi *et al*. 1982).

The use of highly sensitive two site-directed immunoradiometric, immunofluorometric and chemiluminescent assays has revealed that between 7 years of age and adulthood mean LH concentrations increase over a 100-fold, mean FSH concentrations sevenfold and estradiol levels 12-fold (Apter *et al*. 1989). The increase in FSH is gradual, but the rise in LH is steep. The changes in FSH precede the rise in LH concentrations. The mechanisms that keep GnRH secretion in check during childhood and trigger GnRH secretion at the onset of puberty remain unknown.

Changes in reproductive function during aging. There is agreement that serum testosterone levels decline progressively in men with advancing age (Fig. 21.6) (Pirke & Doerr 1973; Dai *et al*. 1981; Gray *et al*. 1991; Simon *et al*. 1992; Zmuda *et al*. 1997; Ferrini & Barrett-Connor 1998; Harman *et al*. 2001; Feldman *et al*. 2002; Matsumoto 2002); almost 25% of men over the age of 70 have serum testosterone levels in the hypogonadal range (Harman *et al*. 2001). As sex hormone binding globulin (SHBG) levels are higher in older men than in younger men, the decrease in free and bioavailable testosterone with aging is greater than the decline in total testosterone levels (Pirke & Doerr 1973; Tenover *et al*. 1987; Ferrini & Barrett-Connor 1998; Harman *et al*. 2001). The diurnal rhythm of testosterone secretion, observed in younger men, is attenuated in older men. While mean serum levels of total, free and bioavailable testosterone fall in the later decades of life, many elderly men retain serum testosterone in the normal male range. There is either no change or a modest increase in circulating estradiol and estrone levels

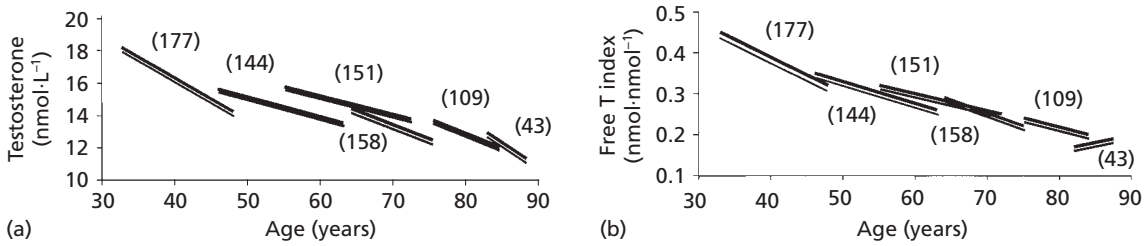


Fig. 21.6 Age-related changes in serum total testosterone levels (a) and free testosterone (T) index (b) in men participating in the Baltimore longitudinal study of aging. (Reproduced from Harman *et al*. 2001.)

with age due to the increased peripheral aromatization of androgen to estrogen (Pirke & Doerr 1973; Ferrini & Barrett-Connor 1998).

The age-related changes in reproductive hormones are compounded by the effects of concomitant illness, changes in body composition and medications. Although the data on the relationship of age to serum androgen levels are mostly cross-sectional in nature, a few longitudinal studies (Zmuda *et al*. 1997; Harman *et al*. 2001; Feldman *et al*. 2002) have confirmed the aging-associated decline in serum testosterone levels. Some studies have been criticized for selecting elderly men who were healthier than the general population. However, even after adjusting for illness, time of sampling, assay variability and medications, testosterone levels are lower in older men than in younger men.

Aging-associated decline in testosterone levels occurs due to defects at all levels of the hypothalamic–pituitary–gonadal axis. Androgen secretion by the testis of elderly men is decreased due to primary abnormalities at the gonadal level. This is supported by their higher basal LH and FSH levels (Kaufman & Vermeulen 1997; Morley *et al*. 1997; Feldman *et al*. 2002), decreased testosterone response to hCG and diminished Leydig cell mass in aging men (Longcope 1973). In addition, secondary defects may exist at the hypothalamic–pituitary level, as indicated by the somewhat blunted LH and FSH responses of older men to GnRH (Harman *et al*. 1982). The circulating levels of free α subunit are also higher in older men as compared to younger men (Harman *et al*. 1982). Older men secrete LH more irregularly than younger men (Pincus *et al*. 1997). The older men also have less synchronicity between LH and testosterone secretion than younger men (Pincus *et al*. 1997). Therefore, aging is associated with abnormalities of the normal feedback control mechanisms that control the flow of information between components of the hypothalamic–pituitary–testicular network, and a disruption of the orderly pattern of pulsatile hormonal secretion (Pincus *et al*. 1997).

Testosterone secretion, transport and metabolism

Testosterone secretion. In males of most mammalian species, 95% of circulating testosterone is derived from testicular secretion. In man, 3–10 mg of testosterone is secreted daily by the testis (Horton 1978). Direct secretion of testosterone by adrenal, and peripheral conversion of androstenedione, collectively account for another 500 μg of testosterone daily. Only a small amount of dihydrotestosterone (approximately 70 μg daily) is secreted by the human testis; most of circulating DHT is derived from peripheral conversion of testosterone (Longcope & Fineberg 1985).

Testosterone is produced in the testis by a heterogeneous group of cells that includes the adult Leydig cells, Leydig cell precursors and immature Leydig cells (Prince 2001). Studies in hypogonadotropic (hpg) mice suggest that fetal development of both Sertoli and Leydig cells is independent of gonadotrophins; however, normal differentiation and proliferation of the adult Leydig cell population requires the presence of gonadotrophins. 46,XY male humans with inactivating mutations of LH receptor have varying degree of genital ambiguity

and Leydig cell agenesis, pointing to the important role of LH in the regulation of Leydig cell development (Dufau 1988; Huhtaniemi & Toppari 1995). Sertoli cell number after birth is regulated by gonadotrophins.

Androgen transport in the body. Ninety eight percent of circulating testosterone is bound to plasma proteins: the SHBG and albumin (Vermeulen 1988; Rosner 1991). The SHBG binds testosterone with much greater affinity than albumin. Only 0.5–3.0% of testosterone is unbound. Although the prevalent dogma assumes that only the unbound fraction is biologically active, albumin-bound hormone dissociates readily in the capillaries and may be bioavailable (Pardridge 1987). Pardridge (1987) has demonstrated that albumin- and SHBG-bound androgens represent the major circulating pool of bioavailable hormone for testis or prostate. Furthermore, these investigators have argued that SHBG-sex steroid complex may be nearly completely available for influx through the blood–testis barrier or prostate plasma membrane; this view is not universally shared (Pardridge 1987).

SHBG is a glycoprotein, synthesized in the liver, that possesses high affinity binding for testosterone and estradiol (Vermeulen 1988; Rosner 1991). Hepatic production of SHBG is regulated by insulin, thyroid hormones, dietary factors and the balance between androgens and estrogens. SHBG is involved in the transport of sex steroids in plasma, and its concentration is a major factor regulating their distribution between the protein-bound and free states. Plasma SHBG concentrations are decreased by androgen administration, obesity, hyperinsulinism and nephrotic syndrome (Rosner 1991). Conversely, estrogen administration, hyperthyroidism, many types of chronic inflammatory illnesses and aging are associated with high SHBG concentrations. A locus that is associated with SHBG concentrations in African-Americans and Caucasians has been mapped to 1q44; several additional loci in African-Americans also exhibit linkage with SHBG concentrations, suggesting that many genes regulate SHBG levels (Larrea *et al*. 1995). The binding of testosterone to SHBG or albumin is not essential for androgen action; rats that are deficient in SHBG and albumin are fertile and have normal mating behavior.

Testosterone metabolism. Testosterone is metabolized predominantly in the liver (50–70%) although some degradation also occurs in peripheral tissues, particularly the prostate and the skin. Liver takes up testosterone from the blood and through a series of chemical reactions, which involve 5-α- and 5-β-reductases, 3-α- and 3-β-hydroxysteroid dehydrogenases and 17-β-hydroxysteroid dehydrogenase, converts it into androsterone, etiocholanolone, both inactive metabolites, and dihydrotestosterone and 3-α-androstanediol. These compounds undergo glucoronidation or sulfation before their excretion by the kidneys. Free and conjugated androsterone and etiocholanaolone are the predominant urinary metabolites of testosterone.

Testosterone as a prohormone: the role of DHT and estradiol in mediating androgen action. Testosterone is converted in many peripheral tissues into its active metabolites, estradiol 17-β and 5-α-DHT (Wilson *et al*. 1993; Grumbach & Auchus 1999). Aromatization of the A ring converts it into 17-β-estradiol. In addition, reduction of the δ-4 double bond can convert testosterone into 5-α-DHT. Testosterone actions in many tissues are mediated through these metabolites. For instance, testosterone effects on trabecular bone resorption, sexual differentiation of the brain, plasma lipid concentrations, atherosclerosis progression and some types of behaviors are mediated through its conversion to estrogen (Grumbach & Auchus 1999). The study of mice that have mutantions in the estrogen receptor-α, estrogen receptor-β, or the aromatase gene has provided considerable insight into the role of estrogen in male mammals (Jones *et al*. 2000). These models of estrogen deficiency exhibit significant disruption of spermatogenesis and fertility, elevated testosterone and LH levels, decreased bone mass and increased adiposity, indicating the important role of estrogens in regulation of bone mass, gonadotropin regulation, body composition and spermatogenesis (Smith *et al*. 1994; Carani *et al*. 1997). A small number of humans with inactivating mutations of the *CYP19* aromatase gene have been reported (Carani *et al*.

1997). Females with *CYP19* gene mutations are masculinized, fail to undergo pubertal development, have elevated levels of androgens and LH and FSH, polycystic ovaries and tall stature. Males with *CYP19* aromatase mutations are characterized by osteoporosis, increased bone turnover, delayed epiphyseal fusion, tall stature and increased testosterone but decreased estradiol levels (Carani *et al*. 1997).

Two isoenzymes of steroid 5-α-reductase have been characterized (Wilson *et al*. 1993; Russell & Wilson 1994). Type 1 steroid 5-α-reductase is expressed in many non-genital tissues, has been mapped to chromosome region 5p15 and has a pH optimum of 8. Type 2 steroid 5-α-reductase is expressed in the prostate and other genital tissues, has been mapped to 2p23 and has a pH optimum of 5.0 (Wilson *et al*. 1993). The biological role of 5-α-reductase type 1 has not been ascertained fully. The gene-targeting experiments suggest that type 1 enzyme plays a role in progesterone metabolism at the end of pregnancy (Wilson *et al*. 1993). The mice lacking 5-α-reductase enzyme type 1 have failure of cervical ripening and fail to deliver (Wilson *et al*. 1993).

Testosterone effects on the prostate and sebaceous glands of the skin require its 5-α reduction to DHT. DHT has been implicated in the pathophysiology of benign prostatic hyperplasia and androgenic alopecia (Wilson *et al*. 1993). Type 2 isoenzyme is the predominant form in the prostate, and has been implicated in the pathophysiology of benign prostatic hypertrophy, hirsutism and possibly male-pattern baldness. During embryonic life, testosterone controls the differentiation of the Wolffian ducts into epididymis, vasa deferentia and seminal vesicles. The development of structures from the urogenital sinus and the genital tubercle such as the scrotum, penis and penile urethra require the action of DHT. Although testosterone and DHT both exercise anabolic effects on the muscle, steroid 5-α-reductase activity is very low or absent in the skeletal muscle, and we do not know whether 5-α reduction of testosterone to DHT is obligatory for mediating androgen effects on the muscle. Similarly, the literature is unclear on whether androgen effects on sexual function in adult men are mediated through testosterone or DHT.

A large body of information about the role of DHT has emerged from the study of patients with autosomal recessive, steroid 5-α-reductase deficiency. 46,XY males with this syndrome contain normal male internal structures including testes, but exhibit ambiguous or female external genitalia at birth (Cai *et al*. 1996; Mendonca *et al*. 1996). At puberty, these individuals undergo partial virilization and normal muscular development (Cai *et al*. 1993). Many, but not all, 46,XY individuals with this disorder develop male gender identity, even if they have been brought up as females. Their development suggests that testosterone itself is able to stimulate psychosexual behavior, libido, development of the embryonic Wolffian duct, muscle development, voice deepening, spermatogenesis and axillary and pubic hair growth. In contrast, DHT is required for prostate development and growth, the development of the external genitalia and male patterns of facial and body hair growth or male-pattern baldness. All 5-α-reductase-deficient kindreds, studied to date, have been shown to have mutations in steroid 5-α-reductase type 2, the predominant form in the prostate (Cai *et al*. 1996; Mendonca *et al*. 1996).

Mechanism of androgen action. Most actions of testosterone and DHT are mediated through binding to an intracellular androgen receptor that acts as a ligand-dependent transcription factor (Zhou *et al*. 1994; Lee, D.K. & Chang 2003). Testosterone binds to the androgen receptor with half the affinity of DHT, although the maximal binding capacity is similar for both androgens. The DHT–androgen receptor complex has greater thermostability and a slower dissociation rate than the testosterone-receptor complex. This may confer greater potency to DHT in mediating androgen effects in some androgen sensitive tissues, such as the prostate.

The androgen receptor has homology to other nuclear receptors, including the receptors for glucocorticoids, progesterone and mineralocorticoids (Zhou *et al*. 1994; Lee, D.K. & Chang 2003). The predominant 919-amino acid, 110–114 kDa, androgen receptor protein has three conserved functional domains: the steroid binding domain, the DNA binding domain and the transcriptional activational

domain; of these, the central, cysteine-rich DNA binding domain is the most conserved. The single copy androgen receptor gene spans a 90 kb region on the chromosomal region Xq11–12. In the absence of its ligand, the androgen-receptor protein is distributed both in the nucleus and the cytoplasm. However, androgen binding to the receptor causes it to translocate to the nucleus; amino acid sequences between 617–633 of the androgen receptor are important for its nuclear migration and transactivation function. There is inconclusive evidence that some androgen effects may be mediated through nongenomic mechanisms through membrane receptors.

The binding of androgens by the androgen receptor results in conformational changes in this protein; there is evidence that the binding of anti-androgens to the androgen receptor might induce a different set of conformational changes (Zhou *et al*. 1994; Lee, D.K. & Chang 2003). The androgen receptor can use two transactivation domains, AF_1 and AF_2, respectively. The transactivation domain AF_1 (including the so-called 1 and 5 regions) is located in the aminoterminal part of the receptor, whereas AF_2 is located in the carboxyterminal, ligand-dependent domain. In the intact receptor, both AF_1 and AF_2 are ligand-dependent and influenced by nuclear receptor coactivators. In contrast, in a truncated androgen receptor, that is missing the ligand-binding domain, AF_1 becomes constitutively active. Hormone binding to the androgen receptor results in assembly of tissue-specific co-activators and co-repressors that determine the specificity of hormone action.

The mutations of the androgen-receptor gene have been associated with a wide spectrum of phenotypic abnormalities (Brinkmann 2001). Patients with complete androgen insensitivity present with male pseudohermaphroditism, characterized by female external genitalia, a blind vaginal pouch and well-developed breasts. Other patients with androgen-receptor mutations may have a male phenotype and milder abnormalities such as hypospadias, gynecomastia and infertility (McPhaul *et al*. 1993).

The lengths of the CAG and GCG repeats in exon 1 of the androgen-receptor gene have been linked to transcriptional activity of androgen-receptor protein. An abnormal length of the polyglutamine tract in exon 1 of the androgen receptor has been associated with spinal and bulbar muscular atrophy, also known as Kennedy's disease. Although some studies have reported an association of polymorphisms in the lengths of polyglutamine and polyglycine tracts with male infertility and risk of prostate cancer, others have not confirmed these findings (Casella *et al*. 2001).

The biochemical pathways that link energy balance and reproductive axis

Humans have known since antiquity that energy balance and nutritional status are intimately linked to the reproductive axis in both men and women. The onset of puberty, the length of the reproductive period, the number of offspring and the age of menopause have all been linked to body weight and composition, particularly the amount of body fat (Frisch & McArthur 1974; Van Der Spruy 1985; Frisch 1989; Foster & Nagatani 1999). Normal reproductive function requires an optimal nutritional intake; both caloric deprivation and consequent weight loss, and excessive food intake and obesity are associated with impairment of reproductive function. The temporal aspects of sexual maturation are more closely associated with body growth than with chronological age (Penny *et al*. 1978; Frisch 1989). In the animal kingdom, during periods of food scarcity, small animals with a short-life span may not even achieve puberty before death (Foster & Nagatani 1999). In animals with longer life spans, sexual maturation may be delayed during food deprivation. Undernutrition, caused by famine, eating disorders and exercise, results in weight loss and changes in body composition and endocrine milieu that can impair reproductive function (Penny *et al*. 1978; Bates *et al*. 1982; Rock *et al*. 1996). As a general rule, weight loss and body composition changes resulting from undernutrition are associated with reduced gonadotropin secretion; the decrease in FSH and LH levels correlates with the degree of weight loss (Fig. 21.7) (Penny *et al*. 1978). However, both hypogonadotropic and hypergonadotropic hypogonadism have been described in cachexia associated with chronic illnesses, such as

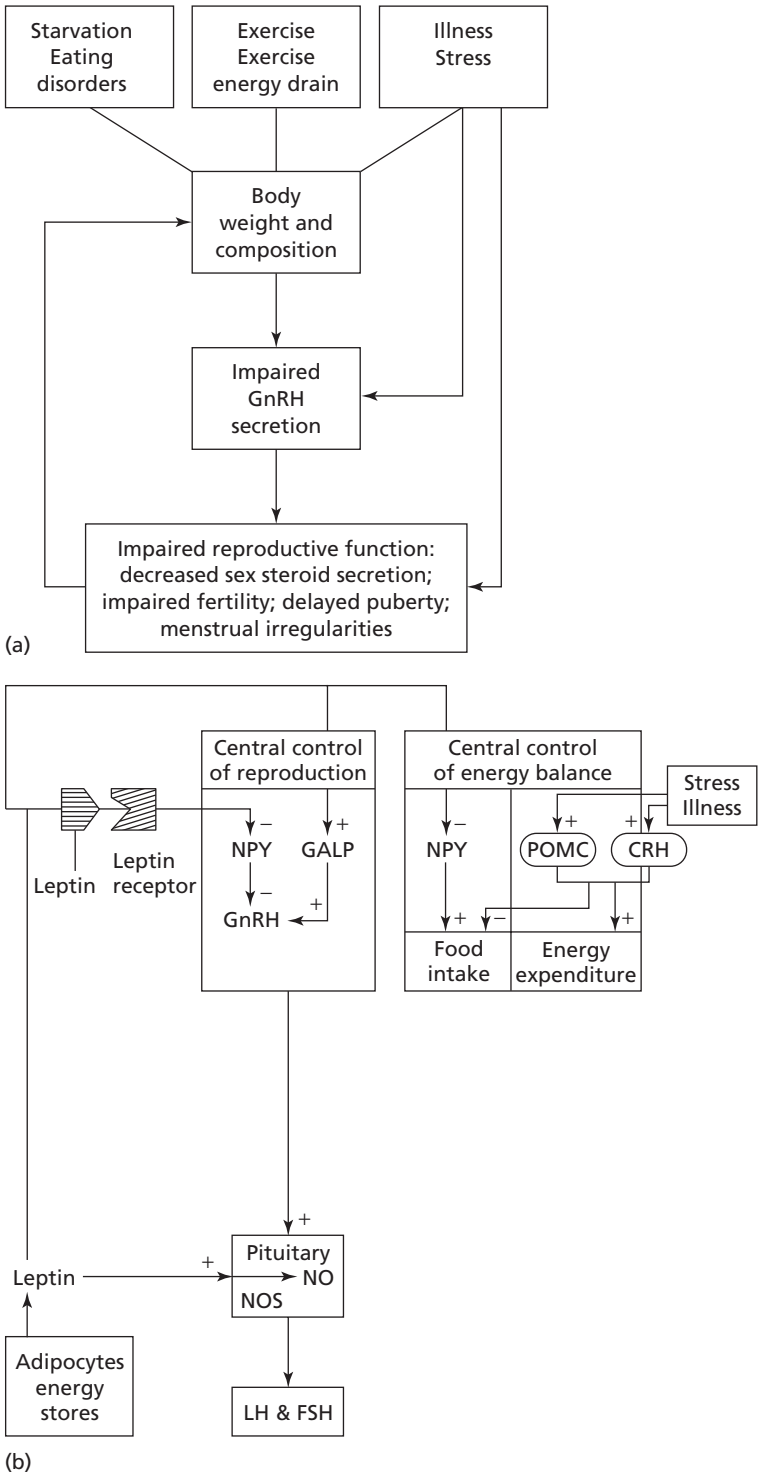


Fig. 21.7 Relationship between energy balance, exercise, illness and gonadotropin secretion. (a) Endocrine signals that emanate from adipocytes and are integrated in the hypothalamus and other central sensors regulate hypothalamic gonadotropin-releasing hormone (GnRH) secretion. In addition, products and mediators of systemic inflammatory and stress responses during illness and exposure to physical, chemical or psychological stress affect the male reproductive axis at multiple levels, resulting in impairment of sex steroid secretion, pubertal development and fertility. (b) Hypothetical depiction of the biochemical pathways that link energy balance and reproductive axis. The prevalent hypothesis is that metabolic signals that link energy stores to hypothalamic GnRH secretion originate in the adipocytes and are communicated to the hypothalamic GnRH secreting neurons through leptin, GALP and other poorly understood chemical signals. Leptin, the product of the obesity gene (*ob*) inhibits neuropeptide Y (NPY) which has a tonic inhibitory effect on GnRH secretion. Leptin also has direct stimulatory effect on luteinizing hormone (LH) secretion by stimulating nitric oxide (NO) production in the gonadotropes. Therefore, the net effect of leptin action is stimulation of LH secretion. Leptin decreases food intake while other mediators of stress response, including products of the *POMC* gene and corticotropin-releasing hormone, inhibit food intake. FSH, follicle-stimulating hormone; NOS, nitric oxide synthase. (Reproduced from Bross *et al*. 2000.)

human immunodeficiency virus (HIV) infection (Arver *et al*. 1999). Collectively, these observations provide compelling evidence that energy balance is an important determinant of reproductive function in all mammals.

We do not know the precise nature of the biochemical pathways that connect energy metabolism and the reproductive axis, two biologic systems essential for the survival of all species. The prevalent hypothesis is that the metabolic signals, that regulate hypothalamic GnRH secretion, are mediated through leptin and neuropeptide Y (Aubert *et al*. 1998; Clarke & Henry 1999; Cunningham *et al*. 1999; Foster & Nagatani 1999). Leptin, the product of the obesity (*ob*) gene, is a circulating hormone secreted by the fat cells that acts centrally to regulate the activity of central nervous system effector systems that maintain energy balance (Schwartz *et al*. 1999). Leptin stimulates LH secretion by activation of the nitric oxide synthase in the gonadotropes (McCann *et al*. 1998), and inhibits neuropeptide Y secretion. Neuropeptide Y has a tonic inhibitory effect on both leptin and GnRH secretion. Leptin also stimulates nitric oxide (NO) production in the mediobasal hypothalamus; NO stimulates GnRH secretion by the hypothalamic GnRH-secreting neurons (McCann *et al*. 1998). More recent evidence suggests that additional pathways including those that involve a galanin-like peptide (GALP) -link energy homeostasis, food intake and GnRH secretion in the hypothalamus (see Fig. 21.7) (Seth *et al*. 2004). The net effect of leptin action is stimulation of hypothalamic GnRH secretion (Schwartz *et al*. 1999).

Caloric deprivation in mammals is associated with reduced leptin levels and a concomitant reduction in LH levels (Schwartz *et al*. 1999). Leptin administration to calorically-deprived mice reverses the inhibition of gonadotropin secretion that attends food-restriction (Schwartz *et al*. 1999). Similarly, genetically *ob*/*ob* mice with leptin deficiency have hypogonadotropic hypogonadism and are infertile; treatment of these mice with leptin restores gonadotropin secretion and fertility (Mohamed-Ali *et al*. 1998; Schwartz *et al*. 1999). Thus, energy deficit and weight loss are associated with impaired GnRH secretion; in part, because of changes in neuropeptide Y and GALP activity and the consequent decrease in leptin secretion. While leptin is an important metabolic signal that links energy balance and the reproductive axis, it remains unclear whether it is the primary trigger for the activation of the GnRH-pulse generator at the onset of puberty. Emerging evidence suggests that leptin is essential but not sufficient for the initiation of puberty.

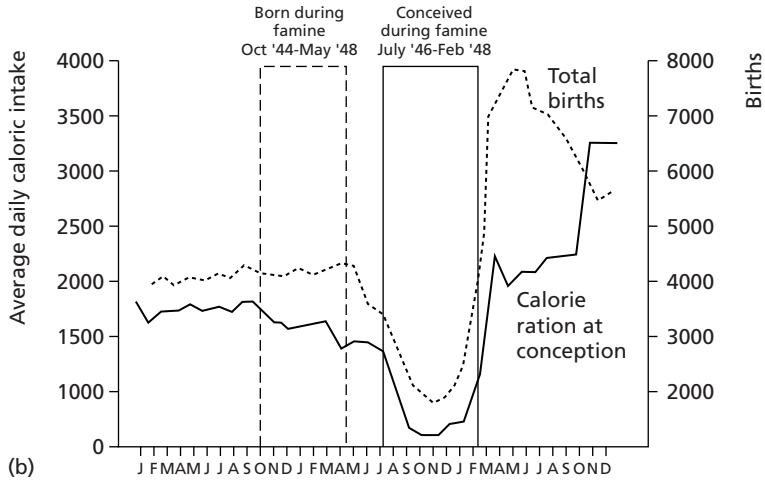
Historical and experimental illustrations of the link between nutritional status, reproduction and fertility

The Dutch Hunger Winter. Between October 1944 and May 1945, during the course of the Second World War, German army restricted food supplies in certain Dutch cities (Fig. 21.8). This resulted in substantial reduction in average daily energy intake to less than 1000 kcal (Stein *et al*. 1973) in cities affected by the German siege. In some adjacent cities, food supplies were not curtailed by the Germans (control cities). Studies by Stein *et al*. (1973) revealed that 50% of women, affected by famine, developed amenorrhea, the conception rate dropped and there was increased perinatal mortality, congenital malformations and schizophrenia. Thus, optimal caloric intake is essential for normal fertility and prenatal growth.

The association of weight change and fertility in the !Kung San of Botswana. The !Kung San of Botswana were a tribe of hunter–gatherers (Fig. 21.9) (Van Der Walt *et al*. 1978). The body weight of men and women in the tribe varied throughout the year depending upon food availability. In summer months, when food supply was abundant, body weight increased, while the nadir of body weight was achieved in winter months. The number of births in the tribe peaked about 9 months after the peak of body weight (Van Der Walt *et al*. 1978). This is another example of how the availability of food regulates fertility patterns in nature.

The Minnesota caloric deprivation experiment. In the late 1940s, Ancel Keys and coworkers studied human starvation in an experiment in which 32 young men volunteered to live on the campus of

(a)



(b)

Fig. 21.8 The Dutch Hunger Winter during the German siege. During the German siege of the Netherlands during the Second World War, residents of many Dutch cities experienced severe curtailment of caloric intake (famine cities). Adjacent cities that did not face curtailment of food supplies were used as control cities (a). (b) The relationship between caloric intake and the number of births. (Reproduced from Stein *et al*. 1973.)

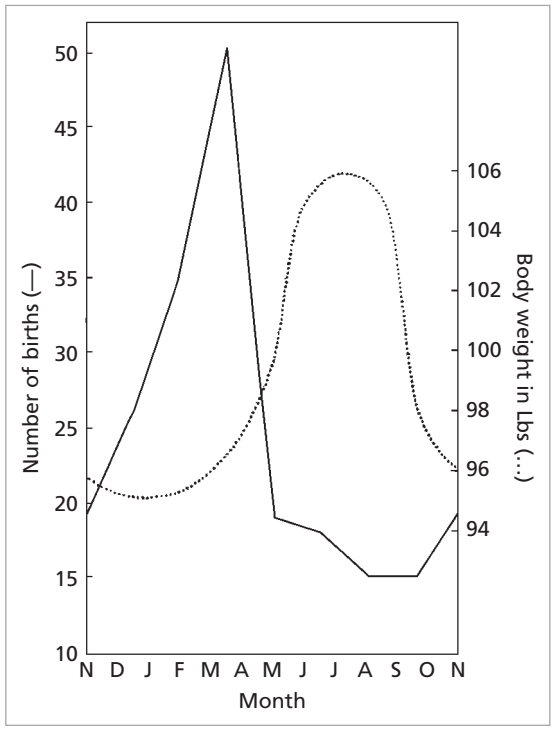


Fig. 21.9 Relationship between feeding pattern and fertility in !Kung San of Botswana. The relationship between weight change and fertility in !Kung San of Botswana. The !Kung San of Botswana were a tribe of hunter–gatherers until about 30 years ago. The body weight of the men and women in this tribe varied greatly throughout the year depending on the availability of food supplies. In the summer months, the food supply was more abundant and the body weight increased; conversely, body weight decreased throughout the winter months. The number of births in the tribe peaked 9 months after the achievement of peak body weight. These anthropological data illustrate how availability of food can regulate fertility patterns. (Reproduced from Van Der Walt 1978.)

the University of Minnesota and consume a diet providing approximately 1600 kcal·day^{-1} (6.688 MJ·day^{-1}), about two thirds of their normal energy requirement (Keys *et al*. 1950). The volunteers lost an average of 23% of their initial body weight; more than 70% of body fat and 24% of lean tissue. Decreased caloric intake and subsequent weight loss first caused a loss of libido and a reduction in prostate fluid, sperm motility and longevity. Sperm production was reduced when men weighed ~ 25% less than the normal weight for their height. Weight gain restored reproductive function.

Reproductive dysfunction in acute and chronic illnesses

There is a high prevalence of androgen deficiency defined solely in terms of low testosterone levels in men with chronic illness. Thus, even after the advent of potent anti-retroviral therapy, androgen deficiency continues to be a common complication of HIV infection in men. In an earlier survey of 150 HIV-infected men who attended our HIV clinic in 1997, approximately one third had serum total and free testosterone levels in the hypogonadal range (Arver *et al*. 1999). Other investigators have reported a similar prevalence of hypogonadism in HIV-infected men (Dobs *et al*. 1996; Grinspoon *et al*. 1996). A recent survey of HIV-infected men found the prevalence of low testosterone levels to be 20% (Reitschel *et al*. 2000). Thus, androgen deficiency continues to be a common occurrence in HIV-infected men.

In our survey, 20% of HIV-infected men with low testosterone levels had elevated LH and FSH levels, and thus had hypergonadotropic hypogonadism (Arver *et al*. 1999). These patients presumably had primary testicular dysfunction. The remaining 80% had either normal or low LH and FSH levels. The men with hypogonadotropic hypogonadism either had a central defect at the hypothalamic or pituitary site or a dual defect involving both the testis and the hypothalamic–pituitary centers. The pathophysiology of hypogonadism in HIV infection is complex and involves defects at multiple levels of the hypothalamic–pituitary–testicular axis.

In a recent study, a majority of men with chronic obstructive lung disease had low total and free testosterone levels (Casaburi *et al*. 1996). Similarly, there is a high frequency of hypogonadism in patients with cancer, end-stage renal disease on hemodialysis and liver disease (Handelsman & Dong 1993; Singh *et al*. 2001). Previous reports suggest that two thirds of men with end-stage renal disease have low total and free testosterone concentrations (Handelsman & Dong 1993). In a recent study, of the

39 men with end-stage renal disease on hemodialysis who did not have diabetes mellitus, 24 (63%) had serum total and free testosterone levels below the lower limit of normal male range (Singh *et al*. 2001). There is a high prevalence of sexual dysfunction and spermatogenic abnormalities in men on hemodialysis (Handelsman & Dong 1993). Muscle mass is decreased and fat mass increased, and muscle performance and physical function are markedly impaired in men receiving hemodialysis (Kopple 1999; Johansen *et al*. 2001). While androgen deficiency might contribute to the complex pathophysiology of sexual dysfunction and sarcopenia in men on hemodialysis, we do not know if any of these physiologic derangements can be reversed by androgen replacement.

The pathophysiology of hypogonadism in chronic illness is multifactorial; defects exist at all levels of the hypothalamic–pituitary–testicular axis (Bross *et al*. 1998). Malnutrition, mediators and products of the systemic inflammatory response, drugs such as ketoconazole and metabolic abnormalities produced by the systemic illness all contribute to a decline in testosterone production.

Low testosterone levels correlate with poor disease outcome. Low testosterone levels correlate with adverse disease outcome in HIV-infected men. Serum testosterone levels are lower in HIV-infected men who have lost weight than in those who have not (Coodley *et al*. 1994). Longitudinal follow up of HIV-infected homosexual men reveals a progressive decrease in serum testosterone levels; this decrease is much greater in HIV-infected men who progress to acquired immunodeficiency syndrome (AIDS) than in those who do not. Serum testosterone levels in HIV-infected men decline early in the course of events that culminate in wasting (Dobs *et al*. 1996). Testosterone levels correlate with muscle mass and exercise capacity in HIV-infected men (Grinspoon *et al*. 1996). Although, patients with HIV infection may lose both fat and lean tissue, the loss of lean body mass is an important aspect of the weight loss associated with wasting. There is a high prevalence of sexual dysfunction in HIV-infected men. With the increasing life expectancy of HIV-infected men, frailty and sexual dysfunction have emerged as important quality of life issues.

Similarly, muscle mass, strength and performance and physical function are markedly impaired in patients on hemodialysis (Johansen *et al*. 2001). Exercise tolerance is attenuated (Kopple 1999; Johansen *et al*. 2001); peak oxygen uptake is typically reduced to roughly half the level predicted for healthy subjects. Although the etiology of sarcopenia in end-stage renal disease is complex, the decrease in testosterone levels, a contributor to loss of muscle mass and dysfunction, is potentially reversible.

Changes in testosterone levels during exercise

The literature on the effects of exercise on testicular function is controversial in part because the published studies differed significantly in the type, mode, intensity and duration of exercise. Furthermore, few studies controlled for the confounding influence of nutritional intake and physical activity level. Therefore, it is not surprising that both increases and decreases in circulating testosterone levels have been reported in men undergoing exercise training. This is in contrast to women undergoing heavy endurance exercise training in whom consistent disruptions of menstrual cycle and ovarian estrogen production have been observed (Frisch & Revelle 1970; Mahna *et al*. 1973; Warren 1980; Baker *et al*. 1981; Veldhuis *et al*. 1985; Loucks *et al*. 1989; Cumming 1996). The onset of menarche is often delayed in ballet dancers and in girls who are engaged in heavy exercise (Frisch *et al*. 1980). Similarly, a high prevalence of menstrual irregularities, attenuated gonadotropin secretion and high cortisol levels has been documented in female runners (Baker *et al*. 1981; Villanueva *et al*. 1986); however, the literature on the literature on the effects of exercise on reproductive function in men is sparse and less lucid (MacConnie *et al*. 1986; Skarda & Burge 1998).

Broadly, exercise-training regimens can be classified into endurance and resistance exercise regimens. Although serum testosterone levels may increase in anticipation of agonistic events or an endurance exercise, most studies are in agreement that mild endurance exercise has little or no clinically significant effect on circulating testosterone

levels (Skarda & Burge 1998; Smilios *et al*. 2003). In contrast, very severe endurance exercise training as exemplified by long distance running, especially if accompanied by significant energy drain and weight loss, is associated with the lowering of serum testosterone levels (Remes *et al*. 1979; Häkkinen *et al*. 1985; MacConnie *et al*. 1986; Dressendorfer & Wade 1991; Skarda & Burge 1998). For instance, male distance runners who ran on average 92 km weekly were found to have 10% lower bone mineral density at the lumbar spine than a control group of men who did not run (Frost 1992). In general, the distance run correlates inversely with vertebral and femoral bone mineral densities; the longer the distance run, the greater the energy drain, the lower the bone mineral density. In one report of men participating in a 15-day 400-km race, serum testosterone levels declined significantly while cortisol levels increased (Dressendorfer & Wade 1991). Therefore, it is not surprising that some of the lowest testosterone levels have been reported in army recruits who are in intense boot camp training.

Remarkably, men who run 15–20 miles weekly have higher bone mineral density than age-matched controls who do not run (Frost 1992; Burrows *et al*. 2003). Similarly, rowers have been reported to have a higher bone mineral density than sedentary controls. In another study, triathletes did not differ from sedentary controls in their bone mineral density. Thus, mild to moderate endurance exercise training may have beneficial effects on bone mineral density with little or no effect on testosterone concentrations; very intense endurance exercise training lowers testosterone concentrations and decreases bone mineral density (Heinonen *et al*. 1995; Burrows *et al*. 2003).

There is agreement that long distance runners may have significant energy deficit and nutritional deficiencies that may affect bone mineral density independent of the effects of exercise on testosterone levels (Burrows *et al*. 2003). Indeed, some studies suggest that only nutritionally deleterious eating behaviors are associated with lower bone mineral density and increased fracture risk. Furthermore, some of the deleterious effects of lower sex hormone levels on bone mineral density may be offset by the direct beneficial effects of exercise and physical activity on bone mineral density.

Frost (1992) has hypothesized that the effect of exercise on bone mineral density depends crucially on the force applied to the limbs during exercise. He has speculated that only exercise regimens that apply forces on the limbs in excess of a certain threshold level are capable of eliciting a bone remodeling response, whereas long distance running that exerts forces of a lower magnitude (5–10 times the body weight) on the limbs does not increase bone mineral density. According to this hypothesis, the magnitude of bone loading is more important than the exercise mode or number of cycles. Thus, lean marathon runners or boot camp trainees, with significant energy deficits, would be expected to have lower testosterone levels, no counterbalancing beneficial effects of bone loading, and consequently lower bone mineral density.

Previous studies have reported either no change or modest increments in serum total testosterone concentrations during regimens of resistance exercise training (Remes *et al*. 1979; Truls *et al*. 2000; Ahtiainen *et al*. 2003). The small changes in testosterone concentrations reported in some studies were not sustained after completion of the exercise; in fact some studies have reported a significant decline in free testosterone levels during recovery from exercise. SHBG concentrations generally did not change during the course of resistance exercise. Some studies have reported increases in the testosterone to cortisol ratio during progressive training for maximal strength gains. There is considerable interindividual variability in hormonal response to resistance exercise training.

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Chapter 22

Hormonal and Growth Factor-Related Mechanisms Involved in the Adaptation of Skeletal Muscle to Exercise

FAWZI KADI

Introduction

Physical exercise can be assimilated to a very complex physiological stimulus that challenges diverse aspects of the cellular function. Skeletal muscle is one of the tissues that respond to exercise by undergoing a series of adjustments at the level of several of its components. The shortening velocity of skeletal muscles, the amount of force they are able to generate and their capacity to resist fatigue are important properties closely related to athletic performances. Thanks to the high degree of malleability of different muscular parameters such as fiber size, fiber type composition and capillarization, skeletal muscles adapt adequately to changes imposed by training. However, skeletal muscles will adapt differently to endurance and strength exercises, suggesting the existence of different sensing systems. Therefore the adaptive process of skeletal muscles to training can be viewed as orchestrated local and peripheral events where hormonal, mechanical, metabolic and neural factors are key regulatory signals. Changes in the rate of synthesis of hormones and growth factors, and the expression of their receptors, are important signals involved in the adaptive process allowing skeletal muscles to meet the physiological demand of different types of physical activities. A brief summary of the role played by some hormones and growth factors in muscle hypertrophy, in the regulation of muscle fiber phenotype and in the remodeling of capillary network form the substance of this chapter.

The enlargement of skeletal muscles: the role myonuclei and satellite cells

Enhanced contractile protein synthesis is an unequivocal condition for the increase in the size of muscle fibers in response to training. Both protein synthesis and degradation rates are altered during the enlargement of skeletal muscles (Goldberg *et al*. 1975). The increase in muscle protein synthesis above resting levels occurs very rapidly, between 1 and 4 h after the completion of a single bout of exercise in humans (Wong & Booth 1990; Chesley *et al*. 1992; Biolo *et al*. 1995; Phillips *et al*. 1997). At the onset of muscle hypertrophy, increased protein synthesis correlates with an increase in RNA activity (Laurent *et al*. 1978; Wong & Booth 1990). The translation of mRNA is enhanced by factors whose activity is known to be regulated by their phosphorylation state (Frederickson & Sonenberg 1993; Wada *et al*. 1996). Paralleling these changes, amino acid transport into exercising muscles is also increased following training. This would theoretically enhance the availability of amino acids for new muscle protein synthesis (Biolo *et al*. 1997).

Following this initial step of muscle fiber hypertrophy, several lines of evidence indicate that increased RNA levels (rather than RNA activity) appear to be essential for muscle fibers to continue to hypertrophy. In this regard, increased amount of mRNA can be attributed to either increased gene transcription per myonucleus or to increased number of myonuclei. Adult muscle fibers contain hundreds of myonuclei, where each myonucleus sustains the protein synthesis over a finite volume

of cytoplasm, a concept called 'nuclear domain' (Cheek 1985; Hall & Ralston 1989; Allen *et al*. 1999). It is important to note that although myonuclei are post-mitotic, they are nonetheless able to sustain the enlargement of fibers up to a certain limit after which the recruitment of new myonuclei becomes necessary. In agreement with this statement, results from animal and human studies showed that the hypertrophy of skeletal muscle fibers was accompanied by significant increases in the myonuclear number (Goldberg *et al*. 1975; Cabric & James 1983; Winchester & Gonyea 1992; Allen *et al*. 1995; Kadi 2000). In well-trained humans, the number of myonuclei in hypertrophied skeletal muscle fibers of power lifters is higher than that of sedentary subjects, and a linear relationship between the number of myonuclei and the cross-sectional area of muscle fibers is found (Kadi *et al*. 1999a; Kadi 2000). The addition of new myonuclei to the enlarged muscle fibers would play a role in the maintenance of a constant myonuclei/cytoplasm ratio, i.e. nuclear domain. The incorporation of additional myonuclei into hypertrophying human muscle fibers was reported both in young and elderly subjects (Hikida *et al*. 1998; Kadi & Thornell 2000).

While the number of myonuclei per fiber is increased during muscle fiber hypertrophy, the converse has also been documented in animal studies. A decrease in the number of myonuclei occurs as a result of an ongoing atrophy of muscle fibers induced by spinal cord transection (Allen *et al*. 1995), space flight (Allen *et al*. 1996) or hindlimb suspension (Hikida *et al*. 1997). Thus, the modulation of the myonuclear population seems to be of great importance in the regulation of fiber size. However, it is important to keep in mind that the enhancement of the myonuclear number would occur only when the transcriptional capacity of existing myonuclei becomes unable to support the enlargement of the fiber. Indeed, significant increases in myonuclear number have been observed in muscle fibers that had hypertrophied by more than 26% (Cabric & James 1983; Allen *et al*. 1995; Roy *et al*. 1999; Kadi & Thornell 2000), but not by 6.8–15.5% (Giddings & Gonyea 1992).

As existing myonuclei in an adult muscle fiber are postmitotic, muscle satellite cells are the major source for the addition of new myonuclei into hypertrophied fibers (Moss & Leblond 1971; Schiaffino *et al*. 1976). Satellite cells are located between the basal lamina and the plasma membrane of muscle fibers (Mauro 1961); they are characterized by a high nuclear to cytoplasmic ratio, a well-developed Golgi apparatus, a prominent rough endoplasmic reticulum and a heterochromatic nucleus (Campion 1984). Several stimuli can activate satellite cells, which then undergo proliferation. The daughter cells then fuse with the underlying adult muscle fiber providing new myonuclei. The involvement of satellite cell-derived myonuclei in fiber hypertrophy is further supported by experiments demonstrating that satellite cell activation and proliferation is required to support the enlargement of muscles in animal models (Rosenblatt & Parry 1992; Rosenblatt *et al*. 1994).

In parallel with muscle fiber hypertrophy, it has been shown that heavy resistance strength training induced a significant increase in the number of satellite cells in human skeletal muscles (Kadi 2000; Roth *et al*. 2001). A 46% increase in satellite cell frequency was reported in skeletal muscle of young women after 10 weeks of strength training (Kadi & Thornell 2000). More recently, an increase in the number of satellite cells was found in skeletal muscles of a group of men aged between 70 and 80 years in response to endurance training (Charifi *et al*. 2003a). Thus, satellite cells contribute to the acquisition of new myonuclei and to the renewal of their own pool (Bischoff 1994; Schultz & McCormick 1994). Finally, the proliferation of satellite cells followed by the fusion of daughter cells together would give rise to new muscle fibers (Kennedy *et al*. 1988; Yamada *et al*. 1989; McCormick & Schultz 1992; Antonio & Gonyea 1993; Kadi & Thornell 1999). The newly formed muscle fibers would replace damaged fibers or contribute to fiber hyperplasia if the number of newly generated fibers exceeds the number of injured fibers upon training (Fig. 22.1).

The effects of androgenic-anabolic steroids

The use of androgenic-anabolic steroids is accompanied by a remarkable increase in muscle size and strength in animal studies (Egginton 1987; Salmons

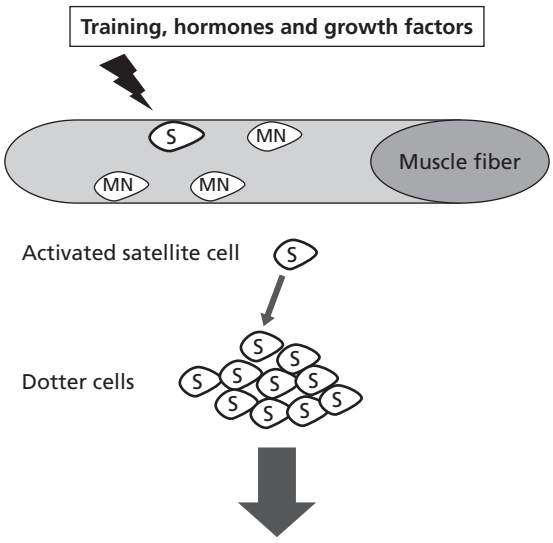

Fig. 22.1 The effects of training, hormones and growth factors on satellite cells. MN, myonucleus; S, satellite cell.

1992). The administration of supra-physiological doses of testosterone for 10 weeks in untrained and trained men produced a significant increase in muscle strength and in the cross-sectional area of the quadriceps (Bhasin *et al*. 1996). Androgenic-anabolic steroids are known to increase the rate of protein synthesis and to promote muscle growth both *in vivo* and *in vitro* (Powers & Florini 1975; Rogozkin 1979). In humans, long-term anabolic steroids usage accentuates the degree of fiber hypertrophy of muscle fibers in well-trained power-lifters (Kadi *et al*. 1999b). Skeletal muscles of power-lifters taken anabolic steroids were characterized by both extremely large fibers and high myonuclear numbers (Kadi *et al*. 1999b). Similarly, using animal models, it was also found that androgenic-anabolic steroids mediate their myotrophic effect by enhancing the myonuclear content of muscles fibers and by increasing the number of muscle fibers (Galavazi & Szirmai 1971; Sassoon & Kelley 1986; Joubert & Tobin 1989; Joubert & Tobin 1995). Thus, androgenic-anabolic steroids would increase the myonuclear number to sustain the protein synthesis of extremely hypertrophied muscle fibers (Kadi *et al*. 1999b). A main mechanism by which androgenic-anabolic steroids induce muscle hypertrophy is by activating and inducing the proliferation of satellite cells, which subsequently incorporate into muscle fibers or fuse together to form new muscle fibers. In agreement with this statement is the immunohistochemical localization of androgen receptors in cultured satellite cells indicating that anabolic steroids can act directly on muscle satellite cells (Doumit *et al*. 1996).

Androgen receptors

Blockade of androgen receptors by oxendolone, an androgen receptor antagonist, suppressed the hypertrophy caused by exercise (Inoue *et al*. 1994). Although several factors are involved in the adaptation of muscle fibers to exercise, this experiment clearly shows that androgen receptors are important mediators of the exercise-induced muscle fiber hypertrophy.

Androgen receptors belong to the family of ligand-responsive transcription regulators. When hormones bind to the receptor, it becomes activated and the hormone-receptor complex is translocated to the hormone responsive element within the nucleus. The binding to selective genes increases the rates of transcription (Luke & Coffey 1994). Early reports indicated that androgen receptors were located in the cytosol of muscle fibers (Krieg 1976; Max *et al*. 1981). Using immunohistochemistry with specific polyclonal and monoclonal antibodies, the nuclear location of androgen receptors has been clearly demonstrated in nearly all tissues (Sar *et al*. 1990; Takeda *et al*. 1990; Ruizeveld-De-Winter *et al*. 1991; Kimura *et al*. 1993; Janssen *et al*. 1994). In skeletal muscles, androgen receptors are expressed in myonuclei (Takeda *et al*. 1990; Kimura *et al*. 1993; Dorlochter *et al*. 1994; Kadi *et al*. 2000b) and in satellite cells (Doumit *et al*. 1996). In normal resting human muscle fibers, androgen receptors are expressed in some but not all myonuclei (Kadi *et al*. 2000b), and differences in androgen receptor content have been reported between human trapezius and vastus lateralis muscles (Kadi 2000). Similar intermuscular differences in androgen receptor

content have also been demonstrated in frog skeletal muscles (Dorlochter *et al*. 1994). In agreement with the intermuscular differences in androgen receptor content, it has been shown that the sensitivity of guinea pig skeletal muscles to testosterone stimulation was greatest in the head and neck region and gradually decreased from head to hindquarters (Kochakian & Tillotson 1957). Intermuscular differences in androgen receptor content might well reflect differences in embryological origins, nerve supply and functional requirements of different muscles.

Training influences the number of androgen binding sites in skeletal muscles. The increase in androgen receptors would lead to an enhancement of the sensitivity of muscles to circulating androgens. A significant increase in the number of androgen receptors has been shown to occur following endurance and strength training, and electrical stimulation in animal studies (Inoue *et al*. 1993; Deschenes *et al*. 1994). The amplitude of changes in androgen receptor content following training is muscle dependent (Hickson & Kurowski 1986; Deschenes *et al*. 1994; Kadi *et al*. 2000b). Long-term strength training is associated with changes in androgen receptor-containing myonuclei in human trapezius but not vastus lateralis muscle (Kadi *et al*. 2000b). Similar differences in the regulation of androgen receptors following exercise exist between rat soleus, extensor digitorum longus, gastrocnemius and plantaris muscles (Hickson & Kurowski 1986; Salmons 1992; Inoue *et al*. 1993; Bricout *et al*. 1994; Deschenes *et al*. 1994).

Deschenes *et al*. (1994) examined the effects of endurance and resistance training on androgen receptor content and receptor affinity to dihydrotestosterone in rat fast and slow skeletal muscles. Neither endurance nor resistance training induced alterations in androgen receptor affinity to dihydrotestosterone. Endurance training induced an enhancement in androgen binding capacity in the slow muscle whereas resistance training induced an enhancement in androgen binding capacity in the fast muscle. Thus, alterations in androgen receptor content are not only muscle dependent, they also depend upon the type of physical activity (endurance/strength exercises). The changes in androgen receptors following training occur rapidly. By 3 days of electrical stimulation, a 25% increase in the number of androgen receptors was reported in the stimulated rat gastrocnemius muscle, and was followed by a progressive hypertrophy of the muscle (Inoue *et al*. 1993).

The effects of androgenic-anabolic steroids on androgen receptor content have also been investigated. In animal models and in cultured muscle satellite cells, it has been shown that androgenic-anabolic steroids may either up-regulate (Doumit *et al*. 1996) or down-regulate (Lin *et al*. 1993; Bricout *et al*. 1994) androgen receptor content. Doumit *et al*. (1996) showed that the administration of testosterone enhanced androgen receptor immunoreactivity in porcine satellite cell nuclei. In contrast, using radio-competition assay, it has been shown that the concentration of androgen receptors was decreased following androgenic-anabolic steroids treatment in rat soleus and extensor digitorum longus muscles (Bricout *et al*. 1994). In fact, the effects of androgenic-anabolic steroids on androgen receptors might also be muscle dependent. Frog skeletal muscle fibers in the shoulder region have been shown to be more sensitive to testosterone than fibers from other regions (Regnier & Herrera 1993a, 1993b). It has also been shown that two rabbit skeletal muscles characterized by similar fiber size, fiber type composition, nerve and blood vessel supply can differ greatly in their response to androgenic-anabolic steroid treatment (Salmons 1992). Finally, long-term self-administration of androgenic-anabolic steroids in humans was associated with changes in androgen receptor-containing myonuclei in the trapezius but not in the vastus lateralis (Kadi *et al*. 2000b). Clearly, further studies are warranted to better understand the modulation of androgen receptors in skeletal muscles in response to physiological and supra-physiological conditions.

Fibroblast growth factors

The fibroblast growth factor (FGF) family comprises of ten members involved in different biological functions (Yamaguchi & Rossant 1995). Some FGF isoforms have been shown to play an important role in the enlargement of muscle fibers in response to a

physiological stimulus and others may exert their myotrophic role during the reparation of muscle fibers following fiber damage. In this respect, FGF6 is an important factor contributing to normal skeletal muscle regeneration following injury (Floss *et al*. 1997). FGF6 would be involved in important regenerative events such as the activation and proliferation of satellite cells and the expression of important myogenic regulatory factors. FGF2 and FGF4, which are localized in the myofiber peripheral matrix in adult skeletal muscles, are up-regulated during stretch mediated hypertrophy in an avian wing-weighting model (Mitchell *et al*. 1999). The up-regulation and specific cellular location of FGF2 and FGF4 support their role in the activation and proliferation of satellite cells (Mitchell *et al*. 1999). Located in the same matrix as satellite cells, it is hypothesized that the release of these FGF isoforms from heparin components might be involved in the generation of new muscle fibers following training (Yamada *et al*. 1989). Alterations in the interaction FGF/heparan-sulfate proteoglycans in exercising muscles would modulate the availability of FGF to satellite cells.

Investigating the mechanisms governing the conversion of a mechanical load into a skeletal muscle growth response, Clarke & Feeback (1996) found that the release of FGF2 increases in parallel with increased mechanical load in a tissue culture model of differentiated human skeletal muscle cells. The growth response was inhibited when the biologic activity of FGF2 was neutralized (Clarke & Feeback 1996). This experiment strongly suggests that FGF2 release is an important autocrine mechanism for transducing the stimulus of mechanical load into a skeletal muscle growth response (Clarke & Feeback 1996).

Insulin-like growth factors and receptor

Insulin-like growth factor (IGF) isoforms are produced by many tissues and are important in both embryonic and postnatal development. IGF-II is essential for normal embryonic development whereas IGF-I is important for both pre- and postnatal growth (DeChiara *et al*. 1990; Baker *et al*. 1993). Exercising skeletal muscles produce and utilize IGF-I (Brahm *et al*. 1997), and IGF-I is considered as an important factor mediating the enlargement of skeletal muscles in response to training. IGF-I is able to stimulate satellite cells proliferation, differentiation and fusion (Dodson *et al*. 1985; Florini *et al*. 1991; Quinn *et al*. 1994; Goldspink, D.F. *et al*. 1995). Immunohistochemical studies showed that IGF-I protein is expressed in satellite cells and in myotubes of regenerating rat skeletal muscles (Jennische *et al*. 1987; Jennische 1989; Jennische & Matejka 1992).

An increase in IGF-I immunoreactivity mostly within the muscle fibers was observed following an acute bout of eccentric exercise in rat tibialis anterior muscle (Yan *et al*. 1993). In humans, 7 days of strenuous exercise induced an elevation of IGF-I immunoreactivity in the vastus lateralis muscle (Hellsten *et al*. 1996). The immunostaining for IGF-I was located on nuclei that might either represent myonuclei or satellite cells (Hellsten *et al*. 1996). Enhanced IGF-I mRNA and protein in parallel with the hypertrophy of muscle fibers indicates that IGF-I is a key factor involved in the process of muscle fiber enlargement via the recruitment of muscle satellite cells (Adams & Haddad 1996). Bamman *et al*. (2001) showed that IGF-I mRNA levels increased 48 h after the completion of a single bout of concentric and eccentric muscle loading in human vastus lateralis muscle (the increase following eccentric loading being more important). In the same study, a substantial increase in androgen receptor mRNA was also found to occur following both concentric and eccentric loading.

Advances in molecular biology allowed the discovery of new IGF-I isoforms (Yang *et al*. 1996; Goldspink, G. 1999; Hameed *et al*. 2002). Two main types of IGF-I expressed in skeletal muscles have been described. The first muscle isoform with a systemic mode of action is known as muscle liver-type L.IGF-I and is similar to the main liver IGF-I (Yang *et al*. 1996). The second muscle isoform, called mechano growth factor (MGF) has been discovered in skeletal muscles subjected to stretch and overload and has an autocrine/paracrine action (Yang *et al*. 1996). In response to stretch by immobilizing the hindlimb in the extended position, a substantial increase in MGF was observed in rabbit extensor

digitorum longus (Yang *et al*. 1996). Later, it has been shown that both muscle L.IGF-I and MGF mRNAs were significantly increased in rabbit extensor digitorum longus following stretch and stretch combined with electrical stimulation at 10 Hz but not after electrical stimulation alone (McKoy *et al*. 1999). These experiments clearly showed that IGF-I isoforms mediate the enlargement of skeletal muscles mainly in response to increased mechanical load. The involvement of MGF in fiber hypertrophy has been investigated in young and elderly subjects. A significant increase in MGF mRNA occured in the young but not in the elderly subjects following 10 sets of six repetitions of single-legged knee extensor exercise at 80% of 1-repetition maximum (1-RM) (Hameed *et al*. 2003). It was concluded that the reduced MGF response to high resistance exercise in elderly subjects might indicate an age-related desensitivity to mechanical loading.

The administration of IGF-I has been considered for the treatment of various neuromuscular diseases characterized by muscular atrophy. In this respect, IGF-I has been successfully used to prevent the development of steroid-induced muscle atrophy (Kanda *et al*. 1999). At the cellular level, it has been shown that daily growth hormone/IGF-I administration combined with functional overload of rat soleus muscle results in the enlargement of muscles and a concomitant enhancement of the myonuclear number of muscle fibers (McCall *et al*. 1998).

IGF-I and IGF-II promote their growth effects through IGF-I receptor. Mice with disturbed IGF-I receptor die soon after birth (Baker *et al*. 1993). The lack of IGF-I receptor induces severe hypoplasia in mice suggesting that IGF-I receptor is essential for the establishment of a mature skeletal muscle (Baker *et al*. 1993). While functional inactivation of the IGF-I receptor induced a marked muscle hypoplasia and a decrease in MyoD and myogenin levels (two important members of the myogenic regulatory factors) (Fernandez *et al*. 2002), the opposite occurs when the IGF-I receptor is overexpressed (Quinn *et al*. 1994). Therefore, IGF-I receptor is currently considered as a key regulator of muscle mass via its action on muscle-specific genes. A single acute bout of exercise has been shown to be accompanied by a significant increase in the IGF-I receptor binding capacity and affinity as well as in IGF-I receptor mRNA in rat skeletal muscle (Willis *et al*. 1997). Similarly, long-term training induced a significant increase in IGF-I and insulin receptor content (Willis *et al*. 1998). Altogether, these findings strongly suggest that IGF-I and IGF-I receptors are essential mediators of muscle fiber hypertrophy.

Capillarization of skeletal muscles

Blood vessels in the skeletal muscles form a rich network of capillaries around muscle fibers. Capillaries consist of a single layer of endothelial cells surrounded by a luminal glycocalyx and an abluminal basement membrane. Located at the end of the cardiorespiratory system, the capillary network plays an important role in nutrients, and oxygen and carbon dioxide exchange with muscle fibers. In response to endurance training, a remodeling of the capillary bed has been shown to occur in different human skeletal muscles (Andersen, P. & Henriksson 1977; Hudlicka *et al*. 1992; Wang *et al*. 1993; Kadi *et al*. 2000a; Charifi *et al*. 2003b).

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a heparin-binding endothelial cell-specific mitogen mediating angiogenesis in different tissues. Both exercise and hypoxia can cause an increase in VEGF mRNA in human skeletal muscle (Gustafsson *et al*. 1999; Richardson *et al*. 1999). VEGF mRNA increased in response to a single bout of exercise at a work load corresponding to 50% of peak work load in normal healthy subjects and in patients with chronic renal failure (Wagner *et al*. 2001). The receptors for VEGF (flt-1 and flk-1) are also up-regulated following both exercise and hypoxia (Takagi *et al*. 1996; Gerber *et al*. 1997; Olfert *et al*. 2001). Thus, VEGF and its receptors are involved in the densification of the capillary network in response to physical activity. There is a strong increase in VEGF mRNA following a single bout of exercise in untrained human skeletal muscles. VEGF response is attenuated in skeletal muscle of trained subjects (Richardson *et al*. 2000). This might reflect an initial intense proliferation of capillaries in untrained muscles occurring at the

beginning of a training program, followed by a period where the enhancement of the capillarization occurs at a slower rate and requires high doses of training as the training status of muscles is improved.

Among factors controlling the expression of VEGF, hypoxia inducible factor 1 (HIF-1) subunit is currently considered as a major regulatory factor. The effects of short-term exercise training on VEGF and HIF-1 have been studied in eight healthy males. Although VEGF levels increased following seven training sessions, no changes in the levels of HIF-1 mRNA subunits were found (Gustafsson *et al*. 2002). This might indicate that an increase in HIF-1 mRNA is not the only factor involved in the regulation of training-induced VEGF enhancement. However, the intervention of different factors controlling the remodeling of the capillary network probably occurs during serial transient time frames making it difficult to assess their importance unless the whole adaptive process is monitored.

FGFs are also believed to play a role in skeletal muscle angiogenesis. However, recent studies suggest that their contribution to angiogenesis is less important than that played by VEGF (Richardson *et al*. 2000; Wagner *et al*. 2001). At present, although it is suggested that VEGF is the most important angiogenic factor involved in the adaptation of the capillary network in human skeletal muscles, further studies are needed to advance knowledge about the importance of all angiogenic factors in regulating skeletal muscle angiogenesis.

Contractile properties of muscle fibers

The existence of numerous types of fibers makes skeletal muscle a very heterogeneous tissue able to perform various functional capabilities. Immunohistochemical and biochemical analysis of skeletal muscles revealed that this diversity in muscle fiber types reflects a broad spectrum of myosin isoforms. The myosin is the molecule primarily responsible, along with actin, for muscle contraction. The myosin molecule is made up of two heavy chains (MyHC) and four light chains (MyLC) (Schiaffino & Reggiani 1996; Pette & Staron 1997). The heavy chain portion of the myosin molecule exists in multiple isoforms and represents a major determinant of the force-velocity properties of muscle fibers. The four most important MyHC isoforms expressed in adult skeletal muscle fibers are: MyHC Iβ, MyHC IIA, MyHC IIX/IID and MyHC IIB. Each isoform is characterized by a specific shortening velocity and force production. Fibers containing MyHC I have a slow contracting velocity and produce less force than fibers containing MyHC IIA, IIX and IIB. Within muscle fibers containing fast MyHCs, the fastest and strongest are those containing MyHC IIB followed by fibers expressing MyHC IIX and MyHC IIA (Bottinelli *et al*. 1994a, 1994b).

The contractile properties of skeletal muscles are able to undergo significant changes in response to exercise. It is generally accepted that endurance training results in a fast to slow transition of MyHC isoforms (Baumann *et al*. 1987; Schaub *et al*. 1989), whereas strength training causes an increase in MyHC IIA and a decrease in MyHC IIX (Staron *et al*. 1991; Adams *et al*. 1993; Andersen, J.L. *et al*. 1994; Fry *et al*. 1994; Kraemer *et al*. 1995; Kadi & Thornell 1999; Andersen, J.L. & Aagaard 2000). It is also suggested that muscle fibers containing MyHC IIX are seldom recruited in the normal daily activities of most people. If they become more recruited, as it is the case during training, they are converted into fibers containing MyHC IIA (type IIA fibers being more fatigue resistant than type IIX fibers) (Goldspink, G. *et al*. 1991; Staron *et al*. 1991; Kraemer *et al*. 1995). During endurance or strength training, the hormonal environment of skeletal muscles is greatly disturbed and these alterations are powerful signals able to trigger changes in myosin expression in exercising muscles.

Effects of testosterone

In some animal studies, a fast-to-slow shift of MyHC isoforms has been observed following androgenic-anabolic steroid treatment (Fritzsche *et al*. 1994; Czesla *et al*. 1997). An increase in fibers containing MyHC IIA and a decrease in fibers containing MyHC IIB has been reported in several skeletal muscles in rodents in response to androgenic-anabolic steroid administration (Egginton 1987;

Dimauro *et al*. 1992). In contrast, an androgenic steroid-induced decrease in MyHC IIA-containing fibers in favor of MyHC IIB-containing fibers has also been reported (Kelly *et al*. 1985; Lyons *et al*. 1986; Salmons 1992). These results suggest that the effects of androgenic-anabolic steroids on the contractile properties might be muscle-dependant and might also vary between species. In fact, there is other evidence indicating that androgenic-anabolic steroids have no significant effects on muscle fiber type composition. For example, muscle overload in animal experiments induces an increase in the expression of slow MyHC I, and the addition of androgenic-anabolic steroids did not alter the pattern of MyHC expression (Boissonneault *et al*. 1987). Likewise, the administration of androgenic-anabolic steroids did not modify the slow-to-fast shift in MyHC isoforms caused by hindlimb suspension experiments (Tsika *et al*. 1987). Finally, in well-trained power-lifters, there were no differences in trapezius MyHC composition between androgenic-anabolic steroid users and non-users (Kadi *et al*. 1999b).

Effects of estrogen

It is well-known that a reduction in force production occurs at menopause (Greeves *et al*. 1999; Dionne *et al*. 2000; Meeuwsen *et al*. 2000). At the cellular level, it has been shown that ovariectomy is associated with a fast-to-slow shift in MyHC isoforms and a reduction in the level of spontaneous running activity in rats (Kadi *et al*. 2002). MyHC changes tend to follow a general pathway of sequential transition in the order MyHC I ← IIA ← IIX ← IIB. This result can be interpreted as an overall transition in the expression of fast isoforms towards slower isoforms, a specific up-regulation of the slower isoforms of *MyHC* genes, or a specific down-regulation of genes coding for fast MyHC isoforms following ovariectomy. When ovariectomized animals are allowed to run and are treated with estrogen, there were no alterations in MyHC composition (Kadi *et al*. 2002). Therefore, it can be suggested that physical activity together with estrogen treatment may help to maintain muscle characteristics of both slow and fast muscles.

Effects of growth hormone

It has been reported that growth hormone administration induced an increase in MyHC IIX in the vastus lateralis of healthy elderly men (Lange *et al*. 2002). The shift in MyHC isoforms towards MyHC IIX has been interpreted as a change into a more youthful MyHC composition as a decrease in MyHC IIX usually accompanies aging in this muscle group (Lange *et al*. 2002). In contrast, it has been shown that the amount of MyHC IIX in growth hormone-deficient patients was higher than in a normal control population (Daugaard *et al*. 1999). Furthermore, treatment of growth hormone-deficient patients with recombinant human growth hormone for 6 months had no effect on MyHC composition (Daugaard *et al*. 1999). Similarly, it has been shown that growth hormone treatment significantly increased the cross-sectional area of rat type II fibers in the soleus muscle with no significant effect on fiber type composition (Aroniadou-Anderjaska *et al*. 1996). Whether increased concentration of growth hormone is associated with a slow-to-fast shift of myosin isoforms remains to be further investigated.

Effects of thyroid hormones

Thyroid hormones exert a powerful action in the regulation of MyHC composition of skeletal muscles (D'Albis & Butler-Browne 1993). Larsson & Yu (1997) showed that the regulation of MyHC isoforms in rat skeletal muscle by thyroid hormone is gender and muscle specific. Administration of 3.5.3′-triiodothyronine (T_3) causes a down-regulation of MyHC I and up-regulation of MyHC IIA in male and female soleus, whereas the up-regulation of MyHC IIX is observed only in male muscles (Larsson & Yu 1997). Treatment with T_3 induces no changes in MyHC isoforms in male extensor digitorum longus (EDL). The same treatment induces a significant transition from MyHC IIA to MyHC IIB in female EDL (Larsson & Yu 1997). Altogether, these results show that the contractile properties of skeletal muscles are under the control of several hormones and growth factors, and that changes in the hormonal environment of skeletal muscles during exercise are

partly responsible for the adjustment of muscle phenotype to the physiological demand. It is now becoming clear that changes in muscle structure and function in response to changes in the hormonal environment can be muscle and gender dependent.

Conclusion

This paper outlined only a few aspects related to role of specific hormones and growth factors in the regulation of some important muscular parameters responsible for the athletic performance. This area of muscle physiology has just begun to develop and there is still much to be discovered in order to better understand the different signals involved in the various adaptive events occurring in skeletal muscles in response to different forms of physical activities. The delineation of the different steps of the adaptation of muscles to exercise would provide the basis for the conception of individualized exercise prescriptions to optimize the quality of training programs both in well-trained and sedentary healthy populations as well as in populations suffering from specific diseases.

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Chapter 23

Resistance Exercise and Testosterone

ATKO VIRU AND MEHIS VIRU

The male sex hormone, testosterone, influences not only sexual activity and emotional behavior (e.g. aggressiveness) but also contributes to metabolic control. Testosterone is considered to be an anabolic hormone; however, its role in metabolic control is actually more extended. Testosterone influences by its contra-action effects on several other hormones. In resistance training, the main role of testosterone is the induction of synthesis of contractile proteins in involved muscles. Beside that, during acute resistance exercises, as well as during competition, testosterone action seems to be essential for mobilizing performance capacity.

The pituitary–testicular system

Testosterone is produced by the interstitial cells of Leydig, constituting of 20% of the mass of testes in adult men. Quantitatively, a much lesser amount of testosterone is derivated from androgenic steroids formed in the adrenal cortex. The Leydig cells secrete testosterone only when they are stimulated by lutropin (LH; also called luteinizing hormone) from the pituitary gland. The quantity of secreted testosterone is in direct proportion to the amount of LH available. The secretion of LH is stimulated by a hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH). In turn, blood testosterone inhibits GnRH secretion (a pronounced negative feedback effect) as well as LH secretion by the pituitary (a weak negative feedback effect). Too little testosterone allows the neurosecretory cells of thalamus to secrete large amounts of GnRH with followed increases in secretion of pituitary LH and testicular testosterone. Thus, the pituitary gonadographs secreting LH and the testes constitute the pituitary–testicular system, and its activity is controlled by nervous influences reaching the pituitary gland from the hypothalamus via neurosecretion and by the feedback action of blood testosterone.

Both LH-releasing hormone and LH are secreted in an episodic manner. Secretory bursts of GnRH last a few minutes at a time, once every 1–3 h. The pulsative manner of testosterone is less pronounced. The blood testosterone level is highest early in the morning and decreases during the day. Most of the testosterone in blood is bound by sex hormone binding globulin (SHBG).

Metabolic action of testosterone

Anabolic action. In 1935 Kochakian reported that, in male castrated dogs, testosterone injection resulted in long-lasting nitrogen retention (Kochakian 1935). Over the next few years, several papers confirmed this result and showed that castration of male rats, guinea pigs or mice induces weakened skeletal muscles due to a decreased protein synthesis rate. Substitution therapy with testosterone abolished these castration effects (Papanicolaou & Falk 1938; Scow & Hayes 1955; Kochakian 1959; Kochakian *et al*. 1964).

According to present evidence, metabolic action of testosterone is mediated through the androgen receptor in the cytoplasm of cell. In the cell cytoplasm testosterone is bound by the receptor protein. The formed complex is activated and transferred into the cell nucleus. Binding of the complex by

chromosomal proteins triggers the production of mRNA specific for the synthesis of necessary protein(s) responsible for actualization of the testosterone effect. In the skeletal muscle the main locus of testosterone action is the synthesis of myofibrillar proteins. This way testosterone contributes to the development of muscular hypertrophy. The receptor protein is common for testosterone and several other androgens. In skeletal muscle fibers, as well as in bone cells, testosterone has a higher affinity to receptor than other androgens. In other cells the highest affinity belongs to 5α-dihydrotestosterone. In these cases, after testosterone enters the cell it has to be converted to 5α-dihydrotestosterone. Conversion of testosterone to dihydrotestosterone is not found in muscle fibers and bone cells. The information contained in the mRNA is translated for the synthesis of related cellular proteins in ribosomes.

The state of cellular receptors is regulated by increasing/decreasing the number of binding sites and by changing the binding affinity. Thus, testosterone metabolism depends on testosterone production as well as the up- or down-regulation of cellular specific receptor. Therefore, the metabolic effect of the hormone augments not only by increased concentration of the hormone in the intercellular fluid but also as a result of the increased number of binding sites and/or increased affinity of receptor to hormone. In conditions of receptor down-regulation, the high hormone level cannot produce a pronounced metabolic effect. Consequently, it is incorrect to put the sign of equality between the blood concentration of a hormone and its metabolic effect.

Ontogenetic development of skeletal muscle is deeply related to the metabolic effect of testosterone causing the male body build to develop from the final stages of puberty, and it is characterized by the increase of musculature over that of the female. Increased testosterone level after sexual maturation also warrants good faculties for strength, power and speed training.

Anti-catabolic effects. Testosterone is capable of inhibiting the catabolic effect of glucocortioids (anti-catabolic action) as well as reducing the suppression of protein synthesis (anti-anabolic action) exerted by them. Anti-catabolic action and the inhibition of anti-anabolic action are founded on the competition between testosterone and cortisol for the specific cellular receptors of glucocorticoids. Depending on the amount of glucocorticoid receptors occupied by testosterone, the cortisol catabolic and anti-anabolic actions decrease (Mayer & Rosen 1977).

Stimulation of bone growth and calcium retention. Testosterone increases the total bone matrix and causes calcium retention. After puberty, bones grow considerably in thickness and deposit additional amounts of calcium salts (Ritzen *et al*. 1981; Krabbe *et al*. 1982). The increase in bone matrix has been related to the anabolic function of testosterone, and the deposition of calcium salts is secondarily caused by the increase of bone matrix.

Other effects. During adolescence and early adulthood, testosterone increases the basal metabolism 5–10%. Testosterone is capable of increasing the rate of erythropoiesis (Palacios *et al*. 1983). It also exerts a modest influence on sodium reabsorption in renal tubules.

Testosterone in women

Testosterone in female blood plasma originates mainly from the adrenal cortex as a byproduct of glucocorticoid biosynthesis. The secretion of adrenal cortex contains androgenic steroids that can be peripherally converted to testosterone. Adrenals begin to produce androgenic steroids at the beginning of the second decade of postnatal life in relation to the adrenarche. The marker of this process is the serum level of dehydroepiandrosterone sulfate (DHEAS) (Wierman & Crowley 1986).

The production of testosterone in women depends on the rate of biosynthesis of glucocorticoids stimulated by adrenocorticotropic hormone (ACTH) from the anterior lobe of pituitary gland. Thus, in women LH has only a minor role, if at all, in the control of the blood level of testosterone. Therefore, when comparing various responses of blood testosterone in men and women, the principal gender difference in the control mechanism has to borne in the mind.

Although the blood level of testosterone in adult women is about 10 times lower than in men, the metabolic effects of testosterone are not so less pronounced. In women, testosterone metabolic effects increase in relation to estrogen production (Danhaive & Rousseau 1988). It has been suggested that increased sensitivity in regard to testosterone metabolic effect is related to up-regulation of androgenic receptor. Another possibility is that in women testosterone has favored conditions for competition for glucocorticoid receptors.

Testosterone responses in resistance exercise

Exercise-induced hormonal responses depend on four main determinants: (i) exercise intensity; (ii) exercise duration; (iii) level of adaptation to the concrete form of exercise; and (iv) homeostatic needs (Viru, A. 1992; Viru, A. *et al*. 1996). The action of these determinants is modified by several modulators, such as emotional strain, carbohydrate and oxygen availability, environmental temperature, biorhythms and fatigue (Viru, A. *et al*. 1996).

Three mechanisms are assumed to constitute the link between exercise and endocrine activities. One of them is related to triggering nervous discharge from cerebral motor centers to spinal motoneurons (central motor command). The importance of this for the activation of endocrine function has been shown in experiments that injected tubocurarine in men. A bolus of 0.015 $mg \cdot kg^{-1}$ α-tubocurarine caused a partial peripheral neuromuscular blockade. As a result, it weakened the skeletal muscles. Therefore, a stronger voluntary effort was necessary to produce a certain work output compared with normal conditions. The increased voluntary effort was confirmed by the higher rate of perceived exertion in this experiment (Galbo *et al*. 1987). The 'stronger' central motor command was associated with exaggerated catecholamine, growth hormone and ACTH responses during exercise performed at a similar level of oxygen uptake without neuromuscular blockade (Kjær *et al*. 1987).

Galbo (1983) assumed that, during continuous exercises, hormone responses were influenced by impulses from receptors sensing temperature, intravascular volume, oxygen tension and glucose availability. However, attention should have first been given to the feedback by nervous impulses from receptors located in skeletal muscles (proprioceptors sensing the muscular tension and metaboreceptors reacting to metabolite accumulation). The use of small doses of epidural anesthesia, to block the thin sensory afferents (mostly from metaboreceptors) and leave almost intact the thicker efferent fibers and, subsequently, motor function, allowed the authors to demonstrate the essential role of nervous feedback from muscles for ACTH and β-endorphin response (Kjær *et al*. 1989).

Significance of exercise intensity, duration and rest intervals for testosterone responses in resistance exercises

In cyclic exercises the level of intensity has been found to be significant for several hormone responses. Exercise intensities higher than the corresponding threshold cause hormonal responses. Intensity thresholds for catecholamines (Lehmann *et al*. 1991), ACTH (Rahkila *et al*. 1988), cortisol (Port 1981), β-endorphin (Rahkila *et al*. 1988) and growth hormone (Chwalbinska-Moneta *et al*. 1996) are close to the anaerobic threshold. In men performing a cyclic incremental exercise test, the exercise intensity has been noticed above that of the elevated blood level of testosterone (Ježova *et al*. 1985). The same was indicated by the results of Galbo *et al*. (1977). However, results of several other studies failed to indicate a clear-cut dependence on exercise intensity.

Taking into the consideration the significance of the 'strength' of the central motor command, most resistance and power exercises should be intensive enough to evoke sufficiently strong central motor command for triggering hormone responses. Nevertheless, an instantaneous single application of great force or muscle power does not mostly induce hormonal responses. The duration of the effort or the number of repetitions seems to be important as well. Kraemer, W.J. *et al*. (1989, 1991b) compared the action of cycling when 100%, 73%, 55% or 36% of leg power was applied for pedaling. The maximal duration of exercise was 6 s at 100%, 10 s at 73%, 47 s at 55% and 3 min 31 s at 36%. In the case of the

highest exercise intensity, the 6 s of duration was sufficient to trigger the increase of norepinephrine in blood serum but not to elevate levels of epinephrine and cortisol. These results demonstrate that, in exercises of high power output, the triggering of hormonal responses depends on the combined effect of the rate of power output and duration of exercise.

Kinetics of testosterone response. The rate of actual appearance of hormonal response is related to: (i) the rate of reaching the triggering signal to the endocrine gland (the highest rate possessed to nervous activation of adrenal medulla; a comparatively high rate is signaling the activation of anterior lobe of the pituitary gland with the aid of hypothalamus neurohormones); (ii) the possibilities for immediate secretion of the already biosynthesized hormone (e.g. catecholamines bound in cytoplasmic granules of adrenomedullary cells); (iii) the rate of biosynthesis of hormone in quantity necessary for increasing the blood level of hormone and maintaining that level during the exercise. The kinetics of testosterone production has still not been investigated in the exercise situation. Padron *et al*. (1980) indicated that a single injection of human chorinic gonadotropin in men was followed 2 h later by a prolonged increase of testosterone level in the blood. These results suggest that the rate of secretory response of testosterone depends on the rate of synthesis of this hormone. Obviously, we have to assume that testosterone secretion cannot be enhanced rapidly. Variability of testosterone changes in exercise of short duration rather shows the normal fluctuations of the basal level of testosterone than the actual secretory response.

One-minute duration of high intensity exercise (consecutive vertical jumps repeated at maximal rate) evoked increases of concentration of anterior pituitary and thyroid hormones 20–36%, but no change of growth hormone, prolactin and insulin-like growth factor concentrations. Total and free testosterone and cortisol level elevated only 12–14% (Bosco *et al*. 1996a). The magnitude of changes of testosterone and cortisol was similar to the reduction of plasma volume in short-term highly intense cyclic exercises (Sejersted *et al*. 1986). Therefore, the observed testosterone and cortisol changes were related, obviously, to the hemoconcentration. Galbo *et al*. (1977) interpreted the increase of testosterone concentration by 13% in incremental exercise as a manifestation of plasma volume reduction. Kraemer, W.J. *et al*. (1992) also pointed to the possibility that in moderate resistance exercise sessions the elevated testosterone levels may be related to changes in plasma volume. Wilkerson *et al*. (1980) measured simultaneously changes of plasma volume and testosterone concentration during cyclic exercise. Their conclusion was that in 20-min steady state exercises the modest increase of testosterone concentration was due to reduction of plasma volume but not due to elevated testosterone secretion. An additional reason for testosterone increase in blood without elevated secretion is the reduced metabolic clearance rate of testosterone during exercise (Sutton *et al*. 1978).

According to these results, the actual increase of testosterone secretion in exercise should be a delayed response. Nevertheless, Ježova *et al*. (1985) found a significant increase of testosterone concentration (by 28%) after cycloergometric testing of 4.5 min duration performed at very high intensity (5 W·kg body weight^{-1}). Obviously, the exercises cause a rather complicated situation including the possibility for altered kinetics of testosterone response.

Results of Ahtiainen *et al*. (2003) showed that after two sets of leg press at 12-repetition maximum (RM) (rest between sets 2 min) free and total testosterone concentrations were significantly higher than initial values. The increased levels persisted up to the end of session, whereas a trend for further elevation of free hormone level was observed (Fig. 23.1). Since plasma volume decreased by 11.8% during the session in one protocol (maximum repetition) and by 14.4% in the other protocol ('forced' repetition), free testosterone response surpassed the hemoconcentration effect after first two sets and total testosterone response after six to eight sets. Another study of this research team demonstrated that four sets of squats of 10-RM (rest pauses 90 s) resulted in increases of concentrations of free and total testosterone as well as of ACTH, cortisol and lactate (Kraemer, W.J. *et al*. 1998a). However, again the contribution of the hemoconcentration effect remains. All in all it is necessary to bear in mind that the

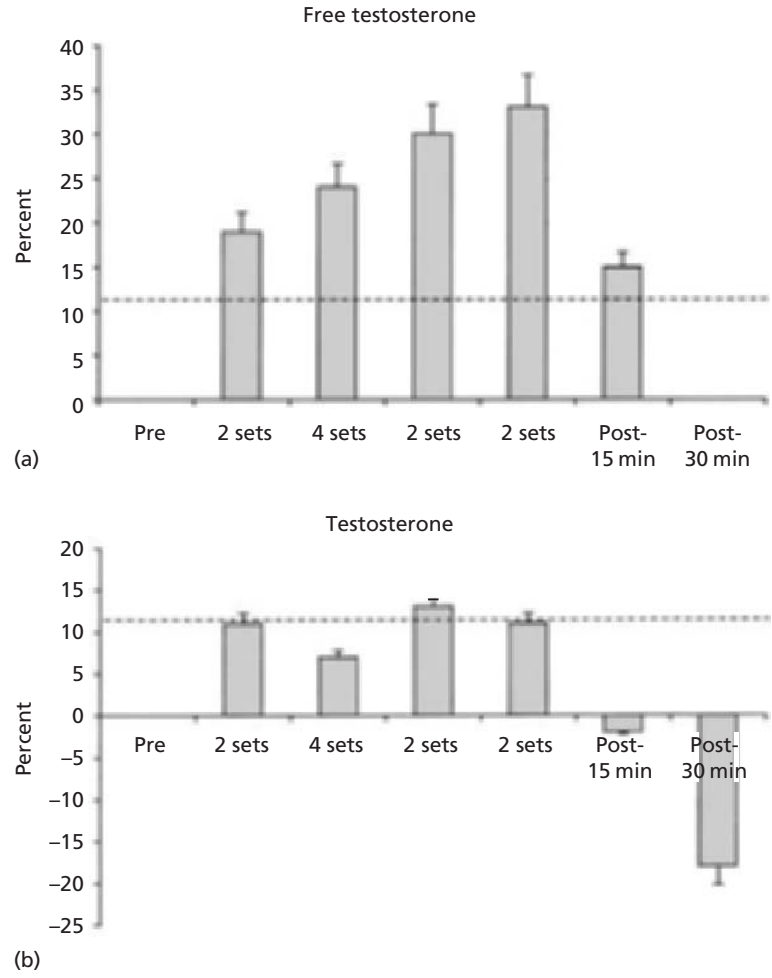

Fig. 23.1 Dynamics of testosterone during a resistance training session. (a) Dynamics of free testosterone. (b) Dynamics of total testosterone. Interrupted horizontal line, magnitude of hemoconcentration. (Data from Ahtiainen *et al*. 2003.)

responses of the blood testosterone concentration do not linearly reflect the secretion responses. Nevertheless, concentration changes are important because metabolic effects of hormones are related to hormone concentration in intercellular fluid. The total hormone amount in the body fluid compartments has significance for maintaining the hormone effects.

Significance of intensity and duration of resistance session. After sessions of resistance exercises a frequent finding is increased testosterone levels. This result has been obtained in results of sessions with a duration of approximately 30 min (Jürimäe *et al*. 1990), or after four exercises consisting of either three to five sets of 5-RM over 3-min rest pauses or three sets of 10-RM over 1-min rest pauses (Kraemer, W.J. *et al*. 1990, 1991a). However, there is also another possibility: six series of eight bench presses at 70% of maximal resistance (total 24 min) did not cause a significant increase of testosterone level. Testosterone response was also not observed when the session was followed by a maximal number of consecutive bench presses at 70% (Guezennec *et al*. 1986).

When two workload protocols (10-RM vs. 5-RM) were involved, testosterone increase in blood was greater after the four exercises using 10-RM (i.e. lower weight, greater total work) protocol as compared with 5-RM (i.e. higher weight, lower total work). After eight exercises no significant difference

in testosterone levels was found between the two protocols (Kraemer, W.J. *et al*. 1991a). Volvek *et al*. (1997a) observed that testosterone concentrations increased modestly (by 7%) using a bench press protocol of five sets to failure with 10-RM but significantly more (by 15%) using a jump squat protocol of 15 sets with 10 repetitions at 30% of 1-RM squat. The significance of training session workload was confirmed by Cotschalk *et al*. (1997): a three-set heavy resistance protocol resulted in a greater increase in testosterone concentration than a one-set protocol. Häkkinen and Pakarinen (1993) reported that the increase of the total workload is responsible for the blood testosterone response despite the necessary reduction of the exercise intensity.

Besides the characteristics of exercise, duration of rest intervals has a significant effect on hormonal responses. The significance of rest intervals undoubtably influenced the results of above-mentioned studies of Kraemer, W.J. *et al*. (1990, 1991a). After a 30-min intensive single circuit weight training session (work/rest ratio 30 s; 30 s at 70% of 1-RM) male university students exhibited testosterone levels 24% higher than the initial values (Jürimäe *et al*. 1990).

Summing up, Fleck and Kraemer (1997) affirmed that the hormonal response in resistance exercise depends on muscle mass recruited, intensity of workout, amount of rest between sets and exercises and previous training. Both concentric and eccentric exercises are able to increase the testosterone concentration in blood (Durand *et al*. 2003).

Jensen *et al*. (1991) compared testosterone changes during and after endurance and strength training sessions in the same men for both forms of training. The mean increase of testosterone concentration was 27% in the endurance session and 37% in the strength session. Differences between responses in the two sessions were not significant but there were large differences in the testosterone responses at the individual level. A high correlation ($r = 0.98$) for individuals was found between increases of testosterone concentration after strength and endurance sessions. The authors suggested that the interindividual differences in testosterone responses might be of importance for individual adaptation to training.

Power sessions. A large number of repetitions with low power output in bodybuilders decreased testosterone and increased growth hormone levels, whereas in weightlifters a high number of repetitions increased testosterone concentrations without changes in growth hormone level. When power output remained close to maximum and the application of force increased (and number of series decreased), no significant hormone changes were found in weightlifters. Sprinters performed exercises at maximal power with a force of 60%. Although the number of series was modest, the total workload was obviously high. In men, blood concentration of testosterone decreased together with the same change in blood levels of LH and cortisol. It is possible to suggest that the reversed hormonal responses were related to pronounced fatigue that developed during the session. This possibility was confirmed by a significant decrease of average power in full squats and by an increase of the total bioelectric activity of contracting muscles to power output in full squats (Bosco *et al*. 2000).

Training and modulators effects

Training effects. Few studies provide results on training effects in resistance training sessions. Guezennec *et al*. (1986) observed no significant testosterone response after six series of eight bench presses at 70% of 1-RM or after the maximal number of repetitions at the same workload. The measurements were repeated each month for 4 months of training, but the results were the same. Because the subjects were male weight-trained athletes, the lack of testosterone response might be related to previous training adaptation to test exercise. In studies on elite athletes, Häkkinen and Pakarinen (1993) found that 20 sets of squats at 1-RM did not increase concentrations of total and free testosterone, whereas the testosterone level rose significantly when 10 sets were performed at 70% of 10-RM. If these results are related to previous adaptation to resistance exercises then, interestingly, adaptation influences the response to high intensity workload but not to voluminous workload.

In weightlifters, increased testosterone concentration has been found after four series of six squats at

90–95% of 6-RM as well as after nine to 10 squats at 60–65% of 6-RM (Schwab *et al*. 1993). In these athletes the exercises did not demonstrate adaptation by disappearance of testosterone responses. However, since the test exercise was adjusted to the individual repetition maximum, the increased actual workload avoided the disappearance of testosterone response.

Summing up, resistance training may remove the hormonal responses when the test exercise is similar to those frequently used in training. At the same time, a possibility exists that long-term resistance training promotes testosterone responses, at least in adolescent athletes. The same weightlifting protocol increased testosterone concentration in 17-year-old juniors who had more than 2-years training experience (Kraemer, W.J. *et al*. 1992).

The effects of resistance training differ from the outcome of endurance training, not only by changes at the level of muscle fibers and of aerobic capacity, but also in regard to influence on hormonal responses in exercises. The combination of strength and endurance training results in an attenuation of the performance improvements and adaptations typical for single-mode training (Kraemer, W.J. *et al*. 1995a). Endurance training suppresses blood testosterone level (Hackney 1996). The endogenous stimulation of testosterone production gives less pronounced change in endurance-trained men compared to sedentary men (Hackney *et al*. 2003).

Modulator influences on testosterone responses in resistance exercise. It has been believed that aggressiveness favors the performance of resistance and power exercises. At the same time, aggressiveness is related to testosterone. Therefore a question arises as to whether emotions of this type influence testosterone responses in resistance exercises. In regard to this question, a new way of thinking arose from the results of Elias (1981). This study showed that judo fighting increased the blood level of testosterone and cortisol more in the winners than in the losers. In accordance, tennis players who exhibited a good mood and self-assurance had higher testosterone levels before competition. After the match, winners exhibited further elevation of the testosterone level, a decrease being observed in losers (Booth, A. *et al*. 1989).

Subjects with a high trait anxiety showed significant increases of testosterone in test exercise similar to normal subjects, whereas concentrations of an adrenal androgen, androstendione, increased less in subjects displaying a high trait anxiety (Diamond *et al*. 1989).

The above results presented that after a hard power training session a pronounced decrease was found in concentrations of testosterone and cortisol (Bosco *et al*. 2000). It was suggested that this change was related to fatigue; more correctly, to a latent fatigue appearing before the actual drop of working capacity. This suggestion has to be verified with the aid of further experiments.

Häkkinen *et al*. (1988b) measured hormone responses in elite athletes performing two strength training sessions a day. Both testosterone and cortisol responses decreased after the first session, but the testosterone increased after the second session. The authors explained these results by the circadian biorhythm: during the morning session the testosterone increase was masked by the decline in hormone basal level, during the afternoon session the lowered initial level of testosterone favored its increase.

Results also became available showing that dietary nutrients (Volek *et al*. 1997b), a protein–carbohydrate supplement consumed 2 h before (Kraemer, W.J. *et al*. 1998b) or post-exercise nutrition (Bloomer *et al*. 2000) may influence the testosterone pattern in resistance training session or during the 24 h of recovery. Amino acid supplementation (Fry *et al*. 1993) or the administration of either creatine (Volek *et al*. 1997a; Op'Teijnde & Hespel 2001) or ginseng (Youl *et al*. 2002) failed to alter testosterone responses.

Relationship of testosterone increase to LH response. Some studies indicated concomitant increases of testosterone and LH levels during long-lasting training (Häkkinen *et al*. 1987, 1988a). However, a possibility of uncorrelated changes of these two hormones has also been shown (Häkkinen & Pakarinen 1991). In resistance exercise sessions increases of testosterone and LH are usually not parallel. In some studies concomitant declines of testosterone and LH have been observed (Häkkinen *et al*. 1988b;

Bosco *et al*. 2000). However, these data cannot be used as evidence against the role of LH in testosterone response during exercises. The LH response consists of a short-term burst of secretion, which is followed by a delayed increase in testosterone secretion. Therefore, in experiments it is a complicated task to 'catch' the LH response. More convenient is to record the prolonged result of LH pulsation in increased testosterone secretion and blood level. The exercise effect on pulsative secretion of LH has been studied in women and in relation to endurance exercises (Cumming *et al*. 1985; Weltman *et al*. 1990) but not in men and in regard to resistance exercise. Endurance training may alter the biological activity of endogenous LH (Di Luigi *et al*. 2002). Corresponding information regarding resistance training is not available.

Gender and age

Testosterone responses in women. Several studies indicated that, differently from men, women do not react to resistance exercises by increased testosterone level in blood. When eight exercises for various muscles were performed with workloads of either 5-RM (rest intervals 3 min) or 10-RM (rest intervals 1 min), testosterone concentration increased in men but not in women despite prior recreational resistance training experience in both contingents (Kraemer, W.J. *et al*. 1991a, 1993). In contrast, Cumming *et al*. (1987) found a 20% increase of testosterone in women after a session consisting of six isokinetic resistance exercises. Although the LH level increased by 60%, it is unlikely that it was the physiological stimulus for testosterone production because women produce this hormone as a byproduct of steroid biosynthesis in the adrenal cortex. Accordingly, those women who did not exhibit a cortisol response failed to show an increase in testosterone concentrations. The other female subjects showed a parallel increase in testosterone and cortisol concentrations (Cumming *et al*. 1987).

During strength exercises, Weiss *et al*. (1983) observed rather similar relative increases in blood testosterone, in men from the high level by 21.6% and in women from the low level by 16.7%. Androstendione concentration increased significantly in both gender groups without difference in the magnitude of response. Resistance exercises during 40 min increased concentration of another adrenal androgen, DHEA, more than endurance exercises in women 19–69 years of age (Copeland *et al*. 2002).

Kraemer, R.R. *et al*. (1995) compared hormonal responses in healthy women in early follicular and luteal phases. A low-volume resistance exercise program caused greater estradiol response in the follicular phase. Growth hormone and androstendione responses appeared only in luteal phase, whereas testosterone and progesterone did not response during this exercise.

A problem is whether the resistance exercises influence the activity of enzymes in the adrenal cortex responsible for forming androgen steroids. Neither confirming nor contradicting evidence is still available. However, individual differences in the production of androgens exist between women. It has been shown that an acute resistance exercise test (ARET) increased concentrations of total testosterone by 25%, free testosterone by 25% and SHBG by 4%. Multiple regression analysis indicated that in young healthy women the testosterone responses to ARET is predicted by the subscapular to triceps ratio in skinfolds and by the ratio of upper-arm fat to mid-thigh fat assessed with magnetic resonance imaging. Waist-to-hip ratio in fat failed to have significance as a discriminator for hormonal concentrations in women (Nindl *et al*. 2001).

Testosterone responses in elderly people. According to the results of Häkkinen and Pakarinenen (1995), a heavy resistance session caused testosterone increase in both 30-year-old and 50-year-old men, but not in 70-year-old men. Women of any age failed to show testosterone response. In another study, Häkkinen *et al*. (1998) compared the acute effect of heavy resistance exercise on blood testosterone in young (mean age 26.5 years) and old (mean age 70 years) men. Exercises for legs, upper body and their combination increased total testosterone level in young men but only leg exercise increased total testosterone level in old men. Kraemer, W.J. *et al*. (1998a) found that four sets of squats of 10-RM caused increased total and free testosterone levels in both 30- and 62-year-old men. The area under the

response curve during and 30-min post-exercise was greater in younger men.

Testosterone responses in adolescents. Appearance of testosterone in blood is related to puberty in boys and to adrenarche in girls. During cyclic exercises significant increase of testosterone level has been detected from the stage 4 of sexual maturation (by Tanner's scale) in boys (Fahey *et al*. 1979) and from the stage 5 in girls (Viru, A. *et al*. 1998).

In resistance exercises, post-exercise as well as pre-exercise testosterone levels were lower in 15-year-old male athletes than in adult male athletes (Pullinen *et al*. 1998).

Pattern of blood testosterone during the recovery period

In men, the testosterone level gradually returns to its initial level during the 1st hour following resistance sessions. In females, low blood levels of testosterone remain without significant alterations both during the session and the 1st post-exercise hour (Kraemer, W.J. *et al*. 1991a). This pattern is different from that found after cyclic exercises. After 2 h of bicycle ergometric exercise the testosterone level dropped during the 6 h post-exercise. Twenty-four hours later it showed a normalizing trend, but the mean testosterone concentration was still below the initial values both in trained and untrained young men (Viru, A. *et al*. 1992). Similar results have been obtained by a Finnish research team (Kuoppasalmi 1980; Kuoppasalmi *et al*. 1980): after running for 13–14 km the testosterone level dropped during the 1st hour and then remained low for 5 h. Ahtiainen *et al*. (2003) also found that 30 min after a resistance training session, free and total testosterone levels were below initial levels. When a 30-min resistance session protocol prescribed a work-to-rest ratio of 30 s, 30-s testosterone concentration decreased during the first 6 h of the post-exercise recovery (Jürimäe *et al*. 1990). Thus, the testosterone pattern after resistance exercises may be similar to that after endurance exercises. In the first and second morning after heavy resistance training, the level of total testosterone was normal but the level of free testosterone increased (Ahtiainen *et al*. 2003).

In rats, seven times 1-min swimming with a high additional weight (of 12% body mass) simulated efforts in resistance training. The blood level of testosterone increased slightly. During the first 2 h of restitution the hormone level decreased. Four hours after exercise a secondary rise in the hormone concentration was detected, exceeding the resting level by 1.5–2.5 times. Testosterone levels in cytoplasm of skeletal and heart muscle fibers increased, with the peak values 72 h after swimming (Fig. 23.2). At that time the specific binding of androgens, as well as the number of binding sites, were increased in the cytoplasm of skeletal muscle fibers (Tchaikovski *et al*. 1986).

It is possible to ask what is important for the post-exercise induction of adaptive protein synthesis in exercised muscles, either testosterone response during the exercise or the secondary post-exercise testosterone increase. The presented results of rat experiments suggest that both increases of testosterone constitute an entire response.

Testosterone in monitoring of resistance training

The fact that something is measured in athletes does not mean training monitoring. It is possible to speak about training monitoring if the following five principles are completely followed (Viru, A. & Viru 2001):

1 It is a process performed for the purpose of increasing the effectiveness of training guidance.

2 It is based on recording changes in an athlete during various stages of the training or under the influence of main elements of the sport activities.

3 It is a highly specific process depending on the sport event, performance level of the athlete and age/gender peculiarities.

4 Any method or measurement is in a sense training monitoring if it provides reliable information related to the task being monitored.

5 The information obtained from measurements has to be understandable so that necessary corrective changes in training can be made.

In the monitoring of the resistance training testosterone basal level, responses to test exercise and the ratio of testosterone/cortisol have been used. The idea is to get information about the contribution

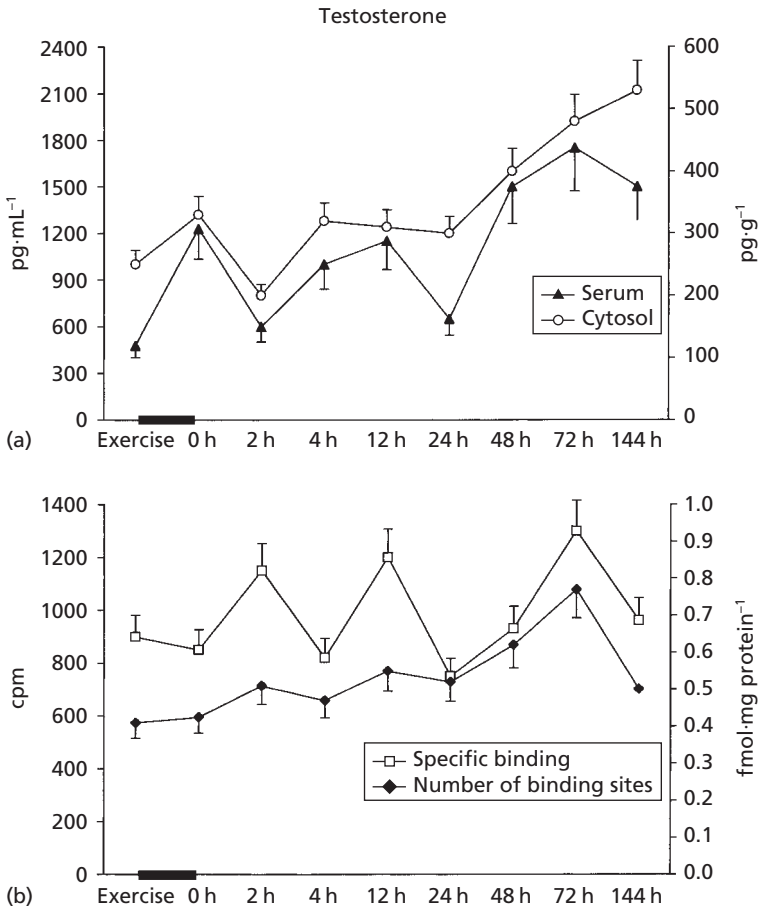

Fig. 23.2 Post-exercise dynamics of testosterone and its binding in skeletal muscle after swimming in rats. (a) Closed triangles, testosterone in serum. Open circles, testosterone in cytosol of muscle fibers. (b) Open squares, specific binding of testosterone. Closed rhombs, number of binding sites. (Data from Tschaikovski *et al*. 1986.)

of testosterone in training adaptations, and to be informed about excessive training workloads.

Training effects

Testosterone basal level during prolonged resistance training. The related studies were initiated by Häkkinen from Finland. In 1985 he and his collaborators reported that heavy resistance training for 24 weeks did not change the basal level of testosterone in blood. Blood cortisol level decreased; the testosterone/cortisol ratio increased. This change was parallel to the increase in isometric force of the leg extensor muscles. During the followed detraining for 12 weeks, both indices decreased to the pretraining values. In combined training using jumping and strengthened exercises (weights of 60–80% 1-RM), during the first 8 weeks the testosterone level increased and the cortisol level decreased. After 16 weeks of training, levels of both hormones decreased. At the end of 24th week the mean concentrations of testosterone and cortisol did not differ from the initial values. The detraining did not accompany hormonal changes. The effect of this training protocol on isometric force was significantly less pronounced than that in the high-resistance training group (Häkkinen *et al*. 1985).

Blood samples obtained every 4 months in elite weightlifters showed testosterone increased from the month 8 to month 12 (Häkkinen *et al*. 1987). During the next year the increased testosterone level persisted (Häkkinen *et al*. 1988a). The authors suggested that increased testosterone level might create optimal conditions for intensive weight

training to increase strength improvement in elite-strength athletes. In 1990 this team reported that, according to 51 weeks of follow-up of six elite weightlifters, the fitness level (evaluated by arbitrary units) and blood testosterone level changed in correlation (Busso *et al*. 1990).

Effects of hard-training stages. Six weeks of preparatory training of elite weightlifters was divided into a high voluminous stressful stage for 2 weeks followed by 2 weeks of 'normal' training and then 2 weeks of taper. During the first 2 weeks testosterone concentration decreased significantly, cortisol concentration elevated slightly and the ratio of testosterone/cortisol dropped. During next 2 weeks changes were modest, if at all. (Häkkinen *et al*. 1987).

The results of Busso *et al*. (1992) confirmed that in elite weightlifters a 4-week stage of intensive training was accompanied with decreases in the blood testosterone level. Fry *et al*. (1994) investigated 1 week of intensive training causing overreaching in junior weightlifters. During the 1st year the intense training week resulted in attenuated testosterone responses in test exercise. Over the 2nd year the intensive training augmented the testosterone responses.

Kraemer, W.J. *et al*. (1995a) compared the effects of hard endurance and strength training and their combination. During endurance training, testosterone was constant but undulations occurred in the cortisol response. In strength training, testosterone again stayed constant while cortisol response decreased. Combined training demonstrated changes of both testosterone and cortisol levels over the training period.

In endurance athletes hard-training stages result in two phases of hormonal changes (Viru, A. & Viru 2001). Typical for the 1st phase was increased cortisol basal level and exaggerated cortisol increase in strenuous test exercise. Testosterone basal level was constant or reduced and responses in test exercise attenuated. The 2nd phase was indicated by suppressed cortisol response in exercise while the basal level might be either increased or decreased. Testosterone levels before and after test exercise were mostly low. Although the material is far from sufficient for conclusions, two phases may be suggested in resistance training under the influence of increased training volume. However, available data suggests that in strength athletes most typical are changes not in the cortisol level but in the testosterone level.

The meaning of 'hard-training stage' is a relative one depending on previous training. Therefore, the lack of testosterone changes in 'intense' training for a couple of weeks (Häkkinen & Pakarinen 1991) or 8 weeks of 'heavy' resistance training (Hickson *et al*. 1994; Potteinger *et al*. 1995) does not contradict the results of other studies. Also 12 weeks of heavy resistance training did not change either the resting level of testosterone or the responses in training sessions (McCall *et al*. 1999).

Kraemer, W.J. *et al*. (1996) suggests that in heavy training hormonal changes are related to early adaptation, which later disappears. A question arises in whether the decrease of hormonal changes indicates the necessity for further increase in the training workout.

Testosterone in the taper stage. During the taper stage after 'stressful' training (causing a decrease in testosterone and a slight increase in cortisol levels), testosterone remained low and cortisol decreased (Häkkinen *et al*. 1987). However, different situations may exist. From the data of Busso *et al*. (1990) it is possible that a sharp reduction of training workload associates with the lowest testosterone level during the training year. Another report of this team (Busso *et al*. 1992) also showed that the reduction of training workload for 2 weeks did not stop the testosterone decline that appeared during the previous 4 weeks of hard training. In power athletes a 2-week detraining after heavy resistance training caused a decrease of cortisol and an increase of testosterone concentration. Detraining does not impair the neuromuscular performance, as indicated by the results of most muscle strength and power tests and surface electromyogram (EMG) activity (Hortobagyi *et al*. 1993). In recreationally strength-trained men, 6 weeks of detraining produced only a minimal change in performance and hormonal levels (Kraemer, W.J. *et al*. 2002).

The taper problem is a rather complicated one because the taper stage may consist of various

degrees of reduction in the training load and in various designs in regard to the choice of exercises and the pattern of their intensity. Further, the body state when the reduction of training is considered to be necessary may be also different. Moreover, the body responses both to overtraining and taper are individually variable. Therefore, summing-up results obtained in various athletes may actually cause a loss of important information.

Discussion of hormonal changes in the taper stage led to an assumption that before competition the taper stage has to be substituted by a short-term training stage for preconditioning the peak (at least high) performance. For power and strength events, a high testosterone basal level may constitute an important precondition for successful performance (Viru, M. & Viru 2000). This is a problem still waiting systematic research.

Overtraining. The main hormonal indicators of overtraining are suppressed production of cortisol, testosterone, growth hormone, nocturnal excretion of catecholamine and decreased sensitivity to hormones (Kuipers & Keizer 1988; Urhausen *et al*. 1995; Lehmann *et al*. 1997, 1999; Viru, A. & Viru 2001). Several overtraining manifestations reflect a general imbalance in the level of hypothalamic neurosecretion and/or in the function of the anterior pituitary gland (Keizer 1998; Lehmann *et al*. 1998; Hackney 1999). In accordance with the listed overtraining manifestations, Fry *et al*. (1998) found that in weight-trained men the overtrained state is indicated by strength decrements and a pronounced decrease in blood cortisol concentration. Overtrained men exhibited a slightly increased testosterone level after the test exercise.

In disagreement, several researchers believe that high cortisol levels in training are related to dysadaptation. The reason for this opinion is the catabolic effect of glucocorticoids that has been set in contrast to the anabolic action of testosterone. However, it should not be forgotten that in stress situations, including strenuous exercise, enhanced catabolism is not a misfortune but ultimately a necessary adaptation response (creation of the pool of free amino acids to be used as additional substrate for oxidation as well as building blocks for the adaptive protein synthesis). Moreover, during acute exercise, cortisol makes an essential contribution to amino acid transamination, and thereby to the formation of alanine, to glyconeogenesis, to the synthesis of urea, to the synthesis of cateholamines and supporting their effects on the post-receptor level, and to the control of sodium–potassium fluxes through cellular membrane, etc. Therefore, insufficient or reverse responses of cortisol should make us think about discrepancies in the metabolic control.

Adlercreutz *et al*. (1986) recommended using the ratio between free testosterone and cortisol as an indication of overstrain if the ratio decreases more than 30% or if the ratio is less than 0.35×10^{-3}. This way, an extreme situation in the balance of anabolic and catabolic stimulation is indicated. However, the proposed quantitative measure was later frequently forgotten and any decrease in the ratio of testosterone/cortisol was considered a bad indication. With this background, conclusions on overtraining were made, although the authors' data demonstrated a good performance level. Even if the ratio decreases more than 30% or is less than 0.35×10^{-3}, indications of overtraining may not exist (Kuipers & Keizer 1988; Urhausen *et al*. 1995).

In essence, the main shortcoming of the use of this ratio is that the hormone level in blood is not linearly related to its metabolic effects. This ratio has been used despite the lack of information about the state of the glucocorticoid and androgen receptor. Actually, the state of these receptors as well as the competition between testosterone and glucocortioid for glucocorticoid receptor are essential for determinants of the outcome in anabolism/catabolism balance. Consequently, the ratio of testosterone/cortisol is too indirect to be used.

Testosterone, training effects and performance

Induction of synthesis of myofibrillar proteins

The main result of strength training is myofibrillar hypertrophy. This process is founded on the induction of synthesis of myosin and actin (Booth, F.W. & Thomason 1991). While various metabolic factors are able to induce this process, a powerful amplifier

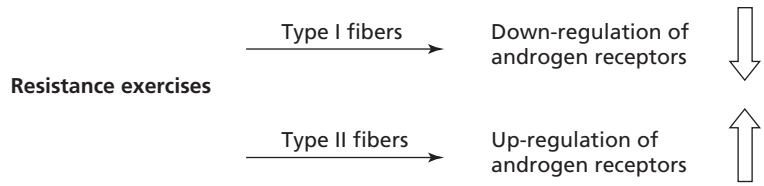

Fig. 23.3 Effect of resistance exercises on androgen receptor in muscle fibers type I and type II.

of the induction of synthesis of contractile proteins is testosterone (Viru, A. 1995). In fact, amplificatory effect of testosterone has been evidenced by the prohibited use of anabolic steroids by athletes. Frequently, although not always, the result was enhanced training effects in strength and power events (Rogozkin 1979; Wilson 1988; Lamb 1989). Rat experiments evidenced that anabolic steroids stimulate the synthesis of myofibrillar proteins, increase RNA polymerase activity and augment the corresponding training effects (Rogozkin 1979; Rogozkin & Feldkoren 1979). Accordingly, it has been assumed that in normal conditions of training the synthesis of myofibrillar proteins is amplified by the endogenous androgens.

It has been mentioned above (pp. 327–8) that, during the recovery period after exercises requiring strong muscle contractions, a number of binding sites increase the cytoplasm of working muscles in association with increased production of proteins in the muscle (Tchaikovski *et al*. 1986). The significance of androgen receptors in training hypertrophy was confirmed in rats: a pharmacological blockade of androgen receptors prevented training-induced muscle hypertrophy (Inoue *et al*. 1994). At the same time muscular activity itself increased the number of androgen binding sites in rat muscles (Inoue *et al*. 1993). Control of testosterone action at the receptor level ensures a fiber-type specific stimulation of protein synthesis in muscles during the recovery period. Resistance exercises induce down-regulation of androgen receptors in slow-twitch fibers and up-regulation of these receptors in fast-twitch fibers (Deschenes *et al*. 1994). Consequently, the tissue's susceptibility to testosterone's effect increases selectively during resistance exercise in fast-twitch muscle fibers (Fig. 23.3).

In humans, the testosterone effect on muscle strength and protein synthesis has been confirmed (Urban *et al*. 1995; Kraemer, W.J. *et al*. 1996). When training induced enhanced testosterone, cortisol and growth hormone responses, the improvement of muscle strength was pronounced (Hansen *et al*. 2001). In disagreement, Hickson *et al*. (1994) reported increased cross-sectional area of fast-twitch fibers as a result of 8 weeks of heavy resistance training without increases of testosterone basal level or its responses in exercises. However, testosterone changes might take part during the recovery period; also possible were alterations at the receptor level.

Thyroid hormones add their action on transcription of oxidative enzymes and myosin (Freeberg & Hamolsky 1974; Konovalova *et al*. 1997). The action of growth hormone, growth factors and insulin on the translation process (Balon *et al*. 1990; Fryburg *et al*. 1991) exerts a general support for the actualization of the adaptive protein synthesis (Fig. 23.4).

Training-induced muscle hypertrophy is controlled at transcription, translation and post-translation level (Booth, F.W. & Thomason 1991), whereas the used exercises may determine the relative significance of contribution of actions at each control level. Therefore, the hormonal effects on muscle hypertrophy also vary in dependence of the exercises used.

After exercise, the catabolic influence of cortisol is essential to provide free amino acids for protein synthesis, to maintain the rate of protein degradation for renewal of protein structures and to adjust the number of protein molecules to the actual need (the post-translation control) (Viru, A. 1995).

Preconditioning effect of testosterone

Data has suggested that performance in muscle power tests relates to the blood level of testosterone in athletes. In professional soccer players, the results of counter-movement jump positively correlated

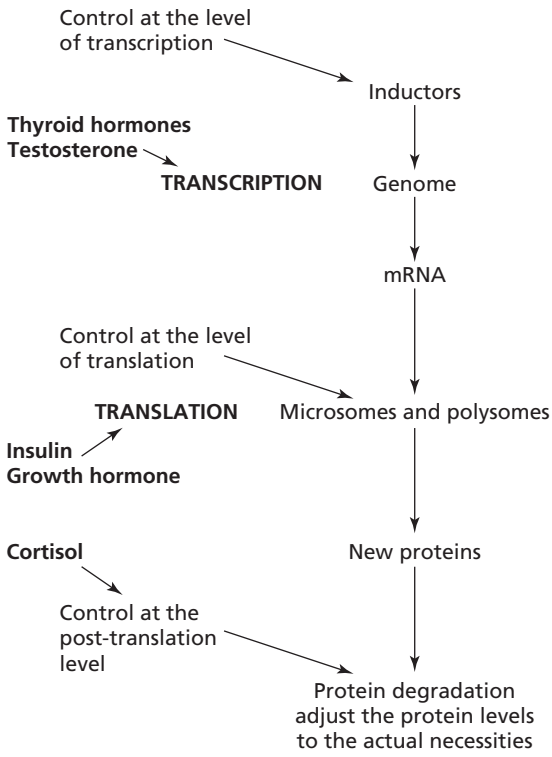

Fig. 23.4 Hormonal control of synthesis of myofibrillar proteins.

with the basal level of testosterone in blood (Bosco *et al*. 1996b). The specific relationship between testosterone level and the explosive strength of leg muscles was supported by the fact that aerobic endurance, determined by Cooper's 12-min runs test, showed negative correlation with testosterone level (Bosco *et al*. 1996b). Comparison of testosterone, determined by morning samples and the rise of the center of gravity in the counter-movement test in 97 high-level athletes, indicated the highest values of testosterone concentration and jumping performance in sprinters and the lowest values in cross-country skiers, whereas soccer players exhibited intermediate values (Bosco & Viru 1998). In accordance, Kraemer, W.J. *et al*. (1995b) reported a positive correlation between testosterone level and performance in double knee extension exercises. Significant correlations were also found between average power output during continuous jumping for 60 s and the change of blood testosterone concentration during this test (Bosco *et al*. 1996a).

Since the metabolic effect of testosterone is a time-consuming process due to the formation of the testosterone-receptor complex, influence of this complex on the genome and synthesis of new proteins (Liao 1977; Mainwaring 1977), it is not believed that, during short-term acute force efforts, jumping exercise or sprint distances, necessary time is provided to activate secretion of testosterone, to transport the augmented amount of the hormone to working muscle and to actualize related metabolic events. Therefore, a hypothesis has been raised that testosterone has a preconditioning action on the application of muscle force and power (Viru, A. & Viru 2001). Competition performance (as well as the main part of a training sessions) is preceded by warming up. Athletes are influenced by the anticipatory state. During competition each athlete can use three or six trials. Therefore, in competition power exercises are performed on the level of altered hormone levels in blood. The significance of these hormonal alterations for performance depends on the time required for the actualization of metabolic effects of hormones. The hormones, which are bound on cellular membrane and acting through formation of cyclic adenosine monophosphate (cAMP) (e.g. catecholamines) need only a couple of seconds to evoke their metabolic effects. The hormones, which are bound by specific receptors in cytoplasm and acting through the induction of protein synthesis (e.g. testosterone and other steroid hormones), in several cases require more than 1 h to show metabolic effects. Consequently, performance in exercises of explosive type of power output may be influenced by hormonal changes before the main performance.

Two types of preconditioning of the performance in power events, exerted by endogenous testosterone, may be distinguished. The long-term preconditioning is related to the influence of testosterone on the development of fast twitch (FT) muscles. Mainly, it is related to the pubertal period. The short-term preconditioning is related to the influence of testosterone either on the central nervous system or peripheral neuromuscular apparatus. The result is 'tuning' of the motor centers of the central nervous system for explosive performance.

Long-term preconditioning. Explosive contractile activity of muscles (jumping, sprinting performance, etc.) is related to the percentage of FT fibers in leg muscles (Costill *et al*. 1976; Bosco & Komi 1979). Thus, the formation of capacity for muscular activities of explosive type depends on the development of FT fiber and fast motor units. Results of several studies indicate that testosterone is, plausibly, responsible for improved anaerobic enzyme systems and structural development of FT fibers in muscles. Bass *et al*. (1971) established that in temporalis muscle of guinea pig, sexual differentiation of enzyme pattern might be converted by testosterone. Dux *et al*. (1982) demonstrated that pubertal castration alters the structure of skeletal muscle. Most of them suffered in the development of FT muscles. Krotiewski *et al*. (1980) confirmed the castration effects in male rats. Testosterone substitution restored development of FT muscles in male castrates. According to these results, it is possible to assume that during puberty inter-individual differences in testosterone action determine the formation and development of FT fibers. Several studies in male adolescents support this assumption. Already at the onset of puberty (in 11–12-year-old boys) area of FT fibers as well as blood lactate level after 15 s all-out exercise correlated significantly with testosterone level (Mero 1988). In circumpubertal boys testosterone levels in blood or saliva correlated with maximal anaerobic power (Mero *et al*. 1990; Falgairette *et al*. 1991), maximal power output in incremental exercise (Fahey *et al*. 1979), blood lactate level after Wingate test (Mero *et al*. 1990) and maximal voluntary strength (Mero 1988). Bosco (1993) indicated that between 8.5 and 14.5 years the rise of center gravity in counter movement jump increases linearly in children of both gender. From the age of 14.5 years a prevalence of boys appeared. Typical for this age is a pronounced increase of blood testosterone concentration.

Thus, in the pubertal period, enhanced increase of testosterone concentration in blood obviously favors the development of FT fibers. Thereby a phenotype is formed which is characterized by high testosterone level and effective performance in exercise of explosive application of forces.

Besides pubertal period, inherent high levels of testosterone may enhance myofibrillar hypertrophy in resistance training. In power events, essential is the influence of high testosterone levels on central nervous structures. Various influences in early postnatal life play a role in the choice of neurons of the central nervous system, which become sensitive to steroids. The spinal nucleus of the bulbocavernosus is highly androgen-sensitive. Testosterone regulates both the size of motoneurons of the spinal nucleus of the bulbocavernosus and also related muscle in adulthood (Kurz *et al*. 1986; Araki *et al*. 1991; Lubisher & Arnold 1995). This neuromuscular subsystem plays an important role in male copulatory behavior. Still, there is no evidence that testosterone influences the neural adaptations in strength training at the level of spinal motoneurons covering the function of major muscles of the body. This may be suggested by the fact that androgens are able to influence the structure of neurons, including dendritic branching and synapse formation in the adult brain (Arnold & Breedlove 1985; Matsumoto 1992). The contribution of testosterone in training-induced long-term neural adaptivity awaits investigation.

Short-term preconditioning. Another way to understand the significance of testosterone in power exercises is the short-term preconditioning effect. In the short-term preconditioning action, the testosterone level in blood is, plausibly, highly significant. Investigation of aggressive behavior showed weak positive correlations between the blood level of testosterone and manifestations of aggressiveness in humans (Archer 1991; Book *et al*. 2001). The testosterone level is not the single determinant of aggressive behavior, which actually depends on the interaction of several factors, including previous experience, environment, danger of the situation, etc. (Mazur & Booth 1998). Thus, the testosterone level is a preconditioning prerequisite for aggressive behavior but not the determinant of the expression of aggressiveness. High levels of testosterone made boys more impatient and irritable, which in turn increased their propensity to engage in aggressive–destructive behavior (Olweus *et al*. 1988). By analogy, it is possible to assume that testosterone promotes changes in neurons, which are not

only related to increased aggressiveness but also to favorable mobilization of neuromuscular capacity for explosive performance in power throwing, jumping and sprint events.

During sport competition, the short-term precondition effect may appear due to the increased testosterone concentration in blood as a result of warming-up exercises, anticipatory state, emotional strain aroused from performance of other competitors, as well as of prolonged concentration for the forthcoming competition. It should be remembered that during sports competition in judo or tennis, winners have higher levels of testosterone than losers (Elias 1981; Booth, A. *et al*. 1989). Therefore Mazur and Booth (1998) suggested that testosterone prepared winners for effective performance.

Some anecdotic examples support the suggestion of the significance of testosterone in the preconditioning of performance in power events. A couple of days before an international competition, low testosterone concentration was found in a discus thrower. His performance was lower than expected. Within 3 weeks following the competition his testosterone concentration normalized. Further, after return from winning an international competition a decathlete showed an unusually high testosterone level (36 $mmol \cdot L^{-1}$).

On the background of extended experimental material, Ingle (1952) affirmed that the permissive effect of hormones consists in enabling changes in body function or metabolic processes (gives the permission to change), although the hormone itself is not the direct cause of the change. The supposed permissive action of testosterone is obviously related to the indirect effect of testosterone, which is actualized without participation of the androgen receptor (Nieschlag & Behre 1998). The manifestations of the indirect effect of testosterone are: production of insulin-like growth factor I; competition for the specific binding sites of glucocorticoids; autocrine release of andromedins; transmembrane influx of extracellular calcium; and activation of extracellular signal-related kinase cascade via binding to a yet unidentified extracellular receptor. Specification of testosterone action on muscle force and power generation in regard of these possibilities, as well as localization of related effect(s) in neurons, synapses or muscle fibers is a matter of further investigation.

In conclusion. The hypothesis on the preconditioning of performance in power events by endogenous testosterone opens a wide spectrum of tasks for further research. Perspectives include testing various aspects of the hypothesis as well as detailed investigations in order to establish the cellular–metabolic foundations for various actions of testosterone on nervous structures and muscle related to power performance.

Conclusions

Testosterone level in blood increases in men during resistance exercises. This response depends on several conditions, among those the primary significance seems to belong to the application of high muscle forces or power output during a sufficiently long time (> 10–15 min) over relatively short rest pauses. During short-term resistance exercises rapid testosterone response is mainly related to hemoconcentration. After resistance training sessions a secondary increase of blood testosterone concentration may appear in the late recovery period concomitantly with increased binding of testosterone by specific sites of androgen receptors in muscle fibers. Resistance exercises, probably also power exercises, cause the up-regulation of androgen receptors mainly in fast-twitch fibers. The most important adaptive effect of testosterone is the induction of the synthesis of contractile proteins, mainly in fast-twitch fibers. This effect is founded on the formation of the testosterone-receptor complex and its action on the genome. The late recovery period after resistance training sessions is the time for actualization of the adaptive protein synthesis induced by testosterone. The anabolic effect of testosterone-initiating transcription of synthesis of myofibrillar proteins (release of related mRNA) is supported by action of growth hormone and growth factors on the translation process.

Action of testosterone on acute muscle performance is also possible without the contribution of androgen receptors. The resulting precondition effect of testosterone is still a suggestion and requires systematic investigation.

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Chapter 24

Exercise Response of β-Endorphin and Cortisol: Implications on Immune Function

ALLAN H. GOLDFARB

Introduction

β-Endorphin (βE) and cortisol are two important neurohormones that influence immune response and glucose levels that ultimately arise from a common molecule. This common molecule (preprohormone) proopiomelanocortin (POMC) (Fig. 24.1) can be cleaved to several peptide components. POMC is not only the precursor of adrenocorticotrophic hormone (ACTH) that stimulates the production of cortisol in the adrenals but also contains the peptide βE. The production of POMC and ultimately both cortisol and βE are regulated by factors that arise from the hypothalamus and the paraventricular nucleus (PVN) in the brain. Corticotropin-releasing hormone (CRH) arises from the hypothalamus and is the major stimulant to activate the release of ACTH from the anterior pituitary gland. Argenine vasopression, which arises from the PVN, is also an activator of ACTH release into the circulation. There are numerous factors that can alter the release of POMC such as diurnal, emotional, physical and biochemical signals. Circulating cortisol will feedback and inhibit the production of POMC although other molecules can influence its synthesis. βE is also synthesized within the brain and spinal cord, has potent opioid actions within the central nervous system and appears to modulate pain.

βE released into the circulation arises primarily from the anterior pituitary gland. The large peptide POMC has a section towards the C-terminus known as β-lipotropin that ultimately is cleaved to γ-lipotropin and βE. Both β- and γ-lipotropin molecules help to mobilize lipid molecules from adipose tissue. βE within the circulation has been implicated in a number of processes including modulation of immune function, pain modulation and assisting in glucose homeostasis. βE receptors have been identified in numerous sites in the body including adipose tissue, pancreas and skeletal muscle. However, the exact role(s) βE may have on these tissues is still being elucidated.

Cortisol, the major glucocorticoid, has a negative feedback impact on its own secretion at the level of the anterior pituitary and the hypothalamus. Cortisol acts by binding to a cytosolic receptor, and this complex is then moved to the nucleus where it binds to modulate gene expression. In this manner,

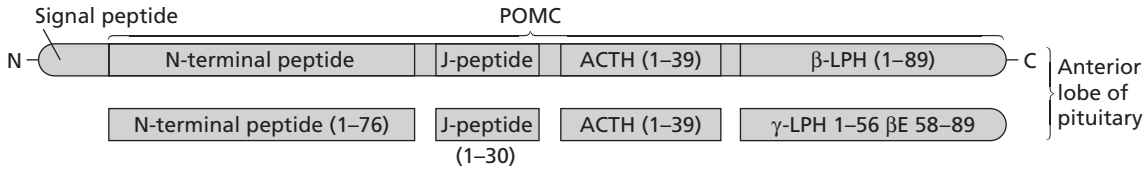

Fig. 24.1 Proopiomelanocortin (POMC) in the pituitary. Anterior pituitary lobe of pituitary. ACTH, adrenocorticotrophic hormone; βE, β-endorphin; β-LPH, β-lipotropin; γ-LPH, γ-lipotropin; J, joining peptide. The numbers coincide to the amino acid sequence for that section.

cortisol is the major inhibitor of the synthesis of CRH and the synthesis of POMC. Additionally, cortisol has been shown to inhibit the release of synthesized ACTH stored in vesicles within the anterior pituitary gland. There is a control of cortisol at the level of the hypothalamus. CRH control from the hypothalamus demonstrates a circadian and pulsatile nature that manifests a pulsatile and varied response throughout the day for these factors. The greatest pulsatile secretion of ACTH typically occurs in the early morning. It should be noted that the suprachiasmatic nucleus of the hypothalamus receives input from the optic nerve and this input has an impact on this circadian rhythm. Elimination of the optic nerve can eliminate the circadian rhythm of ACTH and cortisol.

The formation of cortisol from cholesterol within the adrenal cortex takes some time. Therefore, there will be a delay in the cortisol peak compared to the increased pulsatile peaks of ACTH. The primary function of cortisol is to help maintain glucose levels by enhancing mobilization of amino acids from proteins to the liver to be precursors for glucose. The stimulation of gluconeogenesis by cortisol as well as the stimulation of fat mobilization to enhance fat metabolism helps to raise plasma glucose levels. Cortisol also acts as an immunosuppressive agent and has anti-inflammatory activity.

Exercise influence on circulating β-endorphin and cortisol

βE increases within the circulation have been documented in response to various aerobic and anaerobic exercises (Goldfarb & Jamurtas 1997). Several studies have reported that circulating βE immunoreactivity can increase in response to exercise depending on the intensity of exercise (McMurray *et al*. 1987; Goldfarb *et al*. 1990; Kraemer, W.J. *et al*. 1993). It appears that a critical minimum intensity of > 60% of $\dot{V}O_{2max}$ is needed to result in βE elevation with aerobic exercise (McMurray *et al*. 1987; Goldfarb *et al*. 1990, 1991; Rahkila & Laatikainen 1992). However, this minimum may differ based on the individual (Viru & Tendzegolskis 1995; Heitkamp *et al*. 1996) and nutritional status of the subject. Additionally, the duration of the exercise appears to influence the βE response to exercise (Goldfarb *et al*. 1990; Heitkamp *et al*. 1996).

Incremental exercise and high intensity anaerobic exercise has been reported to stimulate βE increases within the circulation (Metzger & Stein 1984; Farrell *et al*. 1987; Goldfarb *et al*. 1987; Heitkamp *et al*. 1996). Resistance exercise as a stimulus to circulating βE is limited. Conflicting reports exist and may be related to differences in subjects, type of exercise intensity and time of measurement. Kraemer, W.J. *et al*. (1993) reported that βE levels in the circulation increased in response to high total workload. These authors noted that the total work, rest to work ratio and total force needed probably influenced the βE response. These authors also reported an increase in βE in 28 elite male weightlifters after a moderate to high intensity workload (Kraemer, W.J. *et al*. 1992). βE levels were reported to be elevated in females in response to three sets of resistance at 85% of their 1-repetition maximum (1-RM) (Walberg-Rankin *et al*. 1992). An increased βE/β-lipotrophin level was reported in response to weightlifting in five men (Elliot *et al*. 1984). In contrast, low volume resistance exercise did not result in any change in βE levels (Kraemer, R.R. *et al*. 1996). It appears that resistance exercise of sufficient intensity and volume (workload) can result in a transient increase in βE levels within the circulation.

Cortisol response to exercise has varied depending on the type of exercise, the intensity of exercise and the duration of the exercise. Generally, mild intensity moderate duration aerobic exercise does not appear to alter the cortisol level within the circulation, although some reports have indicated a decline in cortisol. In contrast, longer duration exercise and more intense exercise have typically elevated circulating cortisol levels. This may be related in part, to glucose homeostasis. When individuals are given carbohydrate during long duration exercise the cortisol response is attenuated. Exercise of sufficient duration generally will demonstrate an increase in circulating cortisol (Galbo 1983; Petraglia *et al*. 1988). Cortisol levels increased in athletes who ran 1500 m and 10 000 m but not in athletes whom competed in sprints (100 m) or performed the discus throw (Petraglia *et al*. 1988). Short-term exercise may have only minor changes

in the cortisol level in plasma (Galbo 1983). It should be noted that because of diurnal variations in cortisol levels, the increases are not always observed.

Cortisol response to resistance exercise has equivocal responses partly due to the intensity of the exercise and also related to total workload. In a recent study, it was reported that force repetition exercise increased plasma cortisol levels to a greater extent that maximal isotonic exercise (Ahtiainen *et al*. 2003). The results suggest that the workload has an influence on the cortisol response. In support of this finding, resistance exercise using different intensities of 1-RM and varying sets was shown to influence the cortisol response (Smilios *et al*. 2003). The results suggested that cortisol response was similar in the high intensity exercise independent of number of sets whereas in lower intensity resistance exercise four sets increased cortisol more so than two sets. High total work-resistance exercise was reported to increase cortisol as well as βE (Kraemer W.J. *et al*. 1993). It is interesting to note that the elevation in cortisol was fairly rapid and occurred by the middle of the exercise as well as immediately following the activity and during the 15-min recovery period. Not all studies confirm that cortisol levels will increase with high intensity exercise (Volek *et al*. 1997). These equivocal findings in cortisol in response to resistance exercise may be related in part to diurnal variations, nutritional factors and training status of the subjects.

β-Endorphin and immune function

βE influence on immune function has been investigated *in vitro* but has not been adequately investigated *in vivo*. βE (both rat and human) was shown to stimulate T-lymphocyte proliferation (Hemmick & Bidlack 1990). The data suggests that βE mode of action was not through an opioid receptor but might be through the inhibition of the prostaglandin E_1 effect on immune function. Synthetic βE was shown to bind to non-opioid receptors on T-lymphocytes and was not blocked by naloxone or Met-enkephalin (Navolotskaya *et al*. 2001).

βE was shown *in vitro* to stimulate rat spleen lymphocytes by enhancing the proliferative response to several mitogens (Gilman *et al*. 1982). The response was dose dependent and was not blocked by naloxone. Additionally, there was no βE effect on B-lymphocytes. The proliferative response of splenocytes of adult male F344 rats to βE was enhanced 50–100% in a dose-dependent manner on T-cells (van den Bergh *et al*. 1991). It was noted that interleukin 2 (IL-2) was elevated as well as IL-2 receptor expression prior to the βE proliferative effect on the T-cells. Additionally, naloxone was not effective in blocking the βE effect. Further evidence on the βE proliferative effect on human T-lymphocytes was shown using the mitogen concanavalin A (Owen *et al*. 1998). βE stimulated the mitogen response by 121–750% with a bell-shaped curve indicating that too high a dose would actually inhibit the response. It also appears that this response may change with time, dose or mitogen used (Millar *et al*. 1990). These authors also noted that the inhibition of the immune response to cortisol maybe partially reversed by βE. Therefore, the activation of βE may inhibit suppression of the immune response by cortisol *in vivo*.

The effect of βE on human natural killer cell function was studied *in vitro* and was enhanced when βE was present (Kay *et al*. 1984). They also reported that this was a dose dependent response and was inhibited by naloxone. This suggests that the mode of action on natural killer cells appears to be different than the enhancement of T-lymphocyte function. The βE effect on natural killer cell activity (NKCA) and amount with exercise was also studied (Gannon *et al*. 1998). Naltrexone (an opioid-blocking agent) was given 60 min prior to a 2-h moderate intensity exercise (65% $\dot{V}O_{2max}$) and compared to a placebo trial. The βE levels in the blood increased at 90 and 120 min with exercise and NKCA and counts were elevated. Furthermore, the naltrexone treatment did not alter the exercise response in NKCA or counts. These authors suggested that βE probably was not involved in the NKCA increase with exercise. However, it is possible that βE may work independent of this receptor action (Jonsdottir *et al*. 2000). Chronic exercise (wheel running for 5 weeks) in spontaneously hypertensive rats enhanced NKCA. The βE levels in cerebrospinal fluid increased after the running and also enhanced clearance of lymphoma cells from the lungs. The δ-receptor antagonist naltrindole significantly but not completely

inhibited the enhanced NKCA after 5 weeks of exercise. Neither κ- nor μ-receptor antagonists influenced the natural killer cell response. These authors suggested a central δ-receptor mediated response to the exercise training occurred. Peripheral βE given subcutaneously did not alter NKCA *in vivo* (Jonsdottir *et al*. 1996). In contrast, NKCA after central injection of a δ-opioid receptor agonist was enhanced (Band *et al*. 1992). In addition, a single injection into the intracerebral ventricle of a μ-agonist reduced NKCA. Furthermore, a single morphine injection into the periaquaduct area suppressed NKCA (Weber & Pert 1989). This suggests that central mediated βE may act to modulate NKCA via both μ-receptors and δ-receptors. Clearly more research with training programs is needed to substantiate the training response in other populations.

Additional modes of action of βE on the immune response include mononuclear cell chemotaxis (van Epps & Saland 1984; Pasnik *et al*. 1999), immunoglobulin migration (van Epps & Saland 1984; Saland *et al*. 1988) and lymphokine production (van Epps & Saland 1984). Macrophages showed migration to βE injected into the cerebral ventricles in rats (van Epps & Saland 1984). Human neutrophils demonstrated enhanced migration to βE infusion and the response was blocked by prior incubation with naloxone. Analogs of opioids appear to have different responses when injected into the cerebral ventricles (Saland *et al*. 1988). Some may stimulate macrophages and others may influence neutrophils. The chemotaxis response appears to be dose dependent (Pasnik *et al*. 1999). High doses of βE (10^{-3} mol) inhibited the chemotaxis response whereas lower concentrations stimulated up-regulation of neutrophils. Since, physiological βE concentration is below the high dose level even when elevated by exercise or other stressors, it is probable βE would have a stimulatory influence on this immune function.

It has been suggested that the opioid peptides such as βE and the enkephalins have a similar structural component of interleukin-2 (Jiang *et al*. 2000). Interleukin-2 and other interleukins are involved in the inflammatory response and are targets of βE and cortisol. It is highly likely that both βE and cortisol influence the immune response by interacting with interleukins (Zitnik *et al*. 1994). The inhibition may be at a number of levels including the reduction of production of interleukin-1 and 6 in a dose-dependent manner. βE stimulated the production of interferon-γ (IFN-γ) from human mononuclear cells in response to concanavalin A in cultured cells (Brown & van Epps 1986). IFN-γ was enhanced in a dose-dependent manner using physiological βE (10^{-14}–10^{-10} mol) and was not blocked by naloxone.

It appears that βE may act on a number of immune factors both centrally and in the periphery and may act through both opioid and non-opioid receptors. Additionally, the action of βE may work through direct interaction with cortisol.

Cortisol and immune function

Cortisol has been generally thought of as an immunosupressent and anti-inflammatory agent. Corticosteroids given intravenously to humans can induced lymphocytopenia, monocytopenia and neutrophilia but may take up to 4 h to peak (Rabin *et al*. 1996). High doses of corticosteroids can result in cell death of immature T- and B-lymphocytes (Cohen & Duke 1984). Cortisol can regulate the immune system by induction of apoptosis of thymus and blood lymphocytes (Hirano *et al*. 2001). However, the metabolite of cortisol oxidation, cortisone can inhibit the apoptosis of these lymphocytes. Human monocyte cell death was shown to be enhanced by glucocorticoids (Schmidt *et al*. 1999). There was both a time and dose-dependent effect on monocyte apoptosis. IL-1 mediated activation was partially responsible for the enhanced monocyte apoptosis. In addition, cortisol can inhibit tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) by activated monocytes/macrophages (Hart *et al*. 1990). Interleukin-1 may also feedback to the hypothalamus to stimulate ACTH and cortisol synthesis (Besedovsky & del Rey 1987). Incubation of thymocytes and splenocytes with corticosterone *in vitro* resulted in apoptosis and necrosis of these cells after 24 h (Hoffman-Goetz & Zajchowski 1999). The concentration of corticosterone in the medium was similar to the level that near maximal exercise would produce. This suggests that cortisol may

contribute to the apoptosis of lymphocytes and reduced immunity after the intense exercise.

Corticosteroids have been shown to inhibit NKCA *in vitro* (Parillo & Fauchi 1978; Pedersen *et al*. 1984). NKCA was shown to decrease in blood lymphocytes after intravenous methylprednisolone pulse therapy in eight rheumatoid arthritic patients (Pedersen *et al*. 1984). Both *in vitro* and *in vivo* NKCA decreased in response to cortiocosteroids (Parillo & Fauchi 1978). However, the response *in vivo* was different compared to *in vitro.* Adhesion of natural killer cell function to target cells was inhibited in response to pharmacological doses of corticosteroids *in vitro* (Hoffman *et al*. 1981; Pedersen & Beyer 1986). The natural killer cell activity of mononuclear cells was inhibited by methylprednisolone and hydrocortisone in a dose-dependent manner and inhibited adhesion to target cells (Pedersen & Beyer 1986). Inhibition of adhesion to target cells by corticosteroids in mononuclear cells was dose dependent and was related to alteration in the methylation of phospholipids (Hoffman *et al*. 1981).

The decline in immune function associated with cortisol will have a time delay of several hours. The influence of cortisol in response to moderate or low intensity exercise appears to be minimal on immune function. However, intense exercise can induce the cortisol response and this has an impact on immune function. In addition, chronic high intensity exercise can reduce immune function (Pedersen & Hoffman-Goetz 2000).

Summary

Both βE and cortisol influence the immune function, with βE generally enhancing immune function and cortisol acting as an immunosupressent. The interplay of βE and cortisol in regulating immune function in response to both acute and chronic exercise still needs clarification. The exercise effect on central mediated βE and *in vivo* immune function also needs clarification. Adaptation effects to training also need further study. In addition, nutritional factors (i.e. carbohydrate level and antioxidants) have not been adequately examined in relation to both βE and cortisol influence on the immune response.

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Chapter 25

Neuroendocrine Modulation of the Immune System with Exercise and Muscle Damage

MARY P. MILES

The endocrine and nervous systems work together to co-ordinate growth and development, to regulate homeostasis and to co-ordinate the response of the body to stress. The study of the interrelationship between these systems is termed neuroendocrinology. The immune system is controlled locally by chemical signals generated at the cellular level and systemically by the neuroendocrine system. However, complex, bi-directional, anatomical and physiological interactions exist among the three interrelated systems, nervous, endocrine and immune (Mašek *et al*. 2003). All three systems have receptors for a shared set of ligands including cytokines, peptide hormones and neurotransmitters (Haddad *et al*. 2002). Thus, the immune system is capable of exerting influence on the neuroendocrine system as well as vice versa. Exercise is a form of stress to the body, and as such, it elicits the stereotypic, neuroendocrine stress response first described in 1936 by Hans Selye as 'the general adaptation syndrome' (Selye 1936, p. 32). The complexity of the neuroendocrine immune system is such that the immune response to exercise varies according to intensity and duration of exercise, environmental conditions, nutritional factors, level of recovery from previous training and tissue damage (Nieman 1997; Pedersen & Hoffman-Goetz 2000).

The aim of this chapter is to examine the neuroendocrine immune response to exercise stress from several perspectives within the context of: (i) acute exercise; (ii) exercise training; and (iii) exercise-induced muscle damage. The key immune system components are described in Table 25.1. The immune response to acute exercise is influenced by intensity and duration of exercise, the recovery state of the body and the availability of nutrients during the exercise. In many respects, the responses and adaptations to exercise training are the cumulative influence of repeated acute exercise bouts and resources provided to the body for recovery and adaptation. Exercise-induced muscle damage activates the immune system both locally and systemically and provides a model to study the inflammatory arm of the immune system. The neuroendocrine immune response to muscle damage and other stresses to muscle provides insight into the role that this complex system may play in eliciting adaptations to exercise training, for example muscle hypertrophy. Understanding the implications of exercise-induced immune modulations and understanding that the immune system may have a role in producing physiological adaptations to exercise is important to the design of exercise programs for health and for athletic performance.

Sympathetic nervous system

The autonomic nervous system includes the parasympathetic nervous system that controls resting functions and the sympathetic nervous system that enables the body to become physically active, as in the 'fight-or-flight' response. Responses to stress, regardless of the nature of the stress, are coordinated collectively by the sympathetic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis (discussed subsequently) (Tsigos & Chrousos 2002). Epinephrine and norepinephrine, also known as adrenaline and noradrenaline or

Table 25.1 Key components of the immune system. (Data from Liles & Van Voorhis 1995; Shephard 1997; Elenkov *et al*. 2000; Rivest 2001; Suzuki *et al*. 2002; Steensberg *et al*. 2003.)

Component	Production/location	Key functions
Leukocytes		
Neutrophils	Produced in bone marrow and released to the circulation. Many adhere to vascular endothelial cells, particularly in the lungs	Inflammation and natural immune defense against infection. Produce free radical oxygen species that destroy bacteria and injure nearby cells. Remove small debris in the area of infection or inflammation by the process of phagocytosis
Monocytes/macrophages	Monocytes are produced in bone marrow and are found in the blood. After leaving the circulation, monocytes differentiate to become macrophages	Natural immunity protection against viral infection and tumors, phagocytosis of cellular debris, production of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10, IL-12). Following phagocytosis and activation, macrophages are able to 'present' antigens to T-lymphocytes to activate antigen-dependent/acquired immune defenses
Lymphocytes		
Natural killer (NK) cells ($CD3^-$ $CD16^+56^+$)	Production by bone marrow, found in blood and attached to vascular endothelial cells in lymphoid tissues.	Natural immunity via non-major histocompatibility complex (MHC)-restricted cytotoxicity, e.g. killing virally infected cells and some tumor cells. Important for early defense against viruses and some malignancies
Cytotoxic T-lymphocytes ($CD3^+$ $CD8^+$)	Production by bone marrow and maturation in the thymus gland. Mature cells found in lymphatic tissue, spleen and blood	MHC-restricted cytotoxicity. Important for cell-mediated, 'acquired' immune responses to kill infected cells
Helper T-lymphocytes ($CD3^+$ $CD4^+$)	Production by bone marrow and maturation in the thymus gland. Mature cells found in lymphatic tissue, spleen and blood	Co-ordination of immune responses. Undifferentiated (Th0) $CD4^+$ cells will be activated for differentiation to either the Th1 $CD4^+$ cells that regulate cellular immunity or the Th2 $CD4^+$ cells that regulate humoral immunity and some inflammatory functions. A small minority of $CD4^+$ cells produce TGF-β and are designated Th3 $CD4^+$ cells
B-lymphocytes ($CD19^+$)	Produced by bone marrow, upon activation by antigen they differentiate to become plasma cells. Mature B-cells found in many extracellular fluids including blood and mucous, and stored in lymphatic tissues	Stimulation by antigen and cytokines from Th1 type $CD4^+$ T-lymphocytes induces production of immunoglobulins (antibodies)
Immunoglobulins (Ig)	Produced by plasma cells (B-cells activated for antigen production) and released. Found in blood, saliva, mucosal secretions and throughout the body	Bind to molecular and cellular antigens (particularly bacterial) to form an antibody-antigen complex that induces phagocytosis by neutrophils and macrophages to eliminate the antigen

Cytokines		
Interferon-γ (IFN-γ)	Th0 and Th1 $CD4^+$ T-cells, $CD8^+$ T-cells (Tc1 subset), NK cells	Stimulates macrophage activation, neutrophils and NK cells and production of antibodies by B-cells; inhibits Th2 cytokine production by $CD4^+$ T-cells
Tumor necrosis factor-α (TNF-α)	Produced by monocytes/macrophages and NK cells and to a lesser extent by neutrophils, T- and B-lymphocytes and other cells	Anti-tumor activity, initiation of inflammation via chemokine induction, recruitment of neutrophils and monocytes and induction of IL-6 synthesis
Interleukin-1β (IL-1β)	Produced by monocytes/macrophages	Induction of cerebral response to inflammation, e.g. fever, stimulation of prostaglandin E_2 production, stimulation of IL-2 receptor expression, induces IL-6 synthesis
Interleukin-2 (IL-2)	Th0 and Th1 $CD4^+$ T-cells, $CD8^+$ T-cells (Tc1 subset)	Th1 cytokine, potent stimulation of NK cell activity, stimulation of lymphocyte proliferation and antibody secretion by B-cells
Interleukin-4 (IL-4)	Th0 and Th2 $CD4^+$ T-cells, B-cells	Stimulation of Th2 cells, stimulation of immunoglobulin production and proliferation by B cells, stimulation of allergic responses via IgE production, inhibits cytokine production by Th1 $CD4^+$ cells
Interleukin-6 (IL-6)	Th0 and Th1 $CD4^+$ T-cells, $CD8^+$ T-cells (Tc1 subset) and monocytes/macrophages, but nearly all cells can be induced to produce IL-6, most notably muscle cells	Th2 cytokine, stimulates B- and cytotoxic T-cells, induction of IL-2 production, stimulation of acute phase protein synthesis, activation of the HPA axis, inhibition of TNF-α and IL-1β synthesis, stimulation of IL-10 and IL-1ra synthesis
Interleukin-8 (IL-8)	Produced by monocytes, macrophages and endothelial cells	Chemokine, recruits neutrophils to inflammatory sites, stimulates production of reactive oxygen species and degranulation by neutrophils
Interleukin-10 (IL-10)	Produced by Th0 and Th2 $CD4^+$ T-cells, monocytes and B-cells, as well as pituitary and hypothalamic cells	Th2 cytokine, inhibits cytokine production by Th1 $CD4^+$ T-cells, monocytes and macrophages, stimulates B-lymphocyte proliferation and antibody secretion
Interleukin-12 (IL-12)	Produced by monocytes	Stimulates the Th1 immune pathway, promotes cytotoxic activity of $CD8^+$ T-lymphocyte and NK cells, inhibits IgE secretion from B-lymphocytes
Transforming growth factor β (TGF-β)	Th3 $CD4^+$ T-cells, macrophages and other cells	Inhibition of NK cell activity, B- and T-cell proliferation and some macrophage functions; stimulation of IgA secretion by B-lymphocytes

simply catecholamines, are the neurotransmitters released by the sympathetic nervous system. Activation of the sympathetic nervous system via the locus ceruleus–norepinephrine system (LC/NE) stimulates release of epinephrine from the adrenal medulla and norepinephrine from axon terminals of sympathetic neurons. Both of these neurotransmitters increase in the bloodstream during exercise, although the relative concentrations of norepinephrine exceed those of epinephrine by several orders of magnitude (Weicker & Werle 1991; Kjær & Dela 1996). There is a linear increase in the concentration of catecholamines as the duration of exercise increases (Kjær & Dela 1996). In contrast, the increase in catecholamines in response to increasing exercise intensity is of a greater magnitude, more closely approximating an exponential increase (Kjær & Dela 1996).

Leukocyte trafficking

The most dramatic effect of catecholamines on the immune system is to draw leukocytes from extravascular storage depots to the circulation. Research studies involving infusion of epinephrine or norepinephrine to match exercise levels or involving blockade of catecholamine receptors (adrenergic receptors) during exercise clearly indicate that epinephrine (ligand for β_1- and β_2-adrenergic receptors) recruits lymphocytes and neutrophils to the circulation during exercise (van Titts *et al*. 1990; Kappel *et al*. 1991; Benschop *et al*. 1994; Schedlowski *et al*. 1996). Norepinephrine (strong β_1- and weak β_2-adrenergic receptor ligand) has a smaller effect on circulating leukocytes than epinephrine. Thus, it generally is accepted that increased intracellular cyclic adenosine monophosphate (cAMP) induced by epinephrine binding to β_2-adrenergic receptors is a primary stimulus driving lymphocytes and neutrophils into the circulation (Boxer *et al*. 1980; Weicker & Werle 1991; Schedlowski *et al*. 1996).

Tissues where immune cells are produced or reside, including the thymus, spleen, lymph nodes, tonsils, bone marrow and gut-associated lymphoid tissue (GALT), are innervated by the sympathetic nervous system via noradrenergic and or neuropeptide Y (NPY) nerve terminals (Elenkov *et al*. 2000). This 'hard-wire' connection has a major role in functional modulation of immune cells, but it has little effect on exercise-induced leukocyte trafficking.

Leukocyte function

The view that catecholamines have an immunosuppressive net effect is losing favor to a view that recognizes a more complex variety of responses (Elenkov *et al*. 2000). As illustrated in Fig. 25.1, the acute influence of catecholamines on the immune system is complex, but generally leads to suppression of the Th1 system (production of interleukin-2 [IL-2], interferon-γ [IFN-γ] and regulation of cellular immunity), no direct effect on the Th2 system (production of IL-4, IL-5, IL-6 and IL-10, and regulation of humoral immunity) and mixed effects on the inflammatory system. A portion of the proinflammatory response to catecholamines is the result of disinhibition of inflammatory cytokine synthesis that occurs when catecholamines inhibit IL-2 and IL-12 synthesis, i.e. stimulation via removal of inhibition. In contrast to acute exercise, chronic exposure to catecholamines results in desensitization and a cell type-specific down-regulation of β_2-adrenergic receptors or other components of cell signaling (Elenkov *et al*. 2000). Thus, acute and chronic stresses may have divergent effects on the immune system.

Catecholamines also are capable of modulating natural killer (NK) cell function. Both epinephrine and norepinephrine, but particularly epinephrine, have induced increases in circulating NK cells and decreases in the per cell cytotoxic activity of those cells (Schedlowski *et al*. 1993; Klokker *et al*. 1997; Kappel *et al*. 1998). The decrease in activity is likely to be a function of catecholamine-induced decreases in IL-2 and IL-12, cytokines that up-regulate NK-cell cytotoxicity (see Fig. 25.1).

While many cellular responses are induced directly by rises in intracellular cAMP (Borger *et al*. 1998), it has been suggested that the functional modulations of lymphocytes in response to catecholamine increases are mediated by macrophages and nitric oxide (Rabin *et al*. 1996). Evidence for this mechanism has also been presented in the rodent model (Blank *et al*. 1997). An example relevant to

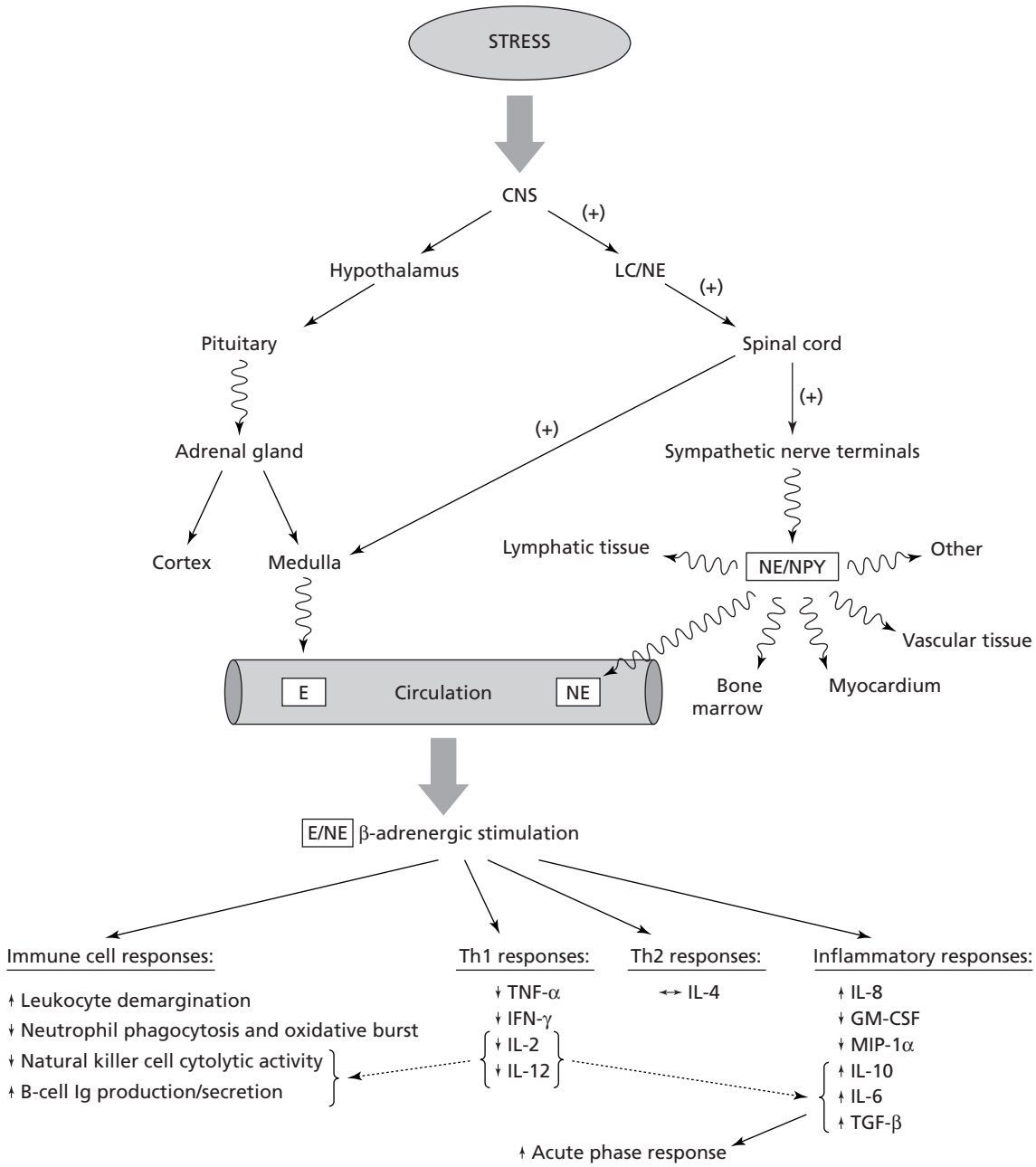

Fig. 25.1 (+) Indicates stimulation; dashed arrows indicate effect induced by removal of inhibition; boxes indicate neurotransmitters. CNS, central nervous system; E, epinephrine; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ig, immunoglobulin; IL, interleukin; LC/NE, locus ceruleus–norepinephrine; NE, norepinephrine; NPY, neuropeptide Y; TGF, transforming growth factor; TNF, tumor necrosis factor. (Data from Sanders *et al*. 1997; Borger *et al*. 1998; Elenkov *et al*. 2000; Kohm & Sanders 2001.)

acute exercise is that Kappel *et al*. (1991) were able to demonstrate that NK-cell activity per cell was lower 2 h following epinephrine infusion. There was a twofold increase in monocytes at this time, and it was hypothesized that prostaglandins produced by the monocytes inhibited the cytolytic activity of the NK cells. As hypothesized, when prostaglandin production by monocytes was blocked using indomethacin, the per cell decrease in NK-cell activity also was blocked. This suggests that prostaglandins produced by monocytes, rather than a direct effect of epinephrine, inhibited NK-cell function. By contrast, it should be noted that Nieman *et al*. (1995a) measured decreased NK-cell activity despite indomethacin administration. Thus, this mechanism may not be consistent across all situations.

Hypothalamic–pituitary–adrenal axis

The HPA axis consists of signals feeding forward from the hypothalamus to the anterior pituitary and then to the adrenal cortex (Tsigos & Chrousos 2002; Beishuizen & Thijs 2003). In response to many forms of stress, the hypothalamus releases corticotropin-releasing hormone (CRH) and vasopressin (AVP). CRP, and to a lesser extent AVP, stimulate the anterior pituitary to produce adrenocorticotropic hormone (ACTH). The main role of AVP is to promote fluid resorption by the kidneys. As an endocrine hormone, ACTH travels through the circulation to stimulate the adrenal cortex to release glucocorticoid hormones, most notably cortisol. As such, cortisol is the final product of the HPA axis. The effects of cortisol on the immune system and the details of the relationships amongst the HPA axis components and the inflammatory cytokines are illustrated in Fig. 25.2. Cortisol acts in a negative feedback fashion to inhibit CRH and ACTH release. As a steroid hormone, cortisol is able to diffuse through plasma membranes and bind to intracellular receptors (Riccardi *et al*. 2002). The cortisol/glucocorticoid receptor complex affects cellular function primarily by up-regulating and down-regulating transcription of various proteins, but also via more rapid means such as a Ca^{2+}-dependent mechanism (Buckingham *et al*. 1996). Cortisol suppresses a number of immune responses and is a key regulatory element preventing the immune system from over responding to immune challenges and becoming destructive. For example, inflammation left unchecked will lead to excessive tissue destruction and possibly death (Northoff *et al*. 1995; Suzuki *et al*. 2002).

The effects of cortisol on the immune system are consistent with down-regulation of immune function, particularly down-regulation of inflammatory functions. For this reason, glucocorticoid analogs are commonly used to treat inflammatory and autoimmune pathologies (Ashwell *et al*. 2000). Binding of cortisol to the intracellular glucocorticoid receptor leads to activation of a glucocorticoid response element (GRE) and an array of transcriptional responses (Pitzalis *et al*. 2002). Of particular interest are the up-regulation of annexin I (formerly referred to lipocortin-1) and anti-inflammatory proteins, for example IL-1 receptor antagonist, and the down-regulation of cell adhesion molecules (CAM) and proinflammatory cytokines (Levine *et al*. 1996; Pitzalis *et al*. 2002).

The level of HPA activity ideally falls within a range that allows for mounting an effective immune/inflammatory response when needed, but does not allow excessive immune activity to become destructive. Excessive stress, in a variety forms, may result in chronically elevated cortisol levels and induce immunosuppression (Buckingham *et al*. 1996). For example, immunosuppression associated with depression has been linked to elevations in cortisol (Leonard & Song 1996). Reciprocally, adrenocortical insufficiency associated with low glucocorticoid release has been associated with increased susceptibility to autoimmune/inflammatory conditions (Buckingham *et al*. 1996). Pathological disorders that result in over and underproduction of glucocorticoids are identified as Cushing's and Addison's diseases, respectively. Within the non-pathological spectrum of adrenocortical reactivity, high and low stress responders have been identified with respect to the release of ACTH in response to stress (Petrides *et al*. 1997; Deuster *et al*. 1999). Thus, variability in the magnitude of the HPA response to stress is expected and a number of factors modulate

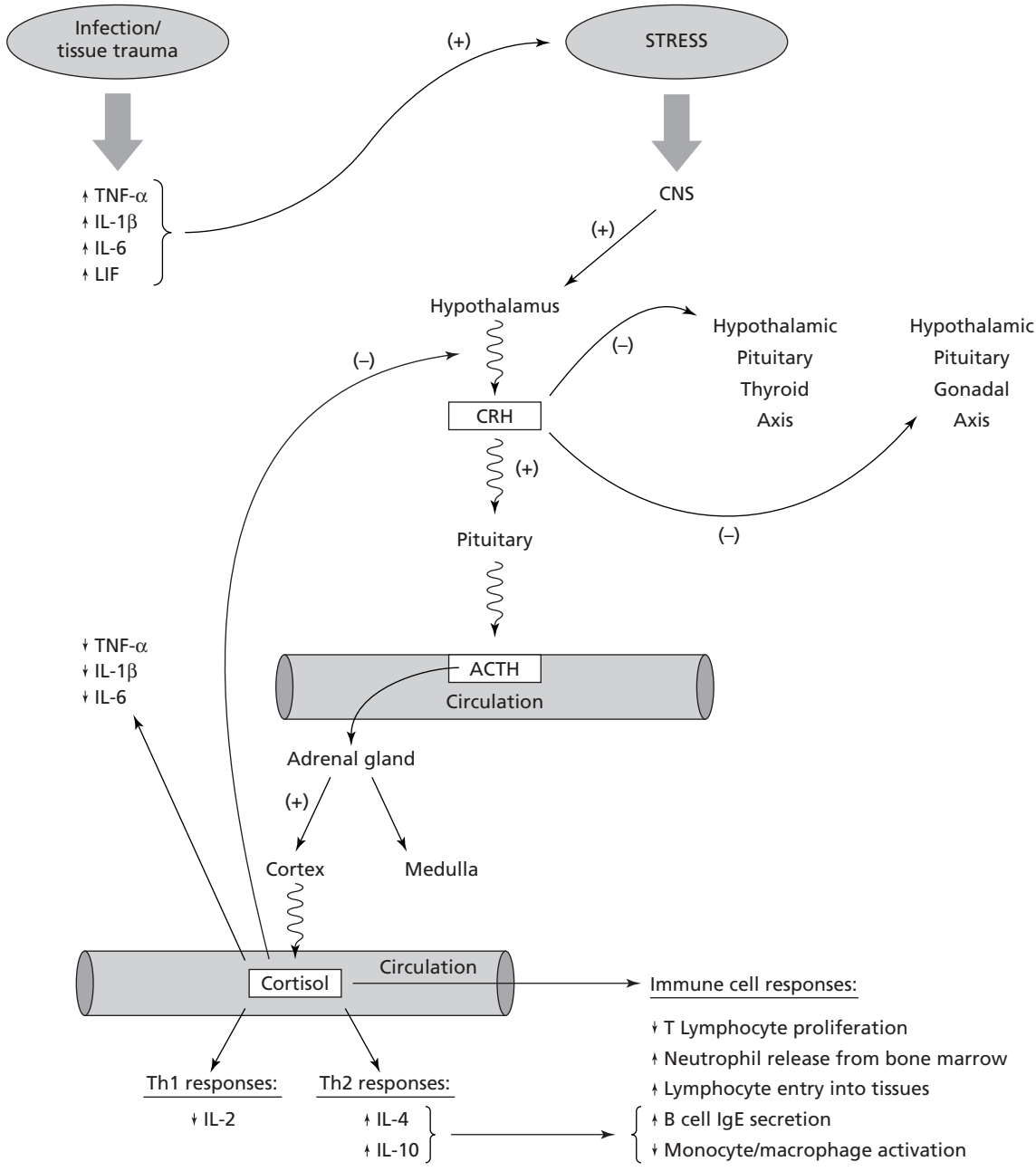

Fig. 25.2 (+) Indicates stimulation; (–) indicates inhibition; boxes indicate neurotransmitters or hormones. ACTH, adrenocorticotropic hormone; CNS, central nervous system; CRH, corticotropin-releasing hormone; E, epinephrine; IL, interleukin; LC/NE, locus ceruleus-norepinephrine; LIF, leukemia inhibitory factor; NE, norepinephrine; NPY, neuropeptide Y; TGF, transforming growth factor; TNF, tumor necrosis factor. (Data from Weicker & Werle 1991; Chesnokova & Melmed 2002; Haddad *et al*. 2002; Riccardi *et al*. 2002; Tsigos *et al*. 2002.)

this variability. This variability also will manifest in variability in the immune response to stress, regardless of the stressor.

The two-way communication between the immune and neuroendocrine systems is such that inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and leukemia inhibitory factor (LIF) can stimulate the HPA axis to induce the release of cortisol (Mastorakos *et al*. 1993; Chesnokova & Melmed 2002; Tsigos & Chrousos 2002). LIF is necessary for inflammation-induced release of ACTH and cortisol, and may be a potentiator of TNF-α and IL-1β induced activation of the HPA axis (Chesnokova & Melmed 2000, 2002; Chesnokova *et al*. 2002). That is, the chemical signals that promote the inflammatory process also initiate a negative feedback loop to turn their own activity down. The reverse of this relationship has also been demonstrated. Specifically, most pituitary hormones can be produced by lymphocytes (Carr & Blalock 1990). For example, lymphocytes stimulated with recall antigen or with IL-12 will produce growth hormone (Malarkey *et al*. 2002).

In addition to modulating the immune system, glucocorticoids play an important role in enabling the body to respond to stress in such a way that increases the likelihood of survival in stressful situations. Glucocorticoids inhibit release of sex steroids and growth hormone (Tsigos & Chrousos 2002), thus removing the likelihood of the need to allocate resources of the body to non-essential growth. Further, glucocorticoids inhibit the hypothalamic–pituitary–thyroid axis, an effect that results in a lowering of basal metabolic rate (Tsigos & Chrousos 2002). To accommodate the need for energy in the tissues forced to respond to stress, glucocorticoids promote gluconeogenesis, glycogenolysis, lipolysis and proteolysis (McMurray & Hackney 2000; Steinacker *et al*. 2004). By promoting gluconeogenesis and glycogenolysis by the liver, cortisol helps to maintain blood glucose levels and it is considered to be a 'glucoregulatory' hormone. Thus, growth processes are turned off, fertility is decreased, metabolic rate is turned down to decrease the overall demand for energy on the body, and energy substrates stored for a 'rainy day' are made available to the body.

Leukocyte trafficking

The effects of glucocorticoids on leukocyte migration from tissue to tissue occur in a delayed time course relative to catecholamines. Shifts in leukocytes typically peak approximately 4 h after elevations in cortisol are induced (Rabin *et al*. 1996). Broken down by leukocyte type in the circulation, the response to glucocorticoid increases are concentration decreases in lymphocytes and monocytes and increases in neutrophils (Rabin *et al*. 1996; Nieman 1997). The net effect is a rise in leukocyte count exclusively owing to an influx of neutrophils. Pharmacological analogs also produce this profile.

Cytokines

Glucocorticoids are capable of inhibiting production of many cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and IFN-α (Ashwell *et al*. 2000; Riccardi *et al*. 2002). The influence on Th1 cytokine production is greater than that for Th2 cytokine production; for example, IL-12 synthesis is profoundly inhibited and IL-10 synthesis is only moderately inhibited (Ashwell *et al*. 2000). As such, glucocorticoids are considered potent inhibitors of cellular immunity and inflammation.

Leukocyte function

The functional consequences of these glucocorticoid-induced genomic and non-genomic effects are widespread through the immune system. Both NK- and T-cell functions are inhibited by cortisol (Ramirez & Silva 1997; Zhou *et al*. 1997; Ashwell *et al*. 2000). The ability of NK cells to lyse target cells is used to determine NK cell activity (NKCA). Cortisol downregulates NKCA by decreasing synthesis of effector proteins (Zhou *et al*. 1997). The cellular immune response is dependent on the clonal proliferation of antigen-specific T- and B-cells. Lymphocyte proliferation assays are a popular functional measurement performed *in vitro* by exposing these cells to cytokines or polyclonal mitogens or antigens to induce proliferation. T-cell proliferation is inhibited

by cortisol (Ashwell *et al*. 2000; Cancedda *et al*. 2002). The effect on B-cells is mixed, with inhibition of immunoglobulin G (IgG) and IgA and stimulation of IgE synthesis (Ashwell *et al*. 2000).

Acute exercise

While it is important to understand the effects of the sympathetic nervous system and HPA axis hormones in isolation, exercise-induced immunomodulation is the result of the collective influences of many physiological responses occurring simultaneously or in sequence. Owing to the complexity of the systems involved and the number of potential factors that can enhance or attenuate the stress to the system as a whole, the array of potential influences on the immune response is virtually infinite. A single bout of exercise can induce the immune system to transiently redistribute leukocytes to the bloodstream and amongst tissues, alter the functional capacity of leukocytes, induce the release of a host of molecules that regulate immune function, produce transient or prolonged inflammation, and in doing so, change the overall immune defense level that the body has for protection against infection and tumor cells. The extent of these changes typically is greater as the magnitude of the exercise stress increases. The typical determinants of stress magnitude are intensity and duration of exercise (McMurray & Hackney 2000). However, the level of stress to which the neuroendocrine immune system must respond is enhanced by additional factors such as insufficient recovery from previous exercise (Ronsen *et al*. 2002a, 2002b; McFarlin *et al*. 2003), low carbohydrate availability (Nieman *et al*. 1998; Green *et al*. 2003), hypoxic conditions (Klokker *et al*. 1995; Niess *et al*. 2003) and heat stress (Brenner *et al*. 1998; Mitchell *et al*. 2002).

Leukocyte trafficking

Activation of the sympathetic nervous system elicits the exercise-induced increases among the different leukocyte subsets. While there is tremendous inter-individual variability in the magnitude of response, the overall pattern of leukocyte redistribution during exercise is robust and consistent (see Shephard 1997 for review). NK cells have the greatest β_2-adrenergic receptor density, followed by $CD8^+$ T-lymphocytes and B-lymphocytes and monocytes, and then by T-helper type 1 $CD4^+$ T-lymphocytes (Maisel *et al*. 1990; Schedlowski *et al*. 1996). T-helper type 2 $CD4^+$ T-lymphocytes are the only lymphocyte subset that does not express β-adrenergic receptors (Sanders *et al*. 1997). Consistent with the catecholamine response, the NK response to exercise increases with intensity and duration of exercise and the reported increases during exercise range all the way from 50% to 900% (Shephard 1997). The remaining lymphocyte subsets respond less dramatically as the density of β-adrenergic receptors decreases. Additionally, the cells that enter the circulation are predominately memory/activated cells with short telomeres in the case of lymphocytes (Bruunsgaard *et al*. 1999; Pedersen & Hoffman-Goetz 2000), and segmented rather than banded nuclei in the case of neutrophils (Miles *et al*. 1998). This indicates that the influx of cells to the circulation is made up of mature cells, rather than of newly produced or released immature/naïve cells.

Cortisol gains increasingly more influence over that of the catecholamines as the duration of exercise continues. Approximately 1.5 h into endurance exercise, the influence of cortisol on leukocyte distribution and function overtakes that of the catecholamines (Nieman 1997). If the duration and intensity of exercise are sufficient to create an increase in cortisol, then a cortisol-driven lymphocytosis occurs for 1–3 or 4 h post-exercise (Nieman 1997).

Neutrophils are similar to lymphocytes in that they increase in the circulation during exercise in a dose–response fashion, and they differ from lymphocytes in that they have a second wave of elevation that is delayed and sustained in the circulation a few hours following exercise that induces a significant cortisol increase (Peake 2002). While epinephrine is credited with a portion of the induced increase during exercise, a number of additional factors have been associated with the rise in neutrophils including IL-6, IL-8, granulocyte-colony stimulating factor (G-CSF), growth hormone and muscle damage (Hetherington & Quie 1985; Suzuki *et al*. 1999; Peake 2002; Yamada *et al*. 2002). The delayed

elevation of neutrophils appears to be the effect of cortisol and includes the entry of both immature band neutrophils from the bone marrow and mature segmented cells from marginal pools (Suzuki *et al*. 1996, 1999).

Monocytes increase in the circulation as part of the transient response to acute exercise (Woods & Davis 1994). This response is likely to be induced by enhanced hemodynamic forces and catecholamine-induced decreases in adhesion to vascular endothelial cells (Woods *et al*. 1999).

Cytokines

The pattern of cytokine production in response to exercise is not entirely consistent with the catecholamine and glucocorticoid response. That is, stimulation of the sympathetic nervous system should inhibit Th1 cytokine production (TNF-α, IL-2, IL-12 and IFN-γ), have little effect on Th2 cytokine production (IL-4), and stimulate inflammatory cytokine production (IL-6, IL-8, IL-10, transforming growth factor-β [TGF-β]). Activation of the HPA axis should inhibit production of some Th1 cytokines (IL-2) and some inflammatory cytokines (TNF-α, IL-1β, IL-6), and stimulate production of some Th2 cytokines (IL-4 and IL-10). These responses are what would be expected if catecholamines and glucocorticoids controlled the cytokine response to exercise. Consistent with this model is the tendency for IL-2 and IFN-γ production not to change or to decrease in response to exercise (Shephard 1997; Ibfelt *et al*. 2002; Suzuki *et al*. 2002). Also consistent with this model is the tendency for IL-8 and IL-10 to increase in response to exercise (Shephard 1997; Nieman *et al*. 2001; Pedersen *et al*. 2001; Suzuki *et al*. 2002). However, exercise elicits an inflammatory or 'inflammatory-like' response in a dose-dependent fashion that includes elevations in TNF-α, IL-1β, IL-6, IL-10 and IL-1 receptor antagonist (IL-1ra) (Ostrowski *et al*. 2000; Pedersen *et al*. 2001; Suzuki *et al*. 2002). Thus, the exercise-induced increase in cortisol occurs in part because the rise in inflammatory cytokines stimulates the HPA axis via the two-way communication network between the immune and neuroendocrine systems (Smith, L.L. & Miles 2000; Steensberg *et al*. 2003).

The single most responsive cytokine to exercise is IL-6 which increases exponentially as the duration of exercise progresses (Pedersen *et al*. 2001) and perhaps in correlation with rises in epinephrine (Papanicolaou *et al*. 1996). However, factors other than epinephrine elicit the vast majority of the exercise-induced rise in IL-6 (Jonsdottir *et al*. 2000; Steensberg *et al*. 2001). With respect to magnitude and time course, IL-6 concentrations increase from two to 100-fold in the blood during endurance exercise and typically return to resting levels within a few hours post-exercise. When exercise is of shorter duration and biased toward the development of high eccentric forces within active muscles, the kinetics are delayed and a relatively small peak of IL-6 has been measured a few hours post-exercise (Bruunsgaard *et al*. 1997a).

A key distinction to be made regarding IL-6 is that, while it has a role in the inflammatory response, it is produced more readily by skeletal muscle during exercise than by leukocytes, adipose, or the liver (Jonsdottir *et al*. 2000; Pedersen *et al*. 2001; Febbraio *et al*. 2003b). A number of physiological roles for IL-6 in the response to exercise have been identified in recent years, and the regulatory role of IL-6 beyond the inflammatory response is an emerging area of research (MacDonald *et al*. 2003; Steensberg *et al*. 2003). For example, as muscle glycogen is depleted, AMP-activated protein kinase (AMPK) increases, and a strong correlation between AMPK and IL-6 release has been measured (MacDonald *et al*. 2003). Thus, IL-6 may be involved in systemic signaling related to energy needs of muscle cells during exercise. Plasma IL-6 increases occur in the absence of muscle damage and are independent of other markers of inflammation. Exercising, but not resting, skeletal muscle cells release IL-6 (Febbraio *et al*. 2003b). Further, *IL-6* gene expression (IL-6 mRNA) was detected in muscle, but not in mononuclear leukocytes (includes monocytes) collected from the blood following strenuous exercise (Ostrowski *et al*. 1998b). Thus, IL-6 will increase in the absence of tissue damage and should be considered both as part of the inflammatory response and as part of an inflammatory-independent response to exercise (Pedersen *et al*. 2001).

Leukocyte function

Intensity and duration of exercise play an important role in the subsequent response of immune cells, including neutrophils. The effects of exercise are somewhat consistent in that exercise that is moderate in duration and intensity transiently enhances functions such as phagocytosis, degranulation of proteolytic enzymes and oxidative burst (Pyne 1994). If exercise is of sufficient intensity and or duration to elicit an increase in circulating glucocorticoids, then there are a number of functional consequences to cells of the immune system. The oxidative burst of neutrophils tends to be enhanced by exercise (Pyne 1994; Suzuki *et al*. 1996, 1999). There is some evidence that exercise-induced increases in IL-6, growth hormone and glutathione may be responsible for priming circulating neutrophils (Atalay *et al*. 1996; Suzuki *et al*. 1999). If the exercise lasts for a prolonged period of time or is extremely strenuous, for example a marathon, then the trend is for these functions to decrease (Müns 1993; Pyne 1994). This decrease may be a function of the diminished capacity of neutrophils to respond to stimuli after they have already been activated (Pyne 1994). Thus, moderate exercise may enhance and strenuous or intense exercise may reduce the first line of defense against bacterial and viral infections.

Exercise of a variety of intensities and durations has been measured to enhance several macrophage functions (Woods *et al*. 1994, 1999). Chemotaxis is the process by which monocytes and other immune cells follow a chemical gradient to move toward sites of inflammation or infection. Phagocytosis and elements of the associated oxidative burst also are stimulated by intense exercise and mediated by corticosteroids (Ortega *et al*. 1996). While the influence of corticosteroids is anti-inflammatory, the net effect of down-regulation of various inflammatory stimuli may be an increase in macrophage activation and activity (Woods *et al*. 1994; Ortega *et al*. 1997). However, Woods *et al*. (1994) have demonstrated in a murine model that the influence may be indirect and related to reactive nitrogen molecules, such as nitric oxide. Thus, the ability of monocytes and macrophages to response to injury or infection is likely to be improved for a period of time after exercise.

One factor attenuating the functional effects of cortisol on immune function is that the sensitivity of monocytes to cortisol is down-regulated by exercise (DeRijk *et al*. 1996). Dexamethasone inhibition of lipopolysaccharide (LPS) -stimulated IL-6 production by monocytes is attenuated following an exercise bout (DeRijk *et al*. 1996; Smits *et al*. 1998). This attenuation allows monocytes to increase IL-6 production, despite the anti-inflammatory influence of cortisol. However, the production of several proinflammatory cytokines by monocytes, including IL-6, is decreased and the production of the anti-inflammatory cytokines IL-10 and IFN-γ is not affected by acute exercise (Smits *et al*. 1998). Thus, the influence of exercise on cytokine production is complex and further research is needed to clarify this issue. It is important to remember that IL-6 synthesis by monocytes is less significant following strenuous exercise than that by skeletal muscle (Steensberg *et al*. 2002).

In response to exercise of light to moderate intensity or short duration, i.e. that in which cortisol does not increase and the effects of catecholamines prevail, redistribution of immune cells is accompanied by a few modest shifts in immune cell function. B-cells are responsible for immunoglobulin (IgA, IgE, IgG and IgM) production and increased serum concentrations of both IgA and IgG have been measured after moderate exercise in some (Nehlsen-Cannarella *et al*. 1991) but not all studies (Eliakim *et al*. 1997). Prolonged exercise decreases the levels of salivary IgA, an indication of decreased mucosal immune defenses (Walsh *et al*. 2002). In the long term, the process leading to development of antibody titers upon vaccination is not influenced by a single stressful bout of exercise (Nieman 1997). Thus, moderate exercise in which the catecholamines prevail do not appear to influence humoral immunity to a significant extent, but prolonged strenuous exercise in which the influence of cortisol prevails does decrease immune defense.

Cellular immunity may be lowered following strenuous exercise that includes the elevation of cortisol. Antigen presentation by macrophages appears to be attenuated owing to suppressed expression of

major histocompatibility complex II and this suppression correlates with cortisol elevations (Woods *et al*. 1997). Consistent with a reduction in the antigen presentation process is the measurement of smaller delayed-type hypersensitivity reactions following a half-marathon (Bruunsgaard *et al*. 1997b). This indicates that stressful exercise results in smaller cell-mediated immunity and antibody production responses to antigenic challenge. Additionally, glucocorticoids are capable of inducing apoptosis (programmed cell death) in lymphocytes and there is some evidence that strenuous exercise promotes T-lymphocyte, lymphocyte, monocyte and neutrophil apoptosis in various compartments in both humans and rodents (Hsu *et al*. 2002; Mooren *et al*. 2002; Hoffman-Goetz & Quadrilatero 2003). Recent research in which carbohydrate and placebo supplementation elicited similar cortisol responses immediately post-exercise measured more apoptosis in the placebo group (Green *et al*. 2003). This suggests that cortisol is not likely to be a causative factor for apoptosis in response to exercise.

The effect of exercise on lymphocyte proliferation is unclear. The only conclusion that can be drawn regarding exercise-induced modulations in the ability of T- and B-lymphocytes to proliferate is that the data are mixed and inconsistent (Nielsen & Pedersen 1997). Similarly, the effects of strenuous exercise on T- and B-cell functions are mixed. However, there are many reports of decreased proliferation responses of T- and or B-lymphocytes to mitogen stimulation following strenuous, prolonged exercise (Green *et al*. 2003). Examination of the proportion of activated $CD4^+$ and $CD8^+$ T-lymphocytes in the circulation expressing the CD69 surface marker, revealed that the proportion of activated lymphocytes was consistent across pre- and post-exercise measurements (Green *et al*. 2003). Based on this, a decrease in T-cell proliferation would not be expected, even following exercise that elicits an increase in cortisol. Dohi *et al*. (2002) found that the proliferation response to a T- and B-cell mitogen was lower in a group of women who had an increase in cortisol compared to those who did not have an increase in cortisol, while there was no exercise-induced change in proliferation to T-cell mitogens. This suggests that B-cell proliferation may be a bit more sensitive to cortisol and or strenuous exercise-induced inhibition.

The NKCA response to exercise must be considered both when NK-cell concentrations are increased, as during and immediately following exercise, and when concentrations are decreased, as in the hours following strenuous or prolonged exercise. The mechanisms controlling the exercise-induced modulation of NKCA are likely to differ during each of these phases. For exercise of moderate intensity and duration, for example 45 min at 50% of $\dot{V}o_{2max}$, the increases in NK-cell concentrations and NKCA are of similar magnitude, suggesting that the increase in NKCA is the result of increased NK cells (Nieman *et al*. 1993b). At higher intensities, reports of the response of NKCA on a per NK cell basis are variable with some reporting an increase in per cell activity (Nieman *et al*. 1993b; Strasner *et al*. 1996), and others reporting a decrease in per cell activity (Nieman *et al*. 1995b; Nielsen *et al*. 1996).

During the delayed phase of decreased NK-cell concentrations, NKCA may be reduced to approximately half of pre-exercise activity for a few hours. The decrease in NK-cell activity has been attributed to decrease in NK-cell numbers (Nieman 1997; Miles *et al*. 2002b), inhibition by prostaglandin E_2 (Pedersen & Ullum 1994; Rhind *et al*. 1999) and inhibition by cortisol (Berk *et al*. 1990). However, the time frame for down-regulation of NKCA by cortisol does not match the time-course of exercise-induced changes in NKCA, thus cortisol is not considered a likely modulator of NKCA beyond the effects of circulating numbers (Nieman *et al*. 1993b, 1995a).

One means of distilling out the influence of cortisol is to examine the research comparing endurance exercise with and without carbohydrate supplementation. Carbohydrate supplementation during prolonged endurance exercise decreases the stress hormone response (Nieman *et al*. 1998, 2001; Green *et al*. 2003). Thus, the difference in immune responses between conditions may reflect the types of functions influenced by cortisol, if the time course of differences in cortisol matches that of functional changes in immune cells. According to this paradigm, it appears that cortisol increases may be

associated with enhanced monocyte and neutrophil oxidative bursts (Nieman *et al*. 1998), phagocytosis (Henson *et al*. 2000), enhanced production of some cytokines, for example IL-8 and IL-10 (Nehlsen-Cannarella *et al*. 1997; Nieman *et al*. 2001), but not with apoptosis (Green *et al*. 2003) or IL-6 (Nieman *et al*. 2001). Reports on the effect carbohydrate supplementation on lymphocyte proliferation are mixed (Henson *et al*. 1998; Mitchell *et al*. 1998). Given the two-way interaction between the immune system and the HPA axis, it may be that the inflammatory responses drive the release of cortisol and not vice versa. Evidence to support this hypothesis is that infusion of recombinant IL-6 to match levels typically measured during exercise elicits a substantial cortisol response (Steensburg *et al*. 2003).

Susceptibility to illness

The down-regulation of various immune defenses following strenuous and prolonged exercise may increase the likelihood that a person will succumb to infection. Athletes are more susceptible to infectious illness, particularly upper respiratory tract infection (URTI), during periods of intense training and in the few weeks following particularly stressful competitive events such as a marathon (Peters & Bateman 1983; Nieman 1998). Reports of incidence of URTI in athletes under these conditions vary, but a rough estimate may be that the risk is approximately double. That is, most athletes do not get URTI following strenuous competitions, but the risk is significantly increased (Nieman 2000). The downtime in training or the potential affect on performance during URTI or other infections are issues of substantial concern for many competitive athletes. Additionally, data from athletes may apply to other physically demanding situations, including occupational or leisure time activities.

The mechanisms contributing to increased susceptibility to illness have not been definitively identified; however, a number of known responses to strenuous exercise are suspected to contribute. The period following strenuous exercise in which lymphocyte numbers and functions are decreased, nasal neutrophil phagocytosis is decreased, there is a decrease in salivary IgA and attenuation of antigen presentation occurs and has been hypothesized to be an 'open window' of opportunity for weakened immune defenses to be overcome by a virus (Nieman 2000). The first line of defense against bacterial or viral pathogens, including mucosal immunoglobulins and the activity of neutrophils in the nasal mucosa, is weakened following strenuous exercise (Müns 1993). Similarly, salivary production of IgA is reduced following strenuous exercise (Mackinnon *et al*. 1989; Mackinnon & Jenkins 1993; Nieman *et al*. 2002), further adding to the impaired first line of defense against URTI. Decreased salivary IgA is the only immune measure that has been associated with the onset of URTI (Mackinnon 2000). The tendency for both catecholamines and cortisol to inhibit type 1 immune responses and promote type 2 responses tilts the scale toward increase susceptibility to URTI. This pattern of T-cell and cytokine responses has been definitively identified following 2.5 h of treadmill running (Steensburg *et al*. 2001). Additionally, IL-6 produced during exercise stimulates release of cortisol (Steensberg *et al*. 2003). Thus, there are a number of mechanisms by which the neuroendocrine and immune systems are induced by strenuous and prolonged exercise to favor Th2 and inflammatory responses at the expense of the Th1 system. This shift decreases viral defenses and may increase the ability of viruses to flourish, particularly those in the upper respiratory tract.

Exercise training

The relationship between exercise training or physical activity and immune status depends on the dose of exercise and likely also on the opportunity for recovery from the stress of exercise. There seems to be general agreement that moderate levels of training may stimulate overall immune defenses while strenuous or severe training may suppress immune defenses (Nieman 1997; Shephard 1997). It is likely that the immune changes induced by training are a component of a systemic response to stress, including the neuroendocrine response. Thus, the ratio of stress to recovery may be a key component dictating the shift from benefit to detriment as the stress of exercise training increases.

Leukocyte trafficking

The number of leukocytes that reside in the circulation at rest may be used as a gross indication of immune status; however, the significance of modest fluctuations within or near the normal range has not been determined. Large deviations from the normal range can be used to identify pathologies. For example, $CD4^+$ T-cell concentrations can be measured to monitor the progression of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Hazenberg *et al*. 2003), and neutrophil concentrations can be measured to determine whether inflammation is occurring (Smith, L.L. & Miles 2000). Both longitudinal and cross-sectional studies have been employed to determine whether exercise training significantly impacts concentrations of leukocyte subpopulations at rest. A number of studies report increased numbers of neutrophils and NK cells in endurance trained compared to non-trained individuals or in response to moderate exercise training (Nieman 1997; Shephard 1997). Longitudinal studies of resistance training have not measured higher NK-cell concentrations in response to training; however, one study measured an elevation after 3 months of training that did not persist through 6 months of training (Flynn *et al*. 1999; Miles *et al*. 2002a). Exercise training does not appear to have a consistent influence on resting concentrations of monocytes, $CD4^+$ and $CD8^+$ T-lymphocytes, or B-lymphocytes (Woods & Davis 1994; Shephard 1997; Miles *et al*. 2002a). With training that is considered strenuous, typically by athletes at the national or international levels, decreased concentrations of monocytes have been reported (Shephard 1997).

The absolute magnitude of increases and decreases in circulating leukocyte populations in response to exercise varies considerably amongst individuals. The influence of exercise training on the catecholamine response to exercise has not been definitively determined. Exercise training induces an increase in work capacity that decreases the relative proportion of maximal work intensity required for any given absolute work effort. For example, the oxygen consumption associated with running at a 6-min per mile (1.609 km) pace may be 90% of $\dot{V}o_{2max}$ before and 75% of $\dot{V}o_{2max}$ after endurance training. Thus, an equivalent absolute work rate requires a lower relative proportion of the maximal effort after training. This is true for virtually all types of training. With respect to the neuroendocrine response to exercise, the rise in catecholamines and other hormones that occurred for this individual working at the same absolute rate will be attenuated before and after training (Kjær *et al*. 1988).

The influence of the neuroendocrine system on leukocyte trafficking during exercise is a function of the relative intensity and duration of the exercise. It is the relative exercise intensity rather than the absolute exercise intensity that dictates the neuroendocrine and consequent immune responses to exercise. Research data consistent with this effect of exercise training on the immune response to acute exercise have been presented for endurance exercise, resistance exercise and high-intensity interval exercise (Moyna *et al*. 1996; Shephard 1997; Miles *et al*. 2002a).

Cytokines

Resting plasma cytokine concentrations typically do not differ between trained and untrained individuals (Smith, J.A. *et al*. 1992; Shephard 1997). However, higher IL-1β and IL-6 concentrations have been reported in endurance trained compared to untrained individuals in some studies (Sprenger *et al*. 1992; Mucci *et al*. 2000). This may be a function of decreased sensitivity to cortisol inhibition in the trained individuals (Duclos *et al*. 1999) or perhaps the cumulative effects of exercise-induced inflammation (Shephard 1997).

Leukocyte function

While basal levels of the HPA axis hormones ACTH and cortisol do not appear to be influenced by endurance training, decreased sensitivity of monocytes to cortisol has been measured in endurance trained compared to untrained men (Duclos *et al*. 1999, 2001). Production of IL-6 by monocytes stimulated with LPS is greater for endurance trained compared to untrained men (Duclos *et al*. 1999). However, following an acute bout of endurance

exercise, the sensitivity of monocytes to glucocorticoids for endurance trained men increased to approximately that of the untrained men (Duclos *et al*. 1999). Thus, the sensitivity of monocytes to cortisol is enhanced by endurance training, but is not a static trait. The ramifications of this may reach a vast array of immune functions, as monocytes produce a number of cytokines that potentially impact all immune cells. In contrast, 12 weeks of progressive resistance training did not influence the stimulated production of TNF-α, IL-1β, IL-2 or IL-6 by peripheral blood mononuclear cells (PBMCs) collected from younger and older healthy subjects or subjects with rheumatoid arthritis (Rall *et al*. 1996). Thus, the ability of leukocytes to produce cytokines does not appear to be substantially impacted by exercise training, with the possible exception of monocytes.

A differential response to exercise has been identified between trained and untrained individuals with respect to neutrophil adherence and oxidative burst (Pyne 1994; Shephard 1997). While this is consistent with increased sensitivity to cortisol, this aspect of the neutrophil response to exercise training has not been investigated. There is no consistency amongst reports of other aspects of neutrophil function in response to exercise training or in cross-sectional comparisons of trained and untrained individuals (Shephard 1997).

Functional activity of T- and B-lymphocytes is not significantly altered in response to exercise training or in cross-sectional comparisons of trained and untrained subjects (Shephard 1997). Longitudinal studies indicate that this is consistent for both younger and older individuals and for endurance and resistance training (Rall *et al*. 1996; Woods *et al*. 1999; Miles *et al*. 2002a), although Woods *et al*. (1999) measured a modest enhancement of T-lymphocyte proliferation following 6 months of aerobic training in an elderly population. With respect to B-cell production of immunoglobulins, levels in serum and saliva are comparable in trained versus untrained individuals across the vast majority of comparisons, both cross-sectional and longitudinal (Shephard 1997; Potteiger *et al*. 2001). One exception to this is that decreased salivary IgA has been measured in athletes undergoing particularly intensive portions of their training, i.e. when training volume and intensity are greatest, in many, but not all, investigations of this phenomenon (Tharp & Barnes 1990; Mackinnon & Hooper 1994; Gleeson *et al*. 1999; Pyne *et al*. 2000). The endocrine system may be partly responsible for this shift, that also may include shifts in cell numbers and reactivity (B-, $CD4^+$ and $CD8^+$ T-cells), the response to tissue damage and overall stress (Shephard 1997).

A number of investigations report that exercise training increases the activity level of individual NK cells such that greater NKCA per NK cell in the circulation is consistently measured (Pedersen *et al*. 1989; Nieman *et al*. 1990, 1993a; Woods *et al*. 1999). Recent research using a rodent model (spontaneously hypertensive rats) suggests that: (i) there is a dose–response in which *in vivo* NKCA is enhanced as exercise volume increases, but this plateaus off and no further gains are achieved beyond moderate volumes; and (ii) the gains in NKCA can be abolished if sufficient rest and recovery do not occur (Jonsdottir & Hoffmann 2000). This information, in conjunction with the research relating to the acute effects of strenuous exercise, suggests that superimposing new exercise stresses before complete recovery has occurred may lead to an apparently 'chronic' suppression. It is difficult to separate out adaptations to exercise training over time from lingering effects of the most recent exercise session for athletes undergoing strenuous training (Shephard 1997).

Overtraining is a situation in which training volume or intensity exceeds the capacity of the body to adapt and a feeling of fatigue and performance decrements are typical (Urhausen *et al*. 1995; Smith, L.L. 2000). Common characteristics associated with overtraining are sympathetic nervous system and HPA axis imbalances and an increased susceptibility to illnesses (Fitzgerald 1991; Urhausen *et al*. 1995). This is not surprising given the interrelatedness of these systems. Smith, L.L. (2000) hypothesized that tissue trauma, particularly muscle damage, may result in increased inflammatory cytokines, followed by activation of the HPA axis, which results down-regulation of immune cell function. Further, she suggests that this represents an advanced stage of the general adaptation model to stress proposed by Selye, and may be the point at which recovery

and survival are more important than adaptation to stress. This model is consistent with the data presented in this chapter; however, more recent research to test this hypothesis did not measure increased levels of inflammatory cytokines in athletes during a period of induced overtraining (Halson *et al*. 2003). The complexities of the neuroendocrine and immune systems make it very difficult to verify or reject several components of this hypothesis.

Susceptibility to illness

The relationship between levels of exercise training and physical activity to incidence of illness is consistent with the overall pattern of changes identified in immune parameters. That is, moderate training may reduce susceptibility to illness somewhat, or reduce the duration and severity of symptoms (Nieman *et al*. 1990). Recent epidemiological studies also confirmed a similar relationship between physical activity and URTI in a diverse, healthy, adult population (Matthews *et al*. 2002) and school children in Poland (Jedrychowski *et al*. 2001). Consistent with this trend toward lower URTI incidence with moderate levels of physical activity or exercise training is the measurement of increased salivary IgA in response to moderate exercise training (Klentrou *et al*. 2002).

When training intensifies and reaches high volumes and or intensities, perhaps coupled with limited recovery, then there is an increase in susceptibility to URTI (Shephard 1997; Mackinnon 2000). Recent research with elite Australian swimmers has advanced IgA levels and stress-induced viral reactivation of dormant viruses, such as Epstein–Barr virus, as likely mechanisms contributing to the incidence of URTI during high level training (Gleeson *et al*. 2002). In a study of training volume and tennis players, salivary IgA levels were inversely related to training volume, but IgA levels were not predictive of URTI incidence. Researchers continue to search for the link between training levels and immunosuppression. Illness occurrence is likely to be a multidimensional function of the temporal accumulation of acute, exercise-induced immunosuppression from multiple training sessions, the level of recovery from bout to bout, nutritional factors, additional stressors and exposure to virus and/or the reactivation of dormant viruses. As noted previously, the 'the general adaptation syndrome' hypothesis of Selye (1936) proposes that all stressors elicit some common responses, including activation of the HPA axis. Thus, exercise may best be viewed as one component of the total stress to which a person must respond.

Cytokine control of muscle responses to stress and damage

Muscle cells release cytokines in response to exercise, whether or not cellular damage occurs. Current research suggests that many of these cytokines have regulatory functions and may be necessary signals for various cellular functions and as systemic signals to the brain, particularly to the HPA axis. That is, inflammation is just one of the functions in which these molecules, particularly IL-6 and LIF, participate, and the production of these molecules within muscle is not dependent on tissue injury. For example, LIF has a hypertrophic effect on skeletal muscle cells and induces satellite cell proliferation (Spangenburg & Booth 2002; Gregorevic *et al*. 2002). IL-6 may act as a systemic signal reflecting low glycogen levels in exercising muscle cells (MacDonald *et al*. 2003). However, when muscle damage does occur, IL-6 and LIF participate in the inflammatory response. As indicated previously, TNF-α, IL-1β, IL-6 and LIF are capable of stimulating the HPA axis in a negative feedback loop to induce production of the anti-inflammatory hormone, cortisol (Mastorakos *et al*. 1993; Chesnokova & Melmed 2002; Tsigos & Chrousos 2002). Unfortunately, the response of LIF to exercise scarcely has been investigated and little is known at this point.

The type of muscle damage most typically associated with exercise is characterized by delayed onset muscle soreness (DOMS); for example, the soreness felt in muscles the day following the performance of exercise to which one is not accustomed. Indirect indicators of this damage are DOMS, loss of strength, swelling and elevations in serum creatine kinase activity (Miles & Clarkson 1994). The exercise-induced inflammatory response involving tissue damage likely occurs in the following sequence:

(i) tissue injury/stress; (ii) resident macrophage activation; (iii) release of inflammatory 'alarm' cytokines TNF-α and IL-1β; (iv) stimulation of local release of chemoattractants, for example IL-8; (v) local production of IL-6; (vi) stimulation of acute phase proteins by the liver; (vii) stimulation of the HPA axis; and (viii) leukocytosis and leukocyte migration to site of injury (Smith, L.L. & Miles 2000). IL-1β and TNF-α initiate the inflammatory cascade, which includes production of IL-6, which in turn stimulates production of a number of counter-inflammatory cytokines and other molecules, including IL-10 and IL-1ra (Turnbull *et al*. 1994; Smith, L.L. & Miles 2000). Local production of IL-1β has been demonstrated in muscle biopsy samples following eccentric exercise associated with muscle damage (Malm *et al*. 2000); however, the increases in blood are short-lived and typically small relative to other cytokines such as IL-6 or IL-10 (Shephard 1997; Suzuki *et al*. 2002). Both IL-1β and IL-6 act as growth factors to promote regeneration at the site of tissue damage (Northoff *et al*. 1995).

Within the realm of inflammation, the process of regeneration is dependent on the removal of damaged cellular debris by phagocytes. The cellular response to exercise-induced muscle damage includes infiltration by and activation of local macrophages (Stupka *et al*. 2001; LaPointe *et al*. 2002). Infiltration of injured tissue by neutrophils has been measured in some (Brickson *et al*. 2001; MacIntyre *et al*. 2001), but not all investigations (LaPointe *et al*. 2002). Muscle biopsy data suggests that this process occurs over a period of weeks (Lieber 1992). This certainly exceeds the duration of any detectable markers of inflammation in the systemic circulation. Thus, much of the response to exercise-induced muscle injury occurs locally rather than systemically.

If exercise is prolonged and strenuous or mechanically stressful, for example containing a high-force eccentric component, then some degree of tissue damage may be expected and the components of the described response are measurable. Marathons and comparable events induce skeletal muscle damage evidenced by disruption of myofibrillar organization and an efflux of the intramuscular enzyme creatine kinase from muscle to blood (Rogers *et al*. 1985; Warhol *et al*. 1985). Following marathon-type races, increases in plasma or serum TNF-α, IL-1β, IL-6, IL-10 and IL-1ra have been measured (Northoff *et al*. 1994; Drenth *et al*. 1995; Nehlsen-Cannarella *et al*. 1997; Ostrowski *et al*. 1998a, 1998b; Henson *et al*. 2000; Nieman *et al*. 2001). High force eccentric exercise also induces skeletal muscle damage (Fridén & Lieber 2001) and smaller increases in IL-1β, IL-6 and IL-1ra (Bruunsgaard *et al*. 1997a; Smith, L.L. *et al*. 2000; Chen & Hsieh 2001; Toft *et al*. 2002). Increases in TNF-α typically are not measured (Bruunsgaard *et al*. 1997a; Toft *et al*. 2002), but this may reflect the difficulty of measuring a systemic response to a local inflammatory response that is relatively small in magnitude. The prolonged period of inflammation within muscles is not accompanied by elevations in cortisol (Pizza *et al*. 1995; Lenn *et al*. 2002). Thus, under reasonable circumstances, the inflammatory response to muscle damage does not appear substantial enough to stimulate the HPA axis for an extended period of time. However, there is a neuroendocrine and immune basis for the cytokine response to muscle damage to stimulate the HPA axis if IL-6, and possibly TNF-α, IL-1β or LIF, production was substantial.

The hypothalamus uses the neuroendocrine–immune network to integrate exercise-induced stress signals and central signals to induce appropriate responses to those stresses (Steinacker *et al*. 2004). Recent muscle biopsy data suggest a link between increases in circulating cortisol and activation of proteolysis within the injured muscle (Willoughby *et al*. 2003). Exercise-induced injury is mediated by the ubiquitin-proteolytic pathway, and cortisol up-regulates gene expression of several components of this pathway, for example ubiquitin itself, ubiquitin conjugating enzyme and glucocorticoid receptors. Consistent with the protective effects known to occur after a single exercise bout is the down-regulation of this system following a second bout of the same injurious exercise. Thus, the two-way network of communication involves cytokine signals generated locally to signal the HPA axis and HPA axis signals generated centrally to signal the muscle to repair and regenerate in response to tissue injury.

The role of inflammatory mediators in the response to exercise-induced muscle damage has been

called into question in light of research in which anti-inflammatory treatment modalities failed to have a substantial impact on DOMS or the recovery of function (Cheung *et al*. 2003). However, the research cited above is a small sample of the evidence of various components of the inflammatory response measured in response to exercise-induced muscle damage. Thus, it may be prudent simply to suggest that the influence of prostaglandin E_2, the typical target of anti-inflammatory medications, is not a major player in the inflammatory response (Semark *et al*. 1999). As a result, anti-inflammatory treatments do not appear to interfere with or enhance the healing process and have only a minor impact on soreness (Cheung *et al*. 2003).

Summary

The immune and neuroendocrine responses to the stress of exercise certainly are linked. However, modulation of exercise-induced immune responses also is influenced by the inflammatory response to tissue injury. Cytokines mediating the local and systemic responses to exercise are often multifunctional, for example IL-6. Many of the functions of these cytokines are unrelated to inflammation or immune modulation per se, for example LIF may be acting as a growth factor and have nothing to do with tissue injury. The two-way communication network between the neuroendocrine and immune systems is such that cytokines produced by muscles are capable of activating the HPA axis. Reciprocal regulation of the neuroendocrine and immune systems is infinitely complex. Regardless, research studies are consistent in measuring enhanced immune function in response to moderately stressful acute exercise or exercise training. The level of exercise stress that the body can tolerate without immunosuppression and increased susceptibility to illness is likely to vary according to the level of other stresses that the body must endure. A number of studies have measured suppressed function in some components of immunity and increased URTI occurrences in athletes when the physical demands of training and competition are extreme. In light of the interactions between the neuroendocrine and immune systems, individuals wishing to maximize their training volumes and intensities may be well advised to reduce the stress response to exercise via adequate recovery and various nutritional strategies and to minimize other forms of stress to which the body must respond.

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Chapter 26

The Impact of Exercise on Immunity: the Role of Neuroendocrine–Immune Communications

ANDREA M. MASTRO AND ROBERT H. BONNEAU

Introduction to exercise–neuroendocrine–immune relationships

The many benefits of regular physical exercise are not only well-documented in the scientific literature but have also received considerable attention in the popular press. It is recognized that exercise has wide-ranging effects on most organ systems of the body but, in general, emphasis has been placed on its impact on cardiovascular and pulmonary function (reviewed in Booher & Smith 2003). However, more recently, the cellular and molecular mechanisms underlying the effects of exercise on several other organs systems, especially the immune system, have begun to be delineated (e.g. Pedersen & Hoffman-Goetz 2000; Pedersen & Toft 2000; Suzuki *et al*. 2002; Woods *et al*. 2002; Lakier-Smith 2003; Nieman 2003). The strong interest in the relationship between exercise and the immune function has lead to the recent publication of a number of excellent review articles devoted to this subject (*International Journal of Sports Medicine* [volume 21, supplement 1, May 2000]; *Immunology and Cell Biology* [volume 78, October 2002]).

It has long been recognized that exercise can have a strong impact on a person's overall mental condition as well as their physical state (Glenister 1996; Fox 1999; Paluska & Schwenk 2000; Salmon 2001). Of particular recent interest is how one's mental state can, in turn, influence a wide range of physiological parameters that together contribute to maintaining homeostasis and overall well-being. Although the physiological benefits of exercise have been recognized for quite some time, the contributions of exercise to physiology from a psychological perspective are less well established. The exact mechanisms underlying these contributions have not been identified; but it is generally accepted that the functioning of both the nervous and endocrine systems are intimately associated with a variety of psychological states. The fact that exercise can affect components of both the nervous and endocrine systems suggests that, in part, these effects may be mediated via a psychological pathway. In recent years, substantial evidence has been collected to support a functional link between not only the nervous and endocrine systems but also among the nervous, endocrine and immune systems (Fig. 26.1) (Conti *et al*. 2000; Ader *et al*. 2001).

Given the many findings that both the nervous and endocrine systems are alone able to modulate a variety of immune functions provides strong

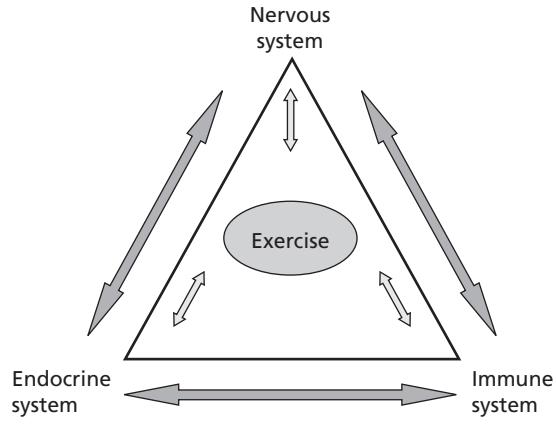

Fig. 26.1 Exercise and the nervous–endocrine–immune network.

support for a psychological link between exercise and immunity. As is outlined below, there is indeed a neuroendocrine-mediated link between exercise and immune function. This link may play an important role in the development and progression of diseases that are either immunologically resisted (e.g. infectious diseases, cancer) or that are caused by an undesirable activation of the immune system (e.g. allergy, autoimmunity).

It is important to note that the information provided in this chapter is *not* intended to represent a comprehensive review of all literature dealing with exercise and immunity. For such information, the reader is directed to several recent reviews in this area (Hoffman-Goetz 1996; Nieman & Pedersen 2000; Pedersen & Hoffman-Goetz 2000; Shephard & Shek 2000a; Hoffman-Goetz & Pedersen 2001). Rather, this chapter is specifically designed to enlighten the reader on the neuroendocrine-mediated mechanisms underlying the relationship among exercise and specific aspects of immune function. It is also intended to provide insight into the areas in which an understanding of the relationship between exercise and immune function can be further expanded as a result of recent advances in our knowledge of immune function and state-of-the-art experimental approaches that can quantify such function. Lastly, we will address the potential impact of exercise on diseases whose prevention or cause is associated with one or more aspects of immune function.

Neuroendocrine–immune communications

Neuroendocrine communications

Before launching into a discussion of the variety of functional relationships that exist between the neuroendocrine and immune systems and their impact on one's level of immunocompetence, it is first necessary to briefly review some basic principles underlying the communications that exist between the nervous and endocrine systems and how these communications are possibly influenced by exercise. However, it should be noted that a comprehensive review of the both the nervous and endocrine systems, as well as exercise physiology, is clearly beyond the scope of this text and is discussed in depth elsewhere (Robergs & Keteyian 2003).

THE LINES OF COMMUNICATION BETWEEN THE NERVOUS AND ENDOCRINE SYSTEMS

It has been clear for quite some time that nervous and endocrine systems function as a single interrelated system within the body. The functional relationships that exist between these two systems and how they regulate a wide range of bodily processes is the basis of the study of neuroendocrinology. This relationship operates bi-directionally in that the endocrine system affects the nervous system and the nervous system affects the endocrine system. Whereas hormones serve as the primary mediators of the endocrine-to-nervous system communication, the nervous system–endocrine communication occurs within the site of direct interface, at the level of the neuroendocrine cell itself.

Hormones impact virtually every tissue in the body as a result of their transport through the blood. Functional interactions between hormones and their target tissues are highly dependent on specific receptor-mediated binding of hormones to the cells which are able to respond. Depending on the hormone, these receptors are located on either the cell membrane, within the cytoplasm, or in the nucleus. The mechanisms of hormone action are varied and include membrane transport, stimulation of gene transcription, and the activation of intracellular second messengers such as cyclic adenosine monophosphate (cAMP).

There are a variety of mechanisms that regulate the release of hormones into the neuroendocrine pathway. Endogenous circadian or diurnal rhythms of release provide a tonic cyclic pattern of hormone secretion that is independent of physiologic perturbation. Superimposed on these endogenous rhythms are a number of highly complex positive and negative feedback loop mechanisms which help to maintain endocrine-associated homeostasis.

FUNCTION AND IMPORTANCE OF NEUROENDOCRINE COMMUNICATIONS

Neuroendocrine connections are important in that

they allow our bodies to maintain a state of homeostasis. For example, neuroendocrine relationships play a critical role in water conservation and maintenance of body fluid osmolarity, blood volume and pressure, growth and development, metabolism, electrolyte balance, ovulation and parturition and behavior. Disorders in the generation, secretion, or response to hormones can lead to a wide range of pathological conditions including diabetes insipidus, osteoporosis and acromegaly, to name a few.

The neuroendocrine system also provides adaptive physiological responses which allow one to respond to and cope with changes in the environment. This ability to adapt to such changes is central to maintaining a number of physiological parameters within a 'normal range' that allows for survival. For example, the hypothalamic–pituitary–adrenal (HPA) axis is among the first physiological response systems to become activated in response to environmental 'stress'. Although 'stress' can be thought of from either a strictly psychological or physiological standpoint, or a combination of both, the ultimate bodily response is the activation of the HPA axis (Chrousos & Gold 1992; Dhabhar & McEwen 2001).

A well-orchestrated communication between the cells and tissues comprising the HPA axis is essential in maintaining homeostasis under conditions of stress. Signals from the limbic system trigger the release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus which, in turn, induces the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH then enters the circulation where it interacts with cells of the adrenal cortex to produce cortisol in humans (corticosterone in rats and mice). It is important to recognize that through the ability of each of these compounds to feedback onto the organs in which they were made, the synthesis of these molecules is well-controlled.

NEUROENDOCRINE ADAPTATIONS TO EXERCISE

The ability of the body to tolerate exercise is regulated by complex interactions between the autonomic nervous system and the endocrine system. The body responds to both neural stimulation and specific chemical and mechanical conditions that help to regulate, through the action of a variety of hormones, a number of physiological functions during exercise. Some of these functions include energy metabolism, fuel mobilization, fluid balance, vascular hemodynamics and protein synthesis. These responses can vary among individuals and be influenced by both exercise intensity and gender.

The exercise-induced, hormonally-based regulation of physiological processes involves a number of hormones including cortisol, growth hormone, vasopressin (antidiurectic hormone), renin, aldosterone, thyroxin, insulin, glucagons, and the catecholamines, epinephrine and norepinephrine. The epinephrine and norepinephrine released from the adrenal gland control changes in muscle metabolism, cardiac output and vascular resistance. There may also be changes in the levels of other hormones (e.g. estrogen, follicle-stimulating hormone [FSH], luteinizing hormone [LH] and testosterone, α- and β-endorphins and enkephalins) that are not necessarily associated with the maintenance of homeostasis. Peptide hormones such as growth hormone, insulin and prolactin increase with exercise as part of the metabolic response. Many, if not all, of these hormones are able to bind to immune cells and elicit a variety of cellular responses.

The above mentioned neuroendocrine responses to exercise have been recognized for quite some time. In addition, many of the hormones that are the products of these responses have been shown to affect various aspects of immune function both *in vitro* and *in vivo* in the context of studies totally unrelated to exercise. However, as outlined in depth later this chapter, it is now recognized the exercise itself may indeed influence immunity through the actions of these neuroendocrine-derived hormones.

Nervous–endocrine–immune communications

INTRODUCTION TO NEUROIMMUNOLOGY

As is briefly outlined above, intricate levels of communication exist between nervous and endocrine systems. In fact, most the other major organs systems of the body (e.g. circulatory, respiratory, gastrointestinal, reproductive) are functionally linked with

both the nervous and endocrine systems and/or with each other in a number of ways. However, from a historical perspective, the immune system had generally been thought to function autonomously with little or no input from any of the other organ systems. However, during the past three decades there have a plethora of both human (Solomon & Moos 1964; Solomon *et al*. 1966; Solomon 1981a, 1981b; reviewed in Glaser & Kiecolt-Glaser 1994) and animal (Solomon *et al*. 1968; Solomon 1969; Ader & Cohen 1975; reviewed in Bonneau *et al*. 2001; Moynihan & Stevens 2001) studies that have provided substantial evidence that the immune system is functionally integrated with both the central nervous system (CNS) and with the endocrine system. Given the fact that the identification of many of the complex interactions among these three systems had their roots in psychology, this area of study has traditionally been known as 'psychoneuroimmunology' (Greer 2000) although a simpler term, 'neuroimmunology' may be equally appropriate.

Defining the relationship among the nervous, endocrine, and immune systems is not easy for several reasons. First and foremost, the immune system itself is very complex. It is comprised of both primary (bone marrow and thymus) and secondary (lymph nodes, spleen) immune organs distributed throughout the body. Although for practical and ethical considerations blood-derived immune cells are studied most often in humans, one can not forget the fact that there are immune cells in other parts of the body that play important regulatory roles. For example, a variety of immune-derived cells line both the respiratory and intestinal systems and contribute to what is known as mucosal immunity. Together with the intraepithelial lymphocytes which reside in the skin, these cells provide an important first line of defense against invading pathogenic microorganisms. Thus, by limiting studies to only blood-borne immune cells we may compromise our ability to truly learn the importance of neuroendocrine–immune interactions in overall immune-mediated defense. A second difficulty in defining the neuroendocrine–immune relationship is that our understanding of immune function continues to grow at a rapid pace, independent of any additional knowledge of the nervous and endocrine systems. At the cellular level, much more is known about the functioning of both the innate and adaptive arms of the immune system and how these two arms are intertwined via the synthesis of and response to cytokines. A better understanding of immune events at the molecular levels have been advanced by developments in molecular biology and through the use of transgenic animals. However, our understanding of how the neuroendocrine systems affect these molecular events is just beginning to be defined.

MEDIATORS AND MECHANISMS OF NEUROENDOCRINE–IMMUNE INTERACTIONS

Recent experimental evidence has proven that the CNS–endocrine–immune axis (neuroendocrine–immune axis) operates in a bi-directional fashion (Chambers *et al*. 1993; Felten *et al*. 1993; Moynihan & Ader 1996; Stevens-Felten & Bellinger 1997). That is, the immune system receives signals from the nervous system and the immune system provides information to the nervous system. This intercellular communication can be mediated by products of the immune system including cytokines, growth factors and even neuropeptides made by the lymphocytes themselves. Therefore, the distinctions that had once been made among lymphokines, growth, factors, hormones and neuropeptides with respect to the organ system in which they function are no longer appropriate.

Studies of the relationship between neuropeptides and the regulation of immune function have focused particularly on those neuropeptides derived from the polyprotein proopiomelanocortin (POMC), particularly ACTH and β-endorphin. Other hormones such as cortisol, growth hormone, prolactin and the catecholamines, epinephrine and norepinephrine are also central to our understanding of the relationship between endocrine and immune function. Overall, the evidence for neuronal, endocrine and immune intersystem communications have formed the basis of numerous studies that have investigated the relationship among physical stressors such as exercise, immune function and the health status of an individual (Fig. 26.2).

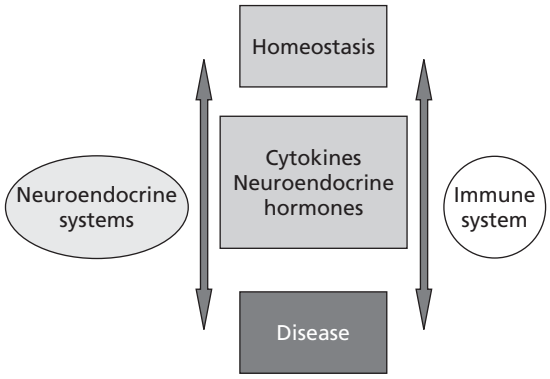

Fig. 26.2 Health and disease. (Adapted from Chambers & Schauenstein 2000.)

The ability of the immune system to respond to neuroendocrine-derived peptides and hormones depends on the presence of functional receptors on the immune cells themselves. Indeed, there are a variety of immune-derived cells (e.g. lymphocytes and monocytes) that possess receptors for neuroendocrine-derived peptides and hormones that are identical to those receptors that are present on cells of the nervous and endocrine systems (Weigent *et al*. 1990; Blalock 1994; DeKloet *et al*. 1994; Garza & Carr 1997). For example, catecholamines, opioids, serotonin, vasopressin, ACTH, growth hormone and prolactin can all influence various aspects of the immune system though receptor-specific binding. Likewise, there are receptors on cells of the neuroendocrine system for immune-derived products such as cytokines.

In order for specific receptors to provide a link between the neuroendocrine and immune systems, the neuroendocrine-derived products must first come in contact with the cells of the immune system. Some products are released into the blood and circulate to the immune cells that are located throughout the body. Alternatively, products of the nervous system may be released from nerve terminals in the direct proximity of immune cells located in the primary (thymus) and secondary (lymph nodes, spleen) lymphoid tissues. The latter mechanism is supported by the finding that nerve fibers of the autonomic nervous system directly innervate the primary and secondary lymphoid tissues (Livnat *et al*. 1985; Felten & Felten 1988; Bellinger *et al*. 1992; Madden *et al*. 1995) in much the same way that other organs of the body, such as the heart, are innervated by the fibers of nervous system. This innervation of the thymus, lymph nodes and spleen is important in overall immune function given that these are sites of lymphocyte development and antigen-specific lymphocyte activation. Such innervation had been referred to as a type of 'hard wiring' of the nervous system with the immune system.

FUNCTION OF NEUROENDOCRINE–IMMUNE CONNECTIONS

The bi-directional levels of communication among the cells, tissues and organs that comprise the nervous, endocrine and immune systems suggests that the overall control and function of these systems is much more complex than had been once thought. Thus, the physiological processes that are critical in maintaining homeostasis and well-being under a number of environmental insults can theoretically be controlled in many ways.

A series of well-orchestrated events at both the cellular and molecular level are necessary for the proper functioning of the immune system. Historically, the orchestration of these events has been generally recognized to be mediated by the variety of cell types that comprise the immune system itself. Cells such as B-lymphocytes, cytotoxic T-lymphocytes, helper T-lymphocytes, macrophages, neutrophils, dendritic cells and natural killer (NK) cells are the most common. In recent years, the identification of the many molecules that each of these cell types synthesize (e.g. cytokines, chemokines, growth factors, etc.) and their critical role in the regulation of overall immune function has allowed for a better understanding of the immune response at both the cellular and molecular levels. However, cells and molecules of the nervous and endocrine systems can also be key contributors to general immune function, thus making it more difficult to fully understand what controls the functioning of the immune system.

To date, it has been shown that nearly every cell type and function within the immune system can be modulated by products of the nervous and

endocrine systems. A comprehensive review of this literature is beyond the scope of this chapter. However, some broad examples include neuroendocrine effects on antigen processing and presentation, antibody production, lymphocyte activation and proliferation, cytokine production and NK cell lytic activity. These effects are mediated at the level of the ligand–receptor interaction, second messenger systems and gene expression. Events such as exercise and stress contribute to these effects by their ability to induce the synthesis of products of the nervous and endocrine systems.

The immune system is intimately involved in our well-being and survival. The ability to ward off the deleterious consequences of infectious pathogens may depend on the generation of an immune response following vaccination either during childhood (poliovirus, hepatitis virus) or as an adult (influenza virus). For infectious pathogens for which no vaccinations currently exist, our ability to mobilize an effective innate immune response through the activation of immune cells such as neutrophils, NK cells and macrophages is essential. For some pathogens, the further development of an adaptive immune response, a response targeted specifically toward the challenge pathogen, may be critical. Such a response is typically mediated by the B-lymphocytes which produce antibodies and the T-lymphocytes which help destroy those cells that the pathogen has invaded and relies on for its propagation and continued insult on the body. Lastly, our ability to generate and maintain immunological memory, as a consequence of vaccination and/or actual infection, is also important in our long-term defense against infection. The fact that products of the nervous and endocrine systems affect all of the above underscores the important role of both the nervous and endocrine systems in the defense against infectious pathogens.

There is also substantial interest in the role of the immune system in the development and progression of cancer. Although the precise contributions of the various components of the immune system in the defense against cancer have not yet been fully defined, there is substantial evidence to believe that immune components such as NK cells and cytotoxic T-lymphocytes (CTL) may indeed play a key role. Recently, there has been experimental data to support the notion that products of the neuroendocrine system can contribute to the immune system's role in the defense against cancer (Berczi *et al*. 1998; Ben-Eliyahu & Shakhar 2001; Turner-Cobbs *et al*. 2001; Sephton & Spiegel 2003). The successful defense against infection and cancer relies on an *enhanced* level of immune function. In contrast, it may be desirable to *suppress* immune function in order to reduce the severity of diseases that result as a consequence of an overactive or inappropriately targeted immune response. A number of autoimmune disorders fall into this category and include well-known diseases such as rheumatoid arthritis, juvenile diabetes, systemic lupus erythematosus (SLE) and scleroderma. Likewise, it is desirable to diminish the magnitude of the immune responses that are involved in mediating allergic reactions and that are responsible for the graft rejection in organ transplant recipients. Exactly how products of the neuroendocrine system contribute to each of these responses is a subject of much interest.

It is important to note that our knowledge of the inner workings of the immune system has rapidly involved during the past three decades. Advances in many areas of biological technology have allowed for a substantially better understanding of immune function at both the cellular and the molecular levels. Concomitantly, there has been an increase in our knowledge of the role of the nervous and endocrine systems at each of these levels, thus advancing the field of neuroimmunology and furthering our understanding of neuroendocrine–immune interactions.

The impact of exercise-induced effects on the immune system

Exercise and immunity

'Exercise helps relieve fatigue and other symptoms of disease', proclaimed a recent newspaper headline. The article (Bertrand 2003) described how exercise helped individuals cope with diseases such as multiple sclerosis and cancer. It included a 'checklist' of approaches to developing an exercise regimen. For example, there were suggestions of

how to monitor pulse and breathing as well as how to pace activity or to modify the intensity and duration of exercise to meet one's needs. Similar articles relating exercise to good health and immunity are common in popular journals, self-help books and newspapers. While it is generally accepted that some physical activity is better than no activity and that exercise is 'good for you', exactly how exercise impacts on the immune system is not clear. Although one researcher in the field of exercise and immunity rather skeptically stated that, '. . . numerous attempts to link exercise to meaningful alterations in immune function have been largely unconvincing (Moseley 2000, p. 128), the potential impact of exercise on immune function can not be ignored nor should studies of such an impact be terminated. This is especially true in light of our increasing knowledge of immunity and the more sophisticated means of quantifying immune function.

The quest to understand the connections between exercise and health is driven by several perspectives (Mackinnon 2000a). If exercise can be used to treat individuals with disease, can exercise prevent disease? On the other hand, can a person exercise too much and actually increase susceptibility to disease? For example, some athletes undergoing intensive and long duration exercise training suffer from upper respiratory tract infections (URTIs). Does this observation imply that exercise can induce immunosuppression and thereby increase the risk of infection? Can exercise protect one from or exacerbate autoimmunity? What happens to the immune system of either bedridden patients who are largely inactive or astronauts while in space? Does moderate exercise modulate the immune system? Questions regarding the relationship between physical activity and good health have been asked for centuries, but the mechanisms by which exercise relates to immunity have only seriously been studied in a systematic approach for the past 30 years or so.

Coupled with the complexity of the immune system is the complexity of the exercise protocol that is being studied. The potential impact of exercise has much to do with the type, mode, intensity and duration of the exercise. The effect of exercise on immunity may also be further confounded by age, gender and prior fitness level among other variables in the humans being studied. Many studies have been carried out using animal models of exercise and immunity. Although such studies have provided valuable information regarding this relationship, the translation of this information to the human condition is not always straightforward. For the most part, the information discussed in this chapter has been limited to that which has been obtained in human studies.

Evaluating the impact of exercise on the immune system in humans

Important in interpreting how exercise affects the immune system are the methodologies that are used to assess immune function. Two commonly used assays in human subject studies are the determination of the subpopulation of the blood leukocytes (phenotyping) and *ex vivo* stimulation of lymphocytes in culture (lymphocyte activation). Due to both convenience and ethical considerations, most human studies rely exclusively on peripheral blood as source of lymphocytes for analysis. However, blood contains only 1–2% of the immune cells in the body, and many of these cells are constantly trafficking throughout the body, entering and exiting various sites where infectious pathogens are commonly encountered. Typically, flow cytometry is used to enumerate the populations of these cells in the blood. This flow cytrometric approach makes use of fluorescently tagged monoclonal antibodies that bind to specific cell surface proteins known as CD, or cluster of differentiation, markers. These surface proteins are used to differentiate and quantitate the various cell types of the immune system. For example, $CD3^+/CD4^+$ cells are T-helper cells while $CD3^+/CD8^+$ cells are T-cytotoxic cells. However, a change in the distribution and number of these cells in blood does not necessarily indicate a change in cell function nor does such a change reflect an immune response at some other location in the body.

A variety of approaches have been used to study immune cell function. One commonly used technique is *ex vivo* culture of lymphocytes in the presence of compounds such as concanavalin A or

phytohemagglutinin, polyclonal T-cell mitogens which stimulate the lymphocytes to produce a variety of cytokines, to express receptors and to divide. Although this technique is fairly straightforward, the results are subject to a variety of experimental manipulations and can be affected by the particular combination of the immune-derived cells that are located in the blood sample being tested. To allow for changes in cell numbers after exercise, the resulting functional data are often mathematically normalized to the number of T-cells in the sample; however, the presence of other cell types in the blood following exercise also can affect the magnitude of T-lymphocyte activation.

The information that can be gained from using the above techniques are necessarily limited by the source of the lymphocyte sample and the fact that measures of lymphocyte number and activation potential *in vitro* are fairly general and may not accurately reflect what is occurring *in vivo*. Newer technologies such as the ability to quantify markers of cellular activation and cytokine synthesis by cells directly *ex vivo* offer the promise of unraveling the mechanisms underlying exercise-induced effects on immune responsiveness.

Acute exercise as a stressor

As both noted above and described in detail below, exercise is known to have many beneficial effects on the body, including the immune system. However, depending on whether or not a given exercise is perceived as a stressor by the body will determine its impact on immune function. The impact of both physical and psychological stressors on the immune system has been studied in great depth and has been reviewed in detail elsewhere (Glaser & Kiecolt-Glaser 1994; Buckingham *et al*. 1997; Rabin 1999; Marsland *et al*. 2002; Moynihan 2003; Padgett & Glaser 2003). Until recently, all forms of stress were thought to be generally immunosuppressive. However, it is now recognized that both the *type* and *degree* of stress can determine its ultimate impact on the immune system. For example, if a stressor is perceived by the body as a 'negative stressor' (distress), then both the type and degree of neuroendocrine activation may result in immunosuppression. However, if the stressor is perceived as a 'positive stressor' (eustress), then the neuroendocrine-mediated effects may actually result in immunoenhancement (Dhabhar & McEwen 2001). Thus, in establishing associations between exercise and the immune function, one has to consider not only whether or not the exercise is even perceived as a stressor but also if the stressor is one which results in immunosuppression or immunoenhancement.

In general, in studies of the relationship between exercise and immunity, researchers consider a bout of acute exercise as a model of induced stress or trauma while a long duration, high intensity exercise is used as a way to bring about immunosuppression (Hoffman-Goetz & Pedersen 1994, 2001). The response of the immune system to a bout of acute exercise has been compared to that of trauma or surgery (an example of an acute bout of exercise is 60 min on a treadmill or bicycle). Following this exercise, numerous changes have been observed including increased leukocyte mobilization, release of proinflammatory and anti-inflammatory cytokines, tissue damage, production of free radicals and the activation of some of the pathways seen in inflammation such as acute phase response, coagulation and fibrinolysis. These observations had led some to conclude that muscle damage associated with exercise initates an inflammatory response which is responsible for these immune changes (Hoffman-Goetz 1996). However, it is now known that muscle damage is not essential for many of these changes in immune parameters (Pedersen & Hoffman-Goetz 2000). Nevertheless, when muscle damage does occur, the immune system, especially the innate system, can effectively bring about healing. There are many changes that occur following a bout of acute exercise regardless of muscle damage. For example, a general leukocytosis (i.e. a several-fold increase in circulating white blood cells) is a common finding. In exercise-induced leukocytosis, all of the white blood cell populations usually increase to some extent. However, cells associated with the innate immune system such as neutrophils, NK cells and monocytes increase more dramatically than do lymphocytes, the subclass of leukocytes that is responsible for the acquired immune response. Within the lymphocyte population, the $CD8^+$

T-lymphocytes (cytotoxic T-lymphocytes) increase more than do the $CD4^+$ T-lymphocytes (T-helper); B-lymphocyte numbers typically change less. Leukocytosis is a rapid and transient event. It occurs within about 30 min following the end of the exercise session but, after a few hours into the recovery phase, cell numbers usually return to baseline. Sometimes there is a biphasic response in which there is a decline in leukocyte numbers followed by a second increase. Because of the rapidity of the increase in leukocytes, it is believed that hemodynamics play the major role in recruiting cells from intravascular marginated pools and storage sites such as the spleen. Cortisol and catecholamines, hormones that are both intimately associated with stress, are believed to be involved in this cell recruitment (Mackinnon 2000a). Moreover, the recruited lymphocytes tend to be the memory cells ($CD45RO^+$) in contrast to newly-differentiated cells ($CD45RA^+$) (Gabriel *et al*. 1993). Memory cells are those which have previously responded to foreign material (antigen) and are programmed to quickly respond to another encounter with the same antigen. Whereas the newly differentiated cells tend to be predominantly located in lymphoid organs, the memory cells tend to home to tissues as well as to lymphoid organs. Thus, this somewhat selective leukocytosis of the two populations which reside in different lymphoid compartments may provide clues to the hemodynamic and hormone driven increases in these cells in the blood. How these exercise-induced, hormone-mediated changes in leukocytosis influences immune responses to pathogens is of significant interest.

An intense bout of acute exercise also affects the endocrine system by elevating the levels of variety of hormones such as the catecholamines (epinephrine, norepinephrine), growth hormone, β-endorphins, sex steroids and cortisol (Hoffman-Goetz & Pedersen 2001) in the blood. Thus, given their location it seems logical that these hormones could also have an influence on immune cell trafficking. In support of this hypothesis, the levels of expression of β-adrenergic receptors on the white blood cells roughly correlates with the increase in the cell numbers seen following exercise (Hoffman-Goetz & Pedersen 2001). Leukocytes have receptors for all of these molecules; i.e. catecholamines, growth hormone, β-endorphins, sex steroids and cortisol, and each of these hormones administered individually has been reported to affect lymphocyte trafficking. These data generally support a model in which catecholamines bind to the β-adrenergic receptors to bring about the immediate acute effects while corticosteroids play a more important role in exercise of greater duration (Hoffman-Goetz & Pedersen 2001).

Exercise may also bring about an increase in circulating levels of compounds that are agents of apoptosis (programmed cell death) for lymphocytes. For example, increased levels of cortisol and reactive oxygen species (ROS) could cause lymphocyte apoptosis which would contribute to loss of lymphocytes from the circulation. However, in a study designed to test this hypothesis, it was noted that increases in cortisol, F2-isoprostanes (indicative of ROS), epinephrine and norepinephrine increased with exercise but the total number of circulating apoptotic lymphocytes did not (Steensburg *et al*. 2002). This finding does not rule out the importance of these molecules but suggests that they may not necessarily influence circulating blood cell numbers.

LYMPHOCYTE SUBPOPULATIONS

T-lymphocytes are an essential component of cell-mediated acquired immunity and play an important role in the defense against a number of viral infections. Both of the T-cell subsets, T-helper (Th; $CD4^+$) and T-cytotoxic (Tc; $CD8^+$), first increase and then decrease after severe, acute exercise (Steensburg *et al*. 2001). The Th cells provide a series of cytokines that are essential to regulate the immune response. Tc cells kill virally infected or tumor cells by direct contact. Each of these T-cell subsets are further classified by the cytokines they produce. A characteristic signature of a type 1 cytokine response, is production of interferon-γ (IFN-γ) and interleukin-2 (IL-2), while type 2 cytokines include IL-4 and IL-6. It is the balance between these sets of T-cells and their cytokines that influence whether the immune response will be more directed to either a cell-mediated (type 1) or a humoral or antibody-

mediated (type 2) response. It is this balance that is essential for assuring an effective immune defense. It has been reported (Steensburg *et al*. 2001) that the Th1 cells decrease while the Th2 are largely unaffected after acute exercise stress. Furthermore, Ibfelt *et al*. (2002) found that the decrease may be due largely to a decrease in memory cells ($CD45RO^+$). Such a decrease in both memory cells and the type 1 cytokines associated with an exercise-stressed immune system might result in the inability of one to mount an effective response against a viral infection.

LYMPHOCYTE ACTIVATION AND APOPTOSIS IN CULTURE

Upon recognition of a specific antigen *in vivo*, both T- and B-lymphocytes become activated, grow into large blast cells and undergo several rounds of cell division. This clonal expansion of antigen-specific lymphocytes assures that the cell numbers will be adequate for mounting an effective defense. This clonal expansion is mimicked *in vitro* by the stimulation of lymphocytes with polyclonal mitogens. The amount of DNA synthesis carried out by these stimulated cells has long been used as an index of *in vivo* cell-mediated immune function. Based on this assay, it has often been observed that intense or prolonged exercise leads to a suppression of the DNA synthesis of lymphocytes sampled from the blood immediately after exercise (reviewed by Mackinnon 2000a). This suppression is usually short-lived and cells from blood samples taken a few hours after the end of the exercise respond normally. This kinetic analysis suggests that the observed suppression may be, in part, explained by the redistribution of cells in the blood as monitored by the phenotype analysis. Furthermore, the measured levels of ^3H-thymidine incorporation by cells (a measure of DNA synthesis) is an average of all the cells in the culture, yet some of the lymphocytes may be non-responders or, in fact, undergo cell death.

Research based on the use of new techniques suggests that lymphocyte cell death via apoptosis may contribute, in part, to the apparent decrease in proliferation associated with exercise (Green & Rowbottom 2003). Apoptosis is a normal and non-inflammatory mechanism for the removal of cells of all types within the body. For example, newly differentiated lymphocytes undergo apoptosis if they do not encounter their cognate antigen. In a recent publication, Green & Rowbottom (2003) suggested that increased apoptosis may account for an apparent decrease in cell proliferation. By combining vital staining with a fluorescent dye (CFSE) and flow cytometric analyses, this group found that exercise did not affect cell division but rather increased cell death. Thus, in this study, the overall observed decrease in lymphocyte expansion was interpreted to be the net result of cell division and apoptosis, with apoptosis being the greatest contributor. This study was limited in the number of samples analyzed so it remains to be seen if the finding is universal.

In another case in which apoptosis was directly examined with the use of annexin V, a protein which binds to the membrane of apoptotic cells, exhaustive but not moderate exercise increased lymphocyte apoptosis (Mooren *et al*. 2002). There are various reports that exhaustive exercise brings about cellular changes which may participate in the apoptotic process such as DNA damage, increased Ca^{2+} levels (Mooren *et al*. 2001), ROS, cortisol and death–death ligand molecules. However, which one of these is *cause* of apoptosis and which is the *effect* of apoptosis remain to be determined. Whether the observed apoptosis is indicative of a state of immunosuppression or is simply a normal regulatory mechanism associated with exercise-induced stress, is not known.

NATURAL KILLER CELLS

NK cells, which make up about 5–10% of the circulating white blood cell population, offer an immediate and major response to the presence of virally infected cells and are believed to offer surveillance against cancer. Both NK-cell number and activity have been examined in numerous acute and chronic exercise studies. As is described below, some studies have described major effects of exercise on NK cells while others do not. Faced with the plethora of information regarding NK cells and their response to exercise, Shephard & Shek (2000b) recently

carried out a meta-analysis of the existing literature. After considering many parameters, including the type of exercise, they concluded that sustained, moderate exercise usually causes an increase in circulating NK cells during exercise, followed by a decrease within about 1 h, and recovery to normal levels within 2 h of the end of the exercise. Both sustained vigorous exercise and a short bout of vigorous exercise brought about the same pattern of changes in NK-cell numbers, but the magnitude of these changes were greater with the short bout of moderate exercise, with no apparent effect of training on these numbers. These changes in circulating NK-cell numbers have been attributed to changes in cardiac output and to increased synthesis of catecholamines associated with the exercise.

There also are reports of increases in NK-cell cytolytic activity as well as increases in their numbers (Mackinnon 2000a). Does this change in activity reflect only the change in cell number or does activity on a per cell basis actually change? For most, but not all studies, it appears that, on a per cell basis, the activity is not affected (Miles *et al*. 2002). In summary, an acute bout of exercise leads to a transitory increase and then suppression of NK-cell counts and associated cytolytic activity. However, there is no evidence that these fluctuations in number and function affects overall health. Authors of the meta-analysis study recommend sampling during exercise and beyond the 24-h recovery period and to measurements of catecholamines, cortisol and prostaglandins in order to more definitively examine how exercise affects NK cells.

HUMORAL IMMUNITY IMMUNOGLOBULIN LEVELS

The humoral immune response is mediated by B-lymphocytes, the immune cells of the body that produce immunoglobulins more commonly referred to as antibodies. These antibodies are glycoprotein molecules that function as very specific cell membrane receptors for foreign molecules (antigens) that are encoded for by all pathogens and, in some cases, may be present on tumor cells. Upon binding of the antibody to the cell-associated antigenic receptor, the B-lymphocyte becomes activated and develops into a plasma cell that, in turn, secretes large quantities of antibodies into the blood, saliva, and other mucosal tissues. These antibodies can protect the host in several ways including neutralizing viruses, targeting bacteria for opsonization by phagocytes, activating complement, and binding to and clearing foreign particles. The immunoglobulin G (IgG) class of antibody makes up the largest proportion of serum immunoglobulins and plays a critical role in each of the above mechanisms of protection.

In general, the circulating levels of IgG or total immunoglobulins have been found not to change following acute exercise or chronic training (Mackinnon 2000a). Although there is a report of low serum immunoglobulin levels in elite swimmers (Gleeson *et al*. 1995), the production of antibodies in response to vaccination is not suppressed and there is no other obvious immunosuppression in these individuals (Gleeson *et al*. 1996; Bruunsgaard *et al*. 1997b). Thus, exercise does not seem to affect the overall B-cell-mediated component of the immune response.

MUCOSAL IMMUNITY

Immunoglobulins of the IgA and IgM subtypes are enriched in mucosal tissues such as the linings of the oral and gastrointestinal cavities. These immunoglobulins provide an important role in the protection from pathogens that enter the body with both air and food. In contrast to total serum immunoglobulin, salivary IgA levels have been found to be reduced after an acute bout of exercise in high performance athletes (reviewed by Gleeson & Pyne 2000). This observation was first published in 1982, by Tomasi *et al*. (1982) who reported decreased secretory IgA in cross-country skiers. The original thought of these investigators was that part of this decrease was due to cold temperatures. However, reduced levels of salivary IgA have since been measured in other athletes with intensive training schedules such as elite swimmers, tennis players, marathon runners, rowers, cyclists, hockey players and kayakers (reviewed by Pedersen & Hoffman-Goetz 2000). Generally, the levels return to pre-

exercise levels within about an hour, but in some cases, chronically depressed levels of salivary IgA were associated with long-term training. Moderate exercise does not appear to bring about these changes. Salivary IgM levels mirrored the changes in IgA. Salivary levels of IgA are of special interest because of the high incidence of URTIs in athletes involved in high intensity training. Yet, it has not been easy to directly link reduced salivary IgA with increased risk of URTI (Pyne *et al*. 2001; Novas *et al*. 2003). There are only a few studies that indicate a general risk associated with exercise and suggest that salivary IgA and IgM can be used to predict infection. The mechanisms by which reduced IgA is related to infection are speculative. The lower levels of salivary IgA may reflect secretion rates and fluid changes brought about by the exercise. These lower levels did not appear to be linked to increases in cortisol caused by the exercise (McDowell *et al*. 1992; Dimitriou *et al*. 2002). Other stress-induced molecules such as prostaglandins may be involved (Tvede *et al*. 1989).

Athlete versus non-athlete

If exercise affects the immune system, is the immune system of a well-trained athlete different than a non-athlete? The simple answer is 'no'. By the various criteria used to assess immunity, no major differences have been identified. The adaptive immune system remains unaffected for the most part (Nieman & Pedersen 1999). Although a heavy bout of acute exercise even in the trained athlete causes transient changes in immune cells in the blood, there is no compelling evidence that these changes are lasting or affect immune system function. Furthermore, although it has been suggested that this stress-like response leaves the athlete with a somewhat suppressed immune system, vaccination, which involves the co-ordinated function of both B- and T-lymphocytes, has been shown not to be affected in male triathletes (Bruunsgaard *et al*. 1997b). On the other hand, a skin test (delayed type hypersensitivity [DTH]) response to recall antigen was shown to be decreased when carried out immediately after completing a half-ironman triathlon competition (Nieman & Pedersen 1999). To interpret these findings it is necessary to remember that the DTH response is a short-term response while an immune response to vaccination involves a much longer period. Furthermore, another study carried out in normal, healthy individuals, indicated that high intensity progressive resistance training did not affect a DTH response (Rall *et al*. 1996).

The idea that training may be immunosuppressive is based on the observation that overtraining or exhaustive training sometimes encountered by marathon runners or long-distance swimmers is often associated with URTIs. These athletes also may show decreased levels of salivary IgA and IgM. Thus, it is concluded that the infection is due to the reduction in the salivary immunoglobulins (see Mackinnon 2000b). In a recent review, Smith (2003) proposed that the URTIs associated with overtraining or excessive exercise, might be associated more with tissue trauma and the production of stress molecules and hormones than with reduced immunoglobulin levels. Smith suggests that the training brings about type 2 (Th2) cytokine production and suppresses the production of type 1 (Th1) -associated cytokines. This balance of cytokines would put the immune system in a compromised position for fighting off viral infections. While this hypothesis is supported by data in the literature, there is still the need to demonstrate that cytokine responses are affected by overtraining and that URTIs are the outcome. In summary, the majority of information suggests that the immune system of the trained athlete is not substantially different from that of a healthy sedentary person. However, excessive or overtraining may, on a short-term basis, make the athlete more susceptible to URTIs but not necessarily due to immunosupression (Weidner *et al*. 1998).

Mechanisms for exercise–immune interactions—the role of cytokines

Although there is a variety of observations that point to exercise as a regulator of immune function, the molecular mechanisms that underlie this interaction of exercise and immunity remain to be fully elucidated. On the other hand, there is evidence that products of the neuroendocrine system

that are elicited by exercise can affect immune function.

It has long been recognized that leukocyte-derived cytokines act as major communicators and regulators of the immune system (reviewed by Vilcek 2003). These low molecular weight proteins or glycoproteins secreted by many cells in the body in response to various stimuli are present in low concentrations in the blood. In contrast to the classic endocrine-derived hormones which act on their target cells and tissues at distant sites, cytokines typically act locally in either an autocrine or paracrine manner. Many of these cytokines retain the name 'interleukin' (e.g. interleukin-2 or IL-2), thus implying their production by a leukocyte to their ability to act on other leukocytes. Because they can act pleitropically and may be redundant in function, it is often difficult to pinpoint their exact mechanism of action. In fact, we now know that cytokines produced by immune cells not only provide communication among cells of the immune system but also serve as a means of communication among the immune, nervous, and endocrine systems. The impact of exercise on cytokine production explains, in part, how exercise can modulate both immune and neuroendocrine systems.

Initially, assays of biological activity (bioassays) were used to identify cytokines and growth factors in the plasma or serum following exercise. In 1983, researchers (Cannon & Kluger 1983) showed that the plasma obtained from subjects following exercise, when injected into rats, caused an increase in body temperature. This bioassay for cytokines that produce fever, indicated the presence of IL-1 or a related cytokine Both the development of more sensitive assays for this and other cytokines and the production of recombinant molecules has suggested roles for many other cytokines following exercise (Pedersen & Toft 2000). Furthermore the plethora of observed exercise-induced increases in cytokines led many to suggest that exercise causes an inflammatory-like response. One such inflammation-related cytokine that is produced in large amounts after exercise, and thus has been the subject of many studies, is IL-6 (Pedersen & Toft 2000). Although IL-6 has been categorized as a proinflammatory molecule it also has many anti-inflammatory properties such as the ability to inhibit tumor necrosis factor-α (TNF-α) and IL-1 and the induction of IL-1 receptor antagonist. At one point, it was believed that exercise-induced IL-6 resulted from muscle damage (Bruunsgaard *et al*. 1997a; Pedersen 2000). While this may indeed be true, muscle damage is not necessary to observe increases in IL-6. Although a correlation has been reported between the exercise-induced increases in both IL-6 and epinephrine, the infusion of epinephrine did not mimic the extent or the kinetics of changes in IL-6 found with exercise (Steensberg *et al*. 2001). IL-6 is one of many cytokines (e.g. IL-1, TNF-α, IFN-γ, IL-12) that are known to stimulate the HPA axis. As is outlined above, the products of the HPA axis, in particular the glucocorticoids, are able to have multiple effects on immune function. IL-6 has a direct link to the HPA axis given that pituitary cells express receptors for IL-6 and respond to its presence (Besedovsky & Del Rey 2001). Receptors for IL-6 and others cytokines have been identified in the brain as well. Interestingly, Nybo *et al*. (2002), found that the brain itself could release IL-6 following a bout of prolonged exercise, albeit at a much lower level (approximately 100-fold less) than skeletal muscle.

In a recent review article, Suzuki *et al*. (2002) summarized the results of a number of studies in which circulating cytokine levels were detected in the plasma following various exercise regimens. However, a major consideration presented in this review was whether or not measuring the plasma levels of cytokines provides sufficient information since both the localization and utilization of cytokines in specific organs and tissues (both immune and non-immune) are also important in assessing cytokine contribution to overall immune function. Regardless, in approximately one-half of these studies, the proinflammatory molecule TNF-α was shown to increase. Plasma IL-6 concentrations also increased with the greatest increases usually occurring with exercise that caused potential muscle damage. The anti-inflammatory cytokines interleukin-1 receptor antagonist (IL-1ra) and IL-10 were seen to increase in many studies. In contrast, none of these studies reported a change in IFN-γ; some even found a decrease in IL-2.

In order to try to reconcile some of these conflict-

ing reports, these same investigators (Suziki *et al*. 2002) selected 16 target cytokines, including pro- and anti-inflammatory, immunomodulatory, multifunctional, colony stimulating and chemokines. They measured both plasma and urine concentrations at 10 min and 2 and 24 h following a short-duration maximal exercise. The findings were surprising. For example, although plasma TNF-α was not detected, the levels of TNF-α in the urine increased fivefold. An opposite finding was seen with IL-1β, where concentrations increased in the plasma but were undetectable in the urine. IL-2, IL-12, IFN-α and IFN-γ either were not detected or did not change in either plasma or urine. The anti-inflammatory cytokines changed more dramatically in response to exercise. For example, IL-1ra increased in plasma and urine as did IL-4. IL-6 increased in both fluids but only significantly in urine. While the proinflammatory cytokines TNF-α and IL-1 increased, they showed a delayed response compared with anti-inflammatory cytokines, particularly IL-1ra. Together, these results indicate that because of the difference in kinetics of production, half-life and clearance, sampling one cytokine at a time either during or after exercise is likely to provide misleading results.

The impact of exercise on diseases that are immunologically resisted

The immune system has long been known to play a key role in the resistance to a variety of infectious pathogens. Although not as well-defined, there is also evidence of a role for immune function in the successful defense against cancer. However, often forgotten are the undesirable contribution of the immune system in conditions such as allergy and autoimmunity. Thus, the impact of exercise on immune function can range from beneficial to detrimental depending on the nature of the exercise and the role of the immune system in the disease of interest. For many years, there was only anecdotal evidence that exercise can affect the development and/or progression of diseases that have an immunological component. However, only recently has there been experimental proof that such a relationship does indeed exist.

VIRAL INFECTIONS

The influence of the neuroendocrine system on immunity to infectious pathogens has been better defined during the past decade. Many of the studies to define the mechanisms underlying this relationship have relied on the use of stress-based models in mice (Sheridan *et al*. 1998; Bonneau *et al*. 2001). Although far fewer studies have explored the impact of stress on the development of infections in humans (Cohen & Herbert 1996; Cohen & Miller 2001), the significance of these studies is broad. Stress and other psychosocial factors have also been shown to influence the ability of vaccinations for hepatitis B virus and influenza virus to elicit vaccine-specific immune responses (Yang & Glaser 2002).

Although there is clearly a role for stress–neuroendocine–immune interactions in the defense against infectious disease, there is little evidence in humans that moderate exercise plays a significant role in the defense against viral and bacterial infections. For example, a recent study (Weidner & Schurr 2003) found that normally sedentary but healthy individuals did not exhibit differences in the type, severity, of duration of cold symptoms with the addition of exercise. Human immunodeficiency virus (HIV) infection is directly related to the immune system since HIV infects immune cells, i.e. T-cells and macrophages. While exercise may help boost the CD4 levels of HIV^+ individuals it does not reverse the disease. Likewise, exercise can increase the general well-being of individuals at all ages but does not reverse or prevent age or disease influenced changes.

There have also been a number of recent murine-based studies conducted in an attempt to define the impact of exercise on the immune response to and susceptibility to viral pathogens. Although the findings of these studies vary depending on the type of exercise and the nature of the pathogen, they have been valuable in dissecting the many effects of exercise on immune-related processes including cytokine (Davis *et al*. 1998; Kohut *et al*. 1998, 2001a, 2001b) and antibody (Kohut *et al*. 2001a) production, antigen presentation (Ceddia & Woods 1999), macrophage function (Davis *et al*. 1997) and

macrophage chemotaxis (Ortega *et al*. 1997). Such animal-based studies will continue to provide a wealth of information in helping to define the cellular and molecular mechanisms underlying the interactions among exercise, immunity and susceptibility to infectious pathogens.

EXERCISE AND CANCER

Does exercise reduce the risk of cancer? The simple answer is 'yes'. Many, but not all, epidemiological studies have indicated that there is indeed a correlation between physical activity and a reduced risk of cancer (reviewed by Shephard & Shek 1995, 2000a; Gammon *et al*. 1998; McTiernan *et al*. 2003). Is this correlation due to a direct affect of exercise on the immune system and its ability to prevent the development and/or progression of cancer? While it is possible, there is no convincing evidence for such a direct connection.

Several epidemiological studies suggest that physical activity correlates with the incidence of various cancers including those of the colon, breast, prostate, testes and lung (reviewed by Gammon *et al*. 1998). However, not all of these studies were carried out in the same way. For example, some studies included activities related to a job or daily living (e.g. lifting boxes or housework) whereas other studies limited assessment to leisure time activities (e.g. weight training, aerobic exercise). Other variables included the nature of the exercise (i.e. strenuous or moderate) and the time of life during which the activity was performed (reviewed by Gammon *et al*. 1998). A recent study (McTiernan *et al*. 2003), to determine the impact of strenuous or moderate recreational physical activity on the risk of breast cancer, indicated that risk decreased with exercise of longer duration and that the exercise did not have to be strenuous. This study was carried out with post-menopausal women but other studies have considered exercise at various ages of life (reviewed by Gammon *et al*. 1998). Whereas some of these studies found a correlation with age (e.g. Thune *et al*. 1997) others did not (e.g. Verloop *et al*. 2000). Age is an especially important consideration for women because age inherently brings about a change in reproductive hormones, a confounding variable for several types of cancer. A reduction in circulating hormones and disruption of the menstrual cycle in some women undergoing strenuous exercise has been invoked as a mechanism by which exercise can directly lessen the risk of breast cancer (reviewed by Hoffman-Goetz *et al*. 1998). However, few women exercise to this extent. Another recent study with women who carry mutations in *BRCA1* or *BRCA2* genes, and thus putting them at a higher risk for cancer, indicated that they may be protected from or delay the onset of breast cancer by remaining active and maintaining a healthful weight while young (King *et al*. 2003).

Depending on the intensity, exercise may also bring about increased levels of catecholamines, glucocorticoids, β-endorphins, growth hormone and prolactin in the circulation. These hormones all have a direct link to cells of the neuroendocrine and immune systems but the link to cancer is much more tenuous. Likewise, the changes in the levels of NK cells or other leukocytes in the circulating blood caused by intense exercise have never been directly linked to susceptibility to any disease, let alone cancer. It should be noted that epidemiological studies are confounded by diet, body build, health history and other countless factors that can contribute to the development and progression of cancer. Thus, there is no simple answer to explain how an active life style may protect against cancer—if it even does!

AUTOIMMUNITY

If exercise can modulate immune responsiveness can it affect autoimmunity? There are many diseases with an underlying autoimmune pathology; for example, diabetes, multiple sclerosis, rheumatoid arthritis. Some of these can be reproduced in animal models. There is anecdotal and experimental evidence to correlate some aspects of exercise stress with autoimmunity, but in general, exercise did not affect the development or cause of autoimmune diseases (Ferry 1996). The impact of stress and the neuroendocrine system on aspects of autoimmunity has been well documented (Ligier & Sternberg 2001; Prat & Antel 2001; Rogers & Brooks 2001; Wilder & Elenkov 2001). Therefore, it would be expected that

exercise, though one or more components of the neuroendocrine system, is able to regulate both the development and progression of autoimmune disease. Exactly how this regulation takes place and how exercise can be used to control this regulation remains to be determined.

The impact of exercise on other systems that may indirectly affect the immune system

The body is clearly a well-integrated set of systems (Fig. 26.3). It would be unreasonable to assume that all effects of exercise on immunity are mediated through neuroendocrine pathways. For one, the muscular system is obviously a prominent target of exercise. During exercise, the muscles compete with other systems of the body for energy and also produce waste. It has been suggested that the depletion of glycogen, glucose and glutamine from muscle makes these compounds less available to the continually renewing cells of the immune system. Moreover, waste products such as lactate may negatively affect lymphocyte metabolism (Miles *et al*. 2003). Muscle damage, likely to occur during certain kinds of intensive exercise, also initiate the synthesis of inflammatory cytokines. Furthermore, the release of heat shock proteins by exercise stressed cells may act as 'danger signals' for the immune system (Moseley 2000) and activate antigen-presenting cells and lymphocytes. The cardiovascular system is responsible for transporting oxygen, nutrients and wastes, and responds very rapidly to exercise. Heart rate and blood pressure increase to assist the flow of blood. As exercise causes fluids to leave the major vascular compartments, the blood and plasma volumes decrease. This hemoconcentration causes an increase in hemoglobin and cells in the blood. Furthermore, blood may be directed away from peripheral immune organs like the spleen with the net effect that the numbers of leukocytes in the blood is increased (reviewed by Nielsen 2003).

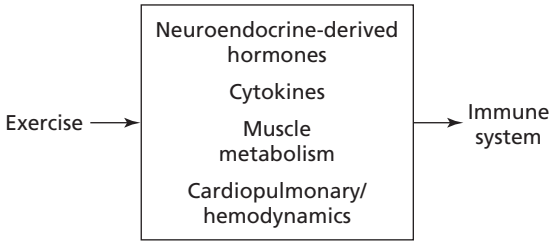

Fig. 26.3 Mechanisms of exercise-mediated modulation of the immune system.

Just as the heart rate rapidly increases, ventilation also rapidly rises and is proportional to changes in intensity in the exercise. The trachea enlarges to allow more, unrestricted air flow. This increased air flow may dry the mouth and have deleterious effects on the lining of the trachea, as well as limit the amount of salivary immunoglobulins. These changes may partially explain why upper respiratory infections increase with intensive, long endurance exercise.

Nielsen (2003) nicely discusses the lymphocyte response to maximal exercise from the point of view of the body's cardiopulmonary responses. He suggests that the transient changes in cell numbers following acute exercise can be explained by increased blood flow, and that increases in ventilation and blood pressure may affect the respiratory epithelium making it more susceptible to infection.

Summary and conclusions

There is no doubt that exercise impacts the neuroendocrine–immune axis. Exactly how, where and to what extent exercise acts on this complex interrelated system, remains to be further defined. Although there is a plethora of indicators of immunomodulation, the ultimate question is how these indicators are related to the functioning of the immune system and how this functioning is related to overall health. Correlations between exercise and changes in immune parameters do not necessarily translate to cause and effect. Even if they did, we still might not understand when it is good to activate or to dampen an immune function, particularly an inflammatory response.

It is difficult enough to evaluate the general status of the immune system in a healthy, homeostatic condition, let alone under stress from exercise. We are able recognize a 'broken' immune system when we see one, for example an immune system ravaged

by the HIV, inherited or acquired immunodeficiencies and autoimmune disorders. In contrast, we do not have a good handle on how to evaluate an apparently intact system. Illness is not necessarily an indicator of a failed immune system. Instead, it may well indicate that the immune system is appropriately responding to fight the acute phase of an infectious disease and is establishing immunological memory in preparation for when the host is challenged with the infectious pathogen once again.

For obvious ethical considerations, we can neither challenge humans with a pathogen nor extensively sample immune compartments where immune responses to such pathogens generally occur. In these respects, studies in animals are valuable in that they provide a means by which to model the impact of exercise on immunity. In addition, newer techniques and an expanding knowledge of the immune system have allowed scientists in the field to begin to unravel some of the mysteries underlying immune function. It will take insightful collaborations between scientists from the disciplines of kinesiology, immunology and neuroendocrinology to move the field forward. The goal is to reach the point where the prescription is no longer a general 'get plenty of exercise', but one in which a very specific recommendation is given of the intensity, mode, duration and kind of exercise needed to complement a standard regimen of medical intervention in an attempt enhance immune function.

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Chapter 27

Exercise Regulation of Insulin Action in Skeletal Muscle

RICHARD C. HO, OSCAR ALCAZAR AND LAURIE J. GOODYEAR

Introduction

Insulin stimulates pleiotropic pathways resulting in increases in both oxidative and non-oxidative glucose disposal, protein synthesis, gene transcription and cellular growth and hypertrophy. It has long been known that exercise is able to elicit similar effects in skeletal muscle. A single bout of exercise can have profound effects on substrate metabolism, and chronic exercise is associated with adaptations that enhance mechanical and metabolic efficiency in skeletal muscle. Among these adaptations are changes in glucose and glycogen metabolism, protein synthesis and hypertrophy, as well as changes in gene transcription.

Efforts have been made to uncover the mechanisms by which exercise is able to mimic and enhance specific effects of insulin, many of which result in clinically relevant adaptations. A fundamental issue in understanding the biological effects of exercise in skeletal muscle is elucidation of intracellular signaling mechanisms that enable exercise to enhance insulin responsiveness. Interestingly, while it has been recognized that insulin and exercise utilize distinct signaling pathways leading to various cellular effects, more recent evidence has emerged showing that exercise and insulin utilize similar signaling intermediates as well.

This chapter will highlight how insulin regulates glucose transport, glycogen metabolism and protein metabolism utilizing the classical insulin-stimulated phosphatidylinositol 3-kinase (PI3K) pathway. We will then review how exercise is able to mimic many of these metabolic effects in skeletal muscle utilizing alternative signaling cascades like the PI3K-independent 5'-AMP-activated protein kinase (AMPK) pathway. While insulin and exercise have been shown to regulate independent pathways leading to the regulation of certain metabolic processes (i.e. glucose transport), we will also discuss how exercise and insulin are both able to regulate common signaling pathways like the family of mitogen-activated protein kinases (MAPKs). Finally, we will discuss the clinical relevance of the various effects of exercise on insulin action in skeletal muscle.

Insulin action in skeletal muscle

Glucose transport

Insulin stimulation has long been known to result in increases in GLUT4 translocation, glucose transport, glycogen synthesis and protein synthesis in skeletal muscle. Insulin elicits many of its metabolic effects in skeletal muscle by activation of the classical PI3K pathway (Fig. 27.1). Under normal physiologic conditions, insulin binds to the α subunit of the insulin receptor (IR). This binding activates autophosphorylation activity in the β subunit and subsequently causes tyrosine phosphorylation of numerous insulin receptor substrates. For example, once activated, the pleckstrin homology domain of the insulin receptor substrate-1 and -2 (IRS-1/-2) can dock with the p85 regulatory subunit of PI3K and activate its p110 catalytic subunit. Catalytic activity of PI3K results in the phosphorylation of phosphoinositide (PI) 4,5-bisphosphate to PI 3,4,5-trisphosphate (PIP_3). PIP_3 is necessary to activate

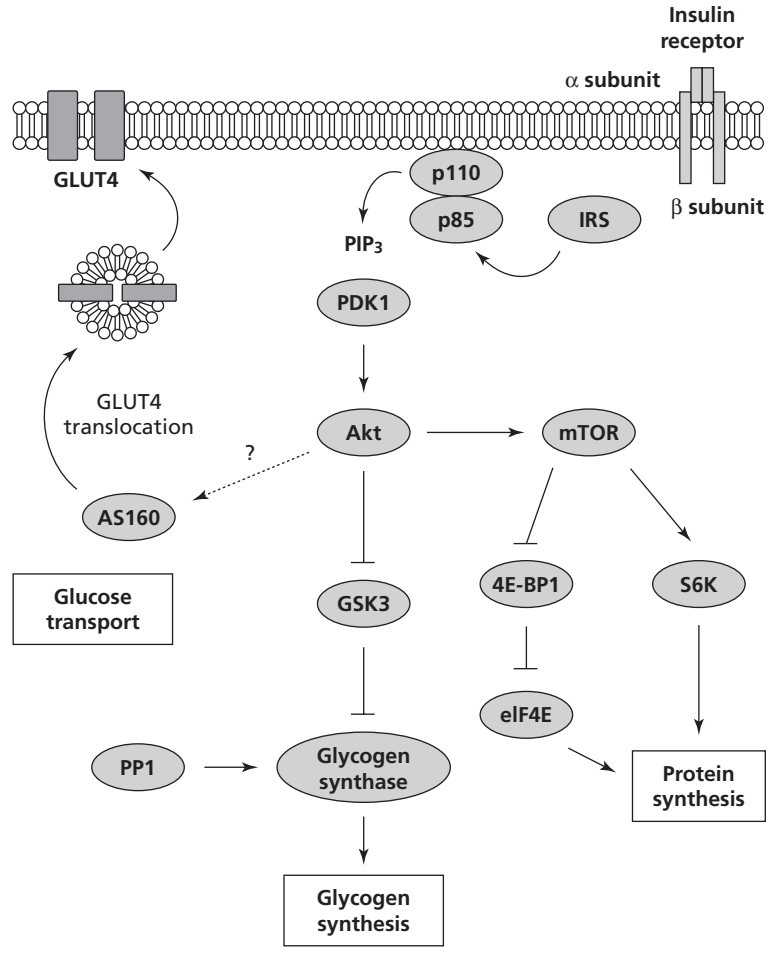

Fig. 27.1 Insulin signaling through the classical phosphatidylinositol 3-kinase (PI3K) pathway. Insulin binding to the extracellular insulin receptor α subunit activates catalytic activity in the corresponding transmembrane β subunit. The β subunit catalyzes the phosphorylation of numerous insulin receptor substrates, some of which (e.g. IRS-1/-2) associate with and activate the p85 and p110 subunits of PI3K. Activated PI3K results in the production of 3,4,5-trisphosphate (PIP_3), and serial activation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt. Akt signaling bifurcates resulting in the modification of proteins involved in the regulation of glucose transporter translocation, glycogen synthesis and protein synthesis.

3-phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates Akt (also known as PKB) on threonine 308. Subsequent phosphorylation of Akt on serine 473 by a yet uncharacterized kinase further activates the enzyme. Glycogen synthase kinase-3 (GSK3), the mammalian target of rapamycin (mTOR), and the 70-kDa S6 protein kinase (S6K) are among the established downstream substrates of Akt. Signaling through this classical PI3K pathway leads to increases in glucose uptake via the translocation of the insulin-sensitive GLUT4 glucose transporter from subcellular vesicles to the plasma membrane. AS160 has recently been proposed to be an Akt-targeted signaling protein mediating GLUT4 translocation (Sano *et al*. 2003).

Glycogen synthesis

The ability of insulin to stimulate glycogen synthesis was believed to involve the activation of protein phosphatase-1 (PP1), and deactivation of GSK3 (Cross *et al*. 1995, 1997; Brady *et al*. 1998; Liu & Brautigan 2000). Through its interaction with glycogen-targeting subunits, insulin activates PP1, which catalyzes the dephosphorlyation and activation of glycogen phosphorylase. However, mice lacking the regulatory subunit of muscle-specific PP1 (PP1G) exhibit normal insulin-stimulated glycogen synthase activation (Suzuki *et al*. 2001), suggesting the involvement of alternative pathways. Under basal conditions, active GSK3 exists in a non-phosphorylated

state. Active GSK3 phosphorylates and inhibits glycogen synthase activity. Upon stimulation by insulin, Akt catalyzes the phosphorylation of GSK3, converting it from its active to inactive form. This, in turn, relieves the negative regulation that GSK3 exerts on glycogen synthase and thus promotes glycogen accretion (Cross *et al*. 1995). Evidence for insulin-induced deactivation of GSK3 has been shown in both muscle cells (Cross 1995) and skeletal muscle tissue (Markuns *et al*. 1999; Wojtaszewski *et al*. 2000).

Protein synthesis

The function of insulin and its mitogenic and anabolic properties in various tissues has been well characterized. Several lines of evidence have suggested that post-prandial increases in insulin are associated with increased protein synthetic rates in skeletal muscle (Shah *et al*. 2000). Studies have suggested that insulin-stimulated increases in rates of protein synthesis are mediated by PI3K-dependent mechanisms, since PI3K inhibitors can attenuate the activation of key regulatory molecules involved in protein synthesis. Presumably, this mechanism involves the sequential activation of PI3K, Akt, mTOR and S6K, and deactivation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (reviewed in Shah *et al*. 2000; Kimball *et al*. 2002). The ability of insulin to promote protein synthesis in skeletal muscle is also partially mediated by PI3K-dependent signaling to eukaryotic initiation factor 2B (eIF2B), a guanine nucleotide exchange protein involved in the regulation of mRNA translation initiation (Welsh *et al*. 1998). Existing data suggest that under non-stimulated conditions, GSK3 phosphorylates eIF2B on an inhibitory serine residue (Welsh *et al*. 1998). Acute insulin stimulation leads to the phosphorylation and inactivation of GSK3, resulting in the activation of eIF2B in skeletal muscle.

Exercise mimics insulin action in skeletal muscle

Like insulin, exercise is able to stimulate GLUT4 translocation, glucose transport, glycogen synthesis and protein synthesis in skeletal muscle. Therefore, it was a logical hypothesis that exercise and insulin utilized similar signaling cascades in skeletal muscle. However, several studies have clearly demonstrated that proximal insulin-stimulated PI3K intermediates are not involved in the mechanism by which exercise elicits its metabolic effects. For example, contractile activity does not lead to increases in autophosphorylation of isolated insulin receptors (Treadway *et al*. 1989) or IRS-1 and IRS-2 tyrosine phosphorylation (Goodyear *et al*. 1995; Sherwood *et al*. 1999; Wojtaszewski *et al*. 1999a; Howlett *et al*. 2002). Consistent with this, muscle-specific insulin receptor knockout mice exhibit normal exercise-mediated glucose transport (Wojtaszewski *et al*. 1999a). PI3K activity has been reported to be unchanged with skeletal muscle contraction (Goodyear *et al*. 1995; Wojtaszewski *et al*. 1996, 1999a). Additionally, wortmannin, a PI3K inhibitor, does not impair contraction-stimulated glucose transport (Hayashi *et al*. 1998). The lack of activation of these proximal PI3K signaling intermediates demonstrates that the underlying molecular mechanisms leading to the insulin- and contraction-induced stimulation of glucose uptake and glycogen synthesis in skeletal muscle are distinct. This is also supported by evidence showing that the effects of acute exercise and insulin on glucose transport are additive (Ruderman *et al*. 1971).

Controversy exists regarding the potential role of Akt in contraction-mediated signaling in skeletal muscle. While early studies reported that exercise and contraction did not result in the activation of Akt (Brozinick & Birnhaum 1998; Lund *et al*. 1998; Widegren *et al*. 1998; Sherwood *et al*. 1999; Wojtaszewski *et al*. 1999a), other studies have shown significant activation or phosphorylation of Akt in intact skeletal muscles in response to contraction (Turinsky & Damrau-Abney 1999; Nader & Esser 2001). Recently, we and others have found that both exercise *in vivo* and contraction *in situ* via sciatic nerve stimulation increases Akt phosphorylation in multiple rat hindlimb muscles (Turinsky & Damrau-Abrey 1999; Sakamoto *et al*. 2002, 2003). It has also been demonstrated that, like insulin, exercise alters the activity of GSK3 in rat skeletal muscles (Markuns *et al*. 1999). Insulin and exercise increased glycogen synthase activity to a similar

extent, however, the mechanisms involved have been shown to be slightly different. Exercise deactivates GSK3α and β activity, and increases phosphorylation to a similar degree as does insulin (Sakamoto *et al*. 2003). Another study showed that bicycle exercise increased glycogen synthase activity immediately following exercise without detectable deactivation of either isoform of GSK3 in vastus lateralis muscle (Wojtaszewski *et al*. 2001). There may be alternative mechanisms in the regulation of GSK3 activity in skeletal muscle. Interestingly, we have recently found that the muscle-specific regulatory subunit of PP1 is required for regulation of glycogen metabolism under basal conditions and in response to contractile, but not insulin, activity (Aschenbach *et al*. 2001). Therefore, insulin-stimulated glycogen synthase activation seems to be regulated by GSK3, while exercise may regulate glycogen synthase via GSK3-independent mechanisms.

Exercise-stimulated AMPK activation

Since PI3K appears not to be required for exercise-mediated glucose transport in skeletal muscle, an independent pathway activated by exercise has been the focus of much attention. The discovery of the AMPK as an enzyme potently regulated by exercise has been revealed as such a pathway. As a member of a metabolite-sensing protein kinase family, AMPK acts as a fuel gauge monitoring cellular energy levels. Under conditions of decreased cellular energy status (increase in the AMP/ATP and creatine/phosphocreatine ratios), AMPK signaling functions to down-regulate adenosine triphosphate (ATP)-consuming pathways and up-regulate alternative pathways for ATP regeneration (Fig. 27.2). Skeletal muscle contraction is known to deplete these intracellular energy stores, and consequently, AMPK has been shown to exhibit robust activation in response to both exercise and contraction (Winder & Hardie 1996; Rasmussen & Winder 1997; Vavvas *et al*. 1997). AMPK is activated by a complex mechanism that involves allosteric modification, decreases in phosphatase activities and phosphorylation by an AMPK recently identified to be LKB1 (Hawley *et al*. 2003; Ossipova *et al*. 2003; Lizcano *et al*. 2004).

Early evidence suggested a role for AMPK in the regulation of fat oxidation (Vavvas *et al*. 1997; Hardie *et al*. 1998). Recently, studies from our laboratory (Hayashi *et al*. 1998, 2000) and others (Hardie *et al*. 1998; Russell *et al*. 1999) have also described a role for AMPK in mediating contraction-induced glucose transport. Both contraction and the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) stimulate glucose transport by insulin-independent mechanisms (Hayashi *et al*. 1998), and AMPK activity is associated with glucose transport in contracting skeletal muscle (Ihlemann *et al*. 1999). Mice overexpressing a dominant inhibitory AMPK mutant were shown to exhibit complete inhibition of AICAR-mediated glucose uptake, but only partial inhibition of contraction-mediated glucose uptake into skeletal muscle (Mu *et al*. 2001). There are two isoforms of the AMPK catalytic subunit (α_1 and α_2), and another study has recently demonstrated that AMPK α_2 knockout mice exhibit significant impairments in AICAR-mediated, yet completely normal skeletal muscle glucose transport, in response to contraction (Jorgensen *et al*. 2004). These data indicate that while AMPK is required for glucose transport in response to AICAR, alternative exercise-responsive signaling pathways are involved in contracting skeletal muscle.

Recent studies suggest that AMPK is involved in the regulation of glycogen metabolism, although existing data are equivocal, suggesting both an inhibitory and activating role of AMPK in the regulation of glycogen synthesis. For example, some studies proposed that AMPK has the ability to phosphorylate key proteins of glycogen metabolism *in vitro*, such as glycogen synthase (Carling & Hardie 1989), which would be expected to inhibit glycogen synthesis (Skurat *et al*. 1994) and phosphorylase kinase (Carling & Hardie 1989), the upstream effector of glycogen phosphorylase. It seems reasonable to speculate that a major role of AMPK in contracting muscle would be to promote glycogen degradation and inhibit glycogen synthesis, as AMPK is known to buffer intracellular ATP levels in various cell systems. In accordance with this hypothesis, there is a study showing an increased glycogen content in skeletal muscle of Hampshire pigs, which

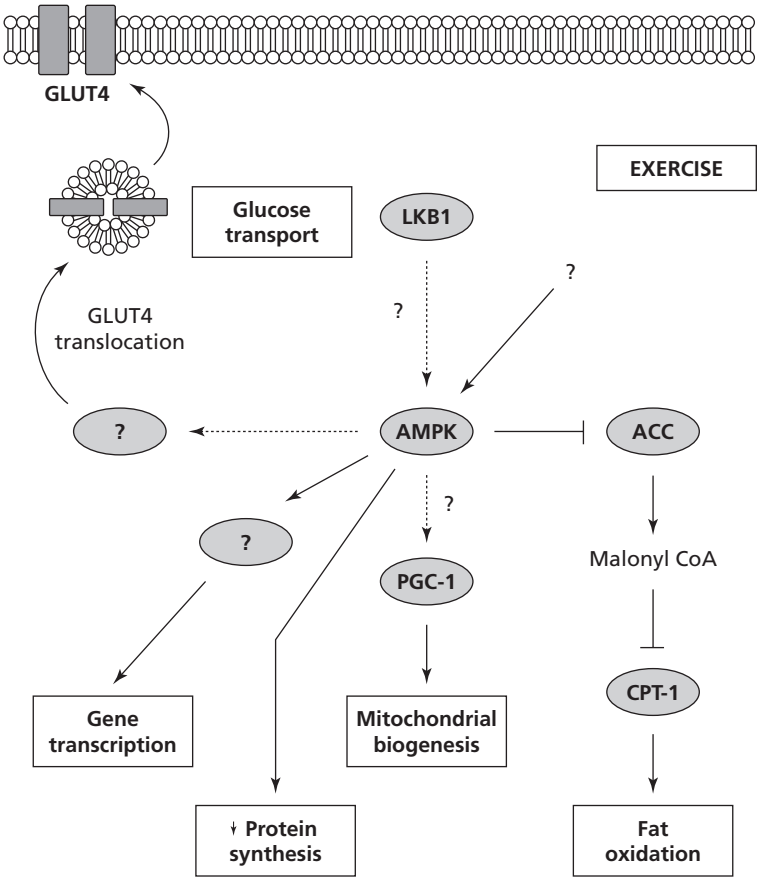

Fig. 27.2 Exercise regulation of 5′-AMP-activated protein kinase (AMPK) in skeletal muscle. Exercise activates AMPK through changes in cellular energy status (i.e. AMP : ATP), and potentially through the recently described AMPK, LKB1. AMPK is known to inhibit ATP-consuming pathways such as protein synthesis, and activate adenosine triphosphate (ATP) regenerating pathways such as fat oxidation. AMPK is also believed to regulate mitochondrial biogenesis via PGC-1α, as well as additional cellular processes such as glucose transport and gene transcription and through mechanisms that have not been fully elucidated. ACC, acetyl-CoA carboxylase.

harbor a point mutation in the γ_3 subunit of AMPK that renders this enzyme less active (Milan *et al*. 2000). In contrast to this, reports have emerged showing that similar mutations in the γ_1 and γ_2 subunits result in constitutively active AMPK in conjunction with elevated muscle glycogen levels (Hamilton *et al*. 2001; Arad *et al*. 2002). Pharmacological activation of AMPK by AICAR in hindlimb muscles has been shown to result in the phosphorylation and deactivation of glycogen synthase in rat soleus, and red and white gastrocnemius muscles (Wojtaszewski *et al*. 2002). In contrast, it has also been reported that AMPK functions to increase glycogen synthesis with chronic AICAR treatment in red, slow-twitch and white, fast-twitch quadriceps and gastrocnemius muscles (Winder *et al*. 2000; Buhl *et al*. 2001).

Exercise is able to regulate glucose transport and glycogen synthesis in skeletal muscle utilizing insulin-independent pathways (Wallberg-Henriksson & Holloszy 1984, 1985). Interestingly, while operating through distinct signaling cascades during exercise, the post-exercise period is characterized by enhancements in insulin sensitivity and responsiveness. These effects of exercise on insulin action and skeletal muscle metabolism persist well into the post-exercise period.

Insulin sensitivity: acute exercise increases insulin action on glucose disposal

In addition to the well-characterized increase in glucose transport during exercise, prior exercise has also been reported to improve insulin action in skeletal muscle. Richter *et al*. (1982) made the initial observation that previously exercised muscles exhibit elevated insulin-stimulated glucose uptake,

even when the effects of the exercise session per se are no longer present. This finding has been verified in numerous animal and human studies using a variety of experimental models. In humans, an increase in insulin-stimulated whole body glucose utilization after exercise has been observed (Bogardus *et al*. 1983; Devlin *et al*. 1987; Mikines *et al*. 1988; Richter *et al*. 1989), and by using the arteriovenous balance technique this effect was shown to be primarily mediated by an increase in skeletal muscle glucose disposal (Ivy & Holloszy 1981; Richter *et al*. 1982).

An early study using a rat model first suggested that the increase in insulin sensitivity with exercise is restricted to the working muscle (Richter *et al*. 1984). One-legged exercise models in humans have also demonstrated that the exercise-induced increase in insulin-stimulated glucose uptake is a local phenomenon restricted to the exercised muscles, and strongly suggested that changes in systemic factors are not the cause of the increase in insulin sensitivity to stimulate muscle glucose uptake (Richter *et al*. 1984, 1989). Interestingly, if isolated epithroclearis muscles from rats are contracted by applying electrical stimulation *in vitro*, no changes in insulin sensitivity are observed following the contraction period (Wardzala *et al*. 1985). This observation and a subsequent study (Kolterman *et al*. 1980) led some to suggest that a factor released into the circulation during contractile activity is necessary for the post-exercise increase in insulin sensitivity. Since there is an increase in insulin sensitivity to stimulate glucose transport in muscles of the perfused rat hindlimb after electrical stimulation (Richter *et al*. 1984), it might be the paracrine action of a neurotrophic factor that initiates the events leading to the increase in insulin sensitivity after exercise (Sasson *et al*. 1987).

Exercise results in increases in blood flow to working muscles, and it has been hypothesized that differences in insulin delivery between exercised and rested muscles could explain the enhanced insulin action observed in the post-exercise period. However, using a euglycemic–hyperinsulinemic clamp, studies have shown that exercised legs exhibit higher insulin sensitivity compared with non-exercised legs, even when no differences are observed between insulin delivery and clearance (Bogardus *et al*. 1983; Devlin & Horton 1985; Richter *et al*. 1989). Furthermore, studies using isolated muscles (*in vitro* or perfused) where insulin levels are controlled show that insulin sensitivity is increased with exercise (Ruderman *et al*. 1971). Additionally, the majority of studies show that exercise does not increase the binding of insulin to its receptor (Bonen *et al*. 1984; Zorzano *et al*. 1985; Treadway *et al*. 1989). Therefore, neither higher insulin delivery nor insulin binding to the receptor explains the increase in insulin action in previously exercised skeletal muscles. Taken together, these data suggest that modulation of a post-insulin receptor event may be involved in the increased insulin action after exercise.

The cellular mechanisms leading to the increase in insulin sensitivity following exercise have been hypothesized to involve enhanced insulin signaling. However, as discussed earlier, studies have shown that exercise does not utilize proximal PI3K signaling molecules during exercise, yet speculations were made that exercise resulted in enhancements in insulin-stimulated PI3K signaling in the post-exercise period. Prior exercise does not change insulin binding to its receptor (Bonen *et al*. 1984; Zorzano *et al*. 1985; Treadway *et al*. 1989) or increase insulin-stimulated receptor tyrosine kinase activity in skeletal muscles obtained from rats (Treadway *et al*. 1989; Goodyear *et al*. 1995) or humans (Wojtaszewski *et al*. 2000). In fact, prior contraction of rat hindlimb skeletal muscles has been shown to cause a paradoxical *decrease* in insulin-stimulated tyrosine phosphorylation of IRS1 and IRS1-associated PI3K activity (Wojtaszewski *et al*. 2000).

In contrast to these reports, other evidence suggests that the increase in insulin responsiveness to stimulate muscle glucose transport immediately following exercise is associated with an increase in insulin-stimulated PI3K activity (Houmard *et al*. 1999; Chibalin *et al*. 2000; Kirwan *et al*. 2000). Furthermore, we have reported that prior exercise resulted in enhanced insulin-stimulated IRS-2-associated PI3K activity compared with insulin stimulation alone (Howlett *et al*. 2002). The increase in insulin-stimulated PI3K activity following exercise is short-lived, since it is not present when

insulin stimulation occurs after 30 min of recovery from exercise (J.F. Wojtaszewski & L.J. Goodyear, unpublished observation). Furthermore, if a physiological hyperinsulinemic clamp is applied 3 h after one-legged exercise in humans, PI3K activity is not higher in the muscle from the exercised leg (Wojtaszewski *et al*. 1997). The lack of enhancement in insulin-stimulated PI3K activity in these later time points after exercise is consistent with findings in humans showing no change in insulin receptor tyrosine kinase activity or IRS-1 tyrosine phosphorylation showing no change in insulin receptor tyrosine kinase activity or IRS-1 tyrosine phosphorylation (Wojtaszewski *et al*. 1997). Thus, although there seems to be an up-regulation of some components of the insulin signaling pathway with insulin immediately after exercise, these changes do not explain the long-lasting influence of exercise on insulin action in skeletal muscle. These studies rule out a role for enhanced insulin signaling as a mechanism for increased glucose uptake after an acute exercise bout, and provide additional support to the hypothesis that exercise and insulin act through distinct signaling mechanisms.

Elevations in post-exercise insulin sensitivity are also associated with the degree of muscle glycogen depletion. It is generally known that increases in insulin sensitivity following exercise can persist if muscle glycogen levels are kept low. Muscle glucose uptake following exercise remains elevated for up to 18 h if rats are fed a carbohydrate-free diet, while rates of uptake return to pre-exercise levels if carbohydrate is provided following exercise (Young *et al*. 1983).

Exercise training increases insulin action on glucose disposal

While the effects of an acute bout of exercise on insulin-stimulated glucose transport in the post-exercise period have been well established, these effects are quite transient with improvements in insulin responsiveness returning to pre-exercise levels, usually within 12 h of the preceding exercise bout. It was reasonable to speculate that chronic exercise would result in adaptations that enable skeletal muscle to exhibit more sustained increases in insulin action. Three decades ago, Bjorntorp *et al*. (1972) suggested that exercise training might increase tissue sensitivity to insulin. This investigation, along with several subsequent studies (Lohman *et al*. 1978; Johansen & Munch 1979; LeBlanc *et al*. 1979, 1981; Seals *et al*. 1984), demonstrated that compared with sedentary individuals, trained individuals tend to exhibit improvements in glucose tolerance and insulin sensitivity. Additional studies using the hyperinsulinemic–euglycemic clamp have demonstrated that exercise-trained people have higher rates of insulin-stimulated glucose disposal than do their sedentary counterparts (Saltin *et al*. 1978; Rosenthal *et al*. 1983; Hollenbeck *et al*. 1984; King *et al*. 1987; Mikines *et al*. 1989). Although these results have been interpreted to indicate that exercise training results in an increase in tissue sensitivity to insulin, this concept has been complicated by the fact that, as discussed previously, an acute bout of exercise produces major effects on both whole body glucose disposal and metabolism (Pruett & Oseid 1970; Bogardus *et al*. 1983; Devlin *et al*. 1985, 1987; Mikines *et al*. 1988, 1989) and skeletal muscle glucose uptake and metabolism (Holloszy & Narahara 1965; Ivy & Hollozy 1981; Elbrink & Phipps 1980; Fell *et al*. 1982; Richter *et al*. 1989) that can persist well into the post-exercise period. Hence many of the effects of regular exercise may be due to the residual effects of the last individual exercise session, rather than long-term adaptations to exercise training. Several studies have attempted to discriminate differences in insulin sensitivity between acute exercise effects and training adaptations (Heath *et al*. 1983; Burstein *et al*. 1985; King *et al*. 1988; Mikines *et al*. 1989). Some of these studies suggest that the increases in insulin sensitivity associated with exercise training are transient (Burstein *et al*. 1985; King *et al*. 1988). One study has reported that while 10 days of detraining resulted in decreases in insulin sensitivity to a level comparable with sedentary individuals, performance of a single exercise bout by the trained subjects on the 11th day of the protocol did not completely return the insulin response to the trained level (Heath *et al*. 1983). These data suggest that effects of an acute bout of exercise cannot completely account for increases in insulin action associated with exercise training.

After comparing trained subjects with untrained subjects who had undergone an acute bout of exercise and after comparing trained subjects before and after 5 days of detraining (Mikines *et al*. 1989), a different group of investigators concluded that an increase in maximal insulin action on whole-body glucose uptake (insulin responsiveness) but not insulin sensitivity is a long-term adaptation caused by endurance exercise training. Additional studies have reported that maximal insulin-stimulated glucose disposal was unaffected by 10 days of inactivity (King *et al*. 1988), suggesting that the reduction in insulin action following short-term inactivity is the result of a decrease in insulin sensitivity and not a decrease in insulin responsiveness. The molecular basis for this phenomenon has not been completely elucidated, but appears to be dependent on multiple factors including humoral factors, muscle glycogen concentrations and alterations in signaling mechanisms.

One study has recently reported that habitual exercise was associated with decreased protein expression of IR, IRS-1 and IRS-2 in trained versus untrained subjects (Yu *et al*. 2001). Another study found that 7 days of exercise training did not elicit changes in PI3K activity, despite increases in insulin action in skeletal muscle (Tanner *et al*. 2002). The enhanced activation by insulin was not associated with a greater insulin-stimulated insulin receptor or IRS-1 tyrosine phosphorylation. These data suggest that the improvement in insulin action associated with exercise training is not associated with increases in PI3K signaling. Contrary to these data, trained rats have been shown to exhibit higher insulin-stimulated glucose transport, IRS-1/-2 activity, PI3K association and Akt phosphorylation compared with sedentary controls (Chibalin *et al*. 2000; Luciano *et al*. 2002). Therefore, increases in insulin responsiveness resulting from chronic exercise may be mediated, in part by alterations in proximal PI3K signaling in skeletal muscle, but most likely involves additional signaling molecules whose characterization remains elusive.

Efficacious exercise training programs can result in changes in plasma lipids and body composition, in particular, decreases in total body fat and increases in lean body mass. These changes, independent of short-term exercise effects are associated with improvements of insulin action in skeletal muscle. However, exercise-mediated improvements in insulin sensitivity have been detected even when studies have controlled for these potentially confounding effects (Oshida *et al*. 1989; Hughes *et al*. 1993). Hence, exercise training appears to improve insulin sensitivity independent of changes in body composition and there may be additive effects of exercise and decreased adiposity.

Glycogen supercompensation: exercise increases insulin action on glycogen metabolism

The increase in glycogen synthesis in response to insulin stimulation has been well characterized. Furthermore, exercise elicits a net breakdown of muscle glycogen despite increases in glycogen synthase activity. In 1966, Bergstrom and Hultman (1966) first described the concept of glycogen supercompensation, whereby glycogen levels increase significantly in response to carbohydrate feeding following a glycogen depleting exercise bout. The preceding exercise bout leads to increases in skeletal muscle glycogen above that which is seen following a meal. This effect is mediated to a large extent by the post-exercise increases in glucose transport, glycogen synthase activity and the degree of glycogen depletion.

Increases in post-exercise glucose transport result in subsequent elevations in the levels of glucose 6-phosphate (G6P), an allosteric activator of glycogen synthase (Price *et al*. 2003). Therefore, the extent to which an exercise bout increases glucose transport in the post-exercise period partially determines levels of glycogen synthesis. However, studies have shown that increases in glycogen synthase activity following exercise cannot fully account for the observed elevations in glycogen accumulation (Bergstrom *et al*. 1972; Conlee *et al*. 1978; Nakatani *et al*. 1997; Greiwe *et al*. 1999).

The degree of glycogen depletion resulting from the preceding exercise bout is a critical factor in determining the subsequent increases in muscle glucose uptake and glycogen synthesis following exercise. In fact, the potentiating effect that exercise exerts on insulin action (glucose transport, glycogen

synthesis) is a function of the degree of glycogen depletion achieved during the bout of exercise. Interestingly, post-exercise increases in glucose uptake and insulin sensitivity can be reversed with carbohydrate feeding and glycogen repletion (Host *et al*. 1998; Kawanaka *et al*. 2000). Similarly, maintaining low glycogen levels following exercise by carbohydrate restriction extends the effect of exercise on insulin action in skeletal muscle (Garcia-Roves *et al*. 2003). However, a study performed in individual tissues from perfused hindquarter muscle suggests that glycogen is not the only regulator in the supercompensation phenomenon (Zorzano *et al*. 1986). In this report, glucose transport returned to near baseline values even though glycogen concentrations were maintained at a low level. This result suggests the existence of alternate compensatory mechanisms that may participate in the regulation of the supercompensation phenomenon together with the glycogen levels. The precise mechanism(s) involved in the supercompensation effect remains to be elucidated. A recent study using AICAR treatment as a pharmacological model to mimic exercise, in fast-twitch (epitrochlearis) and slow-twitch (flexor digitorum brevis) muscles, revealed that AICAR has no effect on either glycogen synthase or glycogen phosphorylase in both muscle types (Aschenbach *et al*. 2002). Therefore, this study suggests that AMPK does not directly regulate glycogen synthase or glycogen phosphorylase in skeletal muscle. Consistent with this finding, a recent investigation using four different phosphorylase kinase substrates demonstrated that glycogen phosphorylase is not a substrate of AMPK *in vitro* (Beyer *et al*. 2000). Further efforts are required in the future to investigate the supercompensation mechanism and its regulation.

Hypertrophy: exercise increases insulin action on protein synthesis

While skeletal muscle is capable of responding acutely to changes in circulating humoral factors (insulin) and metabolic demands (exercise), skeletal muscle also exhibits the ability to adapt to chronic perturbations in such a way that size (hypertrophy), mechanical function (force generation) and metabolic function (oxidative capacity) improve. In particular, resistance exercise is associated with an up-regulation of anabolic processes that allow for skeletal muscle growth and regeneration. One of the main underlying factors responsible for these adaptations is the ability of exercise to elicit transient increases in protein synthesis (Booth & Thomason 1991; Fluckey *et al*. 1996). A protocol involving 4 days of resistance exercise resulted in elevations in insulin-mediated protein synthesis in rat skeletal muscle (Fluckey *et al*. 1996). Other studies have reported no such effect following endurance exercise (Dohm *et al*. 1980; Balon *et al*. 1990). This may partially explain why chronic *resistance* exercise results in muscle hypertrophy, while chronic *endurance* exercise does not. AMPK functions to down-regulate energy consuming pathways, while up-regulating ATP-regenerating pathways. Protein synthesis is an expensive energy consuming process, and subsequently, studies have shown that AMPK is involved in the inhibition of protein synthetic pathways (Bolster *et al*. 2002; Horman *et al*. 2002; Krause *et al*. 2002; Kimura *et al*. 2003). Therefore, increases in protein synthesis in skeletal muscle following exercise appear to occur via AMPK-independent mechanisms.

The capacity of exercise to mimic the anabolic effects of insulin has been attributed to the activation of similar signaling mechanisms. For example, studies have demonstrated that exercise activates MAPK, Akt, S6K and eIF2B, all of which have been implicated in the regulation of skeletal muscle protein synthesis by insulin (Baar & Esser 1999; Farrell *et al*. 2000; Sakamoto & Goodyear 2002). Interestingly, exercise and insulin exhibit a synergy, since studies have reported that the ability of resistance exercise to increase protein synthesis (and eIF2B activity) in skeletal muscle is dependent on permissive levels of insulin (Fedele *et al*. 2000; Kostyak *et al*. 2001). Under moderately hypoinsulinemic conditions, resistance exercise has been reported to stimulate protein synthesis through a mechanism believed to involve compensatory increases in intramuscular IGF-1 signaling (Farrell *et al*. 1999; Fedele *et al*. 2000).

While resistance exercise is known to activate enzymes involved in protein synthesis, maximal

protein accretion is highly dependent on additional factors. Exercise is able to improve the anabolic effects of insulin in skeletal muscle, and interactions with other hormones (e.g. insulin-like growth factor I [IGF-I], testosterone) and substrate availability (amino acids in particular) determine to a large extent subsequent degrees of hypertrophy (reviewed in Tipton & Wolfe 2001).

Caveat: exercise-induced muscle injury can decrease insulin action on glucose disposal

As described above, exercise is generally associated with improvements in insulin-mediated glucose disposal in skeletal muscle. However, acute decreases in insulin-stimulated glucose metabolism have been documented following resistance exercise, a phenomenon that is largely explained by the degree to which the muscle is damaged. The association between whole body injury, trauma and skeletal muscle insulin resistance has been well documented (Black *et al*. 1982). Exercise resulting in muscle damage elicits an inflammatory response, whereby accumulation of inflammatory cells invades the affected tissues (Asp *et al*. 1997). These cells facilitate in the repair, remodeling and removal of damaged tissue. The release of inflammatory cytokines mediate many of these repair mechanisms, however, the ability of cytokines (i.e. tumor necrosis factor-α) to induce insulin resistance is also well known.

Due to force/area dynamics, muscle injury is more likely to occur in response to eccentric (lengthening of contracting muscle) compared with concentric (shortening of contracting muscle) muscle contraction. While a single bout of concentric exercise is associated with improvements in skeletal muscle insulin action (Richter *et al*. 1982, 1989; Bogardus *et al*. 1983), a bout of eccentric exercise was associated with impaired whole body insulin action 48 h post-exercise (Kirwan *et al*. 1992). This effect was also observed in isolated muscle, suggesting that the insulin resistance is a local phenomenon (Asp & Richter 1996). Consistent with differences in insulin-mediated glucose metabolism, concentric exercise is commonly associated with increases in GLUT4 protein levels (Richter *et al*. 1989), while eccentric exercise has been associated with reductions in GLUT4 transcription, mRNA and protein expression in skeletal muscle by up to 65% (Asp *et al*. 1995; Kristiansen *et al*. 1996). Interestingly, while studies have shown that eccentric contraction can impair glucose metabolism, protein synthetic rates remain elevated. This is perhaps due to selective impairments in PI3K signaling and GLUT4 translocation (Fluckey *et al*. 1999) but normally activated MAPK signaling (Haddad & Addams 2002). In fact, MAPKs are not only activated by muscle contraction, but have also been shown to be activated by stretch, muscle damage and injury and inflammatory cytokines (discussed later).

While acute moderate-intensity exercise and endurance exercise training are associated with increases in insulin-mediated glucose disposal, resistance exercise has been shown to elicit opposite effects. An early study showed that high intensity resistance exercise was associated with reductions in glucose disposal (Kirwan *et al*. 1992). Acute resistance exercise has been shown to result in lower insulin-stimulated glucose uptake compared with sedentary controls (Fluckey *et al*. 1999). The degree of eccentric contraction involved in resistance versus aerobic exercise explains, to a large degree, observed differences in insulin-stimulated glucose metabolism. The anaerobic nature of resistance exercise results in significant decreases in muscle glycogen. Interestingly, in addition to decreases in insulin-stimulated glucose transport, eccentric exercise has been shown to impair insulin-stimulated incorporation of glucose into glycogen in various muscles (Asp *et al*. 1997). One study reported that eccentric exercise resulted in a 16% decrease in maximal glycogen synthase activity compared with controls (Asp & Richter 1996). These impairments are often observed in the more glycolytic fast twitch muscles, which are highly recruited during resistance exercise (Asp & Richter 1996).

While muscle damage resulting from a *single bout* of resistance exercise can result in decreases in skeletal muscle insulin action, other data suggest that resistance *training* is associated with improvements in insulin-stimulated glucose disposal (Miller *et al*. 1994; Yaspelkis *et al*. 2002). Several factors help to explain the observed inconsistencies. First, resistance exercise includes both concentric and eccentric

components of muscle contraction. Resistance exercise programs that include more eccentric contractions would presumably lead to increased muscle injury and decreased insulin-action and vice versa. Furthermore, while an acute bout of eccentric-resistance exercise is associated with impairments in glucose metabolism, chronic resistance exercise elicits skeletal muscle adaptations that improve insulin action in skeletal muscle (e.g. GLUT4 expression). Finally, while untoward effects of an acute bout of eccentric exercise are due to local effects (inflammatory response), corresponding changes in body composition (decreased fat mass, increased muscle mass) associated with chronic resistance exercise training are associated with improvements in whole-body insulin action.

Insulin and exercise stimulate MAPK signaling

While it is clear that insulin stimulates glucose disposal utilizing the PI3K pathway, and exercise appears to utilize PI3K-independent pathways (e.g. AMPK), recent evidence suggests that both exercise and insulin are able to activate common signaling cascades in skeletal muscle. While insulin has long been known to activate the family of MAPK, a growing body of evidence suggests that MAPK signal transduction pathways play an important role in exercise signaling in skeletal muscle. The MAPKs represent an important family of signal transduction proteins, which are expressed in all eukaryotic cells (Fig. 27.3). Four MAPK subgroups have been described: (i) the extracellular-signal regulated kinases (ERKs); (ii) c-Jun NH_2-terminal kinases (JNKs); (iii) p38 MAPK; and (iv) ERK5/big MAP kinase 1 (BMK1). ERKs are predominantly activated by growth factors, while JNKs and p38 MAPK are collectively known as stress-activated protein kinases, and have been implicated in a large number of cellular responses, including cell proliferation, differentiation, hypertrophy, inflammation, apoptosis, carbohydrate metabolism and gene transcription (Force & Bonventre 1998; Sweeney *et al*. 1999; Kyriakis & Avruch 2001). In 1996, it was first reported that exercise activates ERK1/2, JNK and p38 signaling in skeletal muscle (Goodyear *et al*. 1996). Since that time there has been intense interest in the regulation of these pathways in skeletal muscle.

ERK signaling

Insulin has long been known to be a potent stimulator of ERK in skeletal muscle. Additionally, activation of ERK1/2 signaling has been reported in rat skeletal muscle with treadmill running (Goodyear *et al*. 1996; Nader & Esser 2001), *in vitro* contraction (Hayashi *et al*. 1999; Wojtaszewski *et al*. 1999b, 2000; Ryder *et al*. 2000; Wretman *et al*. 2000, 2001), *in situ* contraction (Sherwood *et al*. 1999; Martineau & Gardiner 2001; Nader & Esser 2001), muscle overload (Carlson *et al*. 2001) and stretch (Boppart *et al*. 2001); in mouse muscle in response to treadmill running (Dufresne *et al*. 2001); and in human skeletal muscle in response to cycle ergometer exercise (Aronson *et al*. 1997a, 1997b; Widegren *et al*. 1998; Osman *et al*. 2000) and marathon running (Yu *et al*. 2001). Upstream of ERK1/2, both MEK1/2 and Raf1 (Aronson *et al*. 1997a, 1997b; Sherwood *et al*. 1999) activities are increased by exercise and contraction. Molecules downstream of ERK1/2 that have been shown to be activated by exercise include RSK2 (Goodyear *et al*. 1996; Aronson *et al*. 1997a, 1997b; Sherwood *et al*. 1999; Krook *et al*. 2000; Osman *et al*. 2000; Ryder *et al*. 2000; Yu *et al*. 2001) and the mitogen and stress-activated kinase 1/2 (MSK1/2) (Ryder *et al*. 2000; Yu *et al*. 2001). MEK1/2 activation is necessary for ERK1/2 activation since the MEK1/2 inhibitor PD98059 inhibits muscle contraction induced ERK1/2 phosphorylation (Hayashi *et al*. 1999; Wojtaszewski *et al*. 1999b, 2000; Ryder *et al*. 2000) and its downstream substrates RSK2 (Hayashi *et al*. 1999) and MSK1 (Ryder *et al*. 2000).

With one-legged cycling exercise in human subjects, activation of ERK1/2 (Aronson *et al*. 1997b; Widegren *et al*. 1998) and its downstream targets (Aronson *et al*. 1997b; Krook *et al*. 2000) is observed in the muscle from the exercised leg, but not in the resting leg. These data suggest that stimulation of ERK1/2 signaling in response to exercise in skeletal muscle is due to primarily a local, tissue-specific phenomenon, rather than a systemic effect. The molecules involved in this tissue specific stimulation remain elusive.

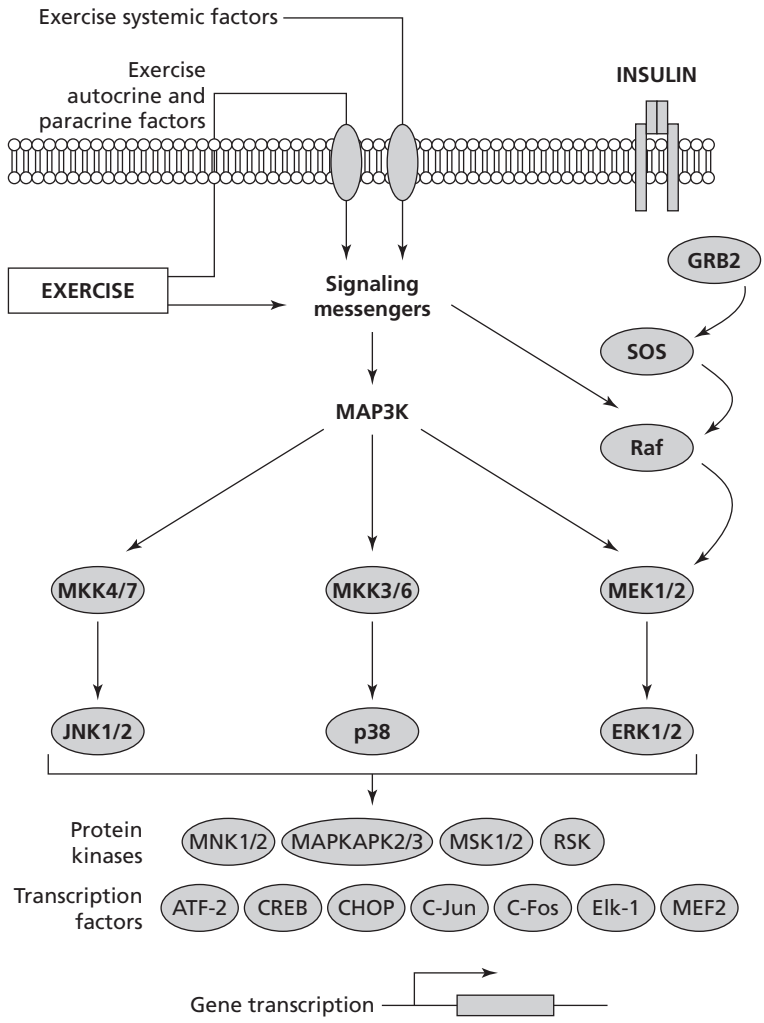

Fig. 27.3 Exercise and insulin regulation of mitogen-activated protein kinase (MAPK) signaling in skeletal muscle. Insulin potently stimulates ERK1/2 activity through a GRB2/SOS pathway. Exercise also regulates MAPK activity via intracellular signaling molecules, as well as systemic and autocrine/paracrine factors. Downstream substrates of MAPKs include various protein kinases, as well as transcription factors. Mechanisms underlying the ability of exercise to induce changes in gene expression are relatively unknown; however, modulation via MAPK signaling represent likely candidates.

JNK signaling

Unlike ERK, JNK is only modestly activated by insulin in skeletal muscle, and some reports show no activation (Aronson *et al*. 1996). Activation of JNK signaling has been reported in rat skeletal muscle in response to *in vitro* contractions (Boppart *et al*. 2001), *in situ* contractions (Aronson *et al*. 1997a; Martineau & Gardiner 2001), treadmill running exercise (Goodyear *et al*. 1996), muscle overload (Carlson *et al*. 2001) and mechanical stretch (Boppart *et al*. 2001). In human subjects, JNK is activated in response to cycle ergometer exercise (Aronson *et al*. 1998), knee extensions resulting in concentric and eccentric contractions of the quadricep muscles (Boppart *et al*. 1999) and marathon running (Boppart *et al*. 2000). In contrast to ERK1/2 signaling, activation of the JNK cascade is sustained during *in situ* muscle contractions, whereas the activation of the ERK cascade is more rapid and transient. This suggests that the upstream proteins that regulate the JNK signaling cascade are distinct from that of ERK1/2 (Aronson *et al*. 1997a). Due to the lack of selective cell permeable inhibitors of JNK signaling at this time, little is known about the physiological function of JNK in contracting skeletal muscle.

p38 signaling

As is the case with JNK, skeletal muscle p38 is weakly activated by insulin, if at all. However, p38 is potently activated in rat skeletal muscle in response to treadmill running (Goodyear *et al*. 1996; Nader & Esser 2001), *in vitro* contractions (Ryder *et al*. 2000; Somwar *et al*. 2000; Wretman *et al*. 2000, 2001; Boppart *et al*. 2001), *in situ* contractions (Nader & Esser 2001), muscle overload (Carlson *et al*. 2001) and mechanical stretch (Boppart *et al*. 2001). It has also been shown that p38 signaling is increased in humans during cycle ergometer exercise (Widegren *et al*. 1998) and marathon running (Boppart *et al*. 2000; Yu *et al*. 2001). Phosphorylation of MAPKAPK-2, a downstream substrate for p38, is associated with changes in p38 activity during exercise/contraction and is inhibited by the p38 antagonist SB203580 (Krook *et al*. 2000; Ryder *et al*. 2000; Yu *et al*. 2001).

Does exercise-mediated MAPK activation regulate gene transcription in skeletal muscle?

Chronic exercise can result in changes in skeletal muscle structure and function. Endurance training leads to increases in capillary density, increases in oxidative capacity and mitochondrial density, etc. The exact mechanisms regulating the adaptive response of skeletal muscle to repeated bouts of exercise are unclear; however, MAPKs are known to activate a number of transcription factors in response to both insulin and exercise. Activation of these transcription factors may contribute to the adaptive effects of exercise in skeletal muscle (Widegren *et al*. 2001). Using various cell systems, activated MAPKs have been shown to translocate to the nucleus and phosphorylate numerous transcription factors such as CREB, Elk, ATF2, c-Jun, c-*fos*, c-Myc, AP-1, MEF2, NFAT and CHOP (Han *et al*. 1997; Gomez *et al*. 2000; Kyriakis & Avruch 2001; Hazzalin & Mahadevan 2002). Not only can MAPKs regulate gene transcription by direct interaction with transcription factors, they can also activate other downstream substrates such as RSK2, MAPKAPK-2/3 and MSK1/2 that can translocate to the nucleus and phosphorylate numerous transcription factors. Downstream consequences of exercise-mediated MAPK signaling remain obscure; however, recent data are emerging which implicate p38 in adaptive responses to increase lipid metabolism in skeletal muscle. Peroxisome proliferator activated receptors (PPARs) have received much attention as transcriptional regulators of lipid metabolism in various tissues. PPARγ is predominantly expressed in adipocytes and has been linked to adipocyte differentiation. PPARα expression is high in skeletal muscle and has been shown to regulate the expression of numerous genes involved in fat oxidation. Interestingly, p38 MAPK has been shown to regulate the activity of PPARα (Barger *et al*. 2001). Furthermore, p38 has also been shown to regulate PPARγ coactivator-1 (PGC-1) (Knutti *et al*. 2001; Puigserver *et al*. 2001). PGC-1 has been shown to regulate mitochondrial biogenesis in skeletal muscle (Puigserver & Spiegelman 2003). Therefore, the adaptive effects resulting from exercise training to improve fat utilization could involve exercise-mediated MAPK activity. Activation of these transcription factors leads to the regulation of gene expression; however, there is a paucity of data regarding the involvement of skeletal muscle MAPK activation with exercise.

Effect of MAPK activation on substrate metabolism

While the regulation of gene transcription is an established function of MAPK, the involvement of MAPK in the regulation of cellular substrate metabolism is unclear. The ERK1/2 signaling cascade was previously proposed to be involved in the regulation of both glucose transport and glycogen metabolism (Merrall *et al*. 1993). However, in subsequent studies, a MEK inhibitor that blocks activation of ERK1/2 had no effect on insulin-stimulated glucose uptake in cultured adipocytes (Haruta *et al*. 1995; Tanti *et al*. 1996) and skeletal muscles (Hayashi *et al*. 1999; Wojtaszewski *et al*. 1999b). Thus, there is substantial evidence that ERK1/2 signaling is not involved in the acute regulation of glucose uptake in response to insulin treatment or contraction in skeletal muscle.

The ERK1/2 signaling cascade has also been proposed to regulate insulin-stimulated activation

of glycogen synthase. This hypothesis was based on the finding that RSK2 could phosphorylate and inactivate GSK3 and phosphorylate and activate the glycogen-bound form of protein phosphatase-1 (PP1-G) *in vitro*, two reported regulators of insulin-stimulated glycogen synthase activity (Dent *et al*. 1990; Sutherland *et al*. 1993). However, inhibition of ERK signaling by a MEK inhibitor resulted in no inhibition of insulin-stimulated glycogen synthase activity (Lazar *et al*. 1995). More direct evidence came from a study using RSK2 knockout mice which revealed that RSK2 is not necessary for insulin-stimulated glycogen synthase activation, and in fact, the RSK2 null mice had greater increases in muscle glycogen synthase activity following insulin treatment (Dufresne *et al*. 2001). However, these studies do not rule out a role for RSK2 in the regulation of glycogen metabolism in the basal state, as the knockout mice had lower levels of muscle glycogen.

A growing body of literature is emerging implicating MAPK pathways in the regulation of lipid metabolism. Recently, a study has suggested that ERK signaling is associated with the plasma membrane fatty acid transporter FAT/CD36 (Todd & Turcotte 2003). Incubation of isolated muscle with PD98059 significantly attenuated the contraction-induced increase in fatty acid uptake in skeletal muscle. ERK has also been recently suggested to play a role in the activation of HSL in skeletal muscle watt (Donsmark *et al*. 2003; Langfort *et al*. 2003; Watt *et al*. 2003). These data suggest that ERK activation is involved in both the uptake of fatty acids and hydrolysis of triglyceride in skeletal muscle. Whether chronic exercise improves insulin-stimulated ERK activity in skeletal muscle remains to be elucidated.

There have been few studies examining the effects of JNK activation in the regulation of carbohydrate metabolism in skeletal muscle. One study demonstrated that activation of JNK by anisomycin, a protein synthesis inhibitor, mimics insulin's action to stimulate glycogen synthesis in mouse skeletal muscle *in vivo* (Moxham *et al*. 1996). Based on their findings, this group concluded that JNK stimulates glycogen synthase activity through the regulation of RSK3 and GSK3. Since exercise and contraction robustly activate JNK activity, we hypothesized that JNK could be involved in the regulation of contraction-stimulated glycogen synthase activity. Overexpression of wild type JNK1 in skeletal muscle *in vivo* dramatically increased basal and contraction-stimulated JNK activity. However, this increase in JNK activity did not enhance basal and contraction-stimulated glycogen synthase activity in mouse skeletal muscle, suggesting that JNK is not involved in the regulation of contraction-stimulated glycogen synthase activity (Fujii *et al*. 2001). Whether JNK is involved in glucose transport regulation in muscle is not known and will be an important area for future investigation.

Recent studies have provided evidence that p38 is involved in the regulation of contraction-stimulated glucose uptake in skeletal muscle. Somwar and colleagues demonstrated that p38 activity and glucose uptake were increased in isolated extensor digitorum longus (EDL) muscles with contraction *in vitro* (Somwar *et al*. 2000), and the p38 antagonist SB203580 abolished the activation of p38 and reduced contraction-stimulated glucose uptake by 40–50%. However, p38 inhibitors are known to have numerous non-specific effects and it is still unclear whether the attenuation of glucose uptake was due to inhibition of contraction-stimulated p38 activity or an indirect effect of the compound on other signaling intermediates (Somwar *et al*. 2000). We have recently showed that p38γ, the isoform highly abundant in skeletal muscle, is a negative regulator of GLUT4 expression and contraction-stimulated glucose uptake (Ho *et al*. 2004).

The impact of exercise on insulin-stimulated MAPK activity remains to be uncovered. Interestingly, cytokines that are released in response to muscle damage and implicated in the negative regulation of insulin-stimulated glucose metabolism (e.g. tumor necrosis factor-α) are also potent stimulators of JNK and p38. Additionally, both JNK and p38 activity have been shown to induce impairments in insulin-stimulated glucose transport in skeletal muscle. However, because exercise results in significant increases in glucose transport in parallel with MAPK activation, the exact role of MAPK in contracting skeletal muscle warrants further investigation.

Clinical implications

Throughout this chapter, we have discussed various effects of exercise on insulin action in skeletal muscle. Exercise clearly has the ability to improve glucose transport, glycogen synthesis and protein metabolism, as well as stimulate adaptive changes through gene transcription. While the majority of studies reviewed in this chapter involve healthy individuals, the impact of exercise on intermediary metabolism also transcends to individuals exhibiting metabolic complications. It has long been recognized that physical exercise has important benefits for people with obesity and diabetes (Trovati *et al*. 1984; Helmrich *et al*. 1991). However, the transient nature of post-exercise insulin sensitivity limits the beneficial impact of physical activity. Chronic exercise, on the other hand, results in multiple physical and metabolic adaptations. Exercise training improves glucose tolerance and insulin action in insulin-resistant humans (Hughes *et al*. 1993) and type 2 diabetic patients (Dela *et al*. 1994). The improvements in insulin sensitivity seem to be multifaceted, including alterations in body composition, plasma lipid profiles, intracellular signaling and protein expression. Furthermore, epidemiological studies have determined that regular physical exercise can reduce the risk of developing type 2 diabetes (Helmrich *et al*. 1991; Manson *et al*. 1991, 1992).

Summary

Significant advances have been made in recent years in the elucidation of mechanisms by which exercise increases insulin action in skeletal muscle. Discoveries have been made showing that both exercise and insulin stimulate increases in glucose transport, glycogen metabolism, protein synthesis and long-term adaptations (e.g. hypertrophy). Interestingly, these effects are elicited through both common and distinct signaling pathways. Furthermore, additive effects of exercise and insulin in the regulation of intermediary metabolism and adaptive responses have a widespread impact in both health and disease. While exercise training can result in adaptations to improve performance, chronic physical activity can also prevent or reverse metabolic defects observed in conditions such as type 2 diabetes.

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Chapter 28

Hormone and Exercise-Induced Modulation of Bone Metabolism

CLIFFORD J. ROSEN

Introduction

Physical activity is an essential component of normal well-being. Within the last decade, more individuals have undertaken activity programs in order to ameliorate chronic medical conditions or to maintain their healthy status than ever before. Some believe that long-term exercises will improve longevity and quality of life, while others contend that physical fitness enhances mental well-being as well as improving muscle function. Regardless of the physiology or the rationale, the long-term effects, either beneficial or harmful, associated with chronic exercise are an area of intense investigation.

By the nature of its components, physical activity affects almost all hormonal systems, from β-endorphins in the brain, to local cytokines and chemokines in bone. Although the vast majority of people believe the effects of long-term exercise are beneficial to virtually every organ system, drastic changes in the normal physiologic state of these hormonal modulators can impact tissues in a negative as well as positive way. Nowhere is this more apparent than in the clinical syndrome of exercise-induced amenorrhea and the female athlete's triad. In predominantly younger women, chronic and intense physical activity results in loss of the gonadotropin pulse generator leading to estrogen deprivation. This in turn drastically affects the bone remodeling unit such that bone resorption exceeds formation resulting in significant bone loss. As such, this interface between homeostatic processes related to hormonal signaling and stress mechanisms induced by exercise are most noticeable in bone, an organ infrequently thought of as a target tissue for systemic modulators. Yet the list of skeletal mediators affected by chronic exercise is nearly endless and includes significant changes in circulating gonadal steroids, adrenal steroids, cytokines, prostaglandins, growth hormone (GH), insulin, insulin-like growth factor I (IGF-I), leptin and others. All these endogenous compounds influence other systems such as metabolic fuel balance, cardiovascular fitness and muscle integrity to induce changes that may either be beneficial or harmful to the organism.

Weightlessness with manned space flight and the accompanying animal experiments provided the first *in vivo* proof of the importance of hormonal effectors on skeletal growth, remodeling and mass. Since those early days of space exploration, our understanding of the effects of physical training on hormonal balance, and its subsequent actions on the skeleton, has grown immensely. But there are still many unanswered questions. In this review, I will focus first on the physiology of bone remodeling since that provides the background for understanding skeletal homeostasis and the importance of circulating, as well as local growth factors in mediating adaptive processes in the skeleton. Next, I will touch on the effects of acute exercise on bone cell function, with a particular focus on the cellular aspects of strain within the basic multicellular unit (BMU) of bone. In the Hormonal and skeletal responses to long-term exercise section below, I will elaborate on the complex skeletal changes associated with sustained exercise programs, particularly as they relate to hormonal balances and imbalances. Finally, I will discuss some potential clinical implications of our

current knowledge about exercise, hormonal modulation and skeletal homeostasis.

Physiology of normal and aberrant bone remodeling

Normal modeling and remodeling

Mammalian skeletons grow and remodel during life. Linear growth is accomplished at the growth plate and is regulated by GH and IGF-I. In rodents linear growth continues over a lifetime although it is most pronounced during puberty. On the other hand, the human skeleton which is more than just calcium phosphate crystals melded together in a protein matrix, grows, models and then remodels. Linear growth in humans occurs from the growth plate, begins at birth and ceases after puberty. It is principally modulated by growth plate chondrocytes. Modeling, which is essentially the process of shaping a bone through resorption and formation, occurs in response to several factors including humoral substances, muscle activity and local factors. It too ceases after puberty in response to several cues including changes in sex steroids and circulating IGF-I. Modeling is very dependent on the direction of the stress vectors that are modulated through muscle such that the actual cross-sectional shape of bone is not perfectly oval but rather slightly eccentric, depending on forces shaping it by loading. Remodeling is a very distinct homeostatic process compared to growth and modeling, even though the cellular players are similar. Remodeling allows the skeleton to reorganize itself without changing it absolute mass and thus serves to enhance skeletal integrity while maintaining metabolic balance, especially for essential ions such as calcium and phosphate (Rosen 2003). During remodeling, the rate of bone resorption or dissolution equals the rate of new bone formation. In contrast, new bone is added by growth and modeling due to linear expansion from the growth plate by chondrocytes, and expansion of lateral surfaces in the diaphysis by periosteal osteoblasts (OBs). Acquisition of peak bone mass requires optimization of all three distinct but overlapping processes. General physical activity affects the growing skeleton, particularly at a time when these three distinct activities are at their highest level; i.e. adolescence. From about the age of 10–18 years, linear growth is very active and modeling of the skeleton is in full force. As noted below, most studies that have examined the role of physical activity (i.e. loading the skeleton in one form or another, such as running or weightlifting) on bone have noted a much more vigorous response in respect to changes in bone mineral density (BMD) during this time period than any other.

Remodeling is a constant process that dictates the metabolic needs of the skeleton and provides for the elasticity necessary for general physical activity. Every 10 years in humans the entire skeleton is remodeled with the greatest turnover noted in the trabecular rich regions of the thoraco-lumbar spine and several areas of the femur (Rosen 2003). Because of this huge undertaking, it is not surprising that the mammalian skeleton is a highly organized and physiologically active organ. Bone basically serves two purposes: (i) to maintain structure; and (ii) to preserve calcium homeostasis for all physiologic processes. As such, the mammalian skeleton is uniquely designed for its protective and structural roles. There is an outer surface of cortical bone that surrounds the inner trabecular elements (Fig. 28.1). Marrow bathes the trabecular skeleton while cortical bone is nourished by periosteal vessels and a

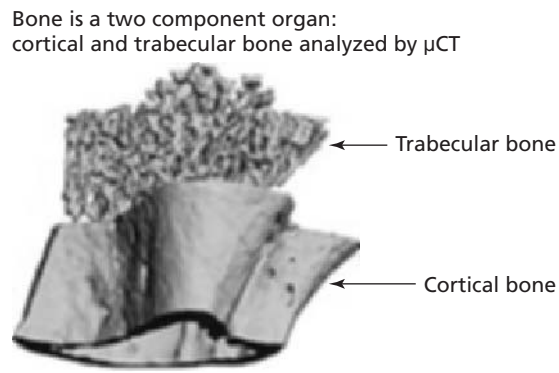


Fig. 28.1 The two compartment model of the skeleton. The inner skeletal complex is made up of trabecular elements with a wide surface area that is bathed in bone marrow. The outer shell is the cortex and on the far outer surface of the cortex is the perisoteal membrane. μCT, micro computed tomography.

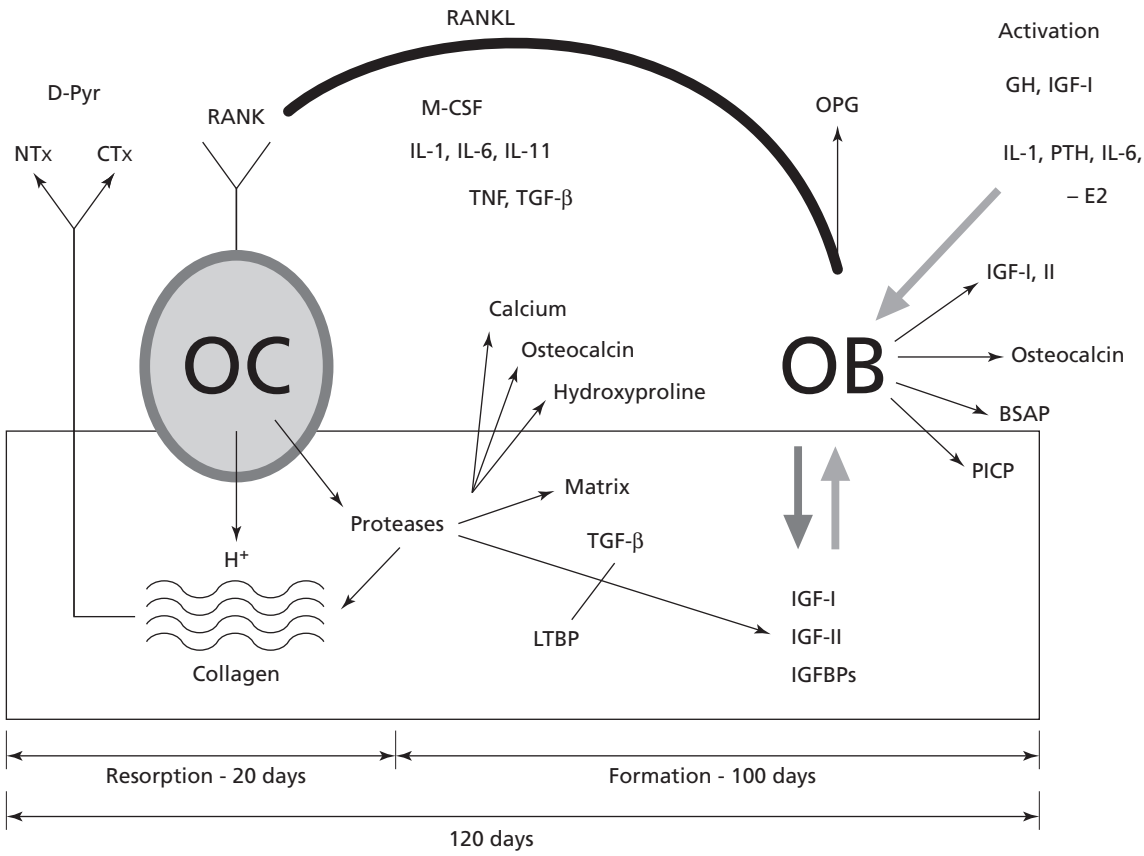


Fig. 28.2 The bone remodeling cycle which is controlled by circulating and local growth factors and cytokines. Bone markers, including NTx, Ctx and D-Pyr, are fragments of collagen released during resorption. Formation markers including osteocalcin, BSAP and PICP are also noted by the osteoblast. BSAP, bone specific alkaline phosphatase; GH, growth hormone; IGF, insulin-like growth factor; IGFBPs, insulin-like growth factor binding proteins; IL, interleukin; M-CSF, macrophage colony-stimulating factor; OB, osteoblast; OC, osteoclast; PICP, procollagen peptide; RANK, receptor activator of nuclear factor kappa B; RANKL, RANK ligand; TGF, transforming growth factor; TNF, tumor necrosis factor.

series of canaliculi connecting osteocytes to lining cells and OBs (Fig. 28.2). The BMU defines the single functional component of bone remodeling and includes lining cells, OBs, osteoclasts (OCs) and osteocytes (Figs 28.2 and 28.3). Gravitary forces influence the BMU and stimulate cortical and trabecular remodeling. In respect to bone growth, periosteal OBs and the underlying growth plate are principally responsible for longitudinal growth and lateral expansion. Both cortical and trabecular bone undergo remodeling, but the frequency of this process is much less in the cortex than in the trabecular components of the spine and distal femur.

Numerous growth factors and cytokines, each of which contributes to coupling bone dissolution (i.e. resorption) to new bone formation, orchestrate bone remodeling within a BMU (Figs 28.2 and 28.3). Pre-osteoblasts (pre-OBs), derived from mesenchymal stromal cells, and under the influence of a key transcription factor (Cbfa1, i.e. core binding factor I or RUNX2) represent target cells for initiation of the remodeling cycle (Martin & Ng 1994; Thissen *et al*. 1994). Systemic and local factors, as well as signals from osteocytes, enhance pre-OB differentiation, and this, in turn, leads to the synthesis and release of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) (Musey *et al*. 1993). These two

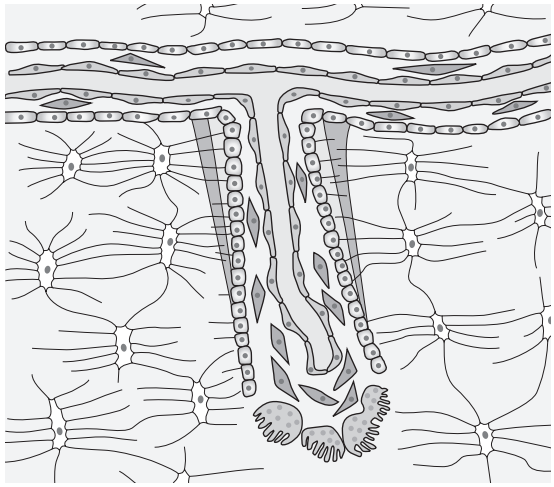


Fig. 28.3 The basic multicellular unit (BMU) consists of osteocytes connected by canaliculi to lining cells (cuboidal) and osteoblasts (ovoid). Osteoclasts are multinucleated giant cells (see at the base of the lacuna) responsible for resorption of bone. The cellular response to loading begins with signals from the osteocytes to lining cells and osteoblasts, resulting in activation of the remodeling sequence.

peptides are necessary and sufficient for the recruitment of bone resorbing cells, i.e. the OCs. Once bone resorption occurs, calcium, collagen fragments and growth factors such as the insulin-like growth factors (IGFs) and transforming growth factors (TGFs), are released from the bony matrix. The latter factors enhance the recruitment of OBs to the bone surface, thereby setting the stage for collagen synthesis and matrix deposition/mineralization (Rosen & Donahue 1998). The entire remodeling cycle in humans takes approximately 90 days, with the majority of time consumed by the elaborate process of bone formation and subsequent mineralization (Fig. 28.2) (Rosen & Donahue 1998). And, at each step, systemic hormones such as parathyroid hormone (PTH), estrogen, thyroxine and GH, influence the timing and direction of remodeling in a three-dimensional space.

One of the most critical local and systemic growth factor influencing bone remodeling is IGF-I (Fig. 28.3). IGF-I and IGF-II, the IGFs, are major components of both the organic skeletal matrix and the circulation. Indeed, the serum of most mammals contains significant concentrations of both IGF-I and IGF-II, bound to high and low molecular weight insulin-like growth factor binding proteins (IGFBPs) (Ketelslegers *et al*. 1995). Similarly, the skeletal matrix also is highly enriched with these growth factors and other non-collagenous proteins, including all six IGFBPs and several IGFBP proteases. In addition, the type I IGF receptor is present on both OBs and OCs.

It is now reasonably certain that skeletal IGFs originate from two sources: (i) *de novo* synthesis by bone forming cells (i.e. pre-OBs and fully differentiated OBs); and (ii) the circulation. In fact, some skeletal IGFs probably make their way into the matrix by way of specialized canaliculi and sinusoids within the bone microcirculation (Rosen & Kessenich 1996; Rosen & Donahue 1998). IGFs, bound to IGFBPs, can also be found within the marrow milieu in close contact with the endosteal surface of bone. But, by most accounts, the vast majority of IGF-I in bone is derived from local osteoblastic synthesis. Yet during active bone resorption, as the matrix is dissolved, significant amounts of IGF-I and IGF-II are released from storage (i.e. binding to IGFBP-5 and hydroxyapatite) (Fig. 28.2). Subsequently, both IGFs recruit precursor OBs, and possibly early OCs, to the bone surface where remodeling is occurring (Rosen & Kessenich 1996; Rosen & Donahue 1998; Heaney *et al*. 1999).

Circulating and skeletal IGF-I are profoundly influenced by nutritional determinants and physical activity. Growth retardation, a major feature of protein calorie malnutrition in children, is associated with significant declines in circulating IGF-I despite enhanced GH secretion. Similarly elderly individuals with poor protein intake have low serum IGF-I levels (Schurch *et al*. 1998). This almost certainly is due to reduced mRNA half-life of IGF-I in the liver. But, regardless of the mechanism, IGF-I is located in the final common homeostatic pathway affected by changes in nutrient intake and energy balance. As such, this peptide emerges as an important mediator of the skeletal response to stress. For example, a recent study of elderly women who suffered a hip fracture (i.e. the end-stage of osteoporosis) support this contention. After a hip fracture there is a profound decline in serum IGF-I levels, probably as result of poor nutrition and significant physical inactivity, as well as a catabolic state (Schurch *et al*.

1998). Levels of IGF-I can be partially restored through the use of recombinant IGF-I administered with IGFBP-3 (Boonen *et al*. 2002). This treatment regimen in elderly patients after hip fractures, resulted in less bone loss and significant improvement in functional outcomes (Boonen *et al*. 2002). This line of evidence supports the importance of a circulating mediator that affects skeletal responsiveness to injury, particularly in relation to energy status.

In children and young adults, exercise stimulates GH secretion, which can result in higher levels of serum IGF-I. This effect is blunted in adults such that serum IGF-I concentrations are not statistically different with sustained exercise regimens. However, in respect to physical activity, anything that reduces dietary ingestion of essential nutrients (i.e. prolonged physical activity with reduced intake, or compulsive undereating) in adults or children will override GH stimulation of IGF-I in the liver and significantly reduce circulating IGF-I concentrations.

Exercise can also impact skeletal IGF-I expression. Several studies have shown that fluid flow in osteocytes and OBs can increase the expression of IGF-I mRNA (Srinivasan & Gross 2000). Repetitive general physical activity not only increases IGF-I expression in muscle but also in the periosteum and probably on the endosteal surface of bone. These changes can have a profound effect on bone formation tipping the remodeling balance in a favorable direction, particularly during peak bone acquisition. On the other hand, unloading removes the stimulus to bone formation, in part by making bone cells resistant to the actions of IGF-I (Sakata *et al*. 2003) (see Hormonal and skeletal responses to long-term exercise section below).

The remodeling cycle is sensitive to changes in other nutrients which can profoundly affect growth factors and cytokines in OBs. Phosphate balance is important for mineralization and low phosphate levels trigger activation of 1α-hydroxylase keying the conversion of 25-hydroxyvitamin D to the active compound 1,25-dihydroxyvitamin D. Conversely, high levels of phosphorus stimulate PTH secretion resulting in marked activation of remodeling and enhanced bone resorption. Low calcium intake, coupled with vitamin D deficiency, inhibits IGF-I expression in bone, can trigger PTH release and almost certainly is a principal factor in the secondary hyperparathyroidism seen in elderly individuals. In addition, poor calcium diets and low vitamin D likely contribute to dampening of the skeletal response to loading (see Hormonal and skeletal responses to long-term exercise section below). Vitamin K is an essential co-factor for γ-carboxylation of osteocalcin, the most common non-collagenous protein in bone. Osteocalcin is produced by bone forming cells that are highly differentiated, and this protein may be important in mineralization. The expression and release of osteocalcin has also been noted in loading studies. Other trace elements such as boron and strontium may affect bone cell function *in vitro*, although their role in the remodeling cycle is uncertain. Similarly, low levels of magnesium may influence bone cell activity *in vitro*, but its role in remodeling and the response to exercise is still debated.

Aberrant remodeling

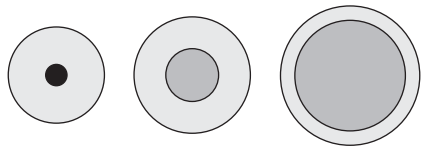
Aging and menopause are most frequently associated with modest uncoupling of the BMU resulting in accelerated bone resorption compared to bone formation. Hence, over a lifespan, women can lose approximately 42% of their spinal and 58% of their femoral bone mass (Rosen 2003). Surprisingly, rates of bone loss in the 8th and 9th decades of life may be comparable or exceed those found in the immediate peri- and post-menopausal period of some women (Lacey *et al*. 1998; Robey & Bianco 1999). This is due to uncoupling in the bone remodeling cycle of older individuals resulting in a marked increase in bone resorption but no change or a decrease in bone formation (Martin & Ng 1994; Rosen & Donahue 1998). However, the mechanisms that lead to an uncoupled bone remodeling unit, especially in the elderly, remain to be elucidated. Almost certainly, major changes in several hormonal factors (estrogen, testosterone, GH) as well as nutrient intake, dramatically affect the skeleton either singly or in combination. The role of physical immobility on age-related changes in the skeleton remains uncertain, but inactivity is likely to accelerate bone resorption in the elderly.

Recent technological advances have made it easier to monitor the remodeling process by defining changes in bone specific turnover markers in pathologic states such as osteoporosis. Alterations in bone turnover can be detected by several biochemical markers including bone resorption indices (e.g. urinary and serum N-telopeptide, C-telopeptide, and urinary free and total deoxypyridinoline) and bone formation markers (e.g. osteocalcin, procollagen peptide, bone specific alkaline phosphatase) (see Fig. 28.2). In general, bone turnover markers are significantly higher in older than younger postmenopausal women, and these indices are inversely related to BMD (Beamer *et al*. 2000). For example in the EPIDOS trial of elderly European females, the highest levels of osteocalcin, N-telopeptide, C-telopeptide and bone specific alkaline phosphatase were noted for those in the lowest tertile of femoral bone density (Thissen *et al*. 1994). Also, increased bone resorption indices were associated with a greater fracture risk independent of BMD (Thissen *et al*. 1994). For those women in EPIDOS with low bone density and a high bone resorption rate, there was a nearly fivefold greater risk of a hip fracture. In respect to exercise-induced changes in the skeleton, bone turnover markers may provide some insight into the role of systemic factors in modulating the adaptive response to loading, particularly in relation to bone resorption.

In contrast to a consistent pattern of high bone resorption indices with aging, bone formation markers in osteoporotic patients are more variable. Serum osteocalcin levels are high in some individuals but this may be indicative of an increase in bone turnover rather than reflecting a true rise in bone formation (Thissen *et al*. 1994). On the other hand, bone specific alkaline phosphatase, and procollagen peptide levels have been reported to be high, normal or low in elderly men and women (Musey *et al*. 1993). Bone histomorphometric indices in some patients are also quite variable. Thus, although there is strong evidence for an age-associated rise in bone resorption, changes in bone formation are inconsistent. Still, with aging and menopause, the major skeletal abnormality is an uncoupling of the remodeling unit that leads to bone loss, altered skeletal architecture and an increased propensity to fractures. These determinants are particularly important when considering the effects of both acute and chronic exercise programs on the aging skeleton.

Weightlessness due to space flight has been reported to induce the most rapid and highest rate of bone loss of any pathologic condition (Neuman 1970). This process is a function of accelerated bone resorption and simultaneous suppression of bone formation which begins immediately after reaching zero gravity. Although hormonal replacement can ameliorate part of this loss, restitution of normal bone mass only occurs with a normal gravity state. The role of physical inactivity that is not due to weightlessness or chronic bed rest, in the progression of osteoporosis is less well defined. Several studies have shown that bed rest in healthy individuals can be associated with uncoupling of formation from resorption, with a high resorptive state and suppressed formation (Chappard *et al*. 1995). A similar process likely occurs after a hip fracture when immobility is significant and bone loss from the contralateral hip can be significant even over a relatively short-time period (Sato *et al*. 2001).

Less attention has been paid to the role that the periosteal surface may play in aberrant remodeling and the effects of inactivity on this region vis à vis hormonal and local factors. A recent 20-year prospective study of post-menopausal Swedish women noted that bone loss from the radius averaged nearly 2% per year. However in these same women, periosteal circumference increased (Fig. 28.4) significantly, such that the cross-sectional moment of inertia, an index of bone strength, actually increased (Ahlborg *et al*. 2003). This suggests that during the process of endosteal bone loss as a result of aging or hormonal deficiency, the periosteum attempts to compensate in order to maintain its inherent strength (Duan *et al*. 2001). Currently it is not known how the periosteal envelope expands in response to loss of the inner aspect of bone, nor how it is signaled (Fig. 28.4) (Beck *et al*. 2000; Nelson *et al*. 2000). However, it should be noted that the two most important regulators of periosteal growth are muscle activity and systemic IGF-I. Since the former can also induce skeletal IGF-I expression in the periosteum and skeletal muscle, this peptide may be very



BMD	1.0	1.0	0.80
X-sectional area	1.77	1.77	1.77
Moment of inertia	0.25	0.64	1.13

Fig. 28.4 The skeletal effects of aging, including the loss of endosteal bone. In response to declining trabecular bone, the periosteum increases in size. This enhances the biomechanical properties of the bone and prevents total failure of the skeleton. BMD, bone mineral density.

critical for the compensatory skeletal response with aging (Adams & Haddad 1996). Indeed, evidence from transgenic and knockout mice tell us that circulating IGF-I is important for modeling the skeleton and providing it with a stimulus for optimal growth, particularly in the medial lateral dimension (Bikle *et al*. 2002). Since the periosteum is highly vascular and pericytes may be the origin of OBs in this compartment, it is not too radical to consider the GH–IGF-I hormonal axis, which may be directly or indirectly modulated by physical activity, as the critical mediator of this compartment. Further studies of this hypothesis are currently underway in several laboratories. Such studies will provide a strong rationale for studying the interface between physical activity, systemic hormonal modulators and bone remodeling.

Hormonal and skeletal responses to acute exercise

The skeleton is an extremely dynamic organ that responds not only to systemic hormonal factors but to locally generated growth factors produced in response to applied forces (Table 28.1). As previously noted, modeling, i.e. the process of growth and shaping, and remodeling, i.e. the process of bone renewal, are responsive to both hormonal mediators and local stresses. More is known about the skeletal response to systemic mediators than it is to loading. However, there are some basic biomechanical properties of the human skeleton that apply to any form of loading and are synergistic with hormonal factors that act on the BMU.

Bone exhibits a remarkable capacity to adapt to changes in bone loading in order to optimize strength without unduly increasing weight. Surprisingly, the skeleton does this by altering its mass, its external geometry and its internal micro-architecture, a

Table 28.1 Systemic and local modulators of bone turnover: response to loading.

Hormone/ growth factor	Systemic/ CNS	Local/ skeletal	Loading effects	Reference(s)
PTH	+++	–	Synergistic	Rosen 2003; Chow *et al*. 1998, Neer *et al*. 2001
GH	+++	–	Synergistic	Forwood *et al*. 2001
IGF-I	+++	++++	+++++	Rosen & Donahue 1998, Rosen & Kessenich 1996, Bikle *et al*. 2002, Lean *et al*. 1996
PGE_2 (PGI)	+++	++++	+++++	Klein-Nulend *et al*. 1997
Leptin	+++	++	??	Takeda *et al*. 2002
Neuropeptides	+++	++?	+	Elefteriou *et al*. 2003, Takeda *et al*. 2002, Mason *et al*. 1997
TGF-β	+	+++++	+	Srinivasan & Gross 2000
Estradiol	++++	++	+/Synergistic	Williams *et al*. 1995, Dueck *et al*. 1996, Otis *et al*. 1997, DiPietro & Stachenfeld 1997, Klibanski *et al*. 1995, Grinspoon *et al*. 2003, Smith & Rutherford 1993
Testosterone	++++	+	Synergistic	Rosen 2003
Cortisol	++++	?	Antagonistic	Chrousos & Gold 1992

CNS, central nervous system; GH, growth hormone; IGF-I, insulin-like growth factor I; PGE_2, prostaglandin E_2; PGI, prostacyclin; PTH, parathyroid hormone; TGF-β, transforming growth factor-β.

concept that has been around for more that 100 years (Boyden *et al*. 2002). What has emerged more recently, however, is a better understanding of the factors that influence the skeletal response to loading, including sensitizing hormones such as PTH and GH (Turner *et al*. 1997). To begin with, loads applied to the skeleton are called stresses (i.e. force per unit area), while strain represents the measure of skeletal deformation in response to a stress (i.e. change in length divided by its original length). It is the strain per se that generates an adaptive response of bone to loading. Adaptation of hard tissues to stress is achieved by changes in bone resorption and bone formation. Bone mass, geometry and trabecular orientation are altered as an adaptive response and these, in turn, are modulated by systemic factors.

Not all of the adaptive responses to stress are equal because of the geometric properties of bone, but there is a direct relationship between the magnitude of strain, and the appropriate skeletal response (Rubin & Lanyon 1985). The cellular mechanism responsible for that relationship is not known but investigators have posited that there must be a 'mechnostat' which regulates both periosteal formation and endosteal resorption. If bone is loaded with more than 2500 microstrain, modeling occurs at both sites, thereby allowing the bone to be more resistant to deformation (Rubin & Lanyon 1985). In contrast, when strain is reduced, modeling is inhibited and endosteal remodeling with resorption occurs. The 'putative' mechanostat has never been identified, but theories abound about as to its location and its mechanism of action. Most agree that osteocytes, buried within the cortex of bone but connected to resting surface cells, sense fluid shifts and gravitary forces (Noble & Reeve 2000). These 'strains' trigger release of cellular factors which travel by way of canaliculi to the surface of bone to activate resting OBs or pre-OBs (see Fig. 28.3) (Turner 1999). This communication allows marrow stromal cells to be recruited and begin the differentiative process necessary for bone formation. Recently, evidence has emerged from genetic studies of a family with high bone mass but normal bone shape, that a previously unidentified pathway in OBs may have 'mechanostat' like properties. Lipoprotein receptor protein 5 (LRP-5) is a ubiquitous membrane receptor which is coupled to the frizzled receptor on OBs and is activated by a family of growth related peptides called Wnts. These growth factors can stimulate bone formation and mineralization as well as cell proliferation, and are now thought to work through a canonical pathway in cells (Gong *et al*. 2001). LRP-5 interaction with the Wnt frizzled ligand receptor complex results in inhibition of β-catenin phosphorylation by glycogen synthetase kinase 3 (GSK-3) (Kato *et al*. 2002). GSK-3 facilitates the ubiquitin mediated breakdown of β-catenin, while LRP-5 acts to prevent this breakdown allowing for translocation of β-catenin into the nucleus where it interacts with a family of transcription factors. An activating mutation of LRP-5 is responsible for the 'high bone mass' phenotype, which has been reported in several families with healthy individuals but with BMD values 3–5 standard deviations above normal, yet normal bone remodeling (Boyden *et al*. 2002; Little *et al*. 2002). Very recently investigators have noted that mutant LRP-5 expression was up-regulated by mechanical stimulation, placing this system in the forefront of candidates for the elusive mechanostat (Bex *et al*. 2003).

The type of load applied defines the particular adaptive response of the skeleton in co-ordination with hormonal mediators. Three critical factors related to physical activity modulate strain in the human skeleton: frequency, duration and intensity (Rubin & Lanyon 1984). The latter is probably the most important since intensity generates load. For example, gymnasts can experience ground forces twelve times their body weight, while runners experience only about three to five times their weight (Grimston 1993; Grimston *et al*. 1993; Bassey *et al*. 1998). As such, the greatest increases in BMD among active exercisers occurs at the point of impact (i.e. the hip) and is most pronounced in gymnasts rather than runners or walkers.

The frequency and duration of physical activity are also important. Individuals who perform jumps from short heights rapidly develop a peak force on impact; these subjects have greater increases in bone density than do runners, and bone formation parameters have been directly linked to strain rate, independent of force (Morris *et al*. 1997; Fuchs *et al*. 2001).

In addition to the rate of change in force, evidence has emerged from both animal and human studies that the frequency in which sound waves are generated by a floor-based machine can affect the rate of bone formation and the acquisition of bone mass (Qin *et al*. 2003). Whether this represents a purely skeletal effect, or one which is co-ordinated through skeletal muscle remains to be determined. However it does provide new insights into the bone modeling process. It is unknown whether there are additional hormonal factors which contribute to or are synergerstic with this effect.

Certain hormones can facilitate the ability of mechanical loading to increase bone formation. The two major ones are PTH and GH. Removal of the parathyroid glands in rats abolishes the skeletal sensitivity to mechanical loading. Daily replacement with PTH restores that mechanical sensitivity although the mechanism responsible for this effect is unknown (Chow *et al*. 1998). One candidate as a local mediator is IGF-I, which is induced by PTH directly through increases in skeletal gene expression. IGF-I null mice do not exhibit any anabolic response to PTH, nor do they show changes in markers of bone formation (Bikle *et al*. 2002). The other important hormonal mediator is GH, another powerful inducer of local IGF-I. Lewis dwarf rats that do not have an intact GH–IGF-I axis do not respond to skeletal loading, but this mechanosensitivity can be restored with GH replacement (Forwood *et al*. 2001). Once again, it is not clear how GH mediates this effect, although replacement induces a significant rise in both local and systemic IGF-I.

The importance of a potent 'modeling' factor such as GH cannot be understated in respect to the growing skeleton of pubertal children. Exercise-induced changes in bone mass are most pronounced during the late prepubertal and pubertal growth phases (Bass 2000). Indeed, post-puberty, exercise-induced changes in bone mass are relatively minor, although the effects on the periosteum require further studies in older individuals. Recently, PTH has been approved for the treatment of post-menopausal osteoporosis based on a large randomized placebo controlled trial (RPCT) of post-menopausal women (Neer *et al*. 2001). It increases bone mass by enhancing periosteal expansion and stimulating endosteal bone formation. Its potential synergy with physical activity has not been tested *in vivo*, but studies are currently being planned.

The acute response to physical activity or loading is systemic and local. The hormonal modulators include changes in classical stress hormones such as cortisol and epinephrine. There are also bidirectional responses in the immune system related to neuroendocrine function. GH levels rise, particularly in younger individuals, although changes in IGF-I concentrations are not noticeable and are balanced by energy needs and nutrient intake. Insulin levels are generally suppressed while glucagon is increased with physical activity. Accompanying the rise in heart rate is the release of central nervous system trophic factors such as neuropeptide Y, as well as systemic and local cytokines tied to the immune response, including interleukin-1 (IL-1), IL-4, IL-6, interferon-γ and IL-11 (Chrousos & Gold 1992). There are also significant changes in circulating white blood cells as a response to these various immune modulators and sympathetic activity is also increased, leading to changes in blood pressure as well as heart rate.

All of the above modulators have also been shown to impact the skeleton in one fashion or another when exposure is chronic. The important role of neuropeptides in controlling OB function has recently emerged, as has the possibility that leptin, an adipocyte-derived factor, plays a role in suppressing bone formation and mediating hypothalamic events related to obesity and starvation (Blum *et al*. 2003; Cock & Auwerx 2003; Elefteriou *et al*. 2003). Sympathetic overactivity is responsible for the syndrome of reflex sympathetic dystrophy, and β-adrenergic blockers can prevent bone loss in rodents following ovariectomy (Takeda *et al*. 2002). However, none of these mediators play a major role in the acute response of the skeleton to loading. That adaptation is part of the mechanostat and is localized to the osteocyte and OB. Moreover, changes that occur in the skeleton as a direct response to loading happen over relatively short-time intervals (e.g. minutes), well before hormonal modulators are active. On the other hand, sustained changes in systemic factors are probably responsible for the

more balanced skeletal response to physical activity seen with long-term physical activity.

Osteocytes, as noted previously, are buried deep within the cortex of the skeleton (see Fig. 28.3). They represent old OBs that have been entombed within the matrix these cells have generated (Noble & Reeve 2000). Despite their relatively modest metabolic rate, these terminally differentiated cells can sense fluid sheer stress as mechanical loading. These cells also communicate with bone lining cells and OBs through gap junctions by way of canaliculi which can carry specific growth factors and cytokines (see Fig. 28.3). As a result, loading can transduce a relatively rapid message to the master control cells of the BMU in order to initiate remodeling. There is some evidence to suggest that lining cells can even make matrix in response to mechanical loading by de-differentiating into OBs.

The earliest changes in the osteocyte after loading, is an increase in ocsteocytic glucose-6 phosphate dehydrogenase activity (Lean *et al*. 1996). This occurs within minutes of the stimulus. At the same time, OBs demonstrate an increase in intracellular calcium, probably as a result of activation of the phosphatidylinositol 3-kinase (PI3K) pathway which mediates intracellular calcium release. These changes result in stimulation of the mitogen activated protein (MAP) kinase signaling pathway, a key circuit for activating gene transcription. At 30–60 min after induction of stress on the bone, lining cells and OBs begin to express c-*fos*, an important proto-oncogene necessary for both OB and OC function. IGF-I expression is up regulated in the OB at 1 h (Lean *et al*. 1996). Other factors clearly contribute to the increase in OB activity, including prostaglandins, TGF-β, neuropeptides and several matrix proteins that can activate integrins and enhance cell movement, particularly in marrow stromal cells.

Prostaglandins likely play an important role in the activation of bone remodeling with loading. Inhibition of prostaglandin synthesis by non-steroidal anti-inflammatory drugs suppresses bone formation *in vivo*. The two most active prostaglandins released during this process are prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) (Klein-Nulend *et al*. 1997). These compounds are synthesized both in the osteocyte and OB and may be major signals for recruitment of OB precursors from marrow stem cells. Selective inhibition of the inducible prostaglandin synthase (COX-2) results in a greater suppression of loading induced bone formation than a non-selective blockade, although the clinical significance of this is currently unknown (Forwood 1996).

As noted earlier, neuropeptides are important systemic mediators of the stress response. For example, neuropeptide Y is downstream of leptin in the hypothalamus and can cause bone loss when administered into the cerebral ventricles of mice. Also neuropeptide Y deficient mice have a marked increase in trabecular bone as do conditional knockout mice that have deletions of neuropeptide Y limited to the hypothalamus. In addition to the systemic and central nervous system effect, recent evidence suggest these proteins may also be important in the acute local response to loading. A glutamate transported has been identified in bone tissue and is down regulated immediately after mechanical loading (Laketic-Ljubojevic *et al*. 1999). Serotonin receptors are present on osteocytes and OBs, particularly in the periosteum suggesting that mechanotransduction may in part be mediated through this pathway (Mason *et al*. 1997).

Two other major processes occur with loading at the cellular level: prevention of apoptosis of OBs and osteocytes as a result of local growth factors (IGF-I, leptin, IL-6, TGF-β) and the release and elaboration of nitric oxide, an important mediator of OB activity and an inhibitor of osteoclastic function. Nitric oxide may be involved in mechanotransduction although the mechanism has not been defined (Turner *et al*. 1996). It has recently been shown to down-regulate RANKL expression in stromal cells, a key factor in osteoclastogenesis (Rubin *et al*. 2003). Nitric oxide also can bind to guanylyl cyclase stimulating cyclic guanosine monophosphate (cGMP), another intracellular mediator that can regulate gene transcription.

In sum, a host of local growth factors relatively rapidly enhance the number of OB progenitors and terminally differentiated cells at the remodeling surface in response to loading. Interestingly, hormonal mediators of the acute stress response that are active during the first minutes of physical activity do not

modulate the adaptive response in the skeleton to loading. But, ironically, their presence and regulation in the local bone milieu plays a major role in the chronic adaptive response to loading. Cytokines, growth factors, neuropeptides, prostaglandins and nitric oxide contribute to an increase in bone formation with physical activity.

Hormonal and skeletal responses to long-term exercise

As noted earlier, the skeleton is ideally suited to respond to mechanical loading by signal transduction resulting in modeling of the periosteum and trabecular reorientation. Hormonal changes as a result of physical activity play a minor role at the tissue level in accommodating the acute adaptive response. However, hormonal cues are particularly critical for regulating the process of bone remodeling, an essential element of bone preservation in adult life. As such, the skeletal adaptation to mechanical loading which results from repetitive muscle activity is balanced by the long-term changes in endocrine modulators such as the gonadal steroids, cortisol and neuropeptides. These changes will be discussed in this section.

Most investigators believe, although there are no long-term prospective data, that a lifetime of physical activity benefits the skeleton (Bouxsein & Marcus 1994). This, indeed is the official position of the American College of Sports Medicine (American College of Sports 1998). However, skeletal adaptation to loading is very site specific, so that generalities about the long-term benefits of physical activity on bone must be avoided. Moreover, as noted earlier, the beneficial effects of loading the skeleton through exercise are much more pronounced before and during puberty than later in life (Bass 2000). Hence, changes in BMD, as measured by dual energy X-ray absorptiometry (DXA), are likely to be much greater in children than adults. In fact, most of the trials related to physical activity in adults have demonstrated only very modest changes in BMD of the spine or hip, despite, in some cases, significant musculoskeletal loading. This difference almost undoubtedly relates to the fact that loading of the adult skeleton preserves bone mass by slowing rates of resorption and increasing slightly bone formation, a result of changes on the endosteal surface of bone. On the other hand, changes in skeletal mass with loading in children can have a profound effect on the growing skeleton, particularly on the periosteal surface. For example, tennis and squash players who begin playing in their preadolescent years have a two to fourfold times greater difference in radial BMD between their playing arm and non-playing arm compared to those who start after puberty (Kannus *et al*. 1995). Similarly, young prepubertal girls who undertook a regimen of regular weight-bearing activity plus jumping for 10 months had a nearly 6% increase in femoral bone mineral content compared to an 8-month intervention in adolescents (age 14.2 years) of a similar nature (Witzke & Snow 2000; Fuchs *et al*. 2001). In that latter study, there was virtually no change in bone mineral content or density at any site except the femoral trochanter. Thus site specificity and age are major determinants of the chronic response to loading. Finally, it should be noted that a major factor that is difficult to quantify, particularly in large-scale trials, is the amount of background physical activity occurring before or during the conduct of the study. Although questionnaires are useful in assessing the amount and frequency of background activity, these are certainly imprecise and subject to considerable variability, particularly in children where normal daily activities are quite pronounced.

However, a word of caution is necessary in relation to the assumption that loading the skeleton has a more pronounced effect in growing children rather than adults. Virtually all of the longitudinal studies showing changes in bone mineral content or BMD in adolescence or preadolescent children utilized areal measurements derived from DXA. This technique is two-dimensional and very size dependent; thus areal BMD would be expected to markedly increase during the peak skeletal growth. This complicates interpretation of the longitudinal data and bespeaks the need for studies looking at volumetric or three-dimensional BMD to determine whether exercise-induced benefits to the skeleton are purely growth mediated or in fact are related directly to greater mineralization and matrix deposition, the critical determinants of BMD.

The chronic hormonal and skeletal response to sustained physical exercise is complex and variable. First it should be noted that there are numerous animal studies demonstrating a sustained skeletal response to chronic loading. Lanyon, Rubin and others have convincingly demonstrated in avian ulna that consecutive cycles of loading resulted in a strain magnitude which enhanced endosteal bone formation (Rubin & Lanyon 1985). In fact, 36 cycles per day (2050 microstrain at 0.5 Hz) increases bone formation and further increases in cycle number do not increase bone mass (Rubin & Lanyon 1984). However, 1000 microstrain at 1 Hz led to a dose-dependent increase in periosteal bone deposition (Rubin & Lanyon 1985). Yet, exercise-induced stimulation of bone cells in the rib following weight-bearing exercise does not induce bone formation. Furthermore, unloading of animal bones with microgravity, hindlimb immobilization, spinal cord injury, or sciatic neurectomy, result in significant bone loss. These studies confirm significant compartmental differences (trabecular versus periosteal) as well as site specificity in regards to chronic loading or unloading of the skeleton.

Human adult studies have confirmed the results in animals, although the size effect related to exercise intervention is much lower. For example exercise training in young premenopausal women enhances areal BMD in a site-specific manner. Resistance training and weight-bearing exercises result in a very modest increase in lumbar spine, femoral and calcaneal BMD (Bassey *et al*. 1998). And, there does not appear to be a significant dose dependency in women for these changes, although those studies are not as neatly designed nor as conclusive as animal studies. Older premenopausal women also show stabilization of areal BMD over a 12-month period in response to chronic loading; however, these changes are not associated with a statistically significant increase in bone mass, suggesting that the effect size of the intervention may decrease with increasing age (Pruitt *et al*. 1992; Bassey *et al*. 1998).

The response of bone to chronic exercise in post-menopausal women is somewhat conflicted. In early post-menopausal women without estrogen replacement, resistance exercise either increased or maintained BMD in the spine and sometimes in the femur, but failed to prevent bone loss at other sites (Kohrt *et al*. 1997). On the other hand, women receiving hormone replacement therapy (HRT) who underwent a series of chronic exercise programs generally showed a marked increase in spine, total body and femoral BMD (Kohrt *et al*. 1997, 1998). The combination of exercise and calcium supplementation in post-menopausal women may be greater than exercise or calcium supplementation alone, as reported in a meta-analysis performed by Specker (1996). As such there appears to be strong evidence that the optimal skeletal response to exercise in post-menopausal women requires adequate calcium supplementation and HRT (Kanders *et al*. 1988).

Exercise intervention studies in men are somewhat more conflicted than in women. Training of recreational male athletes between the ages of 25–52 years for 3 months with walking or running failed to demonstrate an increase in bone mass at the spine, humerus, femur or calcaneus. Similar but smaller studies in older men also failed to show any changes with a 1-year-long exercise regimen. On the other hand, in army recruits completing 14 months of intensive physical training, leg bone mineral content increased 12.4% (Jones *et al*. 1989). This difference may be a function of intensity, age or site of maximal loading. However it does bespeak the tremendous heterogeneity between studies, and the difficulty in performing systematic reviews using meta-analysis for exercise trials.

One of the major limitations of loading trials in humans relates to the endpoint being studied; i.e. areal BMD. DXA measurements of the spine or hip may not capture the true effect of skeletal loading on bone mass. As noted previously, the skeleton is composed of both cortical and trabecular components. Force induced changes in the periosteal and endosteal envelope that surround these compartments are not easily captured by an areal approximation of bone mass. Indeed, changes in shape as a result of skeletal modeling can not be assessed by conventional DXA. Recently, evidence has emerged that changes in areal BMD with anti-osteoporosis therapies, reflect only to a small degree the degree of risk reduction for subsequent fracture (Cummings

et al. 2002). In other words, the variance around fracture risk in relation to any intervention is much greater than that attributed to changes in BMD, and almost certainly reflects qualitative changes in both compartments of the skeleton. Hence, exercise intervention studies that employ BMD as a final endpoint may underestimate the true effect on loading on bone strength and fracture risk. Since there are still no exercise trials with fracture as an endpoint, the degree to which changes in bone density reflect ultimate skeletal strength remains uncertain. Newer imaging technology such as micro computed tomography (μCT), may allow for *in vivo* scanning of trabecular bone, while magnetic resonance imaging (MRI) can precisely delineate periosteal circumference, cortical thickness, trabecular number and connectivity, muscle mass and marrow fat. These endpoints may improve our capacity to define the effects of loading on the axial and appendicular skeleton, particularly in respect to aspects of bone geometry and quality.

Regardless of the imaging tool, BMD represents the sum of many factors over the lifetime of an individual. Particularly relevant to defining overall changes in bone quantity is the role of circulating hormones, cytokines and growth factors. Indeed, in osteoporotic states, delineation of hormonal status (estrogen in women, testosterone in men) represents the first step in establishing the etiology of low bone mass. Moreover, other endogenous hormones such as PTH, 1,25-dihydroxyvitamin D, 25-OH vitamin D and thyroxine, all contribute to changes in bone remodeling and are important in determining the length of the remodeling cycle and its subsequent balance. Similarly, circulating cytokines such as IL-6 have been shown to be important in the pathogenesis of age-related bone loss and primary hyerparathryoidism (Nakchbandi *et al*. 2002). As discussed previously, IGF-I circulates in high concentrations and plays a major role in skeletal modeling and cortical bone mass (Rosen & Donahue 1998). Acromegaly, a condition of excess GH and IGF-I, is associated with large bone volumes and greater BMD (Rosen & Donahue 1998). By contrast, people with conditions such as hypopituitarism have small bones, increased skeletal fragility and reduced BMD. These pathologic states are useful in defining the role of circulating factors on the skeleton and provide some basis for our understanding of the balance between local and systemic factors. The effects of a chronic exercise regimen on systemic factors, such as gonadal steroids, remains a critical aspect of the ultimate skeletal response to loading and must be considered within the context of the entire organism.

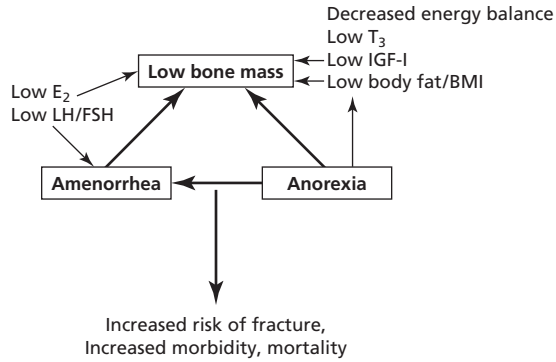


Fig. 28.5 The female athlete triad. This syndrome, common in young athletic women, represents a compelling interface between hormones, exercise and bone adaptation. BMI, body mass index; E_2, estradiol; FSH, follicle-stimulating hormone; IGF-I, insulin-like growth factor I; LH, luteinizing hormone; T_3, triiodothyronine.

Much like the osteoporotic states of aging, the pathologic syndrome now referred to as the female athelete's triad, has provided investigators with significant insight into the interface between circulating hormones, body mass and skeletal adaptation to exercise. This condition is defined by amenorrhea (or hormonal disturbances), anorexia and low BMD (Fig. 28.5) (Drinkwater *et al*. 1984; Marcus *et al*. 1985; Tomten *et al*. 1998). It is most commonly seen in young adult women. To appreciate the adverse effects of this syndrome on the skeleton and its relationship to circulating hormones, an overview of the changes that result from dietary restraint and excessive physical activity is necessary.

The hypothalamic–pituitary axis is the principle responder to stress related to dietary changes and/or physical activity. Two major hypothalamic factors, corticotrophin-releasing hormone (CRH), and gonadotropin-releasing hormone (GnRH) are significantly affected by energy balance associated with activity patterns and nutrient availability.

CRH controls the acute stress response; it in turn stimulates adrenocorticotropic hormone (ACTH) release from the pituitary gland, subsequently leading to cortisol production in the adrenals (Webster *et al*. 2002). CRH is released into the median eminence of the hypothalamus through the portal circulation from neuronal cells of the parvaentricular nucleus. It, combined with vasopressin, stimulates ACTH. Subsequently the increase in cortisol production enhances available energy stores by driving protein catabolism, stimulating fatty acid release from adipose tissue and enhancing gluconeogenesis (Darmaun *et al*. 1988; Chrousos & Gold 1992). Cortisol also modulates the physiological effects of various catecholamines and exerts negative feedback control on the hypothalamic–pituitary axis. All these processes are integral for an individual requiring major energy sources for the brain. But, long-term stimulation of this system can result in excess cortisol release via the hypothalamic–pituitary axis, leading to increased serum cortisol concentrations and deleterious effects on muscle and bone.

The effects of long-term stress or overexercise on the GnRH, luteinizing hormone/follicle-stimulating hormone (LH/FSH) system have also been studied in some detail. GnRH pulsatility is key to the appropriate stimulation of both LH and FSH, the central regulators of ovarian steroidogenesis. A common factor in female athletes with amenorrhea or oligomenorrhea is a disruption in LH pulsatility, almost certainly originating from the GnRH pulse generator (Loucks *et al*. 1992). The precise mechanism for this early loss of pulsatility however remains speculative. But a likely scenario is that a reduction in energy availability prevents the hypothalamic pulse generator from providing the appropriate signal for LH/FSH release. It seems likely that optimal menstrual function almost certainly requires a minimum energy requirement; when that threshold is not attained, loss of gonadotropin pulsatility results in a downstream shutdown in gonadotropin release. Several lines of evidence support this thesis. Loucks & Callister (1993) demonstrated that, in eumenorrheic sedentary women, reduced energy intake over 5 days resulted in impaired LH pulsatility and a fall in trioiodothyronine. This drop occurred at a threshold energy level of 20–25 kcal·kg^{-1} (84–105 kJ·kg^{-1}) of lean body mass. Similarly, Williams *et al*. (1995) showed that active eumenorrheic women experienced suppressed LH pulsatility after only 3 days of physical training when dietary energy intake was reduced. This was completely reversed with appropriate dietary intake. Finally, Dueck *et al*. (1996) showed in three amenorrheic and three eumenorrheic women that increasing energy intake by 350 kcal·day^{-1} (1463 kJ·day^{-1}) and reducing training by 1 day per week was associated with a net increase in energy availability of 250 kcal·day^{-1} (1045 kJ·day^{-1}). At the end of 15 weeks, all six athletes were retested and the amenorrheic athletes had resumption of LH pulsatility as well as a reduction in cortisol secretion.

Chronic changes in the hypothalamic pituitary system centered on GnRH and CRH can have major effects on the skeleton and almost certainly are part of the pathogenetic mechanisms associated with the female athelete's triad (DiPietro & Stachenfeld 1997; Otis *et al*. 1997). Weight loss, intentional or unintentional due to an energy deficit, contributes to a reduction in BMD by altering the mechanical properties of bone in relation to other body components and lowering systemic or local estradiol concentrations. For example, reduced total body weight means less gravitary forces placed on the skeleton, thereby resulting in less strain, and a reduced adaptive response. Moreover, fat is a major source of peripheral estrogen production as a byproduct of testosterone catabolism. Marrow and circulating estradiol levels drop in response to a decrease in the total number of fat cells, or their cell density at peripheral sites such as the bone marrow. Also, a reduction in body fat may reduce the number of available marrow precursor cells that could eventually become preosteoblastic bone forming cells. Regardless of the mechanism, a drop in estradiol in young women results in increased bone resorption, particularly on the endosteal border. If low estradiol levels persist, resorption exceeds formation and bone is lost. Moreover, the beneficial effects of physical activity on the formation side may be diminished or abolished by the lack of estrogen, as noted previously for post-menopausal women.

Surprisingly, not all of the deleterious effects on BMD in amenorrheic athletes can be attributed to low circulating or skeletal estradiol. In fact, restoration of BMD is unlikely to occur in anorexic

women even with HRT unless body weight is restored to at least 70% of ideal weight (Klibanski *et al*. 1995). Thus energy restriction, low body weight and low body fat combine to drastically alter not only the set-point for the two major hypothalamic factors noted earlier (GnRH and CRH) but also for other factors that are nutritionally or energy dependent. These include triiodothyronine, dehydroepiandrosterone (DHEA), GH, IGF-I, IGFBP-1 and several cytokines. In fact, parenteral IGF-I therapy can at least partially reverse the skeletal manifestations of anorexia nervosa, particularly when combined with an anti-resorptive agent (Grinspoon *et al*. 2003).

In summary, the long-term effects of a vigorous exercise program on the skeleton are dependent on the energy threshold, nutrient intake, type of physical activity, as well as local and circulatory hormones. In general, weight-bearing exercises promote bone formation and can, particularly in younger individuals, enhance bone mass through its adaptive response to strain. However, excessive exercise programs that can lead to negative energy balance and weight loss result in a significant decline in estradiol levels such that the skeleton is at significant risk for rapid bone loss. The end result of these processes include a higher risk of fracture and significant immobility and morbidity. Moreover, if the female athlete is young enough (i.e. 15–19 years of age), the loss of bone mass during this interval may not be restorable with return of menses and resumption of normal body weight. Since exercise has become a more frequent habit among all individuals, and thinness is certainly in vogue, particularly in female college-aged students, the combination of several lifestyle factors in young females may have a devastating impact on their long-term skeletal health, despite the short-term benefits related to skeletal adaptation.

It should be noted that there is likely to be an important gender effect on bone related to long-term exercise such that women appear to be more susceptible to the adverse effects of sustained negative energy balance than men. However, the hypothalamic–pituitary–adrenal (HPA)–gonadal axis in long-term male athletes has not been studied as extensively as the HPA axis of women athletes. In fact, the clinical counterpart of the female athlete's triad in men may not exist per se since there are no easy markers for hypothalamic dysfunction short of reduced libido. On the other hand, at least two groups have now demonstrated that male triathletes who are in the age range of 20–40 years have reduced total and free testosterone compared to non-exercising control men (Wheeler *et al*. 1984; Smith & Rutherford 1993). Moreover, although exercise programs in elderly men have virtually no effect on circulating testosterone, sex hormone binding globulin levels are higher in exercisers than controls, thereby affecting free testosterone concentrations (Cooper *et al*. 1998).

Clinical correlates related to the interaction of exercise, hormones and bone remodeling

Exercise is considered by many to be essential for overall well-being. The general benefits clearly outweigh the risks, and therefore prescriptions for healthier bones should include a daily exercise regimen. Unfortunately, the overall impact of an exercise program on fracture risk in older men or women is not known. It is safe to assume that exercise increases muscle performance and possibly muscle mass. This alone may be enough to reduce falls in older individuals, and thereby indirectly reduce fractures. Loading of the skeleton with the resultant adaptive response is optimized in situations where there is adequate gonadal steroids, although there are likely to be skeletal benefits for older individuals that are gonadally insufficient.

Risks of a sustained exercise program are relatively small and relate to problems loading areas of the skeleton that are not adaptable to stress, and crossing a theoretic threshold where energy balance becomes negative in the face of greater musculoskeletal activity. The former issue has become more important for the armed services, where the prevalence of stress fractures among new recruits range from 10–20% (Bennell *et al*. 1996a, 1996b). In many cases these cortical fractures cannot be related to areal BMD, but rather are a function of training and readiness. In addition, for young female army recruits, stress fractures of the pelvis and tibia are much more prevalent than in male recruits. This may be a function of loading in regions of the skel-

eton, particularly the periosteum, that are not used to handling repetitive forces. These fractures cost the armed services dearly in respect to combat readiness, as well as the financial implications related to caring for these individuals. Factors that predict stress fractures in military recruits or elite athletes have not been well delineated, but clearly are somewhat independent of bone density or the strength of the force applied (Bennell *et al*. 1996a, 1996b). Qualitative measures of bone, as determined by newer imaging technologies, may provide some clues for the future identification of those individuals susceptible to injury. Similarly, because there appears to be a heritable component to stress fractures, genetic studies may identify those high-risk individuals prior to the onset of training.

Less clear cut are the clinical correlates related to the deleterious effects of exercise on energy balance, particularly in the young female athlete. Oral contraceptives work in some subjects who have the female athlete's triad; however, restoration of weight remains the best prognostic factor for resumption of menses and improvement in skeletal health. Newer studies have been initiated with DHEA, a weak adrenal androgen, to determine if this compound is more palatable and as effective as estrogen in stopping bone resorption and enhancing bone formation (Gordon *et al*. 2002). Other trials with newer anabolic agents are being considered.

As noted previously, aging is associated with significant bone loss, accelerated bone resorption and a high risk of fractures. Defining the optimal threshold for loading the skeleton and the resultant energy balance will be mandatory in the future if exercise is to be used as a prescription for better bone health. In the meantime, a major push is needed to fully understand the fine balance between local and systemic hormones and skeletal remodeling, particularly in respect to exercise programs and their more global application.

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Chapter 29

Diet and Hormonal Responses: Potential Impact on Body Composition

JEFF S. VOLEK AND MATTHEW J. SHARMAN

Introduction

In order to sustain life, there must be a continuous supply of energy obtained from the diet. The primary energy-providing nutrients are protein, carbohydrate and fat. When food is ingested, there is an acute increase in plasma metabolites and hormones that is dependent, in part, on timing and macronutrient distribution of the meal. Nutrients and hormones then interact at target tissues to regulate cellular processes. In the context of body composition, nutrients and hormones have important roles in regulating skeletal muscle protein and adipose triacylglycerol balance, which over time impacts body composition. Exercise also has independent effects on nutrients and hormone availability and interacts with feeding to create a unique setting that impacts metabolic processes including body composition. This general scenario is depicted in Fig. 29.1. The postprandial phase of metabolism includes the time when nutrients are being absorbed from the gut and appear in the circulation. This time period continues several hours after feeding and therefore most people are in a postprandial phase for the majority of their life. Similar to the situation linking the postprandial lipoprotein response to cardiovascular disease (Patsch *et al*. 1992; Ebenbichler *et al*. 1995), the postprandial hormonal response could be viewed as more physiologically important than the postabsorptive hormonal environment, particularly for enhancing body composition.

Given the important regulatory affects of hormones, it is surprising that a more systematic and comprehensive study of the effects of hormonal responses to feeding has not been undertaken. The studies reviewed in this chapter are from diverse perspectives but share the common design component of measuring the hormonal response to food intake. We synthesized these postprandial hormonal data in an attempt to shed light on the consistency or lack thereof related to the hormonal response to intake of meals with different composition with and without exercise. We discuss separately the effects of meals on growth hormone (GH), insulin-like growth factor I (IGF-I), testosterone, cortisol and insulin because these hormones have a major role in regulating protein and lipid metabolism. For each hormone, we overview the postprandial response to meals of different macronutrient distribution and the exercise-induced hormonal response to feeding before, during, or after exercise. Although theoretical, the implications of diet-induced hormonal responses on body composition are discussed. We focus primarily on human studies and only include animal work to provide supporting information.

Effects of diet on hormones

Growth hormone

GH is a peptide hormone secreted from the anterior pituitary. In skeletal muscle, GH promotes a positive protein balance by increasing protein synthesis and possibly inhibiting protein breakdown (Rooyackers & Nair 1997). These effects are controversial in part because it is difficult to separate out the effects mediated through stimulation of hepatic

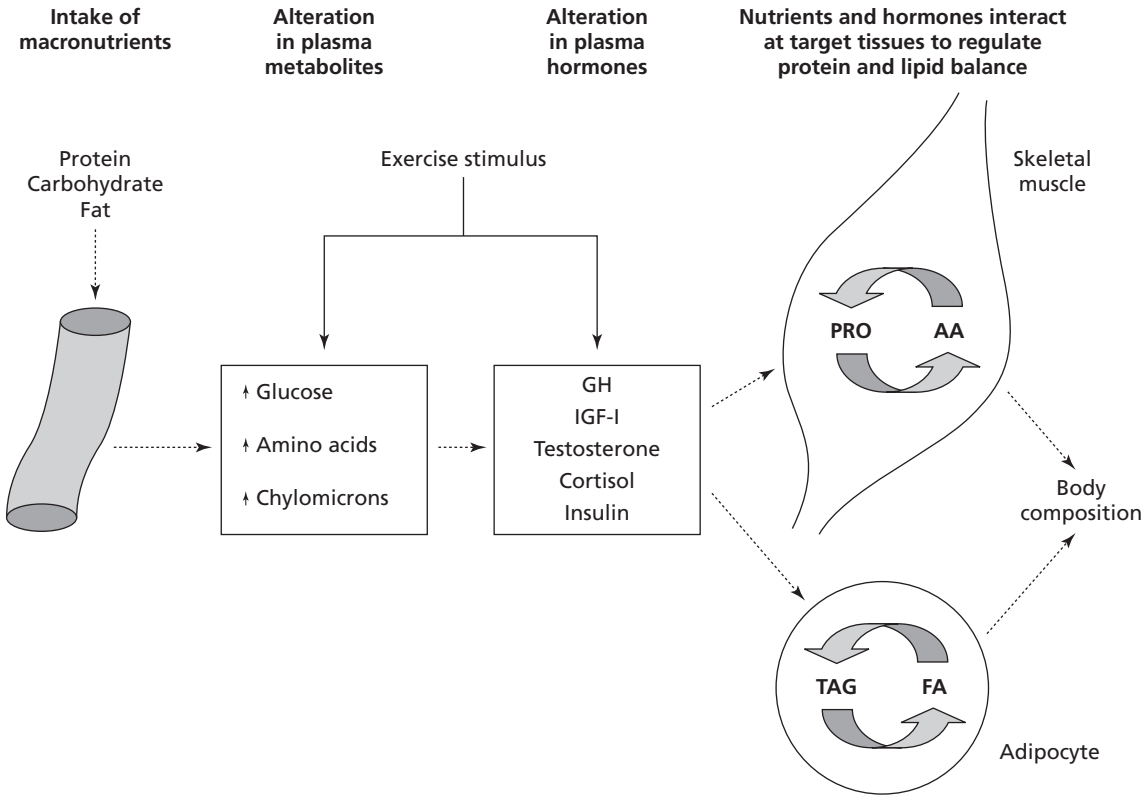

Fig. 29.1 Intake of macronutrients results in appearance of glucose, amino acids and triacylglycerols in the form of chylomicrons in the plasma. These nutrients are also affected by exercise and together contribute to a release of hormones that then partition nutrients and interact with target tissues such as skeletal muscle and adipose tissue to regulate among other processes, protein synthesis/breakdown and adipose tissue lipolysis/lipogenesis, the balance of which over time impacts body composition. AA, amino acids; FA, fatty acids; GH, growth hormone; IGF-I, insulin-like growth factor-I; PRO, protein; TAG, triacylglycerols.

or skeletal muscle IGF-I. In adipose tissue, GH increases lipolysis and there has been specific interest in the C-terminal fragment of GH shown to have marked lipolytic and antilipogenic activity *in vitro* and when administered to animals (Heffernan *et al*. 2000). GH dramatically decreases lipogenesis with a concomitant increase in muscle mass indicating a powerful nutrient partitioning effect (Etherton 2000).

POSTPRANDIAL RESPONSE TO FEEDING

The GH response to meals has been shown to be quite variable (Baker *et al*. 1972; van Loon *et al*. 2003), which may be explained in part by the pulsatile release pattern of GH. Studies have shown that protein, fat and carbohydrate each have independent effects on regulation of GH secretion. An oral glucose tolerance test results in significantly reduced GH levels (Hjalmarsen *et al*. 1996; Bernardi *et al*. 1999; Nakagawa *et al*. 2002). Carbohydrate intake ($0.7\ g{\cdot}kg^{-1}{\cdot}h^{-1}$) alone or in combination with protein ($0.35\ g{\cdot}kg^{-1}{\cdot}h^{-1}$) resulted in declining GH levels over a 2-h period (van Loon *et al*. 2003). An oral glucose tolerance test (75 g glucose) resulted in a slight and gradual decrease in GH for 2 h followed by a significant increase that peaked at 240 min (Frystyk *et al*. 1997). Thus, hyperglycemia is associated with a decrease in GH, which may be followed by

rebound hypoglycemia and a subsequent increase in GH. This is consistent with work showing hypoglycemia is a potent stimulator of GH (Roth *et al*. 1963). Thus in response to carbohydrate, GH tends to be decreased and increased in the early and late postprandial periods, respectively.

Although infusion and, in some cases, oral ingestion of large doses of certain amino acids (e.g. arginine, lysine and ornithine) can increase GH levels, the effect is quite variable and reduced by physical training and high-protein diets (reviewed by Chromiak & Antonio 2002). Ingestion of beef steak resulted in an increase in GH, presumably due to the amino acid content, but the increase was prevented when heparin was injected to acutely elevate circulating fatty acids (Fineberg *et al*. 1972). In line with an inhibitory effect of fatty acids on GH, other work has confirmed that circulating fatty acids, but not triacylglycerols, inhibit GH secretion (Blackard *et al*. 1971). The time course of the GH response to food is not entirely clear. Acute ingestion of a liquid supplement (520 kcal [2177 kJ]) rich in fat or rich in carbohydrate did not affect GH measured 35 min later (Cappon *et al*. 1993) nor did a mixed carbohydrate and protein meal (216 kcal [903 kJ]) affect GH levels measured 90 min later (Carli *et al*. 1992). Alcohol ingestion (0.45 g ethanol·kg^{-1}) consumed each hour for 3 h had no effect on GH responses compared to water (Rojdmark *et al*. 2000).

EXERCISE-INDUCED RESPONSES TO FEEDING

Carbohydrate and protein intake alter the GH response to resistance exercise. A protein and carbohydrate supplement consumed immediately and 2 h after resistance exercise increased GH during late recovery when glucose levels were lower compared to a placebo (Chandler *et al*. 1994), consistent with the known effects of hypoglycemia on GH. Our laboratory reported that a protein and carbohydrate supplement consumed before and immediately after a bout of resistance exercise resulted in an enhanced acute GH response from 0–30 min post-exercise compared to a non-caloric placebo despite similar glucose levels between trials (Kraemer *et al*. 1998). Another study reported that protein and carbohydrate intake after resistance exercise had no significant effect on GH responses (Williams *et al*. 2002).

Meals also alter the GH response to submaximal exercise. Whitley *et al*. (1998) compared the effects of fasting to isoenergetic (956 kcal [40031 kJ]) high-fat (74% fat) and high-carbohydrate (86% carbohydrate) meals consumed 4 h prior to cycling for 90 min on GH responses. The GH response during the fasting and carbohydrate trial were similar and significantly higher compared to the high-fat trial. Consistent with the effects of glucose and fatty acids on GH secretion, the lower GH levels during exercise after the fat-rich meal was associated with higher blood glucose and fatty acid levels. Cappon *et al*. (1993) compared the effects of isoenergetic (520 kcal [2177 kJ]) high-fat and high-carbohydrate liquid meals consumed 45 min before 10 min of intense exercise on post-exercise GH responses. Compared to ingestion of a noncaloric placebo, the post-exercise GH area under the curve was decreased by –54% after the high-fat meal and by –25% after the high-carbohydrate meal. Post-exercise somatostatin was elevated after the high-fat meal and the authors postulate a potential link between dietary fat, somatostatin and GH (Cappon *et al*. 1993). Somatostatin also decreases ghrelin (Schaller *et al*. 2003), a recently identified potent GH-releasing peptide primarily produced in the stomach, providing an alternative fat-induced mechanism to decrease GH.

The finding that a high-fat meal reduces post-exercise GH more than a high-carbohydrate meal is somewhat counterintuitive since carbohydrate feeding would likely lead to higher glucose levels, which should inhibit GH. In line with this role of GH as a counter-regulatory hormone, several studies have shown that compared to placebo, carbohydrate beverages consumed before, during and after exercise result in higher blood glucose levels and lower GH responses to exercise (Bonen *et al*. 1980; Tsintzas *et al*. 1996; Utter *et al*. 1999). Miller *et al*. (2002) showed that carbohydrate provided during 2 h of cycling blunted the post-exercise GH response compared to non-fat milk and a non-caloric placebo despite similar glucose levels among the three trials suggesting that GH response to exercise are mediated by factors other than blood glucose. Studies have also shown that carbohydrate provided before

and during 2 h of cycling (Murray *et al*. 1995) or rowing (Henson *et al*. 2000) do not affect GH levels despite differences in glucose levels. In one of these studies (Murray *et al*. 1995), subjects also consumed the beverages with and without nicotinic acid, an inhibitor of lipolysis. Nicotinic acid consumed with either water or carbohydrate prevented the increase in fatty acids and resulted in increased post-exercise GH levels compared to water and carbohydrate without nicotinic acid (Murray *et al*. 1995). Thus, the GH response to exercise appears to be dependent in part on glucose levels, but fatty acid levels also exert an independent effect.

Exercise-induced elevations in GH to a high-fat diet or fasting are accompanied by a more rapid decline in plasma insulin and glucose concentrations during exercise suggesting that glucose sensitive receptors may modulate the GH response to exercise (Galbo *et al*. 1979). Glucose infusion at the end of exercise does not attenuate the greater GH response observed after a fat-rich diet (Galbo *et al*. 1979; Johannessen *et al*. 1981) again suggesting that other substrates (e.g. glycogen, ketones, fatty acids) or hormones (e.g. insulin, catecholamines) may be involved in the regulation of GH secretion.

Insulin-like growth factor I

IGF-I is an anabolic hormone that stimulates growth in almost all tissues and is likely responsible for many of the effects of GH. IGF-I is primarily produced in the liver but also in other tissues including skeletal muscle under stimulation by GH. In skeletal muscle, IGF-I increases protein balance primarily by increasing protein synthesis (Rooyackers & Nair 1997). In adipose tissue, IGF-I has insulin-like effects stimulating glucose uptake and inhibiting lipolysis (Siddals *et al*. 2002).

POSTPRANDIAL RESPONSE TO FEEDING

Effects of macronutrients on total IGF-I levels. An oral glucose tolerance test (75 g glucose) had no effect on total IGF-I levels measured every 30 min for 330 min in healthy subjects (Frystyk *et al*. 1997). Carbohydrate intake (0.7 $g \cdot kg^{-1} \cdot h^{-1}$) alone or in combination with protein (0.35 $g \cdot kg^{-1} \cdot h^{-1}$) had no effect on total IGF-I levels measured 2 h later (van Loon *et al*. 2003). In another study, total IGF-I levels were not affected when measured 4 h after a carbohydrate-rich breakfast (Bereket *et al*. 1996). A mixed meal had no effect on total IGF-I levels measured over the next 5 h (Svanberg *et al*. 2000) and another study showed no changes in total IGF-I levels over a 24-h period during which subjects were fed mixed meals (Frystyk *et al*. 2003). Consumption of ethanol did reduce IGF-I levels during the late postprandial period (5–7 h after ingestion) (Rojdmark *et al*. 2000). Thus, feeding does not appear to affect total IGF-I levels with the exception of a delayed decrease after alcohol ingestion. However, feeding does appear to alter IGF-I binding proteins.

Effects of macronutrients on other components of the IGF-I system. There are six high affinity insulin-like growth factor binding proteins (IGFBP-1 to 6) that circulate bound to the vast majority of IGF-I. IGFBP-1 shows the most rapid dynamic regulation in plasma in response to meals and it has been suggested that it contributes to glucose regulation by virtue of its role in countering the insulin-like bioactivity of IGF-I (i.e. hypoglycemic effect), most likely by controlling free levels of IGF-I (Lee *et al*. 1993, 1997). After an oral glucose tolerance test, IGFBP-1 gradually decreased reaching a level –52% below baseline after 180 min followed by a gradual increase to values 74% above baseline at 5 h (Frystyk *et al*. 1997). Opposite to IGFBP-1, free IGF-I was significantly decreased (–29% to –38%) during the late postprandial period (270–330 min) and was inversely related to IGFBP-1 at baseline and during the late postprandial period. Other studies have observed decreases in IGFBP-1 in response to mixed meals (Bereket *et al*. 1996; Frystyk *et al*. 2003), oral glucose (Bernardi *et al*. 1999) or intravenous glucose and insulin injections (Nyomba *et al*. 1997). Inverse associations have been shown between IGFBP-1 and glucose, insulin and cortisol levels (Hopkins *et al*. 1994; Holden *et al*. 1995; Frystyk *et al*. 1997; Ricart & Fernandez-Real 2001). Acute ingestion of ethanol resulted in a rapid rise in IGFBP-1 that was independent of changes in glucose, insulin and GH, suggesting its regulation is partially mediated by other mechanisms.

Although there is a significant amount of evidence supporting the role of the GH–IGF-I axis in glucose metabolism (Holt *et al*. 2003), there has been debate whether the IGF-I system is important in glucose regulation in response to metabolic stressors (e.g. feeding and/or exercise). This is because free IGF-I levels are generally not affected during the early postprandial period when IGFBP-1, glucose and insulin are elevated but free IGF-I levels are reduced during the late postprandial period and during the night when glucose and insulin are normalized (Frystyk *et al*. 1997, 2003). Thus, the majority of evidence indicates that the IGF-I system does not partake in meal-related glucose regulation but does contribute to glucose disposal during the post-absorptive state.

Since IGF-I is produced in many tissues including skeletal muscle, it is possible that the IGF-I system is contributing to meal-related glucose regulation through an autocrine and/or paracrine mechanism. However, this hypothesis is unlikely based on findings from a recent study showing that feeding does not affect postprandial skeletal muscle IGF-I mRNA expression in humans (Svanberg *et al*. 2000).

EXERCISE-INDUCED RESPONSES TO FEEDING

Effects of macronutrients on total exercise-induced IGF-I responses. Consistent with the lack of a change of food on total IGF-I levels, studies have shown that pre-exercise and post-exercise meals do not alter the total IGF-I response to exercise (Cappon *et al*. 1994; Hopkins *et al*. 1994; Kraemer *et al*. 1998; Anthony *et al*. 2001). A pre-exercise carbohydrate- and fat-rich meal had the same effect as a non-caloric placebo on IGF-I responses to 10 min of intense cycling exercise, suggesting that pre-exercise feeding and meal composition do not influence exercise-induced IGF-I levels (Cappon *et al*. 1994). There were no differences in total IGF-I levels in a group of men fed a placebo or a glucose polymer solution during cycling exercise to fatigue (Hopkins *et al*. 1994). Our laboratory showed that a protein and carbohydrate supplement consumed 2 h before and immediately after a bout of whole body resistance exercise did not alter the exercise-induced IGF-I response (Kraemer *et al*. 1998). Finally, Anthony *et al*. (2001) showed that a nutritional complete meal fed immediately after 2 h of treadmill running had no effect on exercised induced total IGF-I levels compared to a food-deprived group of rats. Thus, acute feeding of any composition does not appear to affect systemic levels of total IGF-I.

Effects of macronutrients on exercise-induced components of the IGF-I system. Similar to the situation after feeding, it has been proposed that IGFBP-1 may have an important role in glucose regulation during and after exercise (Koistinen *et al*. 1996). Compared to placebo, exercise-induced IGFBP-1 levels were reduced in response to feeding of carbohydrate (Hopkins *et al*. 1994). In this study, IGFBP-1 and glucose levels were correlated in the control condition but not during the carbohydrate feeding trial, suggesting that other factors other than glucose and insulin regulate IGFBP-1 responses to prolonged exercise (Hopkins *et al*. 1994). Anthony *et al*. (2001) showed that provision of a nutritionally complete meal immediately after exercise did not alter the exercise-induced increase in hepatic IGFBP-1 mRNA expression or circulating IGFBP-1 levels despite higher glucose and insulin levels. Again, this is consistent with the hypothesis that exercise-induced increases in IGFBP-1 levels are not directly regulated by circulating glucose levels and do not assist in preventing IGF-I induced hypoglycemia by binding free IGF-I in plasma. Findings from a recent study showed high correlations between liver glycogen and IGFBP-1 responses to exercise, suggesting that the magnitude of liver glycogen depletion may mediate IGFBP-1 responses to exercise (Lavoie *et al*. 2002). Although speculative, it is possible that IGFBP-1 responses to exercise are modulating anabolic (growth), as opposed to the metabolic (glucose-lowering), effects of IGF-I.

Testosterone

Testosterone is a steroid hormone secreted from specialized cells in the testes in men and from the ovaries in women. In skeletal muscle, testosterone increases protein balance primarily by increasing protein synthesis whereas the effects on protein breakdown are unclear (Rooyackers & Nair 1997).

In adipose tissue, testosterone inhibits lipid uptake and LPL activity, and stimulates lipolysis by increasing the number of lipolytic β-adrenergic receptors (De Pergola 2000).

POSTPRANDIAL RESPONSE TO FEEDING

The testosterone response to isocaloric (800 kcal [3350 kJ]) meals that were high-fat (57% fat, 9% protein, 34% carbohydrate) or low-fat (1% fat, 26% protein, 73% carbohydrate) was examined in healthy men (Meikle *et al*. 1990). Testosterone was not affected after the low-fat meal, but postprandial total and free testosterone concentrations were approximately 30% lower for 4 h after the high-fat meal. The fat-induced decrease was not related to changes in other steroids (i.e. estrone, estradiol, dihydrotestosterone, lutenizing hormone [LH], percent free testosterone, or sex hormone binding globulin [SHBG] binding capacity). This study indicates fat-rich meals, but not carbohydrate-rich meals, decrease postprandial testosterone levels. Our laboratory has also observed a significant reduction in total testosterone (–22%) and free testosterone (–23%) after a fat-rich meal in healthy men (Volek *et al*. 2001).

Another study compared testosterone responses to meals with different sources of protein (soy versus meat), amounts of fat (lean versus fatty meat) and sources of fat (animal versus vegetable) (Habito *et al*. 2001). The decrease in testosterone was greater after a low-fat meal consisting of lean meat (–22%) compared to tofu (–15%); a meal consisting of lean meat (–22%) compared to lean meat cooked with animal fat meal (–9%); and a meal consisting of lean meat cooked with vegetable fat (–17%) compared to vegetable oil (–9%). Although these results do not necessarily agree with the findings mentioned above that only fat-rich meals reduce testosterone, the study does provide further evidence that the composition of meals, particularly the amount and type of fat, influences the circulating testosterone response to meals. All the meals resulted in decreases in testosterone, increases in LH and no changes in SHBG.

A number of studies have examined the testosterone response to an oral glucose tolerance test. The majority of these studies have been in women, with only one to our knowledge performed in men (Hjalmarsen *et al*. 1996). In this study, total testosterone and estimated free testosterone levels were significantly decreased during a 2-h oral glucose tolerance test. There were no changes in SHBG but LH was significantly increased (Hjalmarsen *et al*. 1996).

The above studies have all involved men. Studies generally indicate that testosterone is also decreased modestly after an oral glucose tolerance test in healthy normal-weight women (Smith, S. *et al*. 1987; Falcone *et al*. 1990; Tiitinen *et al*. 1990; Aizawa & Niimura 1996; Ivandic *et al*. 1999), which may be attributed in part to the normal diurnal variation in the hormone. There have been a number of studies examining the androgen response to oral glucose tolerance tests in women with polycystic ovarian syndrome (PCOS) because they often exhibit both elevated androgens and insulin, which are associated with insulin resistance. Insulin acts to stimulate testosterone in the ovaries (Cara & Rosenfield 1988), and thus hyperinsulinemia has been hypothesized to play a pathogenic role in women with PCOS (Bergh *et al*. 1993). In hyperandrogenic women or those with PCOS, testosterone responses to glucose tolerance tests also tend to decrease in a similar manner as healthy controls (Falcone *et al*. 1990; Tropeano *et al*. 1994) unless the women are also insulin resistant (Smith, S. *et al*. 1987) or obese (Tiitinen *et al*. 1990), and then there is a slight increase in testosterone.

In summary, testosterone levels consistently drop after feeding and there is evidence this is dependent on the composition of the meal, particularly fat content. Insulin may play a role in explaining some of the variability in testosterone responses to meals because in men insulin and testosterone tend to exhibit an inverse relation whereas in women there is a positive association, especially in women with PCOS (Haffner *et al*. 1994). However, findings from studies using the euglycemic hyperinsulinemic clamp procedure cast doubt on the hypothesis that insulin mediates the testosterone response to feeding because acute elevations in insulin were shown to have no effect on total or free testosterone levels in healthy normal-weight men (Ebeling *et al*. 1995; Pasquali *et al*. 1997) or women (Diamond *et al*. 1991). The mechanism by which fat-rich meals decrease

postprandial testosterone concentrations may be related to the elevated chylomicrons or fatty acids after a fat-rich meal because they inhibit LH-stimulated testosterone production in isolated Leydig cells (Meikle *et al*. 1989, 1990). It is possible that specific nutrients may interact with regions of the testes and impact on the modulation of testosterone. Alteration in the testicular plasma membrane and changes in the responsiveness of Leydig cells and subsequent testosterone synthesis as a result of ingestion of different compositions of lipids has been reported in rats (Sebokova *et al*. 1988, 1990).

EXERCISE-INDUCED RESPONSES TO FEEDING

Resistance exercise has been shown to result in acute elevations of testosterone that peak early after exercise and return to baseline by about 60 min post-exercise; pre- and post-exercise meals alter this response (Kraemer *et al*. 1998). Our laboratory showed that a protein and carbohydrate beverage consumed 2 h before and immediately after a bout of whole body resistance exercise resulted in an increase in testosterone immediately after exercise followed by a sharp decrease to values that were significantly below baseline (Kraemer *et al*. 1998). In this study, the exact same exercise and supplementation protocol was performed on 3 consecutive days. The greater reduction in post-exercise testosterone with pre- and post-exercise protein and carbohydrate intake compared to placebo was observed on all 3 days, emphasizing the consistency and reproducibility of the response (Kraemer *et al*. 1998). Chandler *et al*. (1994) examined the post-exercise testosterone response to supplements containing either protein alone, carbohydrate alone, or a combination of protein and carbohydrate consumed immediately and 2 h after a bout of resistance exercise in healthy men. In agreement with findings from our study, testosterone had decreased to values below baseline by 30 min post-exercise during all the supplement treatments compared to placebo. Testosterone values remained significantly below baseline 5–6 h whereas testosterone returned to baseline shortly after exercise and stabilized throughout the recovery period (Chandler *et al*. 1994). In yet another study, Bloomer *et al*. (2000) compared the post-exercise testosterone responses to a mixed meal, an isocaloric beverage of similar nutrient content, and an isocaloric carbohydrate beverage consumed immediately and 2, 4 and 7 h after exercise. Compared to a placebo, post-resistance exercise testosterone levels were lower during all the meals at 0.5, 2.5, 4.5 and 8 h post-exercise. There is one study that did not show a difference in free testosterone responses to resistance exercise between carbohydrate and a water placebo consumed during exercise (Tarpenning *et al*. 2001).

Collectively, these studies indicate that pre- and post-exercise meals decrease post-exercise testosterone levels compared to fasting. The decrease could be due in part to a decrease in the synthesis/secretion of testosterone and/or an increase in metabolic clearance. Chandler *et al*. (1994) showed that the decrease in post-exercise testosterone was not associated with a decrease in LH arguing against a decrease in the rate of testosterone secretion, however there could still be a decrease in the testicular responsiveness to LH. Since post-exercise meals increase muscle-specific protein synthesis during recovery (Tipton *et al*. 1999a, 2001; Rasmussen *et al*. 2000), the lower testosterone levels could be due in part to increased uptake in active skeletal muscle. In support of this hypothesis, recent work in our laboratory has shown that the meal-induced decrease in testosterone after resistance exercise corresponds with an increase in skeletal muscle androgen receptor content measured 60 min after exercise (unpublished observations).

Cortisol

Cortisol is an adrenal steroid hormone that is regulated by pituitary adrenocorticotropin (ACTH), which in turn is under the influence of hypothalamic corticotropin-releasing hormone (CRH). This hypothalamic–pituitary–adrenal (HPA) axis is sensitive to a variety of different stressors including feeding. At the whole body level, cortisol increases protein breakdown but the effects in skeletal muscle are unclear (Rooyackers & Nair 1997). Physiological concentrations of cortisol increase lipolysis in adipose tissue but the effects are less potent than GH (Divertie *et al*. 1991; Djurhuus *et al*. 2002).

POSTPRANDIAL RESPONSE TO FEEDING

The effects of feeding on cortisol are complicated by the fact that cortisol has a distinct diurnal variation and is sensitive to a number of internal and external stressors. In general, feeding has been shown to increase cortisol levels and the response is particularly evident at the noon meal (Follenius *et al*. 1982; Knoll *et al*. 1984), which may serve to synchronize the diurnal rhythm in HPA axis activity (Leal & Moreira 1997). Although few studies have addressed the influence of meal composition on the HPA axis, it appears that protein has the greatest stimulatory effect on cortisol (Slag *et al*. 1981; Ishizuka *et al*. 1983; Gibson *et al*. 1999) whereas carbohydrate feeding inhibits cortisol (Jezova-Repcekova *et al*. 1980).

In healthy men, salivary cortisol was shown to increase substantially after a mid-day protein-rich meal (550 kcal [2299 kJ] with 39% protein) compared to fasting and this response although variable from subject to subject was highly reproducible within subjects (Gibson *et al*. 1999). In healthy women, salivary cortisol was also significantly increased after a mid-day high-protein (630 kcal [2633 kJ] and 32% protein) compared to an isocaloric low-protein (5% protein) meal, which resulted in a decrease in cortisol (Gibson *et al*. 1999). Other studies have shown that meals containing 20–40% protein enhance postprandial cortisol levels compared to meals with high carbohydrate or fat content and that protein-free glucose or fat-rich meals do not stimulate cortisol release (Slag *et al*. 1981; Ishizuka *et al*. 1983). The stimulation of cortisol by protein may be due to specific essential amino acids such as tyrosine and tryptophan (Ishizuka *et al*. 1983).

The findings from the studies mentioned above that protein has a stimulatory effect on cortisol but carbohydrates have a negligible effect appear to be in conflict with other work that has shown glucose to be important in the regulation of the HPA axis. Gonzalez-Bono *et al*. (2002) fed subjects either glucose, protein, fat or water and then 45 min later were exposed to a psychosocial stress test. There were no differences in cortisol levels measured 45 min after the meals, which the authors suggest may have been due to the timing of the meals. However, cortisol levels after the stress test were significantly higher after the glucose load compared to all other trials and the stress-induced cortisol response was positively correlated with glucose changes before the stress test. Since restoration of the stress-induced HPA response after fasting was limited to the glucose load, which was the only trial to increase blood glucose, the authors suggest that HPA responsiveness is under control of centers sensing blood glucose levels, presumably in the hypothalamus (Gonzala-Bono *et al*. 2002).

Part of the discrepancy between the effects of meal composition on postprandial cortisol may be explained by differences in the pattern of body fat distribution. Vicennati *et al*. (2002) recently showed that women with abdominal fat distribution have a higher cortisol response to a high-carbohydrate meal compared to a high-protein/high-fat meal and that women with a peripheral fat distribution demonstrate the opposite effect, which was more in line with the response of a control normal-weight group of women.

Postprandial cortisol measurements must be interpreted in the context of the normal diurnal variation of the hormone. For example, a morning glucose tolerance test results in a decrease in cortisol for several hours after the meal (Walker *et al*. 2000; Reynolds *et al*. 2001, 2003). However, postprandial cortisol values are higher compared to the normal diurnal drop in cortisol in the morning hours (Reynolds *et al*. 2001).

The findings above showing a stimulatory effect of glucose on cortisol secretion are consistent with the findings of Gonzalez-Bono *et al*. (2002) and the notion that the HPA axis is responsive to increases in circulating glucose, but the precise mechanisms regulating this effect remain unresolved. A role of α_1-adrenorecptors modulating ACTH secretion has been proposed to mediate postprandial cortisol responses (Al-Damluji *et al*. 1987). In these experiments, infusion of methoxamine (an α_1-adrenoreceptor agonist) enhanced and thymoxamine (an α_1-adrenoreceptor antagonist) attenuated the ACTH and cortisol response to a standard meal. These authors also showed that patients with a recent pituitary ACTH deficiency and normal adrenal glands showed no ACTH or cortisol rise after a standard meal (Al-Damluji *et al*. 1987). In further support of a

central mechanism, postprandial ACTH and cortisol levels have been shown to be positively correlated after a meal (Vicennati *et al*. 2002). However, not all data point to a hypothalamic–pituitary mediated mechanism to explain postprandial cortisol secretion (Fehm *et al*. 1983). In summary, cortisol tends to increase after feeding, especially protein, but the response is dependent on the timing and composition of the meal, fat distribution pattern and must be compared to the normal diurnal variation of the hormone.

EXERCISE-INDUCED RESPONSES TO FEEDING

A large number of studies in the last 25 years have examined the effects of feeding before, during and after exercise on cortisol responses. The majority of these have examined carbohydrate feeding and a few have studied how different combinations of macronutrients influence cortisol. In terms of studies that measured the cortisol response to resistance exercise, the majority indicate that intake of carbohydrate or carbohydrate combined with protein before and after resistance exercise does not alter the cortisol response compared to placebo (Kraemer *et al*. 1998; Bloomer *et al*. 2000; Koch *et al*. 2001; Williams *et al*. 2002). One study showed that carbohydrate intake during an acute bout of resistance exercise significantly blunted the cortisol response (Tarpenning *et al*. 2001). This study further showed that the reduction in post-resistance exercise cortisol was significantly related to increases in muscle fiber hypertrophy.

Studies that measured the cortisol response to prolonged endurance or high-intensity exercise are generally mixed with several showing that carbohydrate intake before and during exercise results in lower cortisol responses (Mitchell *et al*. 1990, 1998; Murray *et al*. 1991, 1995; Deuster *et al*. 1992; Nieman *et al*. 1998, 2003; Utter *et al*. 1999; Bacurau *et al*. 2002; Bishop *et al*. 2002; Green *et al*. 2003) whereas others have shown no change in cortisol with carbohydrate feeding (Bonen *et al*. 1980; Tsintzas *et al*. 1996; Bishop *et al*. 1999, 2001; Henson *et al*. 2000; Miller *et al*. 2002).

The reduction in the exercise-induced cortisol response after carbohydrate supplementation may contribute to a more favorable immune response to exercise (Nehlsen-Cannarella *et al*. 1997; Henson *et al*. 1998; Nieman 1998). However, the favorable effects of carbohydrate feeding on immune function during and after exercise may not be mediated through a lower cortisol response (Green *et al*. 2003), but rather some other mechanism such as better maintenance of plasma glucose or glutamine levels, an important fuel source for immune cells (Bacurau *et al*. 2002). A recent study showed that carbohydrate intake reduced the plasma interleukin-6 (IL-6) response to exercise, but IL-6 mRNA expression in skeletal muscle was not affected suggesting that carbohydrate feeding during exercise attenuates IL-6 production by tissues other than skeletal muscle (Starkie *et al*. 2001).

Insulin

Insulin is a peptide hormone secreted by the pancreas. In skeletal muscle, insulin has anabolic effects by increasing amino acid uptake and protein synthesis and inhibiting protein breakdown (Rooyackers & Nair 1997). Insulin is generally accepted as a stimulator of protein synthesis only when adequate amino acids are available (Kimball & Jefferson 2002; Kimball *et al*. 2002). Insulin has long been considered the most important regulator of adipose tissue metabolism. Adipose tissue lipolysis is exquisitely sensitive to insulin at physiological concentrations (Jensen *et al*. 1989). Small-to-moderate decreases in insulin can increase lipolysis several-fold, the response being virtually immediate. Insulin also stimulates lipogenesis by increasing glucose uptake and activating lipogenic and glycolytic enzymes (Kersten 2001).

POSTPRANDIAL RESPONSE TO FEEDING

Carbohydrate ingestion leads to an increase in blood glucose and a relatively similar increase in insulin concentrations (Nuttall *et al*. 1985). A meal rich in fat results in lower insulin responses compared to meals rich in either carbohydrate (Nuttall *et al*. 1985) or protein (Ullrich *et al*. 1985). Carbohydrate ingestion results in elevated glucose and insulin levels that depend primarily on the glycemic index or, more precisely, the glycemic load of the food. The

glycemic index was developed in 1981 and refers to the effect of standard amounts of individual foods containing 50 g of available carbohydrate on blood glucose compared with that of a control food (usually white bread or glucose) (Jenkins *et al*. 1981). Comprehensive tables describing the glycemic index of over 1000 foods exist (Foster-Powell *et al*. 2002). To account for differences in the amount of carbohydrate in foods or meals, the concept of glycemic load was introduced, which is the product of glycemic index and carbohydrate amount (Salmeron *et al*. 1997). The glycemic load can predict glucose and insulin responses to individual foods across a wide range of portion sizes (Brand-Miller *et al*. 2003). Certain amino acids can increase insulin and thus carbohydrate combined with protein can enhance the insulin response (Nuttall *et al*. 1984, 1985).

EXERCISE-INDUCED RESPONSES TO FEEDING

Given the synergistic effects of carbohydrate and protein on insulin responses, there has been some interest in combining protein with carbohydrate to maximize insulin secretion in the hopes of enhancing post-exercise glycogen resynthesis (Zawadzki *et al*. 1992; van Loon *et al*. 2000a) and protein anabolism (Rasmussen *et al*. 2000; Tipton *et al*. 2001). In a study designed to determine the optimal insulinotropic mixture of protein and carbohydrate, it was determined that carbohydrate ($0.7\ g{\cdot}kg^{-1}{\cdot}h^{-1}$) consisting of 50% glucose and 50% maltodextrin combined with protein ($0.35\ g{\cdot}kg^{-1}{\cdot}h^{-1}$) consisting of 50% wheat protein hydrolysate, 25% free leucine and 25% free phenylalanine was most effective (van Loon *et al*. 2000b). Although the effects of carbohydrate combined with protein on glycogen resynthesis after resistance exercise are unknown, there does appear to be a beneficial effect of ingesting some protein and/or amino acids in combination with carbohydrate on glycogen resynthesis after submaximal cycling exercise compared to the same amount of carbohydrate only (Zawadzki *et al*. 1992; van Loon *et al*. 2000a). This effect is likely due to greater insulin secretion after combined carbohydrate and protein intake although it is pointed out that when carbohydrate intake is very high ($1.2\ g{\cdot}kg^{-1}{\cdot}h^{-1}$), additional protein does not further enhance the rate of glycogen resynthesis (Jentjens *et al*. 2001). Enhanced insulin levels resulting from carbohydrate combined with protein could be expected to have a favorable effect on net protein balance because insulin is generally accepted as a stimulator of protein synthesis when adequate amino acids are available (Kimball & Jefferson 2002; Kimball *et al*. 2002).

Implications of diet-induced hormonal changes on body composition

A goal of many athletes and individuals in general is to improve body composition, that is, to decrease adipose tissue (fat mass) and/or increase skeletal muscle tissue (lean body mass). Hormones are major regulators of protein turnover in skeletal muscle and triacylglycerol turnover in adipose tissue. As discussed above, quantity, quality and timing of dietary intake modulates many of the hormones that regulate protein and triacylglycerol turnover. Thus, from a theoretical perspective feeding can be viewed as a strategy to alter the hormonal environment to alter protein and lipid balance, which over time could lead to decreased fat mass and/or increased lean body mass.

Predicting the impact of a defined meal on protein and lipid balance and eventually body composition is however quite complex. Consider that most hormones, at least those discussed in this article, affect both protein and lipid balance through multiple mechanisms (Fig. 29.2), and that unlike the situation *in vitro*, hormones change *in vivo* simultaneously in response to feeding. Thus, the interactions among various hormones determine the overall response in both skeletal muscle and adipose tissue. Because of these multiple interactions, translation of the information known about postprandial and exercise-induced hormonal responses into specific dietary recommendations aimed at improving body composition is a complicated task. Nutritional strategies during the time periods before and after exercise have been particularly studied and shown to have relatively predictable affects on hormones and protein and lipid metabolism (Volek 2004), so this is an appropriate starting point for discussing the

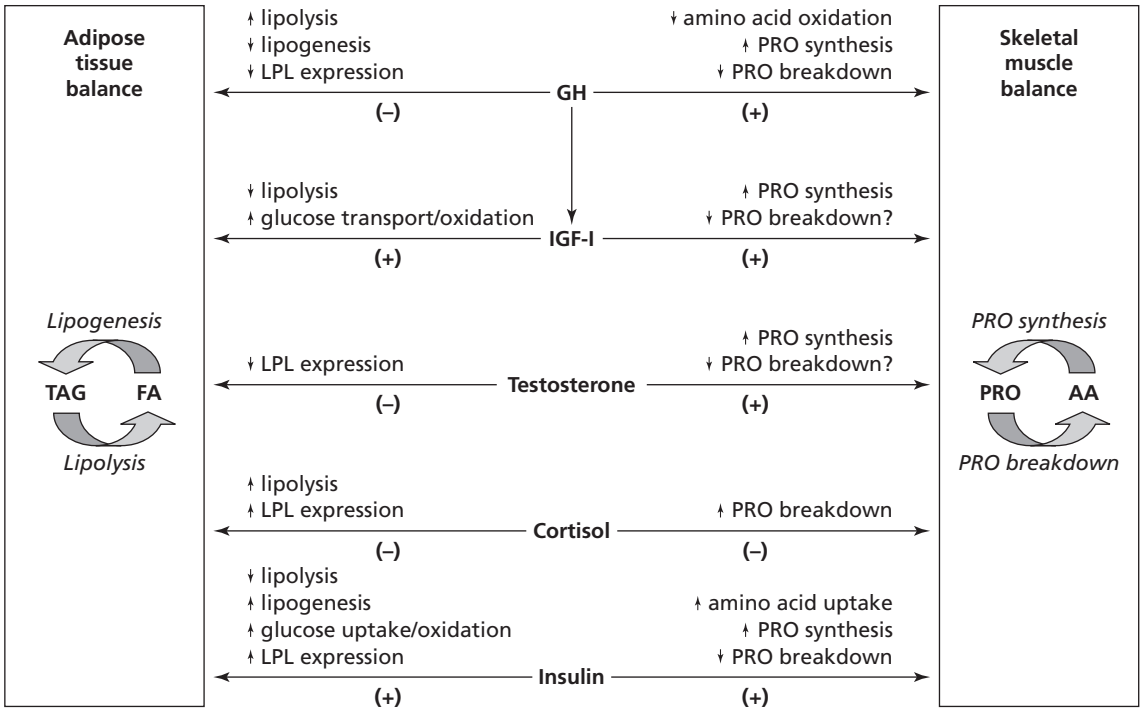

Fig. 29.2 Hormonal regulation of body composition. Several hormones including growth hormone (GH), insulin-like growth factor I (IGF-I) and its binding proteins, testosterone, cortisol and insulin regulate both skeletal muscle protein (PRO) and adipose tissue balance through multiple mechanisms. Feeding affects the postprandial and exercise-induced concentrations of these hormones, which regulate PRO and amino acid (AA) cycling (i.e. protein synthesis/breakdown) in skeletal muscle and triacylglycerol (TAG) and fatty acid (FA) cycling (i.e. lipogenesis/lipolysis) in adipose tissue. These hormones do not change in isolation, rather the simultaneous interaction among hormones must be considered in terms of their ultimate impact on protein and lipid balance and lean body mass and fat mass.

implications of diet-induced hormonal responses. Theoretically, increases in GH, IGF-I and testosterone would all have favorable effects on skeletal muscle and adipose tissue balance; that is, elevations in these hormones would contribute to increased lean body mass and decreased fat mass. Elevated cortisol would be negative in terms of skeletal muscle but might contribute to enhanced lipolysis. In contrast, elevated insulin would have positive effects on skeletal muscle and negative effects on lipid balance.

Nutrients, hormones, and skeletal muscle protein balance

Exercise, particularly resistance training, stimulates protein synthesis and protein breakdown, the balance of which determines the anabolic response of muscle to resistance exercise. Quantity, quality and timing of dietary intake after exercise influences nutrient and hormone availability at specific receptors at target tissues (i.e. skeletal muscle and adipose tissue). The contraction-induced mechanical and chemical events in muscle interact with nutrient and hormonal signals to regulate enzymes (e.g. glycogen synthase) and mediate gene level transcription and translation of proteins (Turner *et al*. 1988). Exercise also results in increased blood flow to the active skeletal muscles, which has important implications for pre-exercise meals that could enhance hormone interactions and the delivery of nutrients to target receptors during and after exer-

cise. The combined effect of muscular contraction and the increased availability of hormones and nutrients have the potential to enhance the rate of amino acid and glucose uptake, and promote an anabolic environment. Nutrient availability is critical during this time as evidenced by studies showing little glycogen resynthesis (Pascoe *et al*. 1993; Roy & Tarnopolsky 1998) and a negative protein balance (Biolo *et al*. 1995, 1997) in the absence of nutritional intake after exercise. An anabolic nutritional and hormonal milieu favorably affects the balance of protein synthesis/degradation, which sets the stage for greater protein accretion and muscle fiber hypertrophy with chronic resistance training. The resulting increased force production capabilities can improve the intensity of subsequent workouts and further enhance the resistance exercise stimuli in a feedforward fashion. Eventually, the repeated exposures to resistance exercise workouts will lead to measurable increases in muscle strength and size.

Feeding has been shown to be a simple and effective method to alter rates of protein synthesis (Svanberg *et al*. 2000). Infusion of amino acids or exogenous administration of amino acids with or without carbohydrate stimulates protein synthesis after exercise (Bennet *et al*. 1989; Biolo *et al*. 1997; Tipton *et al*. 1999a, 1999b; Rasmussen *et al*. 2000). Carbohydrate intake after resistance exercise decreases measures of protein breakdown and slightly increases fractional muscle protein synthetic rate, which is likely due primarily to increases in insulin (Roy *et al*. 1997). Protein synthesis was stimulated ~ 400% above pre-exercise values when a protein and carbohydrate supplement (6 g essential amino acids and 35 g sucrose) was consumed 1 or 3 h after resistance exercise (Rasmussen *et al*. 2000). Consumption of this same protein and carbohydrate supplement immediately before exercise resulted in increased amino acid delivery to muscle and even greater net muscle protein synthesis (Tipton *et al*. 2001). Essential amino acids have been shown to be primary regulators of muscle protein synthesis with little contribution from non-essential amino acids (Smith, K. *et al*. 1998; Tipton *et al*. 1999a, 1999b). The branched-chain amino acids, particularly leucine, appear to be the most important stimulators of skeletal muscle protein synthesis (Kimball & Jefferson 2002). Recent work indicates that it is the extracellular levels of essential amino acids in the blood that regulate muscle protein synthesis as opposed to intramuscular amino acids (Bohe *et al*. 2003). Whether these acute changes in skeletal muscle protein metabolism reflect chronic changes in lean body mass is unknown.

A recent study in elderly men investigated the effect of timing of protein and carbohydrate supplementation on muscle size and strength responses to 12 week of resistance training (Esmarck *et al*. 2001). The supplement (10 g protein, 7 g carbohydrate) was consumed immediately or 2 h after each training session. The group who ingested the supplement immediately after exercise had significantly greater increases in lean body mass, muscle fiber area and quadriceps femoris area. These data indicate that altering the timing of calories, which affects the nutrient and hormonal milieu, can impact chronic adaptation to training. Specifically, early intake of protein and carbohydrate after a workout is more effective at increasing skeletal muscle hypertrophy and lean body mass than a supplement consumed later. These findings are in conflict with a study that showed no differences in acute measures of protein balance when protein was ingested 1 or 3 h after exercise in healthy young subjects (Rasmussen *et al*. 2000). This apparent discrepancy related to timing of protein ingestion highlights the importance of linking acute studies that measure protein kinetics to long-term training studies that assess outcome measures related to muscle size. The only study to date that has done this is work showing that carbohydrate intake during resistance exercise was an effective strategy to lower cortisol levels and that the magnitude of cortisol reduction was directly related to measures of muscle fiber hypertrophy (Tarpenning *et al*. 2001).

Practically no work has examined how acute diet-induced changes in testosterone, GH or IGF-I might contribute to the acute changes in protein balance and chronic changes in lean body mass. Our laboratory has discovered that circulating testosterone is consistently modulated by nutrient intake and that a mixed meal alters post-exercise skeletal muscle androgen content, indicating a possible link

between diet, testosterone and skeletal muscle protein metabolism.

In summary, there appears to be an interaction between increased availability of amino acids, increased insulin and possibly other hormones after exercise and the timing of supplement ingestion (i.e. immediately before exercise) may be important to maximize the anabolic response (Esmarck *et al*. 2001; Tipton *et al*. 2001). Consumption of a protein and carbohydrate supplement at times around exercise (i.e. immediately before and immediately after exercise) may provide the ideal anabolic situation for muscle growth. There does not appear to be any value in including fat in the pre- or post-exercise meals from a hormonal or metabolic perspective.

Nutrients, hormones and adipose tissue triacylglycerol balance

Many of the studies designed to examine how feeding affects hormonal responses to exercise have focused on enhancing protein balance or stimulating glycogen formation with little attention directed to the impact on adipose tissue lipid metabolism. High-carbohydrate intakes are often encouraged to stimulate glycogen synthase and glycogen formation and even protein balance after exercise. The hormonal environment after carbohydrate feeding, dominated by a surge in insulin, would inhibit lipolysis and potentially stimulate lipogenesis in adipose tissue, a scenario not desirable if fat loss is a major goal. A better alternative would be to focus on high quality protein with all the essential amino acids and perhaps low-glycemic carbohydrates during the post-exercise period to stimulate protein synthesis while keeping insulin low to prevent inhibition of lipolysis. Protein intake after exercise may have a slight effect on enhancing GH, which would further support lipolysis and decrease lipogenesis. Subsequent meals throughout the day should focus on foods with a low-to-moderate glycemic load to keep insulin levels low. Since the addition of protein and fat to a meal slow the pace of digestion and lower the glycemic load, including quality sources of protein and healthy unsaturated fat should also be a priority.

The popularity of diets that restrict carbohydrate have increased dramatically in recent years in part because they are advertised to be more effective in promoting weight and fat loss. There were several studies performed in the 1960s and 1970s that showed greater weight loss with a very low-carbohydrate compared to a low-fat diet, even when diets contained the same energy content (Rabast *et al*. 1979), suggesting a metabolic advantage (i.e. a greater weight loss if carbohydrates are low compared to isoenergetic diets of different macronutrient composition). Very little follow-up work was done until recently, as evidenced by several publications in 2003 again showing greater weight loss with very low-carbohydrate diets 3–6 months in duration (Brehm *et al*. 2003; Foster *et al*. 2003; Samaha *et al*. 2003; Sondike *et al*. 2003). Some early reports show that very low-carbohydrate diets result in preferential loss of fat and preservation of lean body mass (Benoit *et al*. 1965; Young *et al*. 1971; Willi *et al*. 1998; Meckling *et al*. 2002), suggestive of a nutrient partitioning effect. In accordance with this notion, we recently reported that a free-living 6-week very low-carbohydrate diet resulted in significant decreases in fat mass and increases in lean body mass in normal-weight men (Volek *et al*. 2002). In a follow-up study we showed that a very low-carbohydrate diet resulted in twofold greater whole body fat loss and threefold greater fat loss in the trunk region compared to a low-fat diet (Volek *et al*. 2004). Carbohydrate restricted diets remain controversial (Blackburn *et al*. 2001; Freedman *et al*. 2001), yet evidence indicates they are very effective in the short-term and are not associated with any adverse effects (Volek *et al*. 2000, 2003; Sharman *et al*. 2002; Volek & Westman 2002).

Very low-carbohydrate diets are low glycemic load diets. Although the mechanisms by which very low-carbohydrate diets benefit weight and fat loss has not elucidated, a reduction in insulin is probably important in explaining a portion of the greater fat loss (Volek *et al*. 2002). Inhibition of lipolysis occurs at relatively low concentrations of insulin with a half-maximal effect occurring at a concentration of 12 $pmol \cdot L^{-1}$ and a maximal effect at a concentration of about 200–300 $pmol \cdot L^{-1}$ (Jensen *et al*. 1989). Thus, even small reductions in insulin may be permissive to mobilization of body fat on a low glycemic diet. A

metabolic advantage, possibly driven by increased protein turnover to supply alanine for gluconeogenesis, is another plausible hypothesis to explain greater weight loss on very low-carbohydrate diets (Feinman & Fine 2004). Although the benefits of following a low glycemic load diet or a low-carbohydrate, moderate protein and fat diet on fat loss look promising, the ideal diet for fat loss and general health remains controversial. However, overwhelming evidence indicates controlling insulin levels through diet is important.

Summary

It is clear that feeding and meal composition significantly alter postprandial and post-exercise hormonal responses. Yet the importance of acute and chronic effects of these hormonal changes on skeletal muscle and adipose tissue balance and, ultimately, body composition remain largely unknown. Given the important regulatory functions of hormones on muscle and lipid balance, the influence of diet on endocrine function is arguably of considerable importance. Optimizing the hormonal environment in favor of an anabolic profile during the recovery period between exercise sessions would be advantageous for individuals attempting to maximize gains in muscle size and strength. Specifically, by elevating the primary anabolic hormones involved in muscle tissue growth (i.e. testosterone, GH, insulin and IGF-I) and/or decreasing major catabolic hormones (i.e. cortisol) a hormonal milieu maximizing protein balance and muscle hypertrophy would be created. If fat loss is a goal, then it would be advantageous to keep insulin levels low and GH and testosterone levels elevated. From a hormonal perspective, meals with a low glycemic load with moderate amounts of quality protein and healthy unsaturated fat would be conducive for fat loss and general health.

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Chapter 30

Neurohumoral Responses and Adaptations During Rest and Exercise at Altitude

BETH A. BEIDLEMAN, JANET E. STAAB AND ELLEN L. GLICKMAN

Introduction

Upon arrival at high altitude, rapid physiologic adjustments occur to compensate for the reduction in ambient oxygen (O_2). Immediate physiologic adjustments, which include an increase in ventilation and heart rate, provide a rapid first line of defense against the reduced ambient O_2 (Stenberg *et al*. 1966; Easton *et al*. 1986). Longer term physiologic adaptations, which include continued hyperventilation, increased production of RBCs, changes in circulation, increased water and sodium excretion, and shifts in substrate utilization, provide a second line of defense against the sustained hypoxic environment (Young, A.J. & Reeves 2002). The autonomic nervous system plays a critical role in mediating many of the immediate physiologic adjustments to altitude but hormonal alterations play a role in fine-tuning many of the body's longer-term adaptations to altitude.

Although several reviews have examined the neurohumoral response to altitude (Hoyt & Honig 1996; Ward *et al*. 2000; Richalet 2001; Swenson 2001; Mazzeo & Reeves 2003), a clear consensus has not been reached regarding the role that hormones play in successfully adapting to altitude. Conflicting results in the literature are primarily due to differences in study design (field versus chamber studies), time point of measurements during exposure (acute versus chronic), altitude (low versus high elevation), environmental conditions (normobaric hypoxia versus hypobaric hypoxia), gender, (male versus female), physical fitness levels (trained versus untrained), duration of ascent (rapid versus slow) and illness status (sick versus non-sick). This review therefore will focus on summarizing the results of numerous studies that were conducted under a defined set of conditions in order to develop a clearer understanding of the neurohumoral responses and adaptations during rest and exercise at altitude.

The studies included in this review were as follows: (a) both chamber and field studies; (b) studies employing hypobaric hypoxia; (c) altitudes ranging from 3500 to 5500 m; (d) both men and women; (e) both physically trained and untrained; (f) both rapid and slow ascent; and (g) if the results of sick and non-sick subjects were separated, only results from non-sick subjects. Acute altitude exposure was defined as < = 3 days and chronic altitude exposure was defined as 4–21 days of altitude exposure. This review will also focus on human studies unless critical points can only be explained by including animal work.

Basic concepts

In order to understand the neurohumoral mechanisms responsible for adaptation to altitude, it is imperative to first understand the basic concepts associated with exposure to altitude. First, barometric pressure (P_B) decreases with increasing altitude (Fig. 30.1). Although the percentage of O_2 in a given volume of air at altitude is the same as at sea level (i.e. 20.93%), the ambient partial pressure of O_2 (Po_2) decreases ($Po_2 = P_B \times \%\ O_2$). Second, the inspired oxygen pressure (P_Io_2) is diminished in direct proportion to the reduction in barometric pressure ($P_Io_2 = (P_B - 47) \times \%O_2$), where the P_Ho_2 is taken to

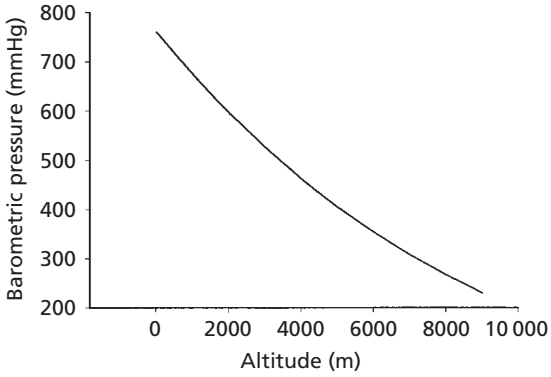


Fig. 30.1 The relationship between barometric pressure (P_B) and altitude (m).

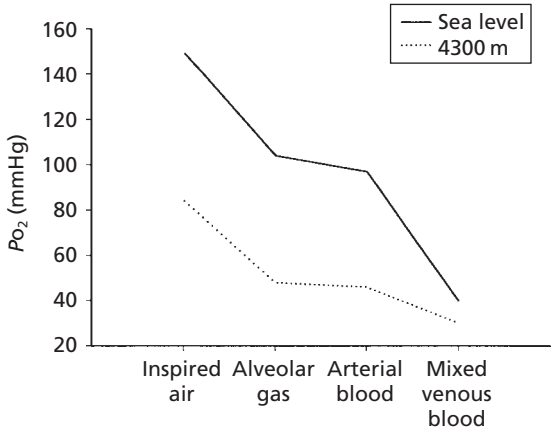


Fig. 30.2 The oxygen transport cascade at sea level and 4300 m. (Modified from Fulco & Cymerman 1988.)

be 47 Torr at a body temperature of 37°C. Thus, the O_2 pressure gradient at each step in the O_2 transport cascade is reduced upon exposure to altitude (Fig. 30.2). As a result, less O_2 is transferred from the environment to the cells as the diffusion of O_2 is directly dependent on the Po_2 gradient, according to Fick's law of diffusion. The result is an impaired ability to deliver O_2 to the tissues of the body upon exposure to altitude. Numerous short- and long-term physiologic adaptations occur to compensate for this hypoxemia.

Physiologic adaptations to altitude

Ventilatory

Upon acute exposure to altitude, there is an immediate increase in ventilation that is dependent on the severity of hypoxia (Rahn & Otis 1949; Easton *et al*. 1986; Bisgard & Forster 1996). This increase in ventilation is primarily the result of hypoxic stimulation of the peripheral chemoreceptors (Weil *et al*. 1970; Lahiri & Delaney 1975; Fitzgerald & Dehghani 1982). Immediately following this increase, ventilation declines over the next 10–30 min of altitude exposure. This response has been termed 'hypoxic ventilatory depression' and is likely due to build up of inhibitory transmitters such as γ-aminobutyric acid or adenosine in the central nervous system (Smith *et al*. 2001). Within the next few hours to days of altitude exposure, there is a progressive increase in ventilation that tends to level off by 7–10 days of altitude exposure (Forster *et al*. 1975). The mechanisms involved in ventilatory acclimatization to altitude are highly debated, but thought to be due to a progressive increase in carotid body sensitivity and central nervous system acid-base changes (Severinghaus *et al*. 1963; Bisgard & Forster 1996; Smith *et al*. 2001). The increase in ventilation with initial and continued altitude exposure mitigates the obligatory fall in partial pressure of alveolar O_2 (P_Ao_2) that follows a reduction in P_Io_2. In fact, if compensatory hyperventilation did not occur, the alveolar gas equation predicts that P_Ao_2 would be 10 Torr lower at 4000 m. This increase in P_Ao_2 with hyperventilation facilitates O_2 loading in the lungs and provides a rapid and continued first line of defense against the reduced ambient Po_2.

Hematologic

Erythropoietin (described below) increases upon acute exposure to altitude, which stimulates the hematological cascade (Jelkmann 1992). Reticulocytes typically appear within 3–5 days (Grover *et al*. 1998; Reeves *et al*. 2001) but red blood cell (RBC) volume remains unchanged following 10 days of altitude exposure (Sawka *et al*. 1996). Although hemoglobin concentration [Hb] clearly increases in

lowlanders sojourning at altitudes for 5–12 days (Maher *et al*. 1974; Horstman *et al*. 1980; Wolfel *et al*. 1991, 1998), this increase in [Hb] is most likely due to hemoconcentration (i.e. decrease in plasma volume) rather than erythrocyte volume expansion (Sawka *et al*. 2000). If the altitude sojourn exceeds 1 month, erythrocyte volume expansion occurs (Reynafarje 1957; Pugh 1964). The higher RBC volumes observed in high altitude natives compared to lowlanders also supports increased RBC production following an extended period of time at altitude (Weil *et al*. 1968; Sanchez *et al*. 1970). The increase in RBC volume and thus Hb, which is the compound responsible for binding O_2, ultimately improves O_2 transport capacity at altitude.

Cardiovascular

Heart rate and cardiac output are increased immediately upon ascent to altitude (Stenberg *et al*. 1966; Vogel & Harris, 1967; Wagner *et al*. 1986). The abrupt increase in cardiac output is due to an increase in heart rate, since stroke volume remains unchanged (Grover *et al*. 1986; Wolfel & Levine 2001). Sympathetic stimulation and parasympathetic withdrawal (described below) both contribute to this increase in heart rate. Normally, sympathetic stimulation would also elicit peripheral vasoconstriction. However, acute hypoxia overrides sympathetically-mediated vasoconstriction and induces vasodilation in all vascular beds except the lung such that total peripheral resistance is decreased (Wolfel & Levine 2001). The specific local vasodilator responsible for overriding vasoconstriction is not known, but nitric oxide and epinephrine have been suggested as potential candidates (Halliwill 2003). Mixed-venous O_2 content also decreases with acute altitude exposure, which indicates increased peripheral O_2 extraction (Wolfel *et al*. 1991, 1998). Thus, increases in both blood flow and peripheral O_2 extraction upon acute exposure to altitude maintain a tight coupling between metabolic supply and demand under limited O_2 conditions.

With chronic altitude exposure, arterial O_2 content (C_aO_2), which is the product of arterial O_2 saturation (S_aO_2) and [Hb] multiplied by 1.34 mL $O_2 \cdot g\,[Hb]^{-1}$, is increased due to ventilatory and hematological adaptations (Wolfel *et al*. 1991, 1998). Stroke volume decreases due to a decrease in plasma volume and/or increase in total peripheral resistance while heart rate remains elevated (Grover *et al*. 1986; Wolfel & Levine 2001; Young, A.J. & Reeves 2002). Cardiac output declines following chronic altitude exposure because of the decrease in stroke volume (Grover *et al*. 1986; Wolfel & Levine 2001; Young, A.J. & Reeves 2002). The decline in cardiac output offsets the increase in C_aO_2 such that O_2 delivery remains unchanged following chronic altitude exposure (Grover *et al*. 1976; Bender *et al*. 1988). Although a declining blood flow and cardiac output would not appear beneficial, the prolonged capillary transit time may minimize the alveolar–arterial diffusion limitation at altitude such that S_aO_2 is not compromised at the expense of increased blood flow (Boushel *et al*. 2001).

Fluid regulation

Water and sodium excretion are increased in normally acclimatizing lowlanders with both acute and chronic exposure to altitude (Hoyt & Honig 1996). This natriureis and diuresis elicits a depletion of plasma volume and increase in [Hb] due to hemoconcentration (Sawka *et al*. 2000), which counterbalances the reduced supply of O_2 to the tissues. The diuresis may also protect against altitude illness (Singh *et al*. 1969) due to a decrease in extracellular fluid volume and brain edema (Hackett 1999). The mechanisms underlying the natriuretic and diuretic effect of hypoxia are not well understood, but suggest that hypoxic stimulation of the peripheral chemoreceptors results in decreased reabsorption of renal tubular sodium (Honig 1989). Tissue hypoxia may also dilate renal vessels directly (Halliwill 2003), which would increase the glomerular filtration rate (Olsen *et al*. 1993) and thus sodium and water excretion. Several hormones may also play a key role in the fluid regulation at high altitude, since the natriuretic and diuretic response is not abolished by renal nerve section (Schmidt *et al*. 1985; Karim *et al*. 1987). In fact, after adrenalectomy, the striking increase in urinary sodium excretion on exposure to hypobaric hypoxia is not observed (Lewis *et al*. 1942).

Metabolic

Upon acute exposure to altitude, carbohydrates may be the preferred fuel since they have a high yield of adenosine triphosphate (ATP) per liter of O_2 consumed (Brooks *et al*. 1991; Roberts *et al*. 1996b). In support of this hypothesis, hypoxia inducible factor-1α (HIF-1α), which is known to increase the transcription of several genes involved in glucose uptake and glycolysis, is up-regulated upon acute exposure to hypoxia (Semenza *et al*. 1994; Firth *et al*. 1995). In addition, lactate accumulation is increased during both rest and exercise upon acute exposure to altitude, which has been attributed to accelerated glycolytic metabolism (Knuttgen & Saltin 1973). However, no differences in muscle glycogen utilization have been observed during exercise upon acute exposure to exercise compared to sea level (Young, A.J. *et al*. 1982; Green *et al*. 1992). With chronic altitude exposure, both increased (Brooks *et al*. 1991; Roberts *et al*. 1996b) and decreased (Young, A.J. *et al*. 1982, 1991; Beidleman *et al*. 1997; Braun *et al*. 2000) utilization of carbohydrates have been reported. However, lactate accumulation is consistently depressed despite a similar O_2 delivery following chronic altitude exposure (Young, A.J. *et al*. 1982; Brooks *et al*. 1991; Beidleman *et al*. 1997). This has been termed the 'lactate paradox' and has been attributed to decreased β-adrenergic stimulation of muscle glycogenolysis (Mazzeo & Reeves 2003).

Acute mountain sickness

Acute mountain sickness (AMS) is a syndrome induced by hypoxia in unacclimatized individuals who ascend rapidly to altitudes exceeding 2500 m and remain there for more than several hours (Singh *et al*. 1969; Hackett & Rennie 1976; Honigman *et al*. 1993). Characteristic symptoms include headache, nausea, vomiting, loss of appetite, fatigue, dizziness and sleep disturbances (Hackett & Rennie 1976; Powles *et al*. 1978; Honigman *et al*. 1993; Sanchez del Rio & Moskowitz 1999; Bailey *et al*. 2000). The onset of AMS usually occurs 4–12 h after ascent, and symptoms become most prominent after the 1st night spent at high altitude (Bärtsch *et al*. 1988). The incidence of AMS increases with altitude; the greater the hypoxemia, the more severe the illness (Maggiorini *et al*. 1990; Honigman *et al*. 1993). Individual AMS susceptibility can also be affected by the rate of ascent (Hansen *et al*. 1967; Honigman *et al*. 1993), prior acclimatization (Hansen *et al*. 1967; Lyons *et al*. 1995), obesity (Honigman *et al*. 1993) and level of exertion to reach a given altitude (Roach *et al*. 2000).

The pathophysiology of AMS is highly debated but in general is thought to be a vasogenic-induced cerebral edema, triggered by the initial hypoxic stimulus (Hackett *et al*. 1998). Vasogenic edema could be the result of increased hydrostatic pressure across blood vessels due to an increase in cerebral blood flow or an altered permeability of the blood–brain barrier. Jensen *et al*. (1990) reported no increase in cerebral blood flow in subjects suffering from AMS. However, increased expression of vascular endothelial growth factor has been reported in humans (Walter *et al*. 2001) and animals (Xu & Severinghaus 1998) exposed to hypoxia or hypobaric hypoxia, which would cause a generalized increased capillary leakage of large molecules due to endothelial activation in forming new blood vessels.

Resolution of AMS usually occurs following 1–2 days of high altitude residence if no further ascent is attempted (Singh *et al*. 1969; Bärtsch *et al*. 1988). Physiological changes that may contribute to this resolution of AMS include an increase in ventilation and diuresis, which can occur over the same few days of altitude residence (Singh *et al*. 1969; Hackett *et al*. 1982; Moore *et al*. 1986; Bärtsch *et al*. 1988). The increase in ventilation causes an increase in P_aO_2, which reduces the initial hypoxic stimulus. An increased diuresis also helps to reduce symptoms of AMS (Singh *et al*. 1969) via a reduction in extracellular fluid volume, which lessens the magnitude of cerebral edema, the causative factor for AMS (Hackett 1999).

Maximal exercise performance

According to the Fick equation, O_2 uptake is the product of cardiac output (heart rate × stroke volume) and the difference in C_aO_2 and venous O_2 content (C_vO_2). Maximal C_aO_2 is decreased upon acute exposure to altitude due to the reduction in ambient

P_{O_2} and corresponding reduction in S_aO_2 (Wolfel & Levine 2001). During maximal exercise at altitude, the body is unable to increase maximal ventilation, heart rate and tissue O_2 extraction beyond maximal values obtained at sea level to compensate for the reduction in S_aO_2 (Stenberg *et al*. 1966; Grover *et al*. 1986). Thus, maximal O_2 uptake ($\dot{V}O_{2max}$) is reduced in direct proportion to the decrease in maximal C_aO_2 upon acute exposure to altitude (Stenberg *et al*. 1966; Vogel *et al*. 1967).

With chronic altitude exposure, maximal C_aO_2 is increased compared to initial ascent due to increases in both S_aO_2 and [Hb] (Wolfel & Levine 2001). Therefore, one would expect an increase in $\dot{V}O_{2max}$ However, research has repeatedly shown that $\dot{V}O_{2max}$ does not change following chronic altitude exposure (Saltin *et al*. 1968; Young, A.J. *et al*. 1982; Bender *et al*. 1988; Wolfel *et al*. 1991, 1998; Beidleman *et al*. 1997). The reason for an unchanged $\dot{V}O_{2max}$ is that maximal cardiac output decreases following chronic altitude exposure due to a decrease in both maximal heart rate and stroke volume, which offsets the increase in maximal C_aO_2 (Grover *et al*. 1986; Wolfel & Levine 2001). Thus, maximal O_2 delivery remains the same upon acute and chronic altitude exposure (Grover *et al*. 1986).

Submaximal exercise performance

Oxygen uptake at rest or during submaximal fixed workloads at altitude does not change from sea level values (Reeves *et al*. 1967; Saltin *et al*. 1968; Raynaud *et al*. 1981). However, since $\dot{V}O_{2max}$ is decreased at altitude, the relative exercise intensity during a fixed submaximal workload can be greatly increased compared to that at sea level (Fig. 30.3). In this example, the same submaximal O_2 uptake (2 L·min^{-1}) represents 50% of a subject's $\dot{V}O_{2max}$ at sea level and 70% of a subject's $\dot{V}O_{2max}$ at altitude. If a fixed submaximal workload is maintained upon acute exposure to altitude, then ventilation, heart rate and tissue O_2 extraction will be increased and submaximal exercise performance will be decreased (Ekblom *et al*. 1975; Fulco *et al*. 2003). If the submaximal workload is adjusted such that an individual is working at the same relative percentage of altitude-specific $\dot{V}O_{2max}$, then ventilation, heart rate, tissue O_2 extraction and submaximal exercise performance will be similar at both sea level and during acute exposure to altitude (Maher *et al*. 1974; Horstman *et al*. 1980).

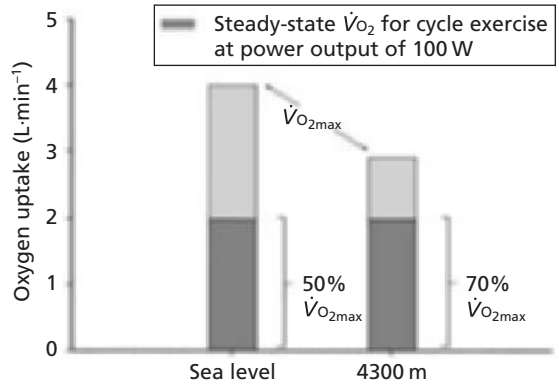


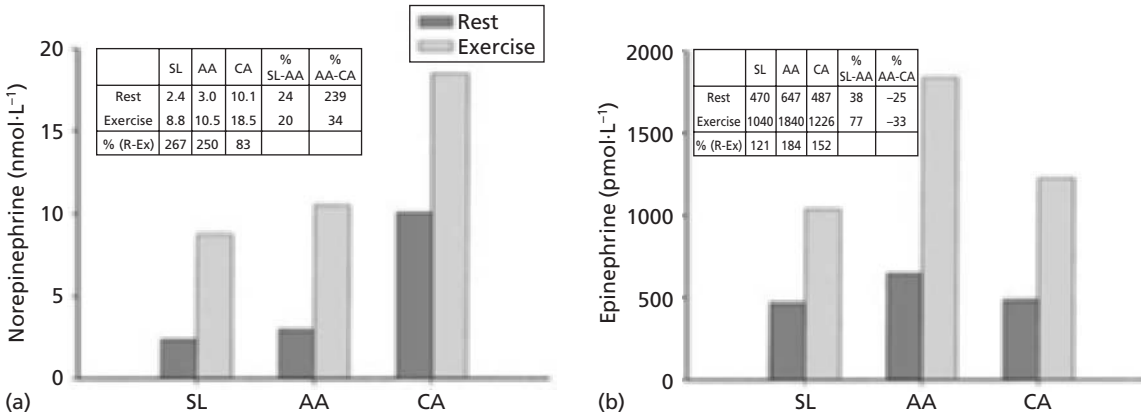
Fig. 30.3 Effect of high altitude (4300 m) on the relationship between absolute work load, oxygen uptake ($\dot{V}O_2$), and relative exercise intensity (% maximal oxygen uptake, $\dot{V}O_{2max}$). (Modified from Young, A.J. & Young 1988.)

With chronic altitude exposure, both small and large muscular endurance performance is improved, even when conducted at the same relative percentage of altitude-specific $\dot{V}O_{2max}$ (Maher *et al*. 1974; Horstman *et al*. 1980; Fulco *et al*. 1994). Although not entirely understood, likely explanations for this improvement include a greater pulmonary diffusion time due to a decrease in blood flow (Boushel *et al*. 2001), a greater O_2 diffusion gradient from the muscle capillary to the mitochondria (Fulco *et al*. 1994; Beidleman *et al*. 2003) and sparing of muscle glycogen due to an increase in blood glucose utilization (Brooks *et al*. 1991; Roberts *et al*. 1996a). Furthermore, AMS symptomatology is decreased following chronic altitude exposure which in and of itself may contribute to improvements in submaximal exercise performance.

Neurohumoral responses to altitude

Sympathoadrenal response

Plasma levels of norepinephrine (NE) are a function of the rate of spillover into the circulation from sympathetic neuronal release (~ 70%), a small amount



	SL	AA	CA	% SL-AA	% AA-CA
Rest	2.4	3.0	10.1	24	239
Exercise	8.8	10.5	18.5	20	34
% (R-Ex)	267	250	83		

	SL	AA	CA	% SL-AA	% AA-CA
Rest	470	647	487	38	–25
Exercise	1040	1840	1226	77	–33
% (R-Ex)	121	184	152		

Fig. 30.4 Summarized results from 30 studies measuring (a) norepinephrine and (b) epinephrine levels during both rest and exercise during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure (Kotchen *et al*. 1973b; Fulco *et al*. 1985; Bärtsch *et al*. 1988, 1991a; Bouissou *et al*. 1988, 1989; Férézou *et al*. 1988, 1993; Kjær *et al*. 1988; Richalet *et al*. 1988; Knudtzon *et al*. 1989b; Mazzeo *et al*. 1991, 1995, 2000, 2001, 2003; Young, A.J. *et al*. 1991, Olsen *et al*. 1992; Ramirez *et al*. 1992; Young, P.M. *et al*. 1992; Loeppky *et al*. 1993; Antezana *et al*. 1995; Larsen *et al*. 1997; Rostrup 1998; Sevre *et al*. 2001; Basu *et al*. 2002; Beidleman *et al*. 2002; Bestle *et al*. 2002; Hasbak *et al*. 2002; Lundby & Steensberg 2004).

secreted by the adrenal medulla (~ 30%), and its rate of removal from the plasma pool. Upon acute exposure to normobaric hypoxia or hypobaric hypoxia, numerous studies have reported an immediate increase in sympathetic nervous system (SNS) activity to skeletal muscle (Rowell *et al*. 1989; Leuenberger *et al*. 1991; Morgan *et al*. 1995) that remains elevated following chronic altitude exposure (Hansen & Sander 2003). Although plasma NE levels do not always track SNS activity (Rowell *et al*. 1989), summarized results from 30 studies also suggest that there is a 24% increase in resting NE levels upon acute altitude exposure that increases a further 240% from acute to chronic altitude exposure (Fig. 30.4a). The mechanism for the initial and continued elevation in SNS activity in hypoxia is not well known, but is unlikely due to the direct effect of hypoxia since SNS activity increases with time at altitude, while hypoxemia decreases with time at altitude (Mazzeo & Reeves 2003). It appears that hypoxia may cause sympathetic activation directly via stimulation of the peripheral chemoreceptors (Marshall 1994) and brainstem neuronal pools (Reis *et al*. 1994). In addition, unloading of cardiopulmonary baroreceptors could be involved due to a decrease in blood volume occurring over time at altitude (Myhre *et al*. 1970; Jung *et al*. 1971; Jain *et al*. 1980).

Similar to exercise at sea level, NE levels increase one to threefold with exercise during acute and chronic altitude exposure (Mazzeo *et al*. 1991, 1995, 2003; Young, A.J. *et al*. 1991), and the magnitude of the response is dependent on the intensity of exercise (Kjær *et al*. 1988; Braun *et al*. 2000; Mazzeo *et al*. 2000; Lundby & Steensberg 2004). Summarized results suggest that the percentage of increase in NE during exercise from resting values is lower following chronic altitude exposure (~ 83%) compared to acute altitude exposure (~ 250%) and sea level (~ 267%). However, NE levels reach higher absolute levels during exercise with acute and chronic altitude exposure due to higher initial starting levels (Fig. 30.4a).

The immediate increase in SNS activity upon acute exposure to altitude likely plays a role in counterbalancing the effects of hypoxia-induced vasodilation on most vascular beds so that blood pressure can be maintained (Wolfel & Levine 2001). The rising NE levels during both rest and exercise following chronic altitude exposure results in increased arterial and venous tone, increased systemic vascular resistance, decreased plasma volume

and decreased cardiac output (Seals *et al*. 2001; Mazzeo & Reeves 2003). These physiologic adaptations serve to maintain a tight coupling between metabolic supply and demand by decreasing blood flow as arterial O_2 delivery is improved following chronic altitude exposure.

Sympathetic stimulation of the adrenal gland upon acute altitude exposure also causes an increase in plasma levels of epinephrine (EPI) that abates following chronic altitude exposure (Mazzeo & Reeves 2003). The summarized results from 30 studies suggest that resting EPI levels increase 38% with acute altitude exposure and return to sea level values following chronic altitude exposure (Fig. 30.4b). The primary mechanism for EPI release does appear to be hypoxia, since both the hypoxic stimulus and EPI levels are increased upon acute altitude exposure and both lessen with chronic altitude residence (Mazzeo & Reeves 2003). Wolfel *et al*. (1994) reported an inverse relationship between S_aO_2 and plasma EPI levels with time at altitude.

Similar to exercise at sea level, EPI levels increase one to threefold with exercise from resting values during both acute and chronic altitude exposure (Mazzeo *et al*. 1991, 1995, 2003; Young, A.J. *et al*. 1991), and the magnitude of the response is dependent on the exercise intensity (Kjær *et al*. 1988; Braun *et al*. 2000; Mazzeo *et al*. 2000; Lundby & Steensberg 2004). Summarized results suggest that the percentage of increase in EPI during exercise from resting values is the same in all conditions (~ 120–180%), but reaches higher absolute levels with exercise during acute altitude exposure due to higher initial starting levels (Fig. 30.4b). The increase and subsequent decrease in EPI levels during both rest and exercise likely contributes to the immediate increase in heart rate, vasodilation, muscle glycogenolysis and lactate accumulation upon acute altitude exposure that subsides following chronic exposure to altitude (Mazzeo & Reeves 2003). These physiologic adaptations serve to increase O_2 delivery and optimize O_2 utilization under limited O_2 conditions and conserve cardiac energy as O_2 supply improves.

Parasympathetic response

Several studies suggest a reduction in resting parasympathetic nervous system (PNS) activity during acute altitude exposure (Richardson *et al*. 1967; Koller *et al*. 1988). In humans acutely exposed to hypoxia, pretreatment with propranolol (a β-adrenergic blocker) fails to prevent the acute increase in heart rate (Koller *et al*. 1988). However, when propranolol and atropine (parasympathetic blocker) are combined, the increase in heart rate upon acute exposure to hypoxia is completely eliminated in both humans (Koller *et al*. 1988) and dogs (Hammill *et al*. 1979). With chronic altitude exposure, most (Hartley *et al*. 1974; Hughson *et al*. 1994; Boushel *et al*. 2001) but not all (Grover *et al*. 1986) studies suggest a reversal of the initial decline in PNS activity such that cardiac vagal activity is increased and maximal heart rate decreased when compared to sea level values. The mechanism for the initial decrease and subsequent increase in PNS activity is not known, but may be induced via a baroreceptor-induced excitation of the vagal nerve in response to a slight drop and subsequent elevation in mean arterial pressure with time at altitude (Hartley *et al*. 1974). The immediate decrease in PNS activity serves to increase blood flow under limited O_2 conditions by increasing heart rate. The increase in PNS activity following chronic altitude exposure may maintain myocardial O_2 demand, which is roughly estimated from the rate-pressure product (heart rate × mean arterial pressure), by decreasing exercise heart rate as mean arterial pressure increases.

Glucocorticoid response

Cortisol (COR) is the major glucocorticoid produced by the adrenal cortex and is secreted in response to increasing levels of adrenocorticotropic hormone (ACTH) released from the anterior pituitary. Although individual study results may differ, the summarized results from 30 studies suggest that resting COR levels increase 33% with acute altitude exposure and return towards sea level values following chronic altitude exposure (Fig. 30.5a). The trigger for the initial increase in ACTH and COR with altitude exposure may be partly related to increased peripheral chemoreceptor input as a result of the initial hypoxic stimulus, since deafferentation of the arterial chemoreceptors results in diminished or abolished increases in ACTH and COR (Raff *et al*. 1982; Honig *et al*. 1996).

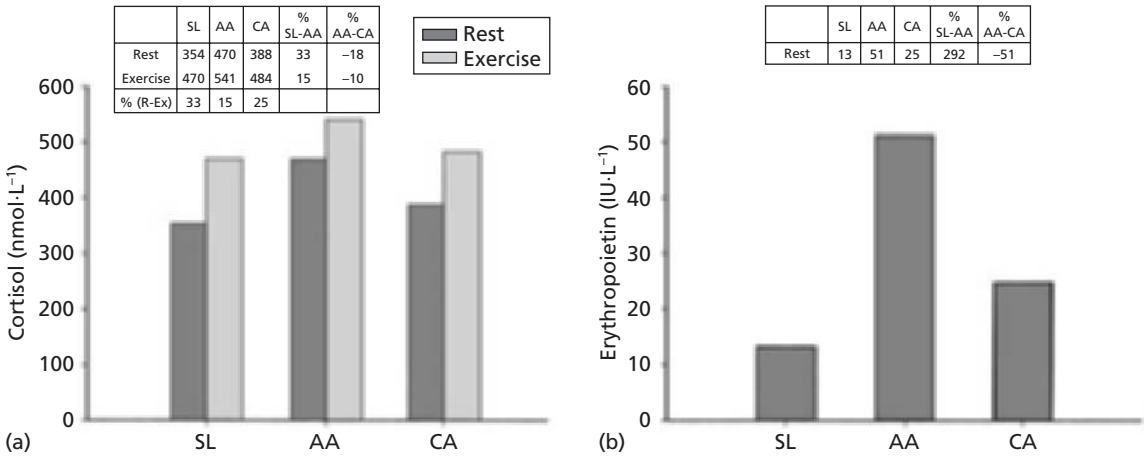


Fig. 30.5 Summarized results from 30 studies measuring (a) cortisol during both rest and exercise (Moncloa *et al*. 1968; Singh *et al*. 1974; Frayser *et al*. 1975; Sutton 1977; Sutton *et al*. 1977; Claybaugh *et al*. 1982; Heyes *et al*. 1982; Mordes *et al*. 1983; Maresh *et al*. 1984; Bärtsch *et al*. 1988, 1991a; Bouissou *et al*. 1988; Friedl *et al*. 1988; Kjær *et al*. 1988; Richalet *et al*. 1989; Rock *et al*. 1989; Tunny *et al*. 1989; Kraemer *et al*. 1991; Sawhney *et al*. 1991; Ramirez *et al*. 1992; Vuolteenaho *et al*. 1992; Westendorp *et al*. 1993; Banfi *et al*. 1994, 1996; Beidleman *et al*. 1997, 2002; Larsen *et al*. 1997; Zaccaria *et al*. 1998; Braun *et al*. 2000; Basu *et al*. 2002) and 15 studies measuring (b) erythropoietin (EPO) at rest during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure (Abbrecht & Littell 1972; Milledge & Cotes 1985; Eckardt *et al*. 1989; Mairbaurl *et al*. 1990; Gunga *et al*. 1994; Klausen *et al*. 1996; Sawka *et al*. 1996; Gleiter *et al*. 1997; Chapman *et al*. 1998; Grover *et al*. 1998; Hudson *et al*. 1999; Bonfichi *et al*. 2000; Koistinen *et al*. 2000; Reeves *et al*. 2001; Ge *et al*. 2002).

Similar to exercise at sea level, COR levels increase 30–100% with exercise during both acute and chronic altitude exposure (Bouissou *et al*. 1988; Kjær *et al*. 1988; Bärtsch *et al*. 1991a; Braun *et al*. 2000), and the magnitude of the response is dependent on the intensity of exercise (Kjær *et al*. 1988; Braun *et al*. 2000). Summarized results suggest that the percentage of increase in COR during exercise from resting values is the same in all conditions (~ 15–33%) but reaches higher absolute values with exercise during acute altitude exposure due to higher initial starting values (Fig. 30.5a). The increase in COR upon acute exposure to altitude during both rest and exercise most likely contributes to increased cardiac contractility, cardiac output, erythropoietin synthesis and energy mobilization through gluconeogenesis (White *et al*. 1995).

Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone that is released primarily from the kidneys and to a lesser degree by the liver. Summarized results from 15 studies suggest that initial ascent to altitude induces a two to threefold increase in resting EPO levels, with peak levels occurring within ~ 24–48 h, that declines toward but does not reach sea level values following chronic altitude exposure (Fig. 30.5b). The EPO response to exercise with acute and chronic altitude exposure has not been well studied. Thus, whether or not the EPO response is potentiated during hypoxic exercise cannot be determined at this time. The initial stimulus for EPO production is related to the reduction in tissue O_2 tension in the renal cortex upon acute altitude exposure (Jelkmann 1992). The actual renal O_2 sensor is probably a heme protein (Goldberg *et al*. 1998), or an enzyme termed hypoxia-inducible factor-α prolyl-hydroxylase (HIF-PH) (Ivan *et al*. 2001; Jaakkola *et al*. 2001). In the presence of decreased O_2 delivery, activation of this sensor appears to lead to the synthesis of HIF-1α, which then binds to the active site on the enhancer region of the EPO gene and activates transcription (Semenza *et al*. 1991). This immediate increase in EPO stimulates the hematological cascade which eventually leads to

an increase in RBC production if the altitude sojourn is prolonged.

The reason for the decrease in EPO to near sea level values despite continuing hypoxemia is difficult to explain. Just like any other hormone, there is a dynamic equilibrium between production and consumption. If increased erythroid activity following chronic altitude exposure leads to increased EPO consumption, plasma levels of EPO may decline in the face of elevated EPO turnover. Furthermore, there may be other stimuli that change following chronic altitude exposure such as increasing 2,3 diphosphoglycerate levels (Miller *et al*. 1973), decreasing sympathetic stimulation (Mazzeo & Reeves 2003) and down-regulation of HIF-1α (D'Angelo *et al*. 2003), which could explain declining EPO levels despite persistent systemic hypoxia.

Fluid regulatory hormones

Renin–angiotensin–aldosterone system. Renin is a glycoprotein released from the juxtaglomerular cells of the kidney. Renin cleaves the circulating substrate angiotensinogen to angiotensin I, which is then further metabolized to its active form, angiotensin II, by angiotensin-converting enzyme in the lung vascular endothelium. Angiotensin II, a potent vasoconstrictor, stimulates secretion of aldosterone (ALDO) by the adrenal cortex. Renin is usually measured by incubating the sample to be assayed and measuring the amount of angiotensin I generated. This measures the plasma renin activity (PRA) of the sample. Summarized results from 25 studies suggest a 9% decrease in resting PRA levels upon acute altitude exposure that declines a further 10% from acute to chronic altitude exposure (Fig. 30.6a). The trigger for the slight and continued decrease in resting PRA and plasma renin with altitude exposure is also unknown, given that renin is typically released in response to β-adrenergic stimulation and decreasing blood pressure (Bouissou *et al*. 1989). Since β-adrenergic stimulation is increased with acute altitude exposure and decreased with chronic altitude exposure, one would expect a parallel

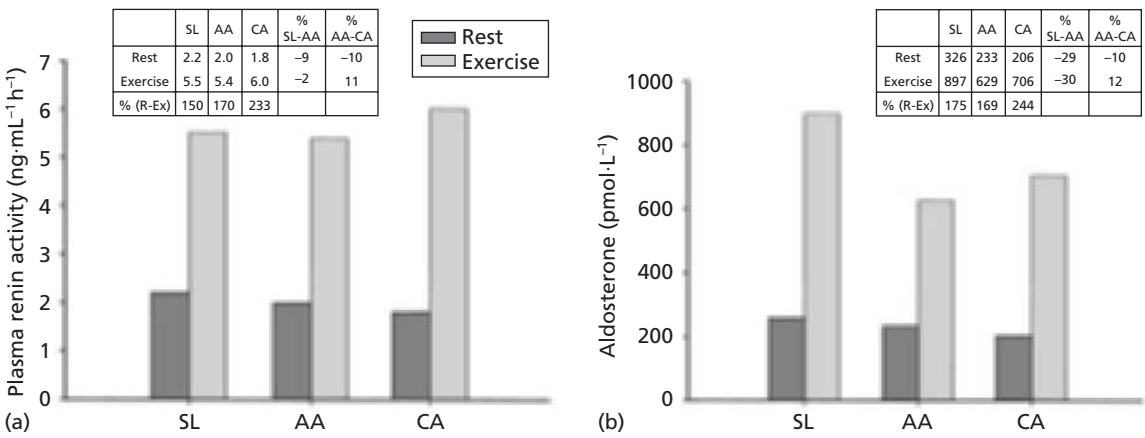


Fig. 30.6 Summarized results from 25 studies measuring (a) plasma renin activity during both rest and exercise (Kotchen *et al*. 1973a; Frayser *et al*. 1975; Maher *et al*. 1975; Sutton *et al*. 1977; Heyes *et al*. 1982; Milledge *et al*. 1983a; Bärtsch *et al*. 1988, 1991a, 1991b; Bouissou *et al*. 1988, 1989; Knudtzon *et al*. 1989b; Tunny *et al*. 1989; De Angelis *et al*. 1992, 1996; Olsen *et al*. 1992; Ramirez *et al*. 1992; Vuolteenaho *et al*. 1992; Rock *et al*. 1993; Westendorp *et al*. 1993; Antezana *et al*. 1995; Angelini *et al*. 1997; Zaccaria *et al*. 1998; Bestle *et al*. 2002; Robach *et al*. 2002) and 28 studies measuring (b) aldosterone during both rest and exercise (Frayser *et al*. 1975; Maher *et al*. 1975; Sutton *et al*. 1977; Claybaugh *et al*. 1982; Heyes *et al*. 1982; Milledge *et al*. 1983a, 1989; Maresh *et al*. 1984; Bärtsch *et al*. 1988, 1991a, 1991b; Bouissou *et al*. 1988, 1989; Knudtzon *et al*. 1989b; Tunny *et al*. 1989; De Angelis *et al*. 1992, 1996; Olsen *et al*. 1992; Ramirez *et al*. 1992; Vuolteenaho *et al*. 1992; Loeppky *et al*. 1993; Rock *et al*. 1993; Westendorp *et al*. 1993; Antezana *et al*. 1995; Angelini *et al*. 1997; Zaccaria *et al*. 1998; Bestle *et al*. 2002; Robach *et al*. 2002) during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure.

increase and decrease in PRA and plasma renin. The hypoxic suppression of PRA with acute and chronic altitude exposure may be related to the increase in atrial natriuretic peptide with acute altitude exposure (Johnston *et al*. 1989), or stimulation of an intrarenal baroreceptor due to increased renal perfusion following chronic altitude exposure (Hogan *et al*. 1973; Olsen 1995).

Similar to exercise at sea level, PRA or plasma renin levels increase one to threefold with exercise during both acute and chronic altitude exposure (Maher *et al*. 1975; Bouissou *et al*. 1988; Bärtsch *et al*. 1991a; Rock *et al*. 1993). Summarized results suggest that the percentage of increase in PRA or renin during exercise from resting values is the same in all conditions (~ 150–233%), and absolute levels reached during exercise are also similar in all conditions (Fig. 30.6a).

Although suspected for some time, it is now generally agreed that the production of angiotensin II and activity of angiotensin-converting enzyme are not inhibited by hypoxia (Milledge & Catley 1987; Raff *et al*. 1989). Thus, ALDO secretion remains under predominant control of the renin–angiotensin system even at altitude (Olsen 1995). Thus, it is not surprising that the summarized results of 28 studies suggest that resting levels of ALDO are also reduced 30–50% from sea level values upon acute and chronic altitude exposure, respectively (Fig. 30.6b). Although the most likely reason for the decreased ALDO levels with acute and chronic altitude exposure is the decreased renin values (Olsen 1995), a decrease in plasma K^+ and ACTH with altitude acclimatization may also contribute (Okazaki *et al*. 1984).

Similar to exercise at sea level, ALDO levels increase one to threefold with exercise during both acute and chronic altitude exposure (Sutton 1977; Bouissou *et al*. 1988; Bärtsch *et al*. 1991a; Braun *et al*. 2000; Beidleman *et al*. 2002). Summarized results suggest that the percentage of increase in ALDO during exercise from resting values is the same in all conditions (~ 169–244%), but reaches lower absolute levels during acute and chronic altitude exposure due to lower initial starting levels (Fig. 30.6b). The decline in ALDO levels during both rest and exercise most likely contributes to the increased natriuresis and diuresis upon acute and chronic altitude exposure. These physiological adaptations would be beneficial for increasing [Hb] and O_2 transport as well as decreasing brain edema and thus AMS (Hackett 1999).

Atrial natriuretic peptide. Atrial natriuretic peptide (ANP) is a polypeptide released by the cardiac atrial muscle fibers in response to overstretch of the atria. Although the literature is conflicting, summarized results of 21 studies suggest that plasma ANP increases 26% upon acute altitude exposure and returns to sea level values following chronic altitude exposure (Fig. 30.7a). The initial stimulus for ANP release may be the hypoxic pulmonary vasoconstriction that occurs upon acute exposure to altitude, but does not explain the decrease in ANP given the sustained increase in pulmonary arterial pressure following chronic altitude exposure (Boussuges *et al*. 2000). Given the stimulatory effects of HIF-1α on transactivation of ANP in hypoxic atrial myocytes (Chen, Y.F. *et al*. 1997), up- and down-regulation of HIF-1α with acute and chronic altitude exposure may explain increasing and decreasing ANP levels (D'Angelo *et al*. 2003).

Similar to exercise at sea level, ANP levels increase 50–150% with exercise during both acute and chronic altitude exposure (Bärtsch *et al*. 1991a; Olsen *et al*. 1992; Rock *et al*. 1993; Braun *et al*. 2000). Summarized results suggest that the percentage of increase in ANP during exercise is lower following chronic altitude exposure (~ 3%) compared to sea level (~ 88%) and acute altitude exposure (~ 72%) (Fig. 30.7a). The immediate increase in ANP likely contributes to the increased diuresis and reduction in plasma volume observed upon acute altitude exposure.

Antidiuretic hormone. Antidiuretic hormone (ADH) is a peptide hormone stored in the posterior pituitary and released in response to increased osmotic pressure. The summarized results of 16 studies suggest that ADH increases 10% with acute altitude exposure and returns to sea level values following chronic altitude exposure (Fig. 30.7b). Due to the limited number of studies, the ADH response to exercise cannot be summarized. Bartsch and

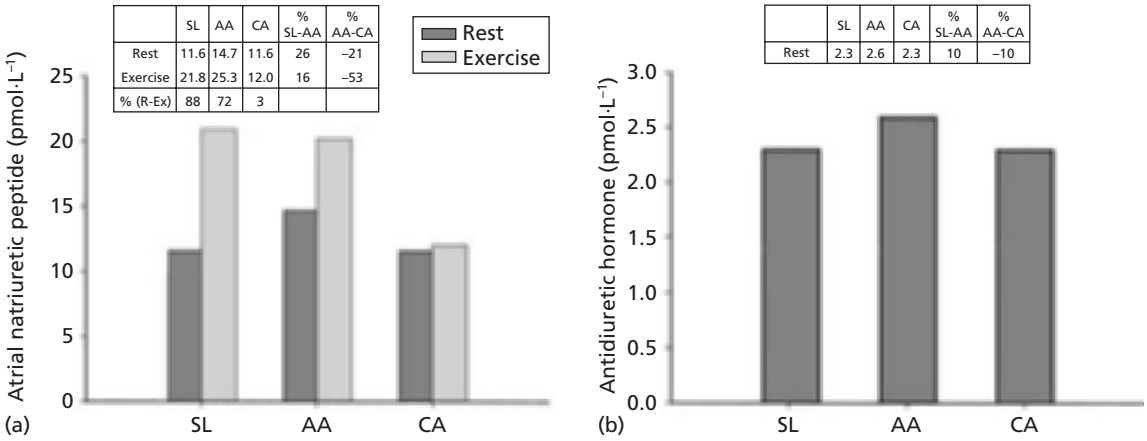


Fig. 30.7 Summarized results from 21 studies measuring (a) atrial natriuretic peptide during both rest and exercise (Bärtsch *et al*. 1988, 1991a, 1991b; Bouissou *et al*. 1989; Kawashima *et al*. 1989; Milledge *et al*. 1989; Tunny *et al*. 1989; Koller *et al*. 1991; De Angelis *et al*. 1992, 1996; Olsen *et al*. 1992; Ramirez *et al*. 1992; Vuolteenaho *et al*. 1992; Loeppky *et al*. 1993; Rock *et al*. 1993; Westendorp *et al*. 1993; Antezana *et al*. 1995; Rostrup 1998; Zaccaria *et al*. 1998; Bestle *et al*. 2002; Robach *et al*. 2002) and 16 studies measuring (b) antidiuretic hormone at rest (Singh *et al*. 1974; Claybaugh *et al*. 1982; Heyes *et al*. 1982; Blume *et al*. 1984; Porchet *et al*. 1984; Bärtsch *et al*. 1988, 1991a, 1991b; Koller *et al*. 1991; De Angelis *et al*. 1992, 1996; Loeppky *et al*. 1993; Rostrup 1998; Sevre *et al*. 2001; Robach *et al*. 2002; Maresh *et al*. 2004) during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure.

colleagues (Bärtsch *et al*. 1991a) reported the same percentage of increase in ADH during exercise both at sea level and upon acute altitude exposure, while Robach *et al*. (2002) found enhanced ADH release during exercise following chronic altitude exposure. Clearly, further research needs to be conducted before definitive insight can be gained on the ADH response to exercise at altitude. The slight increase in ADH upon acute altitude exposure could be the result of an increase in peripheral chemoreceptor activity (Share & Levy 1966) or plasma osmolality (Bestle *et al*. 2002; Maresh *et al*. 2004), but doesn't explain the decrease in ADH as chemoreceptor activity (Bisgard & Forster 1996) and plasma osmolality (Blume *et al*. 1984; De Angelis *et al*. 1992; Bestle *et al*. 2002; Maresh *et al*. 2004) increase following chronic altitude exposure. Heyes *et al*. (1982) found that ADH levels were inversely correlated with blood pressure. Thus, the acute fall in blood pressure upon acute exposure to altitude may stimulate an increase in ADH, while the chronic increase in blood pressure following altitude residence may cause ADH levels to return to normal. Given that an increase in ADH would oppose the beneficial effects of diuresis, the reason for the slight increase in ADH upon acute altitude exposure is not clear.

Glucogregulatory hormones

Insulin. Insulin (INS) is a polypeptide hormone secreted in response to high blood glucose levels by beta cells in the islets of Langerhans. Summarized results from 13 studies suggest that plasma INS increases 12% upon acute altitude exposure and returns toward but does not reach sea level values following chronic altitude exposure (Fig. 30.8a). The initial increase and subsequent decrease in INS secretion with altitude exposure is not entirely known, since resting blood glucose levels are not altered by acute altitude exposure (Young, P.M. *et al*. 1989b; Roberts *et al*. 1996b; Braun *et al*. 2001) and tend to decrease following chronic altitude exposure (Stock *et al*. 1978; Young, P.M. *et al*. 1989b; Roberts *et al*. 1996b). The increase in INS with acute altitude exposure may be due to β-adrenergic stimulation. The subsequent decrease in INS following chronic altitude exposure may be due to rising NE

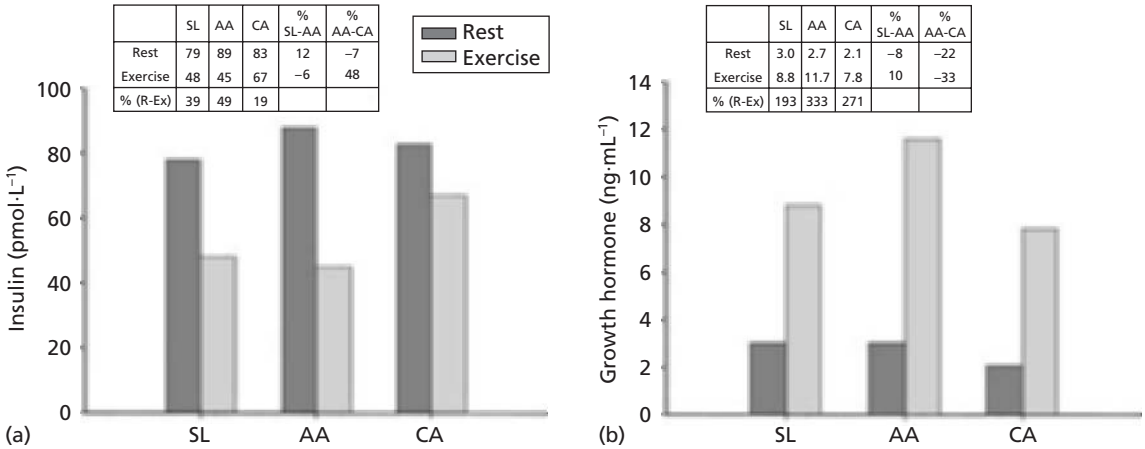


Fig. 30.8 Summarized results from 13 studies measuring (a) insulin during both rest and exercise (Sutton 1977; Stock *et al*. 1978; Sawhney *et al*. 1986; Young, P.M. *et al*. 1989a, 1992; Brooks *et al*. 1991; Sawhney *et al*. 1991; Young, A.J. *et al*. 1991; Roberts *et al*. 1996b; Larsen *et al*. 1997; Braun *et al*. 2000, 2001; Beidleman *et al*. 2002) and nine studies measuring (b) growth hormone during rest (Sutton *et al*. 1970; Sutton 1977; Raynaud *et al*. 1981; Kjær *et al*. 1988; Knudtzon *et al*. 1989a; Sawhney *et al*. 1991; Banfi *et al*. 1994; Larsen *et al*. 1997; Beidleman *et al*. 2002) during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure.

levels, which would suppress INS release via enhanced α_2-adrenergic stimulation (Schmidt *et al*. 1997; Schäfers *et al*. 1999).

Similar to exercise at sea level, INS levels decrease from 10% to 50% with exercise during both acute and chronic altitude exposure (Sutton 1977; Brooks *et al*. 1991; Young, P.M. *et al*. 1992; Braun *et al*. 2000; Beidleman *et al*. 2002). Summarized results suggest that the percentage of decrease in INS during exercise from resting values is slightly less following chronic altitude exposure (~ 19%) compared to sea level (–39%) or acute altitude exposure (–49%) (Fig. 30.8a). However, if the studies were conducted at the same relative intensity of exercise at both sea level and altitude, the percentage of decline in INS during exercise is similar (Braun *et al*. 2000; Beidleman *et al*. 2002). The increased INS levels upon acute altitude exposure may contribute to the preferential use of carbohydrates in order to maximize O_2 fuel efficiency under limited O_2 conditions.

Glucagon. Glucagon (GLG) is also a polypeptide hormone secreted in response to low blood glucose levels by α cells in the Islets of Langerhans. Since summarized results would be skewed by the low number of GLG measurements at altitude, individual study results will be examined. Given the anti-insulin properties of GLG, one would expect a slight decrease upon acute altitude exposure that returns toward normal sea level values following chronic altitude exposure. Most (Kjær *et al*. 1988; Roberts *et al*. 1996b; Larsen *et al*. 1997) but not all (Beidleman *et al*. 2002) studies have reported a slight 5% decrease in GLG upon acute exposure to altitude. This slight decrease in GLG may facilitate enhanced glycolysis. However, only two studies have examined the resting GLG response following chronic altitude exposure, and one reported a 55% increase (Roberts *et al*. 1996b) while the other reported no change (Larsen *et al*. 1997). Thus, whether or not GLG changes following chronic altitude exposure remains unknown.

Similar to exercise at sea level, GLG increases 10–30% during exercise at altitude (Roberts *et al*. 1996b; Beidleman *et al*. 2002), which is most likely related to the exercise-induced depletion of glucose. It does not appear that the GLG response to exercise is potentiated by altitude exposure, but further research is needed in this area.

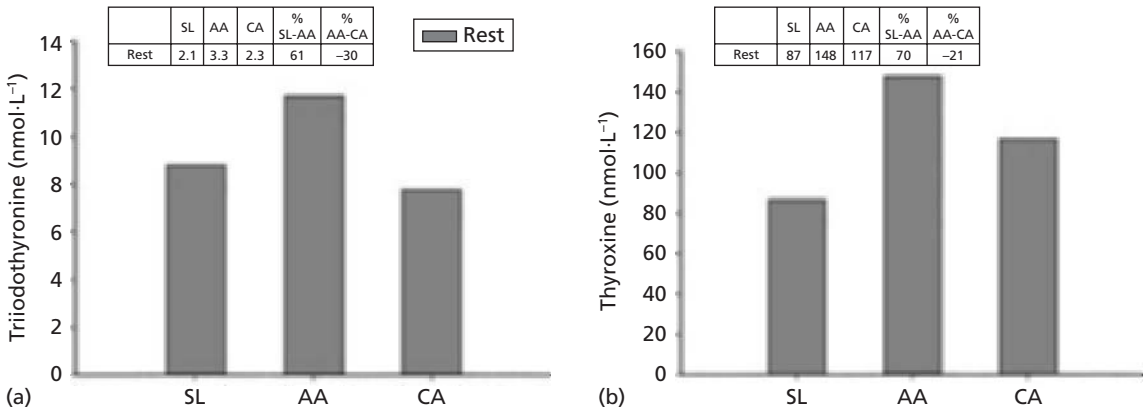


Fig. 30.9 Summarized results from 10 studies measuring (a) triiodothyronine (T_3) and (b) thyroxine (T_4) at rest (Surks *et al*. 1967; Kotchen *et al*. 1973b; Rastogi *et al*. 1977; Stock *et al*. 1978; Wright 1979; Mordes *et al*. 1983; Chakraborty *et al*. 1987; Férézou *et al*. 1988, 1993; Sawhney & Malhotra 1991) during sea level (SL), acute altitude (AA) and chronic altitude (CA) exposure.

Growth hormone

Growth hormone (GH) is a polypeptide that is synthesized and secreted by cells in the anterior pituitary gland. Although the literature is controversial, summarized results from nine studies suggest an 8% decrease in resting GH levels upon acute altitude exposure that decline a further 22% from acute to chronic altitude exposure (Fig. 30.8b). During acute and chronic altitude exposure, the GH response is elevated with exercise conducted at the same absolute intensity (Sutton *et al*. 1970; Sutton, 1977; Raynaud *et al*. 1981) but is actually decreased (Beidleman *et al*. 2002; Gutierrez *et al*. 2003) or unchanged (Kjær *et al*. 1988; Knudtzon *et al*. 1989a) when exercise is conducted at the same relative intensity. The reason for a hypoxia-induced blunting of the GH response to exercise may be related to elevated cortisol levels directly inhibiting GH secretion, or indirectly inhibiting GH secretion through stimulation of somatostatin, an inhibitory stimulus to GH release (Chen, X.Q. & Du 2000). Given the lipolytic actions of GH, a decrease in GH hormone during acute and chronic altitude exposure may enhance glycolysis and carbohydrate utilization.

Thyroid hormones

Triiodothyronine (T_3) and thyroxine (T_4) are both secreted by the thyroid gland in response to thyroid-stimulating hormone (TSH) secreted by the anterior pituitary gland. Summarized results from 10 studies suggest a 61% increase in resting T_3 and T_4 upon acute altitude exposure that returns toward sea level values following chronic altitude exposure (Fig. 30.9a,b). Given that most studies do not find an increase in TSH with altitude exposure, the reason for the acute increase and subsequent decrease in T_3 and T_4 with altitude exposure is unknown. However, since the thyroid gland contains β-adrenergic receptors, the acute increase and subsequent decrease in EPI levels may explain the thyroid hormone response.

Only one study has examined the thyroid hormone response to exercise following chronic altitude exposure, and they reported that the T_3 and T_4 response to exercise was potentiated by altitude exposure (Stock *et al*. 1978). However, the work load was kept constant at both sea level and altitude in this study, and the subsequent higher absolute intensity of exercise at altitude most likely contributed to the greater increase in thyroid hormones. The transient elevation in basal metabolic rate during the first few days at altitude is most likely mediated by increased thyroid hormone levels (Surks *et al*. 1967; Moore *et al*. 1987). Thyroid hormones may also play a permissive role in increasing erythrocyte mass at altitude because an increase in

metabolic rate increases O_2 demand and thus erythropoiesis (Das *et al*. 1975). The physiologic adaptations stimulated by the thyroid hormones serve to increase O_2 delivery under limited O_2 supply.

Reproductive hormones

Estradiol and progesterone. Estradiol (E_2) and progesterone (P_4) are both secreted from the ovaries in response to changing levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted from the anterior pituitary. Resting E_2 and P_4 are not changed with either acute (Beidleman *et al*. 1999; Reeves *et al*. 2001; Rock *et al*. 2001; Takase *et al*. 2002) or chronic altitude exposure (Reeves *et al*. 2001; Rock *et al*. 2001) as long as women remain within the same phase of the menstrual cycle. Similar to exercise at sea level, E_2 and P_4 increase 10–50% during exercise at altitude, and the response is dependent on the intensity of exercise (Beidleman *et al*. 1999).

Testosterone. Testosterone is secreted by the testes in response to changing levels of FSH and LH. Most (Vaernes *et al*. 1984; Sawhney *et al*. 1985; Friedl *et al*. 1988) but not all (Vander *et al*. 1978) studies report that testosterone is decreased by acute and chronic exposure to high altitude. Similarly, most report that LH and FSH decrease with acute and chronic altitude exposure (Humpeler *et al*. 1980; Sawhney *et al*. 1985; Friedl *et al*. 1988). The stimulus for this decrease in testosterone is unknown, but may be due to a direct effect of hypoxia on the testes or indirect effect of lowered LH levels and elevated E_2 levels (Sawhney *et al*. 1985; Friedl *et al*. 1988).

Integration of physiologic function and neurohumoral response at altitude

The integrated neurohumoral response to exercise and adaptations at altitude is complex (Fig. 30.10). Some hormones exhibit an immediate increase (i.e. EPO, EPI, COR, T_3, T_4) upon acute exposure to altitude followed by a decline to baseline sea level values following chronic altitude exposure. Other hormones exhibit a progressive decrease (ALDO, PRA, GH) or no change (ADH, INS, GLG) with time at altitude while another hormone exhibits a slow but continual increase (NE). Regardless of the complexity, it is important to understand how neurohumoral responses influence physiologic function at altitude because the neurohumoral response often precedes physiologic adaptations. If the neurohumoral response is absent, it is unclear whether the physiologic adaptations described previously would occur.

The sympathoadrenal system plays a key role in successful adaptation to altitude. Specifically, the immediate increase in EPI levels upon exposure to altitude improves O_2 transport by increasing heart rate, cardiac output and peripheral vasodilation via β-adrenergic stimulation (Mazzeo & Reeves 2003). Increased EPI levels also augment glycolysis which increases the efficiency of O_2 utilization. Increased

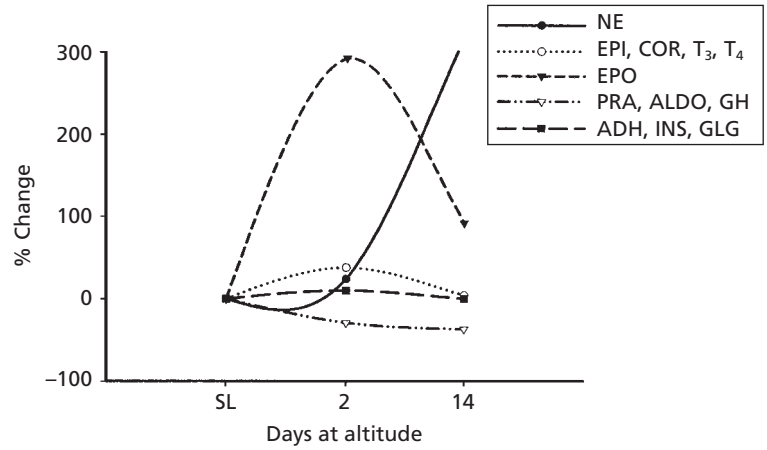


Fig. 30.10 Integrated neurohumoral response to altitude. ADH, antidiuretic hormone; ALDO, aldosterone; COR, cortisol; EPI, epinephrine; EPO, erythropoietin; GH, growth hormone; GLG, glucagon; INS, insulin; NE, norepinephrine; PRA, plasma renin activity; T_3, triiodothyronine; T_4, thyroxine.

COR levels upon acute exposure to altitude also augment the actions of EPI by stimulating muscle and liver glycogenolysis. The cost of these changes is an increased metabolic rate and lactate production (Mazzeo & Reeves 2003). As ventilatory acclimatization proceeds with time at altitude, arterial O_2 content improves, the hypoxic stress subsides, EPI and COR levels fall, and heart rate, cardiac output and lactate production return to baseline sea level values. The progressive increase in peripheral chemoreceptor activity at altitude appears to contribute to the rising NE levels with time at altitude. The immediate increase in SNS activity upon acute exposure to altitude counterbalances the effects of hypoxia-induced vasodilation on most vascular beds so that blood pressure can be maintained (Wolfel & Levine 2001). The progressive rise in SNS activity with time at altitude contributes to increased arterial and venous tone, increased systemic vascular resistance, decreased plasma volume and further decreases in cardiac output (Grover *et al*. 1986; Wolfel & Levine 2001). The cost of increased SNS activity is a decrease in blood flow following chronic altitude exposure. However, this allows for a more precise matching of metabolic supply and demand by as arterial O_2 content improves with acclimatization.

The acute increase in EPO, which stimulates the hematological cascade, also contributes to successful altitude adaptation. Although there is a time lag before RBC mass increases at altitude, the eventual increase allows for improved O_2 delivery. Animal evidence suggests that the acute increase in EPO may also protect against ischemic brain injury and, possibly, AMS (Brines *et al*. 2000). Increases in COR and the thyroid hormones upon acute exposure to altitude may also increase EPO synthesis (Golde *et al*. 1977; Kelly *et al*. 2000). Although the decrease in EPO following chronic altitude exposure does not make intuitive sense, the benefits of expanded red cell volume may be detrimental to blood flow due to an increase in blood viscosity (Young, A.J. *et al*. 1996). Furthermore, HIF-1α, which is down-regulated with continued exposure to hypoxia (D'Angelo *et al*. 2003), exerts a direct effect on transcription of EPO (Semenza *et al*. 1991). Thus, as the body senses improved O_2 transport following chronic altitude exposure, the hormonal response may be down-regulated in an appropriate fashion.

The fluid regulatory hormones are critical for successfully adapting to altitude. The progressive decline in PRA and ALDO contribute to the progressive decline in plasma volume and extracellular fluid volume with altitude exposure. These physiologic changes not only improve O_2 transport but also decrease brain edema and thus AMS. In fact, many studies have reported that when PRA and ALDO are elevated, due to extensive time climbing to altitude, significant sodium and water retention and increased incidence of AMS occur (Milledge *et al*. 1983b, 1989; Bärtsch *et al*. 1988, 1991a). This suggests that if at all possible, exercise should be avoided within the first 24 h of altitude exposure (Roach *et al*. 2000). Given that ADH does not change significantly with altitude exposure, it does not appear to play a significant role in the altitude-induced alterations in fluid balance.

The metabolic hormones likely play a role in substrate utilization at altitude but they are less understood due to conflicting results in the experimental literature. Several studies have suggested increased utilization of carbohydrates upon acute and chronic exposure to altitude (Brooks *et al*. 1991; Roberts *et al*. 1996b) and other studies have reported decreased utilization of carbohydrates following chronic altitude exposure (Young, A.J. *et al*. 1982, 1991; Braun *et al*. 2000). The acute increase in INS, small decrease in GLG, and up-regulation of HIF-1α upon exposure to altitude would support increased carbohydrate utilization. Subsequent down-regulation of HIF-1α and decreased INS levels following chronic altitude exposure would support decreased carbohydrate utilization, which has not been consistently reported (Brooks *et al*. 1991; Roberts *et al*. 1996b). Conflicting substrate results in the altitude literature may be due to differences in study design, intensity of exercise, gender, diet control, level of hypoxemia and other unknown factors. Although it is clear that substrate utilization is altered with exposure to altitude, the timing and mechanisms need further study.

The reproductive hormones do not appear to play a key role in successful adaptation to altitude.

However, they may influence the low fertility rates reported in several high altitude populations (Clegg & Harrison 1971). One study suggested lower luteal-phase progesterone values in sea level natives compared to high altitude natives (Escudero *et al*. 1996). Another study reported decreased testosterone levels and sperm count following a mountaineering expedition to 5100 m that was reversed only after a 3-month stay at sea level (Okumara *et al*. 2003). Although the reproductive hormones may exert effects on ventilation, erythropoiesis, and metabolism at sea level, their effects on these physiologic adaptations at altitude are minimal (Muza *et al*. 2001; Beidleman *et al*. 2002).

In conclusion, many neurohumoral responses contribute to the short- and long-term physiologic adaptations that occur during both rest and exercise at altitude that ultimately allows the healthy lowlander to tolerate the hypoxemia of altitude. By summarizing the neurohumoral responses from a large number of studies under a defined set of conditions, a clearer understanding concerning how these responses affect physiologic adaptation at altitude has been gained. The hormonal trends presented for the sympathoadrenal (EPI, NOR, COR), hematologic (EPO) and fluid regulatory (PRA, ALDO, ANP) response to altitude are consistent and clear due to the large number of studies examined. However, further work examining the metabolic hormones (INS, GH, GLG) and their response to altitude is needed both during rest and exercise to clarify outstanding issues.

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Chapter 31

Neuroendocrine Influences on Temperature Regulation in Hot Environments

LAWRENCE E. ARMSTRONG AND JACK A. BOULANT

The nervous and endocrine systems act in unison to regulate homeostasis of body temperature and other physiological qualities that are essential to life. Because it is not possible to clearly divide the integrated efforts of the nervous and endocrine systems (Galbo 1986), the term *neuroendocrine* refers to the anatomical and functional interrelationships between nerves and endocrine organs. The majority of neuroendocrine research has focused on blood-borne hormone and neuropeptide concentrations in humans, and tissue levels of neurotransmitters in the central nervous system (CNS) of animals. Definitions of these and other relevant terms appear in the following four paragraphs.

A *hormone* is a signaling molecule that regulates physiologic and metabolic functions by acting on receptors located on or in target cells. Hormones can be stimulated by other hormones, nervous reflexes, or chemical transmitter substances. Some are secreted by specific glands, transported by the blood, and control responses in either specific target tissues (e.g. adrenocorticotrophic hormone, ACTH) or in tissues located at multiple sites (i.e. epinephrine, growth hormone, thyroid hormone); these are named *endocrine* secretions. Other signaling molecules are released into the interstitial fluid and act on the receptors of adjacent cells; these are named *paracrine* secretions. When the hormone is released into the interstitial fluid and acts on the originating cell, it is named an *autocrine* secretion (Borer 2003).

A *neurotransmitter* is a chemical mediator, synthesized in the cytosol of the presynaptic nerve terminal, that interacts with receptor molecules in the post-synaptic membrane. A neurotransmitter acts rapidly (i.e. in 1–2 ms) over a small distance to alter ion channel function and the electrical activity of post-synaptic cells. In some cases, these small-molecule transmitters cause acute nerve responses or action potentials that relay afferent sensory signals to the brain and efferent motor impulses to muscles. In addition to norepinephrine (NE), several other neurotransmitters serve to integrate the body's responses to stress (DeSouza & Appel 1991; Toates 1995). Serotonin (5-hydroxytryptamine, 5-HT), for example, directly affects ACTH and prolactin secretion. The brain neurotransmitter dopamine (i.e. a catecholamine precursor of NE) increases with exposure to various stressors, and also alters prolactin secretion. Acetylcholine (ACh) excites the release of corticotropin-releasing hormone from the hypothalamus (and ultimately the release of the hormones ACTH and cortisol, in the hypothalamic–pituitary–adrenal [HPA] axis). Gamma-aminobutyric acid (GABA) inhibits the function of the HPA axis; glycine and glutamate, two other amino acids, also act as neurotransmitters. Nitric oxide, which serves as a neurotransmitter throughout the central and peripheral nervous systems, is important in thermoregulation because it results in blood vessel dilation. Interestingly, nitric oxide regulates endocrine, autocrine and paracrine functions.

Some *neuropeptides* act slowly, over a relatively longer distance, to cause prolonged actions such as changes in the number of excitatory or inhibitory receptors, long-term opening or closing of ion channels, and activation-deactivation of specific genes in the cell nucleus. The following neuropeptides have

prompted considerable research interest among neurophysiologists since the mid-1970s: ACTH, prolactin, met-enkephalin, β-endorphin, human growth hormone (hGH), peptide F, peptide Y, arginine vasopressin (AVP), luteinizing hormone, atrial natriuretic peptide (ANP), vasoactive intestinal peptide (VIP), somatostatin, oxytocin, galanin, α-melanocyte-stimulating hormone, cholecystokinin (CCK), insulin, glucagon, angiotensin II, bradykinin, calcitonin and thyrotropin-releasing hormone (Guyton & Hall 1996; Gibbins & Morris 2000).

Many neuropeptides, released into the blood from the pituitary as hormones, are also released within the CNS as neurotransmitters at much higher concentrations than in plasma. Thus, the same substance may influence different target cells in different organs to a variable extent, depending on the concentration of the neurotransmitter and the sensitivity of the target cells. Receptor density, as altered by DNA-mediated protein synthesis, and receptor subtype also may affect neuropeptide function (Zeisberger & Roth 1996). For example, at least three 5-HT membrane receptors have been identified that differ in binding properties and efferent signaling pathways.

Integrated neural and endocrine functions

The autonomic nervous system (ANS) regulates the visceral functions of the body such as body temperature, sweating and arterial pressure. The ANS is activated by the hypothalamus, cerebral cortex, brain stem and spinal cord, without volitional control (Mosqueda-Garcia 1996). Efferent ANS signals are transmitted through two subdivisions, named the sympathetic nervous system and parasympathetic nervous system, to glands, smooth muscle or cardiac muscle. Sympathetic nerves originate in the spinal cord between the first thoracic and second lumbar vertebrae. About 75% of all parasympathetic fibers exit the brain via two vagus nerves, stimulating the entire thoracic and abdominal regions (Hammel 1965). Both sympathetic and parasympathetic fibers make synaptic contact with neurons in autonomic ganglia located outside the spinal cord. ACh is the most common neurotransmitter at these ganglionic sites. Postganglionic fibers arise from the ganglion neurons, and these fibers then innervate the effector organs. Postganglionic nerve endings of most sympathetic nerves secrete the neurotransmitter NE (designated adrenergic), and most parasympathetic nerve endings secrete ACh (designated cholinergic). An important exception exists: innervation of cutaneous sweat glands, piloerector muscles and a few blood vessels is sympathetic cholinergic.

In a hot environment, activation of the sympathetic nervous system is evidenced by increased plasma NE. This represents an overflow of NE at several peripheral sites, and often is used to represent the activity in the sympathetic–adrenomedullary (SAM) axis (Zeisberger 1998). Sympathetic innervation of the adrenal medulla, as part of the SAM axis, provides an excellent example of the integrated effects of the nervous and endocrine systems. In response to dysequilibria such as hyperthermia, reduced blood volume or blood pressure, and exercise, relatively large quantities of epinephrine and NE are released by the adrenal medulla and circulate to all tissues of the body. The effects of these hormones on organs are virtually the same as direct sympathetic nerve stimulation, but last 5–10 times longer (i.e. 1–2 min after innervation) because these hormones are metabolized slowly (Guyton & Hall 1996). Therefore, organs may be stimulated simultaneously in two ways: directly by sympathetic nerves and indirectly by adrenal medullary hormones. Epinephrine and NE also stimulate organs that are not innervated directly by sympathetic fibers; this is significant because metabolic rate and heat production can be increased in virtually all body cells.

Neurotransmitter roles in temperature regulation: animal research

Early models of thermoregulation that proposed fixed, hard-wired neural circuitry have evolved into complex paradigms that describe hypothalamic integration of different neurotransmitter systems working concurrently (Zeisberger & Roth 1996). Despite numerous anatomical studies, relatively little is known about the functional roles of these neurotransmitters because any given transmitter

may play either a stimulatory or an inhibitory role in temperature regulation, depending on its concentration and the target organ involved.

Peripheral effects. Body temperature is regulated via the neurotransmitters ACh and NE, which act on thermoregulatory effector organs (Zeisberger 1998) in the following ways. First, the tension of muscle contractions and exercise-induced heat production are controlled by the somatomotor system, via release of ACh at neuromuscular junctions. Second, sweat secretion is stimulated by sympathetic-cholinergic nerve ganglia; ACh is involved at all such synapses. Third, skin blood flow changes (i.e. changes in convective and radiative heat loss) are primarily mediated by three neurotransmitters: NE at sympathetic nerve endings and the adrenal medulla, nitric oxide that is released locally and acts as a potent vasodilator, or epinephrine that is secreted by the adrenal medulla then transported to cutaneous blood vessels. In addition to non-peptide neurotransmitters such as ACh and NE, most vasoactive motor neurons contain coexisting neuropeptides. Immunohistochemical evidence indicates that the precise combination of these neuropeptides, in peripheral neurons, varies with the location of the vascular bed and species (Gibbins & Morris 2000).

Although the complex interactions of neurochemical transmitters in the control of body temperature have not yet been unequivocally defined, experimental evidence indicates that peripheral systemic injection of neurotransmitters and neuroendocrines produce changes in body temperature. The picture is complex, however, because these changes vary, not only with the animal species and environmental temperature, but also with the site, time and mode of injection (Clark & Fregly 1996). For example, histamine, administered peripherally into animals, affects core body temperature (Brezenoff & Lomax 1970). The direction and magnitude of these responses are a function of the ambient temperature. A hypothermic response occurs in cool environments, whereas a hyperthermic response occurs when environmental temperatures exceed skin temperature (Lomax & Green 1981). Because circulating histamine and other central neurotransmitters (i.e. dopamine and NE) do not cross the blood–brain barrier freely, histamine likely mediates these changes in core body temperature via peripheral nerves that affect heat loss (i.e. skin blood flow, sweating, or metabolic heat production). In contrast, peripherally administered serotonin (5-HT) evokes whole-body hyperthermia (Feldberg & Myers 1964). Indeed, peripheral administration of several neuropeptides (i.e. angiotensin II, CCK, β-endorphin, α-melanocyte-stimulating hormone, neuropeptide Y, AVP, thyrotropin-releasing hormone and substance P; see Clark & Fregly 1996) increases or decreases heat storage. Until consensus is reached regarding the individual peripheral effects of each, the pharmacological evidence will remain unreconciled.

Central effects. The paramount role of the rostral hypothalamus, particularly the preoptic-anterior hypothalamus (POAH), as the major temperature regulating center of the brain, has been established in many animal species (Lomax & Green 1981; Boulant 1996). The POAH receives and integrates nerve impulses from the skin and interprets the circulating blood-borne compounds and endogenous substances. Signals from the POAH are integrated with impulses arising in the spinal cord, brain septum and CNS motor pathways. The following sections describe several neurotransmitters and endogenous substances that are thought to be involved in both thermal afferent pathways and in the POAH control of thermoregulation.

Glutamate. Glutamate is the primary excitatory neurotransmitter in the brain and an important neurotransmitter in thermoregulatory pathways. In afferent pathways, glutamate is involved at spinal (Nishiyama *et al*. 2001) and supraspinal levels (Salt & Turner 1998) in the integration and relay of cutaneous thermal information. In the hypothalamus, microinjection studies indicate that glutamate injections have variable effects on body temperature (Clark 1979). Since glutamate excitation may play a role in both heat loss and heat production responses, these variable responses might be expected depending on the injection site, dose and ambient temperature. An important microinjection

study was conducted by Kanosue *et al*. (1998), employing glutamate microinjections at various POAH sites in anesthetized rats. These investigators showed that POAH glutamate had effects similar to hypothalamic warming; i.e. eliciting tail vasodilation in a thermoneutral environment and suppressing shivering (Zhang *et al*. 1995) and non-shivering thermogenesis in a cold environment (Chen, X.-M. *et al*. 1998).

Glutamate is also an important transmitter in efferent thermoregulatory pathways that descend from the rostral hypothalamus. For rat tail blood flow, POAH projections descend to the midbrain where they release glutamate as a neurotransmitter. In the midbrain, glutamate excitation in one area (i.e. periaqueductal grey) elicits tail vasodilation, while glutamate in another area (i.e. ventral tegmental area) elicits tail vasoconstriction (Zhang *et al*. 1997). Therefore, it is likely that there are two subpopulations of preoptic-anterior hypothalamic neurons having descending axons controlling tail blood flow: one population controlling vasoconstriction and the other controlling vasodilation. Finally, there is also evidence that another population of POAH neurons send glutamate fibers to the caudal midbrain and medulla to control non-shivering thermogenesis by brown adipose tissue.

GABA. Gamma-aminobutyric acid is the primary inhibitory neurotransmitter in the brain. Most studies indicate that POAH microinjections of GABA-like drugs produce hypothermia (Minano *et al*. 1989). Conversely, increases in body temperature occur with administration of antagonists of two different types of GABA receptors; i.e. either $GABA_A$ (Horton, R.W. *et al*. 1988) or $GABA_B$ (Jackson & Nutt 1991). Studies also suggest that there are central interactions between $GABA_A$ receptors and body temperature changes associated with prostaglandins and opioids (Eguchi *et al*. 1999). In addition, GABA is an important transmitter in the efferent thermoregulatory pathways that descend from the rostral hypothalamus. Studies indicate that GABA is the inhibitory transmitter in axons of some POAH neurons that project to the midbrain to control tail blood flow (Kanosue 1998) and to other brain stem areas controlling non-shivering thermogenesis by brown adipose tissue (Uno & Shibata 2001).

Corticotropin-releasing factor (CRF). There is strong evidence that CRF release in the POAH is important in fever and thermogenesis (Nakamori *et al*. 1993). There are several distinct pathways for the induction of fever. One well-documented fever pathway involves interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) causing increases in a central mediator, prostaglandin E_2, which alters the activity of POAH neurons to induce fever. CRF appears to be a mediator in a separate fever pathway that is associated with inflammation, stress and neural trauma (Tache *et al*. 2001). Different cytokines (IL-1β, IL-6, IL-8) and a different prostaglandin mediator ($PGF_{2\alpha}$) serve in this separate pathway, and it is proposed that these substances increase the hypothalamic release of CRF to alter the activity of thermoregulatory neurons (Nakamori *et al*. 1993).

Serotonin. Early studies found that (either induced or naturally-occurring) changes in hypothalamic serotonin (5-HT) are associated with body temperature changes (Clark & Lipton 1986). While opposite responses occurred (depending on dose, site, species, ambient temperature) many early rat studies indicated that POAH serotonin caused thermogenesis, heat retention, and increased body temperature. In support of this, a recent microdialysis study of various neurotransmitters in the rat POAH found a strong correlation between serotonin metabolites and the rise in body temperature (Yasumatsu *et al*. 1998). In some studies, hypothalamic 5-HT has been linked with either temperature increases or decreases, possibly depending on the type of 5-HT receptor stimulated. For example, pharmacological studies link activation of $5\text{-}HT_{1A}$ receptors with hypothermia (Malone & Taylor 2001), while $5\text{-}HT_{2A}$ receptor activation has been linked with hyperthermia (Sugimoto *et al*. 2000).

Neuroimmune system. The traditional view of immune function has been replaced by a new paradigm that recognizes that immune function is co-ordinated with CNS function by the hypothalamus. This paradigm relies on the following facts: (a) immune

cells and neurons share membrane receptors for a variety of neuropeptides, corticosteroids and cytokines (i.e. immune system messenger proteins that amplify immune function and modify physiological responses); (b) the organs of the immune system are innervated by sympathetic and parasympathetic nerves, under hypothalamic control; (c) production of phagocytic cells and lymphocytes (i.e. B and T) are regulated by neuropeptides and corticosteroids emanating from the HPA axis; and (d) circulating cytokines bind to several brain regions, including the neural pituitary gland, and stimulate these regions to produce releasing hormones. Such interconnections explain why infection and other immunological stimuli influence thermoregulation (fever), nutrition (anorexia) and psychological state, collectively termed 'sickness behavior' (Zeisberger & Roth 1996).

The cytokines, a class of chemical messengers, are released into the blood in response to tissue injury, infection, and other hormones (e.g. epinephrine, NE, hGH, prolactin, β-endorphin). These messengers are critical to survival and include the subcategories interleukins (IL-1α, IL-1β, IL-6), tumor necrosis growth factors (TNF-α), chemokines and interferons (IFN-α, IFN-β). Cytokines are released by a variety of cells, including immune, endothelial and fat-storing cells (Smith 2000). The most commonly described actions of cytokines are: (a) their release from immune cells; and (b) their suppression of the early phase of inflammation, via feedback to the brain (Armstrong & VanHeest 2002). As illustrated in Fig. 31.1, they act in a way that resembles a diffuse sensory organ, providing the hypothalamus with information about a variety of processes occurring in the periphery of the body (Maier & Watkins 1998; Smith 2000). In healthy humans, cytokines have relatively little impact on body temperature regulation, but during illness they influence heat balance in two ways. First, they increase metabolic rate and heat production, as described in the Human heat balance: production versus loss section below. Second, in response to microbial (i.e. viruses, bacteria, mycobacteria, fungi) and non-microbial agents, cytokines (i.e. IL-1β, IL-6 and TNF-α) act as pyrogens, by altering the effector responses of the POAH and shifting the equilibrium

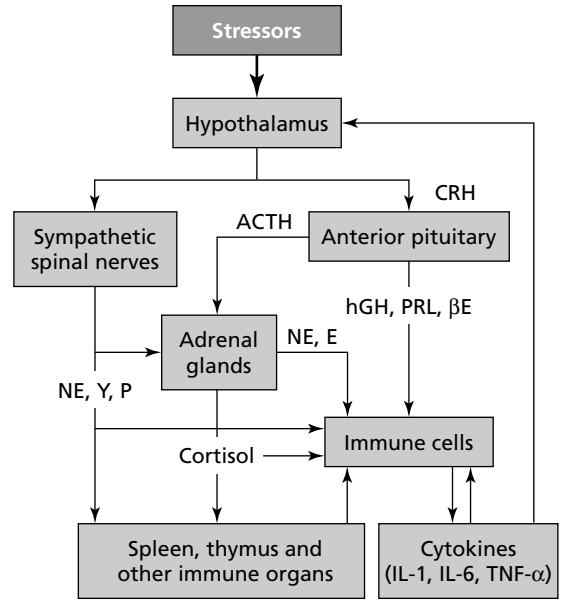

Fig. 31.1 Brain–immune system interactions. Cytokines are chemical messengers, produced by a variety of cells that complete a bi-directional communication network between the periphery and the brain. ACTH, corticotropin (adrenocorticotropic hormone); βE, β-endorphin; CRH, corticotrophin-releasing hormone; E, epinephrine; hGH, human growth hormone; IL-1, interleukin-1; IL-6, interleukin-6; NE, norepinephrine; P, substance P; PRL, prolactin; TNF-α, tumor necrosis factor-α; Y, neuropeptide Y. (Reprinted from Armstrong & VanHeest 2002 with permission.)

body temperature upward (Blatteis 1998). The resulting increase in body temperature can occur by various means, including increasing heat production responses (e.g. shivering and non-shivering thermogenesis) and heat retention responses (e.g. cutaneous vasoconstriction), as well as decreasing heat loss responses (e.g. sweating). It is likely that this is accomplished by inhibiting or enhancing the activity of specific types of hypothalamic neurons, as described in the following section.

Influences on hypothalamic neurons. As mentioned above, the POAH plays an important role in all physiological and behavioral responses that maintain body temperature constant. Some of the neurons in the POAH are thermosensitive and, therefore,

sense changes in body core temperature. These same neurons synaptically control other neurons that elicit heat loss responses (e.g. sweating), heat retention responses (e.g. cutaneous vasoconstriction) and heat production responses (e.g. shivering and non-shivering thermogenesis). Exercise, for example, causes core temperature (and hypothalamic temperature) to rise, and this is sensed by hypothalamic thermodetectors which initiate appropriate responses, such as sweating and an increase in skin blood flow.

While most POAH neurons are relatively insensitive to temperature, about one-quarter of the spontaneously firing neurons are considered to be warm sensitive. Spontaneously firing neurons produce action potentials and have firing rates determined by the number of action potentials per second. Warm sensitive neurons significantly increase their firing rates when the hypothalamus is warmed above 37°C, and these neurons also tend to decrease their firing rates when the hypothalamus is cooled below 37°C. Thus, warm sensitive neurons sense both increases and decreases in body temperature. POAH warm sensitive neurons also receive synaptic inputs from ascending somatosensory pathways conveying information from thermoreceptors in the skin and other body locations (e.g. spinal cord) (Boulant & Hardy 1974). For example, some POAH warm sensitive neurons increase their firing rates during increases in either hypothalamic temperature or skin temperature. In this way, POAH neurons can integrate central and peripheral temperatures.

POAH neurons form synaptic networks that control thermoregulatory responses. Various neuronal models have been postulated to explain these networks (Hammel 1965; Boulant 1996). For the neurons controlling heat loss responses like sweating, most models suggest that these effector neurons are synaptically excited by warm sensitive neurons and possibly inhibited by temperature insensitive neurons. Accordingly, hypothalamic warming would increase the firing rate of warm sensitive neurons which, in turn, would synaptically increase the firing rate of nearby neurons controlling sweating. A different synaptic arrangement is proposed for neurons controlling heat production and heat retention. Neurons controlling these responses are thought to be excited by temperature insensitive neurons but synaptically inhibited by the warm sensitive neurons. These neurons would appear to be 'cold sensitive' because hypothalamic cooling would decrease the firing rates of warm sensitive neurons which, in turn, would decrease their inhibition on the innervated effector neuron. As a result, a heat production effector neuron would increase its firing rate during hypothalamic cooling. While only a small proportion of POAH neurons are considered to be cold sensitive, POAH warm sensitive neurons could synaptically inhibit other effector neurons located in various brain stem locations.

The neuroendocrines and neurotransmitters mentioned in the above sections generally have predictable effects on the different types of POAH neurons. Neurochemicals that produce hypothermia often have excitatory effects on POAH warm sensitive neurons. Excitation of warm sensitive neurons increases heat loss responses and, thus, lowers body temperature. In contrast, neurochemicals that produce hyperthermia (such as fever-producing pyrogens) tend to inhibit warm sensitive neurons and excite cold sensitive neurons. This produces decreased sweating, decreased skin blood flow, but increased shivering and non-shivering thermogenesis; and all of these responses increase body temperature.

In addition to regulating body temperature, the POAH also is important in reproduction. Some POAH neurons contain and are sensitive to reproductive neuroendocrines such as testosterone, estrogen or progesterone. Moreover, there are overlaps and strong interactions between temperature sensitive neurons and neurons that are sensitive to reproductive endocrines. In a study of rat POAH tissue slices, about 30% of warm sensitive neurons were found to be excited by either testosterone or estrogen. If warm sensitive neurons enhance heat loss responses, this may explain why animal studies sometimes link testosterone or estrogen administration to lowered body temperatures (Silva & Boulant 1986). The menstrual cycle of women may also illustrate the interactions between thermosensitive and endocrine sensitive POAH neurons. Midway through the menstrual cycle, just before ovulation,

some women show a brief drop in body temperature that is associated with peak estrogen levels (Stephenson & Kolka 1999). Following this, there often is a rapid rise in body temperature that stays elevated for much of the later half of the cycle; and this later period is associated with elevated progesterone levels (Kolka & Stephenson 1997). As mentioned for the rat tissue slice study above, some POAH warm sensitive neurons are excited by estrogen. If warm sensitive neurons facilitate heat loss and suppress heat production, this may explain the transient, mid-cycle drop in body temperature during the estrogen peak. Moreover, similar neuronal experiments (Boulant, unpublished) have discovered some POAH warm sensitive neurons that are inhibited by progesterone, which may explain the sustained elevation in body temperature during the last half of the cycle.

Human heat balance: production versus loss

To maintain internal body temperature at about 37°C during waking and sleeping hours, humans constantly adapt to changes in air temperature, humidity, air movement, solar radiation, barometric pressure and clothing insulation. Further, as food is digested and metabolized, approximately 80% of all nutrient energy eventually is transformed to heat. If strenuous muscular exercise is undertaken, the rate of energy utilization can increase well above the resting metabolic rate. For example, an elite marathon runner (body mass, 66.9 kg; height 185 cm) required 5862 kJ (1400 kcal) energy·h^{-1} (97.6 kJ·min^{-1} [23.3 kcal·min^{-1}]; running speed, 314 m·min^{-1}), continuously for 2.2 h, to complete the 1984 Summer Olympics in Los Angeles (Armstrong *et al*. 1986); this was approximately 8–12 times greater than his energy utilization at rest (i.e. 8.3–12.6 kJ·min^{-1} [2–3 kcal·min^{-1}]). Such a heat load must be removed or serious hyperthermia (i.e. body temperature above 39–40°C), tissue damage and death may result from exertional heat-stroke. Indeed, this athlete experienced this malady twice during his running career.

Environmental–heat stress increases the need for evaporative and radiative-convective cooling. Therefore, the preoptic area of the anterior hypothalamus senses rising blood and organ temperatures, and initiates sweat gland secretion concurrent with cutaneous blood vessel dilation. Elsewhere in the CNS, the heated spinal cord also influences temperature regulation by inducing complimentary effector responses in skin sweat glands and blood vessels (Jessen 1996). Although prolonged strenuous exercise in a hot environment typically results in sweat losses of 0.8–2.0 L·h^{-1}, the aforementioned marathon runner produced 3.71 L·h^{-1} of sweat and incurred dehydration that was detrimental to his running performance and health. The body dissipates 2428 kJ (580 kcal) for each liter of sweat that is evaporated from the skin. Because 85–90% of all heat dissipation occurs via sweat evaporation in a hot-dry environment (Adams *et al*. 1975), and because over 50% of heat loss in a hot-humid environment may occur via radiation and convection from dilated skin blood vessels, the autonomic and neuroendocrine control of metabolism, sweating and blood flow are vital to thermoregulation, health and exercise performance.

During strenuous exercise, neuroendocrine responses within the ANS support temperature regulation, ventilation, cardiovascular responses, fluid-electrolyte balance and immune functions. These widespread effects involve the SAM axis, the HPA axis, endogenous opioid messengers (i.e. endorphins, dynorphins, enkephalins) and fluid-electrolyte regulating hormones (Borer 2003). During exercise and rest, the ANS affects temperature regulation primarily via sudomotor (i.e. in sweat glands) and vasomotor (i.e. in blood vessels) responses. The sudomotor response facilitates heat dissipation through sweat production and evaporation. An increase in temperature is detected by central and peripheral thermoreceptors, and the eccrine sweat glands are activated via sympathetic cholinergic neurons. These same thermoreceptors also affect vasomotor responses (i.e. cutaneous vasoconstriction or vasodilation) controlling skin blood flow, which determines radiative and convective heat loss to the environment.

Exclusive of exercise, the term *thermogenesis* refers to adaptive or regulatory increases in metabolic rate that are associated with excess food consumption

(i.e. diet-induced thermogenesis); cold exposure, cold adaptation and arousal from hibernation (i.e. non-shivering thermogenesis); or responses to disease, injury and stress (often referred to as hypermetabolism). While the preoptic anterior hypothalamus exerts strong control over thermogenesis, several other neural structures can influence heat production. These other structures may include the, ventromedial hypothalamus, posterior hypothalamus, paraventricular nucleus, cerebral cortex, hippocampus and brain stem structures such as the raphe nucleus and locus coeruleus (Rothwell 1994). Furthermore, animal research has verified that several peptides (i.e. CRF, thyrotrophin-releasing factor [TRF], insulin, β-endorphin, angiotensin, somatostatin, γ-melanocyte-stimulating hormone, CCK) and cytokines (i.e. IL-1α, IL-1β, IL-6, IL-8, IFN-γ, TNF-α) increase metabolic rate.

Acknowledging that the peripheral and central effects of specific neurotransmitters and neuropeptides remain largely unknown, the remainder of this chapter focuses on neuroendocrine responses and adaptations to whole-body hyperthermia (i.e. core body temperature > 38°C) in humans, at rest and during exercise in a hot environment.

Passive, intense heat exposure in humans

The hot-dry Finnish sauna bath is taken in a paneled room that contains wooden benches and a rock-filled heater. The air temperature ranges from 80–100°C at face level and 30°C at floor level, with a relative humidity of 10–20%. A single exposure lasts 5–20 min, but this exposure may be extended or repeated by experienced bathers. Human sauna bathing provides an interesting model to study the body's neuroendocrine responses to extreme hot-dry environments.

Heat production and dissipation. Mean resting metabolic rate increases 20%, ranging to 40% above the pre-exposure level (Hasan *et al*. 1966). This increased heat production, coupled with an environment that stifles radiative and convective heat loss, causes adult rectal temperature to rise 0.2, 0.4 and 1.0°C during exposures of 15 min (72°C), 20 min (92°C) and 30 min (80°C), respectively (Hannuksela & Samer 2001). After the sauna, metabolic rate returns to baseline at about the same rate as body temperature.

In response to this rise in core body temperature, hypothalamic efferent signals cause visible sweat production within 8–12 min. The mean sweat rate ranges from 0.6–1.0 $L \cdot h^{-1}$ at 80–90°C, resulting in an average total loss of 300–500 mL during a typical sauna bath. Despite this sweat secretion, skin temperature rises from 35–36°C (pre-exposure) to 40°C within a few minutes (Hannuksela & Samer 2001).

Cardiovascular responses include a large increase in skin blood flow, from 0.5 to 7.0 $L \cdot min^{-1}$ (i.e. from 5–10% to 50–70% of total resting cardiac output), which is accomplished by a decrease of peripheral autonomic nerve activity (i.e. withdrawal of sympathetic vasoconstriction), causing a decrease in total peripheral resistance (Zeisberger 1998). This vasodilation in skin involves local production of the neurotransmitter nitric oxide (Kellogg *et al*. 1999). But, because air temperature is much greater than skin temperature, radiative and convective heat is gained through skin, not lost. Simultaneously, the increase of sympathetic activity in thoracic organs (i.e. increased vasoconstrictor tone) reduces splanchnic blood flow by 0.6 $L \cdot min^{-1}$. As a result, most studies consistently report a decline in diastolic blood pressure (6–39 mmHg), but variable pressure responses at systole. Because cardiac stroke volume remains constant, the increased cardiac output at rest is solely accomplished by increased frequency of contraction. Mean resting heart rate increases to 100 $b \cdot min^{-1}$ in experienced bathers, and as high as 150 $b \cdot min^{-1}$ in some unaccustomed adults who experience intense heat stress (Hannuksela & Samer 2001). Dehydration, due to sweat losses, further reduces central blood volume. To maintain cardiac output, vasomotor reflexes decrease blood flow to splanchnic organs and increase heart rate. In addition to the release of epinephrine and NE, cardiovascular stability is enhanced by increased secretion of renin, angiotensin II, aldosterone, AVP and cortisol (Hannuksela & Samer 2001). All of these hormones act to increase blood pressure and/or heart rate (Borer 2003).

Table 31.1 Acute human thermoregulatory and stress hormone responses to severe heat exposure (80–100°C, 7–15%rh) in a sauna bath.

Hormone	Exposure duration (min)	Human subjects		Change of blood concentration	References
		Male	Female		
ACTH[†††]	30	7		Increased	Jezova *et al*. 1985
	20		11	Unchanged	Laatikainen *et al*. 1988
	30	8		Increased	Vescovi *et al*. 1990
	30	8		Increased	Vescovi *et al*. 1992
	20		8	Increased	Jezova *et al*. 1994
	20	8		Unchanged	Jezova *et al*. 1994
β-Endorphin[†‡]	30	7		Increased	Jezova *et al*. 1985
	20		11	Increased	Laatikainen *et al*. 1988
	30	8		Increased	Vescovi *et al*. 1992
	30	8		Increased	Vescovi *et al*. 1990
Growth hormone[§]	20	6		Increased	Aldercreutz *et al*. 1976
	20–40	37	18	Increased	Lammintausta *et al*. 1976
	30	10	7	Increased	Leppaluoto *et al*. 1986
	30	8		Increased	Vescovi *et al*. 1992
Prolactin[§¶]	20	6		Increased	Aldercreutz *et al*. 1976
	30	10	7	Increased	Leppaluoto *et al*. 1986
	20		11	Increased	Laatikainen *et al*. 1988
	30	8		Increased	Vescovi *et al*. 1992
Cortisol[††]	20	6		Increased	Aldercreutz *et al*. 1976
	30	7		Unchanged	Jezova *et al*. 1985
	20		11	Unchanged	Laatikainen *et al*. 1988
Epinephrine[*‡§**]	10	5		Increased	Hussi *et al*. 1977
	20		11	Unchanged	Laatikainen *et al*. 1988
	20		8	Increased	Jezova *et al*. 1994
	20	8		Increased	Jezova *et al*. 1994
Norepinephrine[*‡§**]	10	5		Increased	Hussi *et al*. 1977
	20		11	Increased	Laatikainen *et al*. 1988
	20		8	Increased	Jezova *et al*. 1994
	20	8		Increased	Jezova *et al*. 1994
Triiodothyronine (T_3)[**‡‡]	30	10	7	Unchanged	Leppaluoto *et al*. 1986
	30	10		Increased & unchanged	Strbak *et al*. 1987

ACTH, adrenocorticotropic hormone.
*Responds to stressors as part of the hypothalamic–pituitary–adrenocortical (HPA) axis.
[†]ACTH (adrenocorticotropic hormone) and β-endorphin are anterior pituitary products of a common precursor (proopiomelanocortin) (Vescovi *et al*. 1990).
[‡]Mediates skin blood flow (i.e. radiation and convective heat dissipation).
[§]Stimulates secretion of sweat in eccrine glands (i.e. evaporative heat dissipation).
[¶]Prolactin may alter Na^+–K^+ and water transport in eccrine sweat gland membranes.
**Increases metabolic rate and heat production.
[††]Responds to stressors as part of the sympathetic–adrenomedullary (SAM) axis.
[‡‡]The active form of thyroid hormone.

Peripheral hormone responses. Because present technology does not allow direct measurement of human brain neurotransmitters, and because these neurotransmitters do not cross the blood–brain barrier freely, Table 31.1 describes changes of blood-borne hormones (all of which are neurotransmitters or neuropeptides except cortisol and triiodothyronine, T_3), following sauna exposure. These hormones are secreted by the anterior pituitary (ACTH, β-endorphin, hGH, prolactin), adrenal cortex (cortisol), adrenal medulla (epinephrine, NE) and thyroid gland (T_3) in response to a variety of stressors (i.e. hyperthermia, intense exercise, dehydration, hypoxia, hypoglycemia, hypovolemia and psychological stress; Borer 2003). The stress hormones in Table 31.1 indicate that a portion of the hormone response to sauna bathing (80–100°C) may result from SAM axis or HPA axis responses to psychological or environmental stresses.

Three factors complicate the interpretation of Table 31.1. First, although all blood samples were collected at rest and all of these hormones increase when animals are experimentally heated (Borer 2003), it is impossible to attribute changes in human hormones to heat storage alone, unless central hyperthermia was verified via measurements of core body temperature. Unfortunately, only one of these hormone studies (Jezova *et al*. 1994) measured core body temperature validly (i.e. rectal, esophageal or tympanic membrane temperature), and none included simultaneous measurements of sympathetic nervous system effector responses such as sweat rate, cutaneous blood flow, or metabolic heat production. Indeed, the variant findings of both unchanged and increased hormone concentrations (column 5 in Table 31.1) may be due to different degrees of body temperature elevation. Second, hormones (column 5, Table 31.1) may increase or decrease at different rates, in response to hyperthermia, depending on the time that elapsed between heat exposure and blood sample collections (Jezova *et al*. 1985, 1994). Figure 31.2 illustrates this phenomenon, in that peak plasma concentrations of ACTH and cortisol were observed at different time points after sauna exposure. This likely was due to continued release of cortisol by the adrenal glands, well after the peak pituitary secretion and plasma concentration of ACTH had occurred. Third, repeated exposures to intense environmental heat and repeated bouts of whole-body hyperthermia alter the secretion patterns of some hormones. Thus, the neuroendocrine responses in Table 31.1 may not be observed among experienced bathers who have taken saunas throughout their lives (Kukkonen-Harjula *et al*. 1989).

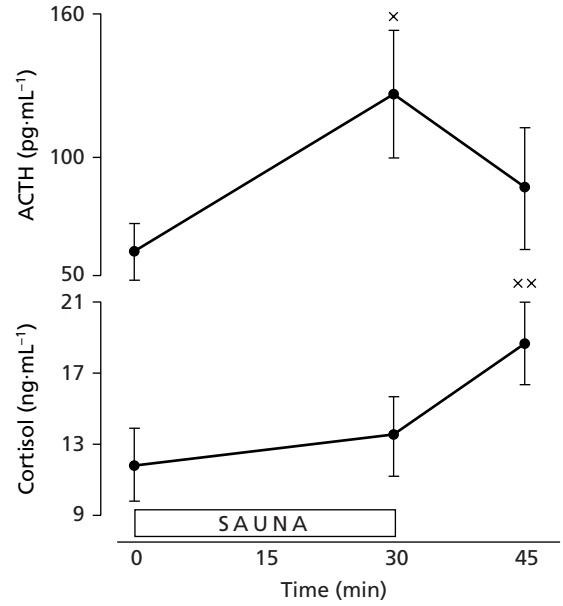

Fig. 31.2 Mean changes of plasma cortisol and adrenocorticotropic hormone (ACTH) (in seven healthy men) in response to intense heat exposure in a sauna for 30 min. X, $p < 0.05$; XX, $P < 0.01$. (Redrawn from Jezova *et al*. 1985 with permission.)

In light of these considerations, we believe that the following statements provide a reasonable summary of Table 31.1, regarding changes in circulating hormones after resting sauna exposure:

(a) hGH, β-endorphin, prolactin and NE were elevated in blood samples, following sauna exposure, in all investigations.

Notes:

- Research evidence indicates that β-endorphin may mediate blood flow (Navaratnam *et al*. 1992), lowers the hypothalamic set point temperature and reduces metabolic heat production (Kraemer, W.J. *et al*. 2003).

- Secretion from eccrine sweat glands is peripherally mediated by the neurotransmitter ACh in sympathetic cholinergic nerve fibers, circulating NE, plus anterior pituitary hormones hGH (Boisvert *et al*. 1993) and prolactin (Follenius *et al*. 1979; Kaufman *et al*. 1988).
- An increased plasma NE concentration represents increased sympathetic and adrenal medullary activity that maintains thermoregulatory effector responses (Zeisberger 1998).

(b) Circulating ACTH, cortisol and epinephrine may or may not increase following exposure to heat, depending on the duration/intensity of exposure and the degree of whole-body hyperthermia experienced.

Notes:

- ACTH and cortisol represent a neuroendocrine response to stress as part of the HPA axis.
- Circulating epinephrine potentiates sweat secretion, but plays a secondary role to direct innervation of eccrine sweat glands by sympathetic cholinergic nerve fibers (Zeisberger 1998).

Summary statements (a) and (b) agree with previously published observations that NE (Laatikainen *et al*. 1988), prolactin (Aldercreutz *et al*. 1976; Mills & Robertshaw 1981; Leppaluoto *et al*. 1986; Laatikainen *et al*. 1988) and hGH (Leppaluoto *et al*. 1986) are sensitive markers of physiological strain in the severe heat of a sauna (80–100°C, 7–15%rh).

Exercise–heat stress in humans

Neuroendocrine responses during exercise in a hot environment (defined here as ≥ 35°C) are distinctly different from those that occur while resting in a sauna bath. This is true because there are multiple stimuli for neurotransmitter or hormone release and, unlike rest, exercise alters the activity of several neurotransmitter pathways concurrently or in succession (Giustina & Veldhuis 1998). Although most of these perturbations can be attributed to increased sympathetic nervous tone (i.e. increased ventilation, oxygen consumption, heart rate) or increased activity in the HPA and/or SAM axes, the endocrine responses to concurrent heat and exercise stress are not always straightforward. For example: (a) exercise and thermal stressors have a synergistic effect on the plasma concentrations of some hormones (i.e. NE, epinephrine, cortisol) but not others (i.e. dopamine, growth hormone) (Brenner *et al*. 1997); (b) plasma prolactin concentration increases more during passive heating than during exercise (Kukkonen-Harjula 1989); and (c) plasma cortisol may increase or decrease during low intensity exercise, depending on its circadian rhythm (Thuma *et al*. 1995).

During exercise–heat stress, numerous hormone, neuropeptide and neurotransmitter interactions complicate a thorough description of human thermoregulation. Growth hormone is a prime example. For example, the release of growth hormone is mediated by neuropeptides (i.e. galanin, thyroid-releasing hormone, neuropeptide Y, calcitonin, β-endorphin), neurotransmitters (i.e. ACh, NE, dopamine, 5-HT and GABA), metabolic substrates (i.e. blood glucose, L-arginine) and hormones (i.e. gonadal testosterone, estrogens, somatostatin and thyroid hormones) (Giustina & Veldhuis 1998). Several isoforms of growth hormone have been identified, but their distinct physiological roles have not been clarified (Baumann 1999). Both temperature and exercise (i.e. exercise intensity, intermittency, duration and length of rest periods) are recognized as stimuli for growth hormone release. Thus, it is likely that multiple neurotransmitter pathways, as well as a variety of peripheral feedback signals, regulate growth hormone secretion either by acting directly on the anterior pituitary gland and/or modulating the hypothalamic release of growth-hormone releasing hormone or somatostatin. This is relevant to body temperature regulation because a deficiency of growth hormone reduces sweat secretion from cutaneous eccrine glands (Lobie *et al*. 1990; Borer 2003), decreases evaporative heat loss and increases the risk of serious hyperthermia during exercise (Hjortskov *et al*. 1995).

Figure 31.3 depicts the complex interaction of additional factors that may influence the neuroendocrine regulation of human body temperature during exercise–heat stress. The heart of this figure (see shaded rectangle) involves neuroendocrine release, clearance and membrane receptors, transporter protein binding and release, as well as sympathetic effector responses, that enhance heat

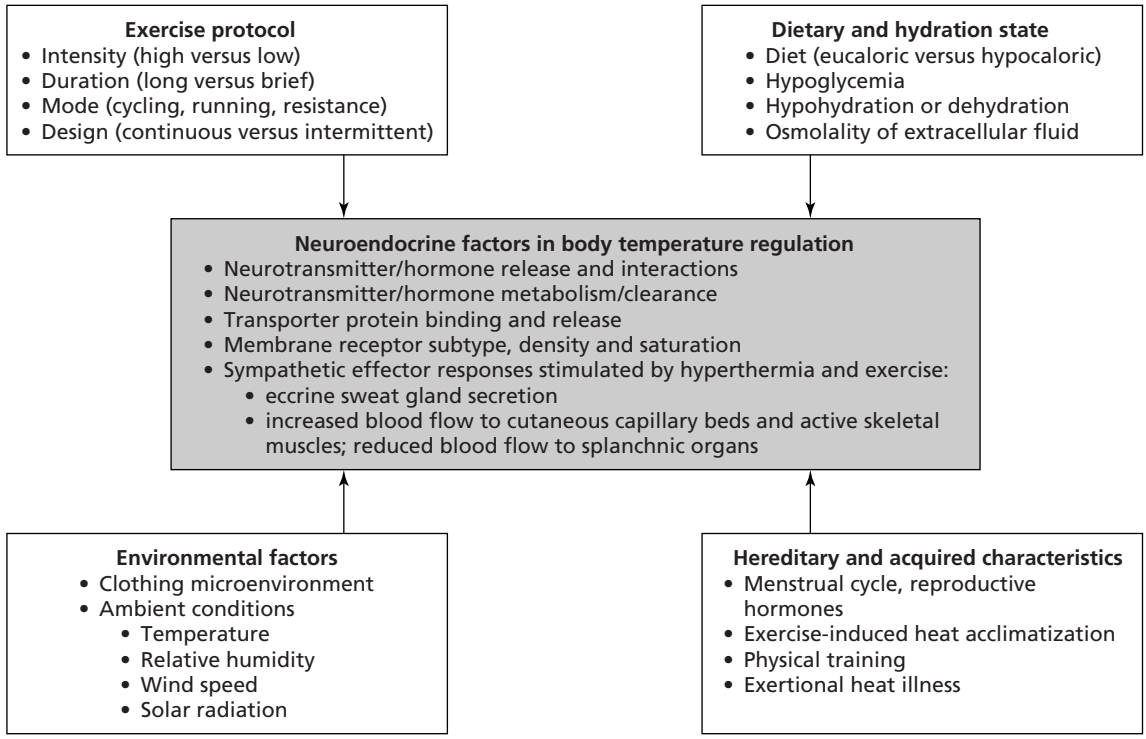

Fig. 31.3 Interactions of numerous factors that may influence the neuroendocrine regulation of human body temperature during exercise–heat stress.

dissipation via sweating and cutaneous vasodilation. These components of neuroendocrine function are influenced by other stimuli (see unshaded rectangles) including features of the exercise protocol, diet/metabolism/fluid balance, hereditary/acquired characteristics and environmental factors. Although today it is virtually impossible to explain this multifactorial problem simply, the following section describes our present understanding of these factors, as they influence neuroendocrine responses to exercise–heat stress.

Exercise protocol. The research of Hoffman *et al*. (1994) advanced NE as the preferred neuroendocrine index of human exercise–heat strain. This arose from the fact that neither epinephrine, testosterone nor cortisol increased in response to low-intensity treadmill exercise at 33°C, regardless of baseline hydration state or *ad libitum* water intake during exercise. Mora-Rodriguez *et al*. (1996) and Castellani *et al*. (1997) also identified NE as a more sensitive neuroendocrine marker, versus epinephrine, of physiologic strain in the heat (33°C in both; 150, 90-min cycling exercise; and 65, 50% $\dot{V}o_{2max}$, respectively). Similarly, Nybo *et al*. (2002) reported that NE responded markedly, whereas epinephrine did not, when young men wore insulated clothing (i.e. establishing uncompensable heat stress) and cycled in a 20°C environment (60 min, 50% $\dot{V}o_{2max}$). However, during an intense (80% $\dot{V}o_{2max}$) 16.1-km outdoor run in the heat (30°C), both plasma epinephrine and NE concentrations increased (Hartung *et al*. 1987), suggesting that either exercise intensity or posture (upright versus seated) may alter catecholamine responses, thereby affecting thermoregulation and body heat storage. This raises the question, 'What influences do the components of an exercise protocol have on human neuroendocrine and thermoregulatory responses?'

Borer (2003) and other authorities state that

pituitary secretion of ACTH and β-endorphin, adrenocortical release of cortisol and adrenomedullay secretion of the stress hormones epinephrine and NE increase above exercise intensities of 80–90% $\dot{V}O_{2max}$, in a mild environment. However, reality may be more complex than this statement suggests. Borer's concept evidently does not include investigations that reported: (a) no change in ACTH, β-endorphin and cortisol at three supramaximal exercise intensities (175, 230, 318 $\dot{V}O_{2max}$; 6–46 s duration; seated cycling; 23°C) but an increase in these hormones at 115 $\dot{V}O_{2max}$ (Kraemer, W.J. *et al*. 1989); (b) constant plasma β-endorphin levels during the initial hour of cycling exercise (50% $\dot{V}O_{2max}$; 35°C), that increased during the final 75–120 min (Kelso *et al*. 1984); (c) a decrease in cortisol concentration during five 15-s maximal anaerobic power tests, separated by 30 s of active recovery (22 and 35°C) (Hoffman *et al*. 1997); and (d) an increase in circulating epinephrine, cortisol and NE concentrations during 120 min of cycling at 40% $\dot{V}O_{2max}$ (22°C), with a plateau in NE from 60–120 min (Horton, T.J. *et al*. 1998). Two of these research teams concluded that hormonal responses were not duration- or intensity-dependent (Kraemer, W.J. *et al*. 1989), and were perhaps more duration-dependent than intensity-dependent (Hoffman *et al*. 1997).

Although the aforementioned findings are difficult to interpret, they likely are due to differences in experimental designs. In support of this interpretation, the investigation conducted by Kraemer, W.J. *et al*. (1995) observed that: (a) different heavy-resistance training protocols resulted in different blood β-endorphin and cortisol kinetics; and (b) the duration of force production and the length of rest periods between sets were the critical design features. Few studies have attempted to separate the independent effects of exercise mode (i.e. cycling, running, resistance exercise), design (i.e. continuous versus intermittent exercise), intensity, or duration on neuroendocrine factors. One investigation (Brenner *et al*. 1997) offers a noteworthy exception, in that it distinguishes the physiological responses to air temperatures of 23°C and 40°C from responses to exercise. A thorough understanding of the neuroendocrine regulation of body temperature, as it is influenced by various exercise protocols, requires much additional study, especially with environmental temperatures above 35°C.

Diet and hydration state. Because the release of hormones and neurotransmitters may stimulate the mobilization or storage of biochemical substrates, dietary adequacy or insufficiency may alter neuroendocrine function. In this regard, research has focused on two independent variables: energy availability and body hydration state. First, decreased blood glucose concentrations (< 3.3 $mmol \cdot L^{-1}$), during prolonged exercise, result in an increased activation of the HPA axis in humans, as evidenced by increased blood ACTH and cortisol concentrations. Representative of increased physiological strain, this phenomenon can be reversed by maintaining blood glucose at a constant level (Tabata *et al*. 1991).

Second, a loss of body water also affects human thermoregulatory responses. For example, during low-intensity walking (140 min total; four 25-min exercise bouts; 28–30% $\dot{V}O_{2max}$) in a 49°C (20%rh) environment, test subjects exhibited increased plasma cortisol and growth hormone concentrations (versus euhydration) when they were dehydrated to –5% of initial body weight (Francesconi *et al*. 1984). Further, reduced plasma volume per se (–14.6%, versus 0% in a control trial) affects heat dissipation during prolonged cycling, resulting in increased rectal temperature during dehydration (Roy *et al*. 2000) and increased whole-body sympathetic nervous drive (i.e. plasma NE increased but not epinephrine). Dehydration apparently affects sweating, via sympathetic cholinergic innervation and the neurotransmitters ACh and NE; it reduces the sensitivity of eccrine sweat glands to hyperthermia and increases the temperature at which sweating begins (Armstrong & Maresh 1998). Thus, sweating begins later in exercise when an individual is dehydrated, versus euhydrated.

Senay (1979) concluded that these hypothalamic responses are largely due to changes in the osmolality of brain tissue or extracellular fluid osmolality. Three publications from our laboratory support this concept. When healthy males performed 90 min of continuous treadmill exercise (5% grade, 36% $\dot{V}O_{2max}$) in a heated chamber (33°C, 56%rh), dew

point sensor measurements indicated that local sweat rate decreased as plasma osmolality increased (from 281 to 324 $mOsm \cdot kg^{-1}$) and as body water loss (from −0.2 and −6.7% of body mass) increased (Armstrong *et al*. 1997). During these experiments, Hoffman *et al*. (1994) observed that *rehydration* with plain water during exercise significantly reduced the plasma NE response. This was true whether test participants began exercise in a euhydrated or hypohydrated state. In a separate study from our laboratory, Castellani *et al*. (1997) observed that rehydration via either oral or intravenous fluids (both 0.45% NaCl) reduced the plasma NE (i.e. sympathetic nerve activity), ACTH and cortisol (i.e. HPA axis activity) increases that occurred in a separate experiment that involved no fluid and exercise –heat stress. These findings are consistent with the concept that rehydration decreased the osmotic pressure (i.e. 307 $mOsm \cdot kg^{-1}$ without water versus 293 $mOsm \cdot kg^{-1}$ with water), this was sensed by hypothalamic neurons, which in turn reduced both the sympathetic nervous drive for sweating and the anterior pituitary release of ACTH. This reasoning links hydration state and osmolality to neuroendocrine control of body temperature.

Individual ions also may influence thermoregulation. The thorough review of hypothalamic regulation of body temperature by Myers (1980) concludes that whole-body hyperthermia can be reversed, while laboratory animals exercise, by infusing calcium ions into the hypothalamus. This suggests that individual ions (i.e. calcium, sodium, potassium and magnesium) play a role in thermoregulatory effector responses and heat dissipation.

Gender and reproductive hormones. After reviewing numerous publications, we concluded that gender influences on human neuroendocrine regulation of body temperature are incompletely defined, especially because no studies involved exercise in the heat. However, a few gender-comparison studies involved either exercise in a mild environment (22°C; Horton, T.J. *et al*. 1998; Kraemer, R.R. *et al*. 1989; Davis *et al*. 2000) or rest in intense heat (90°C; Jezova *et al*. 1994), and generated variant findings. Reproductive hormone fluctuations also may set the stage for gender differences. For example, growth hormone responses to exercise vary at different points in the menstrual cycle (Hansen & Weeke 1974) and oral contraceptives potentiate the exercise-induced secretion of growth hormone (Bernardes & Radomski 1998). These effects likely are due to the interaction of estrogen with the hypothalamic regulation of growth hormone release. Estrogen also may affect radiative and convective heat loss by enhancing the bioavailability of nitric oxide, a potent vasodilator (Chen, Z. *et al*. 1999). Further, estradiol and progesterone are known modulators of sweat rate, cutaneous blood flow and metabolic heat production, having the effect of altering core body temperature throughout the menstrual cycle (Kolka 1999). Although not perfectly aligned with the question at hand, these studies provide direction and testable hypotheses for future research.

Heat acclimatization adaptations. During 5–14 days of exposure to environmental stress (i.e. heat, cold, high altitude), a complex of systemic, ultrastructural and biochemical adaptations occur that are named *acclimatization* in nature or *acclimation* in an artificial environment (Armstrong & Maresh 1991). Obviously, CNS and neuroendocrine adaptations are involved in heat acclimatization, in the form of decreased rectal temperature (i.e. enhanced peripheral effector responses that increase heat dissipation), heart rate, sweat/urine sodium concentrations (i.e. aldosterone secretion from the adrenal cortex) and onset temperature for sweat production (Armstrong *et al*. 1987; Armstrong & Maresh 1998); these occur concurrently with increased total sweat rate (i.e. sympathetic cholinergic innervation) and plasma volume (i.e. sympathetic vasodilatory control via α- and β_2-adrenergic receptors in precapillary blood vessels; see Horowitz & Gival 1989).

An animal model of heat acclimation demonstrated central neuroendocrine modifications, in the form of increased NE and dopamine levels in the POAH (Christman & Gisolfi 1985), in concert with enhanced peripheral heat-dissipating capacity. This agrees with observations that heat-acclimated rats consistently show enhanced thermoregulatory sensitivity to POAH injections of various neuromodulating agents including NE and 5-HT (Gordon 1993).

Ultrastructural and metabolic adaptations during heat acclimation have been identified by Horowitz (1998). First, central adaptations have been observed in the POAH, including altered membrane components, that accompany peripheral changes in membrane receptor density, receptor affinity for specific molecules, and cytosolic Ca^{2+} concentration. Second, whole-body measurements of lower oxygen consumption (i.e. lower $\dot{V}o_2$ at a given workload) have been reported in humans who participated in exercise–heat acclimation programs (Sawka *et al*. 1983). Little was known about this phenomenon until recently. Animal studies have shown that improved biochemical efficiency (i.e. lower metabolic rate) occurs in cardiac tissue, in the same time frame that increased glycogen content, enhanced cellular uptake of glucose and increased glycolytic potential occur (Horowitz 2003). These biochemical changes enhance the energy-generating potential of the heart, allowing it to deal with the increased cardiovascular strain imposed by environmental–exercise stress.

A recent review (Armstrong & Stoppani 2002) describes the emerging paradigm of central nervous system control of adaptations during repetitive thermal stress, and emphasizes neural network plasticity that is synchronized with altered biochemical pathways. The authors describe several plausible mechanisms for human physiological adaptations, including: (a) CNS control of habituation (i.e. diminished synaptic transmission), the set point of core body temperature or neural circuitry; (b) biochemical and ultrastructural adaptations (i.e. altered cell membrane characteristics; changes in the intracellular concentration of organelles and rate-limiting enzymes of energy metabolism); and (c) effector organ responses (i.e. change of the threshold temperature for increased blood flow or sweat rate; morphological changes in sweat gland or neuromuscular junction size). Because many neurobiologists believe that information storage is associated with the specific processing areas that are engaged in homeostatic control, the anterior hypothalamus (i.e. thermoregulatory integration), medulla or pons (i.e. heart rate, blood pressure, ventilation) and the reticular formation (i.e. integration of neural inputs) are theoretically implicated in the development of heat acclimatization (Armstrong & Stoppani 2002).

Regarding thermoregulatory and stress hormones, Table 31.2 summarizes the adaptations that have been reported in humans and animals, subsequent to 7–10 days of exercise–heat acclimation in 35–49°C environments. Acknowledging that numerous factors influence chronic neuroendocrine adaptations during heat acclimation (see Fig. 31.3), the primary benefit of Table 31.2 is that it emphasizes future research that is needed.

Physical training adaptations. Because exercise–heat acclimation involves consistent, repeated exercise, it is logical that heat-acclimation adaptations would overlap those that are derived from exercise training (Armstrong & Pandolf 1988). Indeed, an endurance training program in a cool environment results in a lowered threshold temperature for the onset of sweating, increased plasma volume, increased cardiac stroke volume, decreased heart rate, decreased skin temperature, decreased rectal temperature and increased $\dot{V}o_{2max}$. These similarities support numerous reports that highly-trained endurance athletes, training exclusively in cool environments, respond to exercise in a hot environment as though they were heat acclimatized (Armstrong & Maresh 1991). A few studies have evaluated hormonal responses to multiweek physical training programs in cool environments. In general, these studies have shown an improved functional endocrine capacity, expressed as increased blood levels of ACTH, epinephrine, NE and β-endorphin (Viru 1992). Unfortunately, the research regarding the influence of physical training on neuroendocrine regulation of human body temperature, in any environment, is limited and does not allow generalizations to be advanced at this time.

Exertional heatstroke and heat exhaustion. Exertional heat exhaustion involves an inability to continue exercise in the heat because of cardiovascular insufficiency. It is the most common heat-related disorder in military, athletic and civilian settings and recovery is complete within 24–48 h. It results from either water depletion, salt depletion or mixed salt–water depletion (Armstrong & Anderson 2003).

Table 31.2 Summary of thermoregulatory and stress hormone adaptations during exercise–heat acclimation (35–49°C) in humans and animals.

Circulating hormone*	Research findings	Exposure duration (days)	References
ACTH	Undetermined		
β-Endorphin	HA had no effect on β-endorphin levels in normal males†	8	Kraemer W.J. *et al*. 1987; Kraemer, W.J. *et al*. 2003
	HA decreased β-endorphin levels in former heatstroke patients†	7	Kraemer, W.J. *et al*. 2003
hGH	HA had no effect on hGH levels in 35°C and 49°C environments when subjects were euhydrated	10	Francesconi *et al*. 1984
Prolactin	Undetermined		
Cortisol	HA (intense intermittent exercise, 50 min) had no effect on cortisol levels	8	Kraemer, W.J. *et al*. 1987
	HA (intense intermittent exercise, 56 min) decreased cortisol levels	8	Armstrong *et al*. 1989
	HA (mild intensity 100 min) decreased cortisol levels when subjects were hypohydrated‡ in a 35°C, but not in a 49°C, environment	10	Francesconi *et al*. 1984
Epinephrine	Undetermined		
Norepinephrine	HA resulted in increased brain NE levels in rats	14	Christman & Gisolfi 1985
	HA enhances thermal effector responsiveness to NE injected into the POAH	21	Christman & Gisolfi 1980
Dopamine	HA resulted in increased brain dopamine levels§ in rats	14	Christman & Gisolfi 1985
Serotonin	HA enhances thermal effector responsiveness to 5-HT injected into the POAH	d.n.p.	Gordon 1993
Thyroxine	HA resulted in a sustained decrease of thyroxine in rats	14–30	Horowitz 1998; Horowitz 2003

ACTH, adrenocorticotropic hormone; d.n.p., data not provided; 5-HT, serotonin; hGH, human growth hormone; HA, exercise–heat acclimation in humans; NE, norepinephrine.
*The physiological significance of these hormones appears in Table 31.1 footnotes.
†β-Endorphin levels increased during each daily exposure to exercise–heat stress.
‡Hypohydration (–5% body weight loss) increased plasma cortisol levels (versus euhydration) on day 1 but not day 10 of HA.
§As determined by analysis of a dopamine metabolite (3,4-dihydroxyphenylglycol).

Exertional heatstroke, a medical emergency, occurs when the combined thermal stresses from the environment and muscular metabolism exceed the body's heat dissipation capacity. A dangerous hyperthermia may lead to profound CNS involvement, multisystem failure and death if the body is not cooled promptly (Casa & Armstrong 2003).

Ethical principles dictate that investigators shall not induce exertional heat illness in test participants. Therefore, virtually all investigations of the neuroendocrine responses to exertional heat illness arise from retrospective analyses. Table 31.3 summarizes the scientific literature regarding exertional heatstroke and exertional heat exhaustion. In addition to the SAM-axis or HPA-axis stress responses to hyperthermia and exhaustive exercise, these investigations indicate that β-endorphin, dopamine and 5-HT participate in the pathophysiology of exertional heat illnesses.

Future research

Many factors influence the neuroendocrine regulation of body temperature during exercise in a hot environment. In addition to the factors delineated in Fig. 31.3, these include interactions among specific

Table 31.3 Thermoregulatory and stress hormone responses of exertional heat-illness patients and laboratory animals.

Circulating hormone*	Illness†	Experimental protocol	Research findings	References
ACTH			Unknown	
β-Endorphin	Exh	8-day laboratory HA‡; Exh in one male on day 8	β-Endorphin level ↑ 20-fold (versus baseline)	Armstrong *et al*. 1988§
	HS	7-day laboratory HA¶	β-Endorphin level ↑ within-day (patients & controls); β-Endorphin response ↓ by day 8 of HA (patients only)	Kraemer, W.J. *et al*. 2003
	HS	Religious trek to Mecca, in desert	β-Lipotropin/β-endorphin at admission ↑ 42.7-fold (versus later recovery)	Appenzeller *et al*. 1986
	HBI	24 rats; passive heat exposure for 4 h, 38°C	Blockade of opioid peptide** receptors ↓ edema, cell damage and ischemia	Sharma *et al*. 1997
GH	Exh	Religious trek to Mecca, in desert	No change during treatment	Kashmeery 1995§
	HS	Religious trek to Mecca, in desert	GH at admission ↑ 4.1-fold (versus later recovery)	Appenzeller *et al*. 1986
Prolactin	HS	Religious trek to Mecca, in desert	Prolactin at admission ↑ 3.9-fold (versus later recovery)	Appenzeller *et al*. 1986
Cortisol	Exh	Religious trek to Mecca, in desert	No change during treatment	Kashmeery 1995§
	Exh	8-day laboratory HA‡; Exh in one male on day 8	Post-Exh cortisol level ↑ Twofold (versus day 1 and 4)	Armstrong *et al*. 1988§
	HS	Religious trek to Mecca, in desert	Cortisol at admission ↑ 1.7-fold (versus later recovery)	Appenzeller *et al*. 1986
Epinephrine			Unknown	
Norepinephrine			Unknown	
Dopamine	CIND	Rats; passive exposure, 42°C	Depletion of brain dopamine†† ↓ CIND and ↑ survival time	Lin 1997
Serotonin (5-HT)	CIND	Rats; passive exposure, 42°C	Depletion of brain serotonin‡‡ ↓ CIND and ↑ survival time	Lin 1997

ACTH, adrenocorticotropic hormone; CIND, cerebral ischemia and neural damage in rats; Exh, human exertional heat exhaustion; GH, growth hormone; HA, exercise-induced heat acclimation in a laboratory; HBI, hyperthermic brain injury in rats; HS, human exertional heatstroke.

*The physiological significance of these hormones appears in Table 31.1 footnotes.

†To distinguish exertional heatstroke from exertional heat exhaustion, see text.

‡HA program involved 8 days of high-intensity treadmill running (50-min exercise, 100-min total exposure to 41°C).

§Fluid-electrolyte regulating hormones also were measured (not shown).

¶HA consisted of 90-min walking at 40°C; nine former HS patients versus eight healthy, matched-control subjects.

**Findings represent opioid peptides as a class, including β-endorphin; naloxone and naltrexone were used to block receptors.

††Dopamine depletion was accomplished by 6-hydroxydopamine injection.

‡‡Serotonin depletion was accomplished by injection of either methysergide or lysergic acid dithylamide.

neurotransmitters and neuropeptides, multiple afferent influences on hypothalamic and pituitary function, pulsatile release (i.e. growth hormone) or circadian rhythms (i.e. ACTH, cortisol, hGH, prolactin) of some hormones, as well as feedback and feedforward controls (Veldhuis & Yoshida 2000). Future research certainly is needed to interpret this complex matrix. Acknowledging that today's technology is inadequate, we propose the following areas of investigation regarding neuroendocrine influences on thermoregulation during exercise in a hot environment:

• Determine the differences between central (i.e. brain) and peripheral (i.e. blood) neuroendocrine responses.
• Examine the specific effects of putative neurotransmitters and neuropeptides on the different types of POAH neurons (e.g. warm sensitive, cold sensitive).
• Distinguish the individual effects of different stressors (i.e. exercise, intense environmental heat) on neuroendocrine responses.
• Identify the unique effects of exercise mode, duration, intensity and intermittency on neuroendocrine responses.
• Clarify the course of chronic neuroendocrine adaptations that occur during heat acclimatization.
• Investigate the influence of nutritional influences, including dehydration, on neuroendocrine responses.
• Evaluate the changes in neuroendocrine function following exertional heatstroke and heat exhaustion, to determine if permanent disabilities result.

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Chapter 32

Alterations in Arginine Vasopressin with Exercise, Environmental Stress and Other Modifying Factors

CARL M. MARESH AND DANIEL A. JUDELSON

Introduction

In the healthy human body a complex set of endocrine and behavioral mechanisms are present to control fluid and electrolyte balance. The interactions and co-ordinated effects of arginine vasopressin (AVP), the renin–angiotensin–aldosterone system, thirst and drinking behavior and normal kidney function are central to the preservation and maintenance of fluid and electrolyte homeostasis at rest. Similarly, in the context of exercise and other perturbations (e.g. environmental stress) AVP is a major factor responsible for water conservation. In this chapter we focus our attention on the AVP response to various physical and pathological situations. We begin with a basic review of AVP physiology followed by a review of the current knowledge related to the AVP response to exercise, training, exercise and hydration status, modifying factors (including menstrual phase and status, pregnancy, gender and aging) and environmental influences.

Basic endocrinology of arginine vasopressin

In humans the major determinant of fluid balance is the ability of the body to regulate the intake and excretion of water. For the most part this is achieved by the stimulation and suppression of osmotically sensitive cells in the hypothalamus that control the thirst mechanism and the release of AVP. AVP is a neurohypophyseal hormone that plays an important regulatory role in water conservation and the maintenance of body fluid osmolality, and in concert with aldosterone and atrial natriuretic peptide, control of blood volume and blood pressure (Sampson 1992).

Synthesis of AVP mainly occurs in the cell bodies of magnocellular neurosecretory neurons within the supraoptic nuclei and to a lesser extent in the paraventricular nuclei (Ludwig 1998). It is transported bound to a specific precursor molecule, neurophysin II, as neurosecretory granules along nerve axons. When bound to its neurophysin, the half-life of AVP is increased fivefold, from about 2 min to more than 10 min. There are at least four nerve tracts that deliver AVP to different parts of the brain that are thought to be the major sites of its observed central nervous system actions, but the major pathway terminates in the posterior pituitary. From here AVP and neurophysin II are released via calcium-dependent exocytosis into the systemic circulation in equimolar quantities. It also should be noted that the high levels of AVP in portal blood provides the means by which it serves to modulate anterior pituitary function (Sampson 1992). AVP circulates in the blood unbound to other proteins and is rapidly removed, mainly by the kidney, from the circulation.

Three distinct AVP receptor subtypes have been identified. Each of these has seven transmembrane spanning domains and each is G-protein coupled. However, they are encoded by different genes, and differ in tissue distribution, down-stream signal transduction and function (Hurbin *et al*. 1998). The V_2-receptor is most specific to the actions of AVP in the collecting duct of the distal nephron of the kidney.

AVP has many actions but its primary physiological effect is regulation of water reabsorption in the

Table 32.1 Selected functions of arginine vasopressin (AVP).

Increases the passive water permeability of the cell membrane of nephron collecting ducts:
increases water retention
reduces serum osmolality
A potent vasoconstrictor of splancnic, hepatic and renal vessels
A neurotransmitter in CNS regulation of:
the secretion of ACTH
the cardiovascular system
temperature and other visceral function
Promotes hemostasis:
release of endothelial coagulation factors
increases platelet aggregation
Increases blood volume and blood pressure

ACTH, adrenocorticotropic hormone; CNS, central nervous system.

distal nephron. Table 32.1 presents some of the other functions attributed to AVP. The structure and transport processes of the distal nephron allow the kidney to both concentrate and dilute urine in proportion to the circulating AVP concentration. By way of selective water channel proteins (aquaporins) present in the wall of the distal nephron, water is reabsorbed from the duct lumen along an osmotic gradient resulting in excretion of concentrated urine (Marples 2000).

Regulation of arginine vasopressin release

Under resting conditions the major stimulus of AVP secretion is plasma osmolality (P_{osm}) (Baylis & Robertson 1980). The osmoregulatory systems for thirst and AVP secretion, and in turn the actions of AVP on renal water excretion, serve to maintain P_{osm} within narrow limits: 284–295 mOsm·kg^{-1}. In general, the mean osmolar threshold for AVP release approximates 284 mOsm·kg^{-1}, above which plasma AVP increases in response to increases in P_{osm} in a linear manner. Considerable variations in both the threshold and sensitivity of AVP release have been noted between individuals, but these are quite reproducible for a given person over time (Maresh *et al*. 1985; McKenna & Thompson 1998). Nevertheless, the normal relationship between P_{osm} and AVP concentrations will breakdown due to: (i) rapid increases in P_{osm} resulting in an exaggerated AVP release; (ii) drinking rapidly, which suppresses AVP release through afferent pathways originating in the oropharynx (Arnauld & du Pont 1982); and (iii) aging, which increases sensitivity of AVP to an osmotic stimulus (Helderman *et al*. 1978; Bevilacqua *et al*. 1987). The influence of aging is presented in greater detail later in this review.

It is important to note for this discussion that similar to AVP, there also is a linear relationship between thirst and P_{osm} in the physiological range (Stevenson 1969). Mean osmotic threshold for thirst perception approximates 281 mOsm·kg^{-1}, such that thirst occurs when P_{osm} rises above this threshold. Furthermore, as with AVP, there are specific physiological conditions in which the relationship between P_{osm} and thirst will break down. These include: (i) drinking, which will reduce osmotically stimulated thirst; (ii) an extracellular volume depletion, which will stimulate thirst through volume-sensitive cardiac afferents and the generation of circulating and intracerebral angiotensin II, a powerful dipsogen (Reid 1984); and (iii) aging, when both thirst and fluid intake can be blunted in the elderly.

Under specific conditions physiological factors other than osmolality can increase AVP release. Some of these are presented in Table 32.2.

Exercise

Endurance exercise

By the early 1980s, research had confirmed that whole body endurance exercise increases the concentration of plasma AVP (Wade 1984). This has been repeatedly shown with cycle ergometry (Convertino *et al*. 1980a, 1983; Melin *et al*. 1980; Brandenberger *et al*. 1986, 1989; Grant *et al*. 1996; Roy *et al*. 2001a) and treadmill running (Beardwell *et al*. 1975; Wade & Claybaugh 1980; Maresh *et al*. 1985; De Souza *et al*. 1989; Melin *et al*. 1997; Mudambo *et al*. 1997). Physiologically, exercise stimulates AVP release through several mechanisms. Although

Table 32.2 Non-osmotic factors that can cause an increase in arginine vasopressin (AVP) release.

- A fall in blood pressure
- Hemorrhage
- Nausea/vomiting
- Pain/anxiety
- Increased body temperature
- Hypoxia
- Hypercapnia
- Humoral factors: thyrotropin-releasing hormone, catecholamines, renin–angiotensin–aldosterone system, endogenous opioids, prostaglandins, estrogens, progestins
- Drugs: morphine, narcotic analogs, nicotine, anesthetics, tranquilizers, barbiturates

exercise-induced increases in P_{osm} (Convertino *et al*. 1980b; Brandenberger *et al*. 1989; De Souza *et al*. 1989; Freund *et al*. 1991; Montain *et al*. 1997) and decreases in plasma volume (Wade & Claybaugh 1980; Melin *et al*. 1980; Freund *et al*. 1987; Shoemaker *et al*. 1998; Roy *et al*. 2001b) have each been exclusively implicated as the sole cause of increased AVP, significant evidence exists against either as individual phenomena (Beardwell *et al*. 1975; Wade & Claybaugh 1980; Convertino *et al*. 1983; Maresh *et al*. 1985; Shoemaker *et al*. 1998; Melin *et al*. 2001; Roy *et al*. 2001a, 2001b). Rather, it is the combination of increased P_{osm} and decreased plasma volume that dictate the AVP response to exercise (Beardwell *et al*. 1975; Convertino *et al*. 1981; De Souza *et al*. 1989; Grant *et al*. 1996; Melin *et al*. 1997).

Other than these primary influences, exercise affects several other characteristics that may subtly adjust the AVP response. By enhancing sodium reabsorption (and therefore increasing P_{osm}) plasma renin activity and aldosterone may stimulate AVP (Convertino *et al*. 1980b; Wade & Claybaugh 1980), although conflicting reports exist (Convertino *et al*. 1981; Shoemaker *et al*. 1998). Decreased blood pressure (Wade & Claybaugh 1980), increased sympathetic nervous system activity (Beardwell *et al*. 1975; Convertino *et al*. 1981) and decreased renal metabolism of AVP (Wade & Claybaugh 1980) may also be influential. Thus, exercise stress globally stimulates AVP through changes in P_{osm} and plasma volume and modifies this response with aortic baroreceptors, afferent sympathetic impulses and renal metabolism.

Not all exercise, however, stimulates AVP secretion. Although exercise mode and duration are relatively unimportant determinants of AVP concentration, AVP release is highly intensity dependent (Wade 1984) and is only stimulated above 40–65% of $\dot{V}o_{2max}$ (Convertino *et al*. 1981, 1983; Montain *et al*. 1997). Exercise at intensities below this % $\dot{V}o_{2max}$ threshold may be incapable of stimulating the necessary changes in P_{osm} and plasma volume required to elicit an AVP response. Above the threshold, a direct correlation between exercise intensity and AVP secretion has been frequently observed, although the linearity (Wade & Claybaugh 1980; Convertino *et al*. 1981) or curvilinearity (Convertino *et al*. 1983) of the relationship is still debated. Interestingly, exercise below the intensity threshold may also affect AVP, but conversely. Freund *et al*. (1991) showed reduced AVP after 20 min of cycling at 25% $\dot{V}o_{2max}$, and attributed this decrease to exercise-induced increases in thoracic blood volume and resultant negative feedback by atrial and aortic baroreceptors.

While the physiological causes describing exercise-induced AVP release are reasonably clear, the ultimate effect and importance of increased AVP remains obscure. In his review, Wade (1984) suggested AVP secretion during exercise could affect several physiological factors, including initiation of antidiuresis, regulation of plasma volume, regulation and secretion of sweat and training-induced hypervolemia. His conclusion, however, was that the physiological importance of magnified AVP was unclear. Unfortunately, much of this conclusion remains true requiring further research for complete clarification.

Endurance training

As a single bout of appropriate exercise effectively stimulates AVP acutely, repeated exercise bouts (i.e. endurance training) may chronically alter the control of AVP, both at rest and during exercise. Studies employing repeated measures and parallel designs to evaluate training consistently demonstrate similar resting AVP concentrations regardless

of training status (Geyssant *et al*. 1981; Freund *et al*. 1987, 1988). Exercise responses, however, are more equivocal; AVP reaction to exercise is either unchanged (Convertino *et al*. 1983; Freund *et al*. 1987), decreased (Melin *et al*. 1980; Mudambo *et al*. 1997; Shoemaker *et al*. 1998) or increased (Geyssant *et al*. 1981) after training. These contrasting results may be explained by differences in exercise protocol. Specifically, Convertino *et al*. (1983) demonstrated that AVP responses were dependent on whether exercise was assigned as absolute or relative workloads. Because exercise elicits a lower osmotic stimulus at any given absolute workload after training, the magnitude of AVP stimulation at a specific absolute exercise intensity should decrease. At a given relative work rate, however, training should have little influence. Alternately, endurance training may change exercise responses and regulation of plasma volume (Convertino *et al*. 1980b; Shoemaker *et al*. 1998), modify body weight and/or hematocrit (Melin *et al*. 1980) and improve osmotic sensitivity (Freund *et al*. 1987), any of which may influence AVP regulation.

To eliminate the confounding effects of exercise intensity, several studies have assessed training using non-exercise protocols with fluid regulatory demands. Freund *et al*. (1988) examined the results of a water load (1% of lean body mass drunk in 2 min) in trained and untrained subjects. Despite similar P_{osm}, trained subjects experienced smaller decrements in AVP and less diuresis than untrained subjects. They concluded that exercise training: (i) altered the osmotic regulation of AVP by blunting oropharyngeal inhibition; and (ii) led to overall fluid conservation. These findings contradict Convertino *et al*. (1993), who detailed the effects of 5 h of water immersion on runners, swimmers and untrained subjects. In that study, runners exhibited larger decreases in AVP and greater diuresis than untrained subjects, leading the authors to suggest dry-land endurance training enhanced baroreceptor sensitivity and/or reduced renal tubule sensitivity to AVP. Thus, while we are confident that endurance training has little effect on resting AVP, further research is necessary to determine if and how chronic exercise modifies responses to hydrodynamic stress.

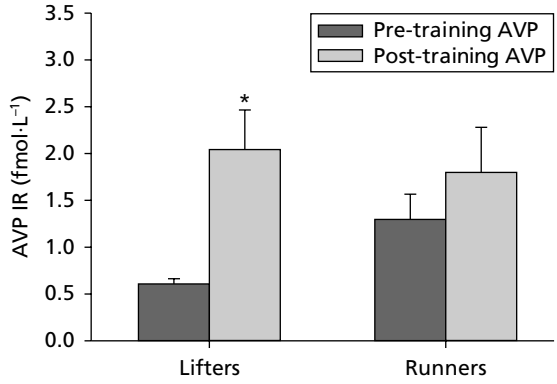

Fig. 32.1 Resting arginine vasopressin (AVP) responses before and after 12 weeks of high intensity resistance training (lifters) or 12 weeks of endurance training (runners). * Indicates a significant difference pre- to post-training within a group.

Resistance exercise and training

Because weightlifting exceeds the intensity threshold required to stimulate AVP, resistance exercise, similar to acute endurance exercise, may induce an AVP response (Kraemer *et al*. 1999). In the only study to test this theory, Kraemer *et al*. (1999) examined the effect of leg pressing at 80% of maximum force production to fatigue in trained power lifters and untrained subjects. In response to the high intensity exercise, AVP was unchanged in either group, as were other potential endocrine regulators of AVP (aldosterone, atrial natriuretic peptide and angiotensin II). The authors attributed this complete lack of a fluid regulatory hormonal response to the protocol's insignificant impact on plasma volume.

Unpublished data from our laboratory, however, indicate that chronic resistance training may alter AVP regulation. Although Kraemer *et al*. (1999) showed power lifters and untrained subjects had similar resting AVP, we observed a significant increase in baseline AVP after 12 weeks of heavy resistance training (Fig. 32.1). Resistance training had no effect on aldosterone, cortisol, renin activity or lactic acid at rest or in response to the exercise challenge. Based on this enhanced resting AVP, we have speculated that the increased fat free mass resulting from resistance training may be related to

exaggerated tissue fluid retention, not just enhanced myofibrillar protein content. Needless to say, the dearth of research in this area affords several exciting opportunities to further examine this topic.

Exercise and hydration status

As total body water is closely tied to AVP control, pre-exercise hydration status has strong influences on the concentration of AVP elicited by exercise. Dehydration is a powerful stimulant to AVP release and increases the quantity of AVP observed with exercise (Brandenberger *et al*. 1989; Melin *et al*. 1997, 2001; Montain *et al*. 1997; Roy *et al*. 2001a); likewise, initiation of exercise in a hyperhydrated state (via water supplementation) (Wade & Claybaugh 1980; Brandenberger *et al*. 1989) or plasma volume expansion (Grant *et al*. 1996; Roy *et al*. 2001b) limits the AVP concentration. AVP response to exercise appears to be preserved regardless of total body water, rather, hydration state alters baseline AVP and this modification persists throughout exercise (Grant *et al*. 1996; Montain *et al*. 1997; Roy *et al*. 2001a). The effects of hydration on AVP are due to the changes in plasma volume, blood pressure and/or osmolality (Wade & Claybaugh 1980; Grant *et al*. 1996; Melin *et al*. 1997; Montain *et al*. 1997; Roy *et al*. 2001b).

Although pre-exercise hydration state modifies the AVP concentration during exercise, a prolonged bout affords the opportunity to significantly alter hydration state during exercise. Rehydration during prolonged (3–4 h) low intensity exercise blunts the AVP response (Mudambo *et al*. 1997), even if subjects are initially hypohydrated (Brandenberger *et al*. 1986, 1989). Concomitant rehydration with water or an isotonic carbohydrate–electrolyte solution is more effective in limiting AVP than pre-exercise hyperhydration (Brandenberger *et al*. 1986, 1989). Combinations of pre- and during-exercise rehydration are also potent inhibitors of AVP during exercise (Melin *et al*. 1997).

Recent data from our laboratory (Maresh *et al*. 2004a) also highlight the relevance of hydration state in modifying AVP during exercise. During that study, subjects completed four 90-min walking trials in the heat; exercise bouts differed in pre-exercise hydration status (euhydrated or hypohydrated) and water intake during exercise (*ad libitum* or no fluid intake). Subjects dehydrated before the appropriate trials by intermittently exercising the previous day and ~ 17 h of subsequent water deprivation. Immediately post-exercise, no significant differences existed in AVP, plasma osmolality, or plasma volume among the euhydrated + water, euhydrated + no water and hypohydrated + water trials, confirming that hydration prior to and/or rehydration during exercise inhibited the release of AVP during low intensity exercise in the heat. Additionally, as displayed in Fig. 32.2, changes in thirst

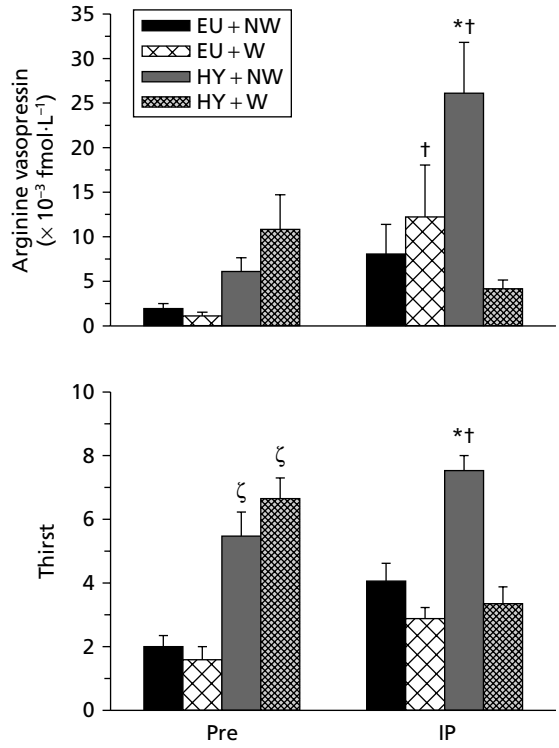

Fig. 32.2 Changes in arginine vasopressin (top panel) and thirst (bottom panel) before and after 90 min of walking in the heat. * Indicates significant difference compared to all other values at the same time point. † Indicates significant difference compared to corresponding Pre value. ζ Indicates significant difference compared to both EU values at the same time point. EU, euhydrated; H, hydrated; IP, immediate post-exercise; NW, no water intake; Pre, pre-exercise; W, *ad libitum* water intake. (From Maresh *et al*. 2004b.)

(and resultant drinking) mirrored AVP, a relationship previously suggested by several authors (Verney 1947; Andersson *et al*. 1980; Nose *et al*. 1988).

Modifying factors

Menstrual phase and status differences

The vast majority of research reports that baseline AVP is similar across menstrual phases (Spruce *et al*. 1985; Vokes *et al*. 1988; De Souza *et al*. 1989; Trigoso *et al*. 1996; Stachenfeld *et al*. 1999a, 1999b; Claybaugh *et al*. 2000; Calzone *et al*. 2001). In one of the only studies to document serial measures throughout the menstrual cycle, however, Forsling *et al*. (1981) noted significant cyclical variations in resting AVP (AVP peaked at ovulation and minimized during menstruation). Figure 32.3 shows that studies collecting data only on 2 specific days may have missed these peaks and nadirs because samples were generally obtained at the mid-point of each phase, when the concentration of AVP is moderate. This conclusion and its physiological significance, however, remain to be established.

Significant research has also compared females' AVP response to a variety of exercise, osmotic and dehydrating stimuli between menstrual phases.

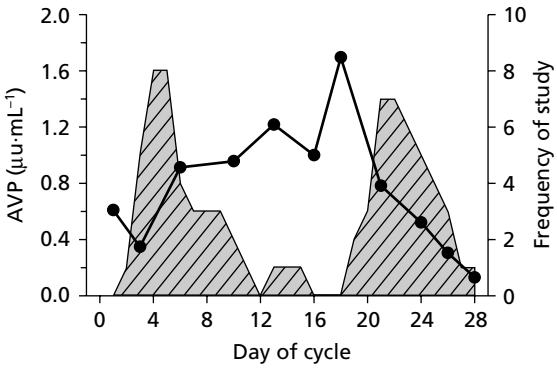

Fig. 32.3 Arginine vasopressin (AVP) measures collected throughout the menstrual cycle in eight healthy, eumenorrheic women. Shaded areas indicate the number of studies examining resting AVP on a given day of the menstrual cycle and, hence, why significant differences in baseline AVP may have not been found. (Portions of the figure redrawn from Forsling *et al*. 1981.)

Table 32.3 Challenges stimulating an arginine vasopressin (AVP) response.

Challenge	P_{osm}	Plasma volume	SNS activity
Exercise	↑	↓	↑
Hypertonic saline infusion	↑	↑	–
Water deprivation	↑	↓	–
Water load	↓	↑	–
Posture change	↑↓	↑↓	–

SNS, sympathetic nervous system.

Although the magnitude and osmotic regulation of the AVP response elicited by exercise is similar in the luteal phase, the follicular phase (De Souza *et al*. 1989; Stachenfeld *et al*. 1999a) and amenorrhea (De Souza *et al*. 1989), the osmolar threshold for AVP release decreases during the luteal phase in response to hypertonic saline infusion (Spruce *et al*. 1985; Vokes *et al*. 1988; Trigoso *et al*. 1996; Stachenfeld *et al*. 1999b, 2001), potentially reducing free water clearance and maintaining hypotonic plasma. In the majority of cases, sensitivity of the AVP to a unit change in P_{osm} was unchanged across phase (Vokes *et al*. 1988; Trigoso *et al*. 1996; Stachenfeld *et al*. 1999b, 2001), although reductions in the AVP sensitivity during the luteal phase have been observed (Spruce *et al*. 1985). Different results observed with exercise vs. infusion challenges are most likely due to the stimuli they present. Table 32.3 shows that although both challenges increase P_{osm}, exercise reduces plasma volume and stimulates sympathetic nervous activity, while hypertonic saline infusion increases plasma volume and has little effect on sympathetic nervous activity.

Further research has examined the effect of dosing estrogen, progesterone or combinations of both on AVP response to a stimulus. These studies are challenging because subjects' normal hormonal patterns must be non-existent (i.e. post-menopausal women), artificial and controllable (i.e. oral or intramuscular contraceptive users) or pharmacologically eliminated. Similar to luteal phase alterations, estrogen administration reduces the threshold P_{osm} required to induce an AVP response (Forsling *et al*. 1982; Stachenfeld *et al*. 1998, 1999b; Calzone *et al*.

2001; Stachenfeld & Keefe 2002), increasing AVP release for any given P_{osm} (Forsling *et al*. 1981; Spruce *et al*. 1985; Claybaugh *et al*. 2000; Calzone *et al*. 2001; Stachenfeld *et al*. 2001) and preserves the slope of the P_{osm}–AVP curve (i.e. AVP sensitivity) (Stachenfeld *et al*. 1998, 1999b; Calzone *et al*. 2001; Stachenfeld & Keefe 2002). As it readily crosses the blood–brain barrier, estrogen's influence is most likely mediated by a direct effect on the central nervous system (Stachenfeld *et al*. 1998, 1999b, Calzone *et al*. 2001; Stachenfeld & Keefe 2002). Progesterone, on the other hand, typically has minor, inconsistent effects on AVP (Forsling *et al*. 1982; Stachenfeld *et al*. 1999b; Calzone *et al*. 2001).

If, as suggested by the previous studies, estrogen mediates menstrual control of AVP, why is the osmolar threshold for AVP release reduced during the luteal phase (when endogenous estrogen is high), but not the late follicular phase (when endogenous estrogen is also high)? Several plausible hypotheses exist. Use of synthetic, exogenous steroids in many drug studies is potentially confounding; natural and artificial hormones have different bioavailabilities and binding characteristics (Stachenfeld *et al*. 1998; Calzone *et al*. 2001). Alternately, estrogen likely interacts with other ovarian hormones: the increased luteinizing hormone or follicle-stimulating hormone characteristic of the late follicular phase may inhibit, or the enhanced progesterone of the luteal phase may facilitate estrogen's full effect on AVP. The possibility that data collection has been mistimed (see Fig. 32.3) also cannot be eliminated. Finally, although improbable, estrogen may not drive menstrual alterations in AVP regulation, as cyclic fluctuations in AVP clearance (Forsling *et al*. 1981, 1982) and/or volemic status (Trigoso *et al*. 1996) could modify AVP control.

Pregnancy

In one of the only studies examining AVP during pregnancy, Davison *et al*. (1984) compared gravidas' responses to several experimental challenges during their third trimester and again 8–10 weeks postpartum. AVP responded appropriately to all challenges, but the osmolar threshold for AVP release was significantly reduced during pregnancy. Considering the similar hormonal milieus of pregnancy and the luteal phase, this finding is not surprising.

Gender differences

Based on limited research, few conclusions about gender-related control of AVP have been reached. Baseline AVP has been reported as similar between genders (Stachenfeld *et al*. 2001) and increased in men (Stachenfeld *et al*. 1996). Although exercise appears to elicit a similar response regardless of gender (Maresh *et al*. 1985; De Souza *et al*. 1989; Stachenfeld *et al*. 1996), females have demonstrated greater AVP during recovery than males, potentially due to higher concentrations of endogenous estrogen (Stachenfeld *et al*. 1996). In response to a 12 $mL \cdot kg^{-1}$ lean body mass water load, AVP was similar between genders, but differences in P_{osm} led authors to suggest that women experienced a reduced osmotic threshold for AVP release and/or an enhanced AVP release compared to men. The importance of this shift was questioned, however, as women experienced reduced renal responsiveness to AVP (Claybaugh *et al*. 2000). A combination salt infusion–water load elicited a greater slope in the P_{osm}–AVP curve (i.e. greater AVP sensitivity to changes in P_{osm}) in men, potentially due to gender differences in arterial resistance, renal sensitivity, androgen concentration, plasma volume and/or cardiopulmonary baroreceptor sensitivity (Stachenfeld *et al*. 2001). These varied, sometimes conflicting results preclude firm conclusion and will hopefully be clarified with future research.

Aging

Examination of subjects from 52 to 95 years of age has shown resting concentrations of AVP are similar regardless of age (Helderman *et al*. 1978; Rowe *et al*. 1982; Phillips *et al*. 1984, 1991, 1993; Bevilacqua *et al*. 1987), although confounding reports exist (Kirkland *et al*. 1984; Davies *et al*. 1995). The response to AVP challenges, however, is more diverse. Overnight fasting (Duggan *et al*. 1993; Faull *et al*. 1993) and 24 h of water deprivation (Phillips *et al*. 1984), have

produced contrasting results; elderly AVP response was either similar to (Phillips *et al*. 1984; Duggan *et al*. 1993) or lower than (Faull *et al*. 1993) younger subjects, sometimes independent of P_{osm}. Water loads up to 20 mL·kg^{-1} demonstrate that elderly subjects fail to diminish their AVP as promptly as younger subjects (Phillips *et al*. 1993; Davies *et al*. 1995). Finally, although AVP response to hypertonic saline infusion is at least maintained (Phillips *et al*. 1991) and may be enhanced (Hong *et al*. 1977; Davies *et al*. 1995) in the aged, their responses to a change in body position (from recumbency to standing) are significantly less than young subjects (Rowe *et al*. 1982; Bevilacqua *et al*. 1987).

Physiologically, these apparently conflicting results may be explained by an age-related divergence in the sensitivity of the primary receptors controlling AVP release. Based on several studies, authors have suggested that aging increases the sensitivity of AVP to an osmotic stimulus (Helderman *et al*. 1978; Phillips *et al*. 1984; Bevilacqua *et al*. 1987; Davies *et al*. 1995). Research on changes in body position indicates that volume–pressure input, however, is decreased in the elderly (Rowe *et al*. 1982; Bevilacqua *et al*. 1987). Thus, aging may sensitize the osmoreceptor and desensitize the baroreceptor (Rowe *et al*. 1982; Bevilacqua *et al*. 1987). Additionally, oropharyngeal control of AVP may be altered with aging (Phillips *et al*. 1991). Exactly how these considerations explain the dehydration problems of the elderly is currently unknown; however, a generalized reduction in AVP secretion is not to blame (Kirkland *et al*. 1984; Phillips *et al*. 1984, 1993).

Environmental influences

Temperature

The effects of temperature on AVP control are primarily mediated via changes in intrathoracic blood volume and resultant baroreceptor stimulation. Cold-induced cutaneous vasoconstriction increases central blood volume and results in central pressure–volume receptor activation, typically diminishing AVP and increasing urine output. This AVP inhibition has been shown after exposures of 30–60 min at temperatures of 4–13°C (39.2–55.4°F), sometimes overriding stimulatory changes in plasma volume, plasma sodium and P_{osm} (Segar & Moore 1968; Wittert *et al*. 1992). Conversely, central blood volume decreases during exposure to high ambient temperatures due to peripheral vascular vasodilation, resulting in decreased baroreceptor stimulation and enhanced AVP output. This AVP stimulation has been shown after exposures up to 2 h at temperatures of 42–50°C (107.6–122.0°F) and exceeds the AVP stimulus resulting from hypovolemia and hyperosmolality associated with sweating (Segar & Moore 1968; Convertino *et al*. 1980b; Melin *et al*. 1991).

Altitude

Unfortunately, the release and control of AVP with high altitude exposure is still not completely understood. Studies show that resting measures of AVP increase (Hackett *et al*. 1978; Porchet *et al*. 1984; Rostrup 1998), decrease (Ramirez *et al*. 1992; Bestle *et al*. 2002; Robach *et al*. 2002) or do not change (Rostrup 1998; Robach *et al*. 2002; Maresh *et al*. 2004b) in response to significant elevation (2900–4559 m), frequently disconnected from P_{osm}. Adding further confusion are findings that high altitude natives have higher resting AVP than lowlanders (Ramirez *et al*. 1992, 1993, 1998). At altitude, hypertonic saline infusion still increases P_{osm}, but has inconsistent effects on AVP (Ramirez *et al*. 1992, 1993, 1995; Bestle *et al*. 2002). Furthermore, although 30 min of moderate intensity cycling elicited a similar AVP response at sea level and after 7 days exposure to 4350 m, AVP responses were magnified when identical exercise was repeated 2 days after return to sea level (Robach *et al*. 2002).

Besides differences in protocol, AVP is difficult to evaluate at altitude because acclimatization dynamically changes physiological responses, expanding the importance of measurement timing. Recent work by our laboratory (Maresh *et al*. 2004b) has clarified the role of acclimatization by examining the effects of a 24-h water deprivation trial at sea level, after 2 days exposure to 4300 m and after 20-days exposure to 4300 m. The primary findings of that study are shown in Fig. 32.4: progressive altitude acclimatization modifies the relationship between P_{osm} and AVP in a time-dependent fashion.

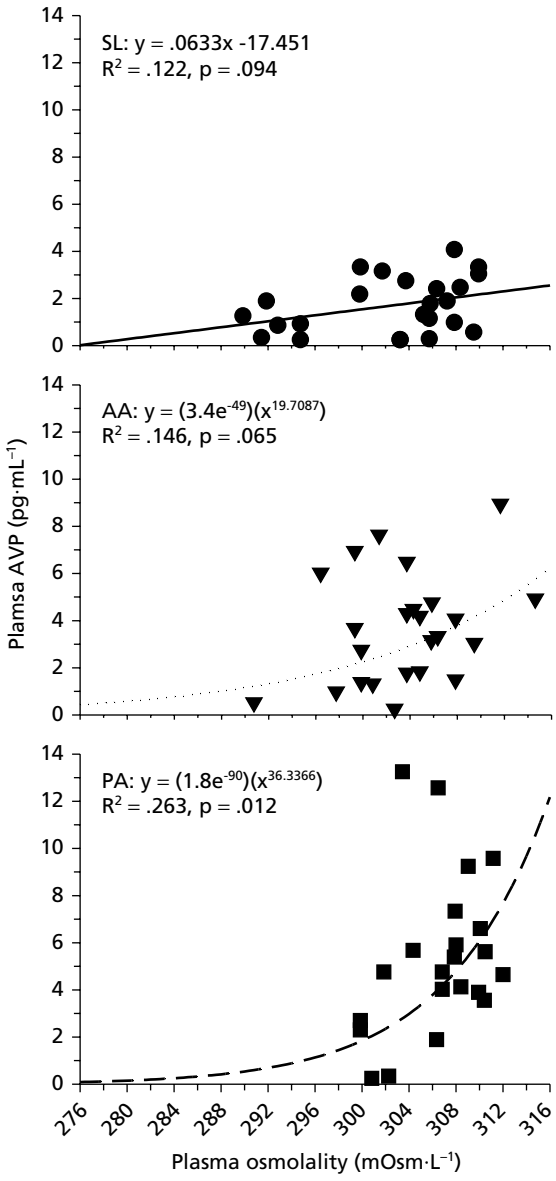

Fig. 32.4 Best-fit curve estimations of the relationship between P_{osm} and P_{AVP} for sea level (SL), acute altitude exposure (2 days at 4300 m) (AA) and prolonged altitude exposure (20 days at 4300 m) (PA). (From Maresh *et al*. 2004a.)

Additionally, if the relationship between P_{osm} and AVP is forced into a linear model, the osmotic threshold of AVP release increases after prolonged altitude exposure. Physiological hypotheses explaining this change include increases in AVP as a compensatory mechanism to defend blood pressure in the face of decreased catecholamines (Rostrup 1998), renal resistance to AVP (Ramirez *et al*. 1998) or altered control of AVP by P_{osm}, blood pressure and plasma volume (Bestle *et al*. 2002; Robach *et al*. 2002; Maresh *et al*. 2004b).

Hyperbaria

Those studies finding a typical hyperbaria-induced diuresis show hyperbaric exposures decrease resting AVP (Hong *et al*. 1977; Claybaugh *et al*. 1984, 1987, 1992, 1997; Matsui *et al*. 1987; Takeuchi *et al*. 1995; Torii *et al*. 1997; Park *et al*. 1998). Unlike the time-dependent response to high altitude exposure, this phenomenon is preserved whether the exposure is as minor as 1 h at 3 ATA (atmosphere's absolute pressure) (Torii *et al*. 1997) or as intense as 14 days at 31 ATA (Claybaugh *et al*. 1984). Limited data on AVP responses to stimulating challenges during hyperbaria confirms resting results. Although a 1 L water load appropriately (i.e. similarly to 1 ATA) reduced AVP at 31 ATA (Tekeuchi *et al*. 1995), head-up tilt (31 ATA) (Matsui *et al*. 1987), 20 min of exercise at 80% of maximum heart rate (46 ATA) (Claybaugh *et al*. 1997) and maximal exercise (37 ATA) (Claybaugh *et al*. 1997) elicited reduced, or no, AVP response. Minimized AVP at hyperbaria may result from pressure-induced magnification of negative intrathoracic pressure and resultant increases in thoracic blood volume. Through its actions on central baroreceptors, increased thoracic blood volume decreases AVP and promotes diuresis (Hong *et al*. 1977; Claybaugh *et al*. 1984, 1987; Tao *et al*. 1992; Torii *et al*. 1997). Alternately, reduced AVP may be caused by altered AVP receptor responsiveness (Raymond *et al*. 1980; Claybaugh *et al*. 1997), hypoosmolality (Matsui *et al*. 1987; Torii *et al*. 1997) or as a direct effect of increased pressure on the central nervous system (Claybaugh *et al*. 1987, 1992; Matsui *et al*. 1987).

Summary

AVP plays an important regulatory role in water conservation and the maintenance of body fluid

osmolality. Exercise stimulates AVP release through several mechanisms, but principally through a combination of increased P_{osm} and decreased plasma volume. Endurance training may alter control of AVP at rest and during exercise, but recent findings suggest that resistance training also may alter AVP regulation. Pre-exercise hydration status has a strong impact on the plasma AVP response to exercise. The majority of research reports that baseline AVP is similar across menstrual phases and few conclusions about gender-related control of AVP have been reached. Aging increases the sensitivity of AVP to an osmotic stimulus. High ambient temperatures tend to enhance AVP secretion. The AVP response to high altitude depends on several factors, but most assuredly on the length of altitude exposure. Typically, AVP concentrations are reduced with hyperbaric-induced diuresis. Several of these results require further clarification.

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Chapter 33

Human Endocrine Responses to Exercise–Cold Stress

JOHN W. CASTELLANI AND DAVID W. DEGROOT

Introduction

The combination of cold exposure and exercise performed in this environment elicits profound physiological responses. These include increases in metabolic heat production and vasoconstriction in order to maintain body temperature, changes in fluid balance and changes in substrate mobilization in order to fuel increased metabolic activity. Many of these physiological responses are associated with endocrine secretion and concentration changes. It is acknowledged that differences in plasma levels as a result of cold exposure could be due to secretion, clearance and volume of distribution that cannot be differentiated in these studies. Most of the studies examining the role of cold exposure on hormone responses have been completed during resting exposure and these are still relatively few in number. The interaction of exercise and cold on the endocrine system is even less studied. The primary purpose of this chapter is to survey the endocrine responses to exercise–cold stress in humans. Animal studies will not be considered. Sedentary cold exposure responses are also reviewed to provide background information and for use as a point of comparison to exercise studies. Table 33.1 presents the hormones that will be discussed and presents a general overview of the changes caused by resting and exercise–cold stress.

Catecholamines

When people are exposed to the cold, metabolic heat production increases via shivering thermogenesis and cutaneous blood flow is decreased, limiting heat loss to the environment. These physiological responses are mediated by the sympathetic nervous system (SNS) in order to defend against a drop in body temperature. Plasma norepinephrine (NE) concentrations are used as an acute marker of SNS activity since NE is released from peripheral nerve endings during acute cold exposure. NE exerts its thermoregulatory effects via β-adrenergic receptors on skeletal muscle (metabolic heat production) and α-adrenergic receptors on smooth muscle (vasoconstriction). Plasma NE increases two to sixfold during sedentary cold exposure (Wilkerson *et al*. 1974; Johnson *et al*. 1977; O'Malley *et al*. 1984; Wang *et al*. 1987; Weiß *et al*. 1988; Frank *et al*. 1997; Armstrong 1998; Castellani *et al*. 1998, 1999a, 1999b) and also increases urinary NE excretion (Arnett & Watts 1960; Lamke *et al*. 1972), a 24-h marker of SNS activity. The weighting/contribution factor of core and skin temperature for the NE response to resting cold exposure is ~ 2 : 1, that is ~ 67% of the increase in NE is due to decreases in core temperature and 33% is due to the fall in skin temperature (Frank *et al*. 1999).

NE concentrations increase during exercise–cold stress (Castellani *et al*. 2001), not surprisingly, but the responses between cold and temperate environments are likely dependent on whether core temperature declines. When high intensity exercise (~ 60% $\dot{V}O_{2max}$) was performed for 2 h, plasma NE was not different between a dry, 15°C environment and a wet, windy, 5°C environment (Weller *et al*. 1997a). However, as soon as the intensity was lowered to less than 30% $\dot{V}O_{2max}$, and core temperature subsequently fell, NE concentrations were 240% higher in

Table 33.1 Summary of hormone responses during exercise–cold stress.

Hormone	Physiological effects	Response to resting cold exposure	Response to exercise–cold stress	Other observations
Catecholamines	↑ Cutaneous vasoconstriction ↑ Heat production ↑ Glycogenolysis ↑ Lipolysis	↑ 2–6-fold	↑ At exercise intensities below 40% $\dot{V}o_{2max}$ compared to temperate; ↑ Epinephrine during long duration exercise (> 6 h)	
T_3 & T_4	↑ Metabolism vasodilator	↓ In 30 min to 3-h exposures	↑ Levels after 6–9 h at 30% $\dot{V}o_{2max}$	
Plasma renin activity	Converts angiotensinogen to angiotensin I	↓ Or no change	↓ Following graded exhaustive exercise	
Arginine vasopressin	↑ Water conservation	↓ In water or air	No change with graded exercise	
Atrial natriuretic peptide	↓ Sodium reabsorption	No change	↑ After graded exercise to exhaustion	
Cortisol	Catabolic hormone; promotes lipolysis vasoconstriction	↑ Or no change	↑ After 5-h cold–wet exposure at 30% $\dot{V}o_{2max}$; ↓ After 1-h swimming at 68% $\dot{V}o_{2max}$	Responses may be related to time of day & intensity-duration interaction
Insulin	Anabolic hormone; promotes glucose uptake	No change when core temperature does not fall; ↓ when core temperature ↓ by 1°C	↓ With cold-water exercise but not different from temperate; no change during cold-air exercise	
Glucagon	Catabolic hormone; ↑ glycogenolysis ↑ gluconeogenesis ↑ lipolysis	No change	No change	
Growth hormone	Anabolic hormone; ↑ free fatty acid mobilization ↑ gluconeogenesis	No change	No change after 1-h swimming at 68% $\dot{V}o_{2max}$	β_2-Adrenergic stimulation may suppress GH levels; released in pulsatile manner
Prolactin	Lactation	↓ During and after exposure	Small increase with exercise–cold stress but lower than exercise–heat stress	Possible marker of cold acclimation
Testosterone	Anabolic hormone; spermatogenesis	No change	Variable responses depending on exercise stress	
Luteinizing hormone	Promotes testosterone estrogen & progesterone secretion	Chronic exposure to cold lowers basal values	No effect from acute exercise	

T_3, triiodothyronine; T_4, thyroxine.

the cold environment. However, if the exercise intensity is relatively high (60% $\dot{V}o_{2max}$) but performed over an extended duration (4 h) that causes core temperature to fall, plasma NE concentrations are 45% higher in a cold–wet environment. Galbo *et al*. (1979) found that swimming in 21°C water for 1 h (rectal temperature decreased by 0.8°C) increased NE concentrations 87% above that compared to swimming in 27°C. As well, when both core and skin temperatures are low during submaximal and maximal exercise, absolute NE responses are the greatest (Bergh *et al*. 1979).

The interaction between skin temperature and exercise intensity may also influence plasma NE concentrations during exercise–cold stress when there are no differences in core temperature. Plasma NE is doubled in 5°C air (versus 21°C) during cycle ergometry at 50 W but not at 150 W (Stevens *et al*. 1987). Furthermore, Weller *et al*. (1997b) found that when exercise initially begins at a low intensity (30% $\dot{V}o_{2max}$) in a cold–wet environment, plasma NE concentrations are three-times higher after 2 h even though rectal temperature is not different compared to dry–temperate conditions. Skin temperatures in these low intensity studies were 5–8°C lower in the cold. However, when cycle exercise is performed at 50–100% $\dot{V}o_{2max}$, there are no differences in NE concentrations between temperate and cold environments (Quirion *et al*. 1989; Anderson & Hickey 1994).

The effect of cold exposure on epinephrine (EPI) is less clear. In contrast to NE, plasma EPI responses do not appear to increase during resting cold-water immersion (Weiß *et al*. 1988), cold-saline infusion (Frank *et al*. 1997) and cold-air exposure (Wang *et al*. 1987; Armstrong 1998). However, Frank *et al*. (2002) found that EPI levels increased when the core temperature decreased by 1.0°C using cold-saline infusion (skin temperature remained normal). That study did not collect peripheral venous (antecubital) blood but instead sampled central venous blood and suggested this source is closer to the site of EPI release and is not affected by clearance. They suggest that the lack of an EPI response in other studies is due to the sampling methodology. If EPI is increased during cold exposure, it likely would increase shivering thermogenesis and fat mobilization via β-adrenergic receptors.

Plasma EPI concentrations during exercise–cold stress, compared to temperate conditions, are elevated if core temperature falls, similar to plasma NE. EPI levels were elevated after 4–6 h of cold–wet exposure, compared to a 15°C dry condition when the rectal temperature difference was 0.4°C (Weller *et al*. 1997b). Likewise, Galbo *et al*. (1979) observed 71% higher EPI levels after swimming in 21°C water versus 27°C. On the other hand, if core temperatures are not different between cold and temperate environments, EPI concentrations are the same (Weller *et al*. 1997a, 1997b) or lower (Parkin *et al*. 1999).

It is important for the reader to be aware that cold acclimatization might influence the NE response to exercise–cold stress, although no studies have directly tested this. However, there have been several studies that have examined the effect of cold acclimation on NE levels during resting cold exposure.

There are three distinct types of cold acclimation: habituation, metabolic and insulative acclimation (Young 1996). Cold habituation, characterized by blunted shivering and vasoconstrictor responses, typically occurs after cold exposures not severe enough to elicit falls in body core temperature. Metabolic acclimatization is defined as a higher metabolic activity. This has been observed in one study and occurred after moderate cold exposure for 6 weeks. Insulative acclimatization is induced by repeatedly lowering the body core temperature, which causes greater peripheral vasoconstriction, lower skin temperatures and perhaps a shift in the onset of shivering thermogenesis so that shivering does not begin until a lower mean body temperature is reached. When multiple cold-air exposures were used to induce cold habituation (with little fall –0.5°C in core temperature), plasma NE values declined during standardized cold air tests (Hesslink *et al*. 1992; Leppäluoto *et al*. 2001). On the other hand, when 25 cold-water immersions (18°C water) were used for acclimating subjects (causing significant falls in core temperature –1°C) that led to an insulative acclimation (lower core and skin temperatures), plasma NE was significantly higher after acclimation, compared to preacclimation, during a standardized cold-air exposure (Young *et al*. 1986).

Thyroid hormones

The thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), are very important for maintaining basal metabolism and thermogenesis. The calorogenic effect of these hormones is believed to be caused by increasing energy consumption of the sodium–potassium pump. Also, thyroid hormones have been shown to be vasodilators. Thus, these hormones may affect peripheral heat loss during cold exposure.

Sedentary cold-air exposure (4–10°C) from 30 min to 3 h has no effect on plasma thyroid-stimulating hormone (TSH), T_3 and T_4 levels (Hershman *et al*. 1970; Wilson *et al*. 1970; Nagata *et al*. 1976; Tuomisto *et al*. 1976; Leppäluoto *et al*. 1988). In contrast, several authors (Golstein-Golaire *et al*. 1970; O'Malley *et al*. 1984) have noted increases in these hormones after short-term resting cold exposure whereas others (Solter & Misjak 1989) found that TSH, total T_3, reverse T_3 and total T_4 were all lower following an 8-h work day in a cold environment. Short-term cold stress should not bring about large increases or decreases in plasma concentrations because most of the hormone is bound to plasma proteins that provides a substantial reservoir of thyroid hormone (Goodman 1994).

Only a few studies have examined thyroid hormone responses during controlled exercise–cold stress experiments. The response pattern could be related to a number of factors including exercise duration or intensity. T_4 is elevated following 6–9 h of cold–wet exposure during low intensity exercise (Dulac *et al*. 1987; Castellani *et al*. 2002) whereas T_3 responses did not change or increased. Thyroid hormones likely increased during prolonged exercise–cold stress to support lipid metabolism, since it amplifies β-adrenergic responses of the SNS. Core temperature was not a likely mediator of this effect since it reached only 36.9°C in one study (Castellani *et al*. 2002). TSH was not elevated during these low intensity exercise studies, but increased by 90% during a 30-min moderate intensity performance swim in 20°C water (Deligiannis *et al*. 1993). Likewise, free T_4 levels increased by 46% during cold-water swimming. Whether this effect was a result of central (core) or peripheral (skin) input is not known since these values were not reported. The elevated T_3 and T_4 following long duration exercise–cold stress is more likely due to the exercise stimulus per se, rather than the cold exposure, since acute cold exposure has little effect on these hormones.

Since cold habituation leads to blunted shivering and higher skin temperatures, thyroid hormones could also change since they are involved in thermogenic and vasodilatory responses. Hesslink *et al*. (1992) exposed subjects to 4.4°C air for 30 min twice a day for 8 weeks (80 total exposures). They also supplemented a group of subjects with T_3 to artificially suppress TSH and T_4 levels in order to determine the relative importance of these hormones for inducing cold acclimation. The repeated cold exposure program did indeed induce habituation as demonstrated by reductions in oxygen uptake, mean arterial pressure and plasma NE values subsequent to acute cold exposure. No differences were observed between the supplemental T_3 group (low TSH and T_4) and the non-supplemented group and there were no changes in thyroid hormone concentrations. Similarly, Leppäluoto *et al*. (2001) exposed subjects for 11 straight days for 2 h·day^{-1} in 10°C air and found no changes in T_3, T_4 and TSH, even though skin temperatures were higher after acclimation. Savourey *et al*. (1994) studied eight men who underwent a 1°C cold-air test for 2 h before and after cold acclimation. Acclimation was induced by 20 ice-water immersions to the thigh over a 1-month period and resulted in lower rectal temperatures during cold-air exposure after acclimation, compared to preacclimation. T_3, T_4 and TSH were not different after the acclimation period. These studies demonstrate that thyroid hormones do not have a role in cold acclimation induced over a short-time period.

The pattern observed in fairly short acclimation studies is somewhat different than that seen in personnel over-wintering in Antarctica for 8–12 months. Reed *et al*. (1986, 1988, 1990) characterized that Antarctica residence leads to an elevated TSH response subsequent to thyroid-releasing hormone stimulation, lower T_3 levels and no changes in T_4. Sawhney *et al*. (1995) related lower T_3 values to the period of the Antarctic summer when physical activity levels are high and observed higher T_3 in the

winter, when is was extremely cold and dark. Interestingly, if personnel from Antarctica are given supplemental T_4, declines in cognitive performance are attenuated (Reed *et al*. 2001). Likewise feelings of fatigue and confusion are lessened in the supplemental group. The reader should be aware that Antarctic sojourners are exposed to a multitude of factors including low ambient temperature, extreme light conditions, highly variable physical activity levels, high electromagnetic radiation and isolation. Thus, it is difficult from these studies to discern if the cold per se has an effect on thyroid levels or if it is due to the combination of all these environmental factors.

Thyroid status is classically linked to cold sensitivity, i.e. people with thyroid insufficiency typically complain about feeling cold (Larsen *et al*. 1998). Nagashima *et al*. (2002) recently showed that T_4 concentrations were 29% lower in women who complained about feeling cold, compared to matched controls. Associated with this increased cold feelings were lower finger temperatures and metabolic rates. However, the relative importance of thyroid hormones during acute cold exposure in humans is still not well understood. Surprisingly, there are little data (one subject, Thompson *et al*. 1971) on acute thermoregulatory responses during cold exposure in people who are hypothyroid. After T_4 administration, this subject exhibited a higher oxygen consumption, but a blunted fall in skin temperature, so that the core temperature response was about the same after 2 h in 10°C air. Thus, thyroid hormones appear to have little effect on thermal balance as it simultaneously increases heat production and heat loss.

Fluid regulation

Fluid balance is regulated by several hormones, including renin–angiotensin–aldosterone, atrial natriuretic peptide (ANP) and arginine vasopressin (AVP). These hormones monitor blood volume and plasma osmolality in order to precisely regulate water and electrolyte losses to maintain normal levels. Angiotensin and aldosterone are regulated by the enzyme renin, which is released from the juxtaglomerular cells in the kidney. Renin is regulated by decreased pressure (volume) and decreased sodium and chloride flux in the kidney. Higher angiotensin and aldosterone levels increase sodium and water retention. ANP is released from right-atrium cardiac myocytes in response to high central blood pressure/volume and causes greater sodium and water excretion from the kidney. AVP is released from the posterior pituitary in response to increased plasma osmolality as well as decreases in blood volume sensed by high-pressure (carotid) and low-pressure (right atrium) receptors. AVP directly increases water reabsorption in the collecting duct of the kidney.

Exposure to cold temperatures lowers total body water levels via several mechanisms including cold-induced diuresis (CID), sweating, respiratory water loss, blunted thirst, poor water availability and conscious under-drinking (O'Brien *et al*. 1998). These body water losses are associated with changes in plasma volume and electrolyte concentrations that can have a profound effect on fluid regulatory hormones. Cold exposure also increases central blood volume and right atrial pressure. Exercise causes profound changes in plasma volume and electrolytes. However, relatively few studies have examined fluid regulatory hormones during rest in cold environments, and only one study has examined these responses during exercise.

The most commonly studied effect of cold exposure on body fluid balance is CID. Over 50 years ago, Bader *et al*. (1952) suggested that CID is caused by a fall in AVP levels, subsequent to higher central blood volumes. Administering pitressin, an AVP analog, abolished the higher urine flows observed in the cold, without affecting the glomerular filtration rate, although this has not always been observed (Lennquist 1972). If cold exposure causes an essential water diuresis then plasma tonicity should go up, but it does not. A series of studies (Lennquist *et al*. 1974; Wallenberg & Granberg 1974; Atterhog *et al*. 1975; Knight & Horvath 1985; Deuster *et al*. 1989) demonstrated that urine electrolyte excretion is also elevated with cold exposure suggesting that CID is due to an isosmotic fluid loss. What is not known is whether an elevated renal solute excretion (primarily sodium) leads to water loss, or if cold increases both sodium and water

excretion via mechanisms independent of each other. Some evidence supports that salt losses are primary in CID. When NaCl supplementation was used during a 48-h field exercise (Rogers *et al*. 1964) in extreme cold, 30% less urine was secreted compared to a non-NaCl group even though both groups drank the same amount of water.

Resting studies that have examined fluid-regulatory hormone responses during cold exposure (Segar & Moore 1968; Hiramatsu *et al*. 1984; Hassi *et al*. 1991; Wittert *et al*. 1992; Hynynen *et al*. 1993; Nakamitsu *et al*. 1994; Jansky *et al*. 1996; Arjamaa *et al*. 1999, 2001; Sramek *et al*. 2000) commonly find that plasma renin activity either does not change or falls, plasma aldosterone and ANP are not changed and AVP levels decrease. Urinary sodium excretion was elevated in these studies. Cold-induced natriuresis was not mediated via changes in aldosterone or ANP, but possibly through the paracrine influence of urodilatin, an ANP-like compound secreted from the kidney (Nakamitsu *et al*. 1994; Bestle *et al*. 1999). Lower AVP concentrations lead to increased water excretion, secondary to increases in central blood volume and right atrial pressure.

Only one study has examined fluid-regulatory hormones during exercise–cold stress. Therminarias *et al*. (1992) found lower plasma renin activity and higher ANP levels during graded exercise to exhaustion in 10°C air, compared to 30°C air. No differences were observed for AVP. The higher ANP and lower plasma renin activity levels in 10°C air were due to greater cardiac filling caused by a central redistribution of blood volume.

Cortisol

Plasma cortisol modulates many physiological processes that are important for responding to cold exposure. These include increasing resting energy expenditure, inhibiting vasodilation, increasing free fatty acid availability for substrate utilization, and functioning of the SNS. Cortisol secretion is regulated by adrenocorticotrophic hormone (ACTH), which is secreted by the anterior pituitary. Plasma cortisol concentrations are lowest in the evening hours and highest just before awakening. This point is important as stress-related changes in cortisol concentrations are blunted when plasma levels are highest in the morning.

The effects of resting cold exposure on plasma cortisol concentrations are equivocal as both increases (Suzuki *et al*. 1967; Wilkerson *et al*. 1974; Kauppinen *et al*. 1989; Hennig *et al*. 1993; Tikuisis *et al*. 1999) and no change (Golstein-Golaire *et al*. 1970; Wilson *et al*. 1970; Ohno *et al*. 1987; Wittert *et al*. 1992; Frank *et al*. 1997; Marino *et al*. 1998) have been observed.

Exercise–cold stress studies, as with resting studies, show both increases and decreases. Potential reasons include the exercise duration and the time of day. Galbo *et al*. (1979) studied swimming exercise, whereas McMurray *et al*. (1994) and Castellani *et al*. (2002) used cycle ergometry and walking, respectively. Only one study corrected for changes in plasma volume and reported the use of standardized blood draws controlling for factors such as arm position and posture (Castellani *et al*. 2002). Differences in the change in core temperature between Galbo *et al*. (1979) (–0.8°C) and McMurray (1994) (0°C) could explain why cortisol declined in the swimming study but not following cycling. However, Castellani *et al*. (2002) also observed a fall in core temperature (0.2–0.5°C) and found that exercise–cold stress increased cortisol concentrations. Perhaps the long duration cold exposure (~ 5 h) combined with moderate intensity exercise (50% $\dot{V}O_{2max}$) leads to a greater stress response compared to high intensity, short duration exercise bouts (Galbo *et al*. 1979). Pandolf *et al*. (1992) also did not find any changes in cortisol after exercising in cold water for 50 min at 50% $\dot{V}O_{2max}$. Interestingly, in Castellani *et al*. (2002), cortisol increases occurred in the absence of an ACTH response suggesting that cortisol secretion during prolonged exercise–cold stress is ACTH-independent.

Time of day may be the most important factor in determining whether plasma cortisol changes as a result of exercise–cold stress. Castellani *et al*. (2002) studied their subjects in the late afternoon/early evening when cortisol levels are typically lower and stress responses are discriminated easier, whereas other studies exercised their subjects in the morning, when cortisol levels are high. Also, if exercise studies are initiated in the morning and are fairly

long and not intense (Ainslie *et al*. 2002), lower cortisol concentrations post-cold exposure could just reflect the normal diurnal rhythm. Utilization of proper control groups will allow for correct interpretation of exercise–cold stress studies.

Insulin and glucagon

Insulin and glucagon are the two primary fuel regulatory hormones. Insulin is an anabolic hormone that promotes fuel storage throughout the body whereas glucagon acts on the liver to secrete glucose and β-hydroxybutyrate. Since moderate sedentary cold exposure increases plasma glucose oxidation by 138%, muscle glycogen oxidation by 109% and lipid oxidation by 376% (Haman *et al*. 2002), it would appear that these hormones would have a role in mobilizing substrates to fuel shivering thermogenesis.

Plasma insulin does not appear to change as a result of acute cold exposure where there are minimal changes in core temperature (Martineau & Jacobs 1989; Vallerand *et al*. 1995; Tipton *et al*. 1997; Haman *et al*. 2002; Koska *et al*. 2002), but during a 10°C cold-water immersion that elicited a 1°C decline in core temperature and higher metabolic rates, Jacobs *et al*. (1984) found a 32% decline in insulin concentrations. Cold-air exposure does appear to enhance insulin sensitivity or insulin responsiveness in skeletal muscle. Following an intravenous glucose tolerance test, plasma glucose falls more rapidly with lower plasma insulin concentrations in 10°C air compared to a temperate environment (Vallerand *et al*. 1988).

Exercise–cold stress studies are difficult to interpret due to different research designs, exercise modes and intensities. It appears that, unlike rest, lower insulin levels are not related to lower core temperatures during exercise (Galbo *et al*. 1979) and that the response is not mediated via β-adrenergic receptors (Lehtonen *et al*. 1984). Ten weeks of cold-water exposure causes insulin to fall during light intensity cold-water swimming (Hermanussen *et al*. 1995). It is unclear if this is related to changes in body temperatures or to the higher basal insulin concentrations. The catabolic state also may influence the insulin response. Following a 36-h fast, insulin was significantly lower at baseline, but exercise–cold stress caused no further decline in serum insulin levels after fasting whereas insulin fell 68% in the non-fasting trial (Weller *et al*. 1998). Similarly, 5 h of exhaustive exercise lowers baseline insulin levels and remains low during subsequent cold–wet exposure (Tikuisis *et al*. 1999).

Glucagon concentrations have been shown to both increase and decrease as a result of resting cold exposure. In the two studies that found increased levels (Seitz *et al*. 1981; Tikuisis *et al*. 1999), plasma hormone values were not corrected for plasma volume changes (decreased by 9–10%), which could account for the higher values. In the other studies, conducted either in cold water or cold air, glucagon did not change as a result of cold exposure (Jacobs *et al*. 1984; Martineau & Jacobs 1989; Vallerand *et al*. 1995).

There are limited data on the response of glucagon to exercise–cold stress. During moderate intensity swimming in cold water (21°C) that elicited a 0.8°C decline in core temperature (Galbo *et al*. 1979), glucagon levels did not change during or after exercise, while the same exercise elicited an increase in glucagon in warm water. These findings suggest that either cold-water exercise suppresses glucagon release or that a threshold core temperature needs to be reached before glucagon secretion occurs. Free fatty acids levels rose throughout the exercise period and during recovery, but the same response was observed during swimming in 27° and 33°C water, arguing against cold exposure increasing the need for fat metabolism. In a study of diabetic patients (Ronnemaa *et al*. 1991), glucagon levels did not change during exercise in either warm (30°C) or cold (10°C) conditions. Exercise was limited to three 15-min bouts of cycle ergometry at 60% $\dot{V}o_{2max}$. Considering that the magnitude of the glucagon response is dependent on the intensity and duration of exercise, the lack of a glucagon response is not surprising.

Growth hormone

Growth hormone (GH) is secreted by the anterior pituitary and defends glucose concentrations by sustaining lipolysis and inhibiting glucose metabolism.

Therefore, it may be important for regulating fuel homeostasis during prolonged exercise–cold stress. For example, by defending glucose concentrations and preventing hypoglycemia, GH may have a role in maintaining shivering thermogenesis.

GH has been studied using various methodologies to induce cold stress, including cold-air exposure, ice ingestion and cool-water immersion combined with ice ingestion. The findings from these studies are highly variable. Sitting in cold air for 2-h has been shown to either cause no change (4°C air, Golstein-Golaire *et al*. 1970) or lower GH concentrations (10°C, Leppäluoto *et al*. 1988), with transient increases 30 min following exposure (Okada *et al*. 1970). Ice-water ingestion that lowered tympanic temperatures by 0.5–0.8°C demonstrated either no change (Berg *et al*. 1966) or a significant decrease (Weeke & Gundersen 1983). The reader must be cognizant that many of these studies did not employ a control group and also are measuring GH at one point in time. This is important because GH is secreted in a pulsatile fashion and depending when the sample is taken, GH can be relatively high or be undetectable. Serial sampling over many hours has not been done using repeated subjects designs during or after cold exposure.

Only three studies have examined GH responses during or after exercise–cold stress. Galbo *et al*. (1979) found that exercising in 21°C water (decreased rectal temperature by 0.8°C) for 60-min elicited no change in GH, whereas GH was elevated following exercise in 27°C and 33°C water. These data suggest that lower body core temperatures suppress GH release or that a core temperature threshold is needed before plasma GH levels rise. Lehtonen *et al*. (1984) exercised subjects for 1 h at –3°C at a heart rate of 140–150 b·min^{-1} on one of three treatments: placebo, atenolol (β_1-adrenergic blocker) and pindolol (blocks β_1 and β_2). They found that GH was significantly higher during the pindolol trial compared to placebo, suggesting that β_2-adrenergic receptors modulate GH responses to exercise–cold stress. Studies (Hermanussen *et al*. 1995) also show that GH does not change in the post-exercise period after swimming in 2–7°C water for 1.5–15.0 min. Since these studies only provide a 'snapshot' in time, it is unknown how the normal pulsatility of GH release is affected by the interaction of exercise and cold stress and what these changes mean for overall fuel metabolism.

Reproductive hormones

Brain serotonin is linked to temperature regulation; increases in brain serotonin levels are associated with feelings of sleepiness and lethargy; and there is evidence implicating serotonin in producing central fatigue during exercise (Cheuvront & Sawka 2001). Since central serotonin levels in humans cannot be measured, plasma prolactin (PRL) concentrations are an accepted marker for brain serotonergic activity (Cheuvront & Sawka 2001) due to anatomical relationships within the brain as well as the finding that plasma PRL concentrations change in response to serotonin agonists and antagonists. Thus PRL changes during exercise–cold stress may be indicative of changes in temperature regulation or useful as a marker of fatigue.

Resting cold exposure significantly decreases prolactin concentrations during and up to 90 min post-exposure (Mills & Robertshaw 1981; O'Malley *et al*. 1984; Leppäluoto *et al*. 1988), although the studies varied slightly in exposure temperature and duration.

The literature is equivocal concerning exercise–cold stress and acute changes in PRL, and there appears to be influences of both exercise and cold-stress intensity. No clear relationship exists between PRL changes and temperature regulation. One study (Frewin *et al*. 1976) found no change in PRL levels during moderate intensity treadmill exercise in 10°C air, but an increase when the same exercise was performed in 40°C air. Another study (Ronnemaa *et al*. 1991) reported an increase in PRL during exercise in 10°C air in patients with diabetes, but, again, the PRL response at 10°C was lower compared to exercise at 30°C in these patients. The increase in PRL during 10°C exercise in diabetics may be due to the subject population, although PRL is lower in type I diabetics compared to healthy subjects (Ramires *et al*. 1993). A third study reported a decrease in PRL during an 8-h shift in meat-processing plant workers exposed to –20°C to –40°C temperatures (Solter & Misjak 1989). Other research

suggests that PRL levels only increase during exercise when core temperature increases above 38°C (Cheuvront & Sawka 2001). Therefore, PRL as a sensitive marker of temperature regulation or fatigue during exercise–cold stress is not supported by the literature.

There is evidence, however, that PRL may be a marker of cold acclimation. Ten weeks of a winter swimming program (2–7°C water) doubled the baseline PRL compared to preswimming concentrations while non-exercise control group PRL levels did not change, indicating the possibility of seasonal variation (Hermanussen *et al*. 1995).

Testosterone is an anabolic hormone that is responsible for growth and development of tissues that characterize males, including skeletal muscle growth. With respect to cold environments, this hormone has been studied in relation to sperm production. McConnell and Sinning (1984) found exercise–cold stress (running at 80% maximal heart rate in 6.2°C air for 5 consecutive days) had no effect on sperm count, semen sample volume, or total sperm per sample. They also found testosterone increased on days 4 and 5 compared to baseline following daily cold-air exercise. However, the same response was found whether the exercise was conducted in warm and hot conditions, indicating that the testosterone results are caused by exercise and not cold stress. On the other hand, resting cold exposure does not change testosterone concentrations (Leppäluoto *et al*. 1988) and other exercise–cold stress studies suggest testosterone decreases. During 10 days of outdoor military training in the winter (Hackney & Hodgdon 1991), subjects who slept in tents at night had a lower testosterone on day 5 compared to subjects who slept in warmer barracks at night. Testosterone in the outdoor-sleeping group returned to normal on day 10. Testosterone decreased after an 8-h shift in meat processing plant workers, but only in the group exposed to the lowest ambient temperatures (Solter & Misjak 1989). This group was the only one who experienced intermittent exposure and the effect of the alternating 1-h work and 1-h rest (in normal room temperature conditions) is not known. Core temperature was not reported during this study, but it is likely that it did not change due to clothing worn by the workers. In a 42-day field study (Johansen & Norman 1991), three of the four male subjects gained total body mass and lean mass and lost body fat during a period of severe testosterone deficiency (the authors remarked that testosterone values during the last 22 days were in the 'female range'). This is in contrast to the remaining subject, who lost a significant amount of total body mass, lean mass and body fat yet had a similar testosterone profile during the trek. The authors speculated that GH may have been elevated during the trek and was responsible for the increased lean mass, but they present no data to support this hypothesis.

Luteinizing hormone (LH) stimulates Leydig cells in the testes to synthesize and secrete testosterone. Sedentary exposure for 2 h to 10°C air does not change blood LH values (Leppäluoto *et al*. 1988). The exercise studies are difficult to interpret due to environmental/field conditions and methodologies and there is no clear linkage to testosterone. Several minutes of swimming in ice water has no effect on plasma LH (Hermanussen *et al*. 1995). Solter and Misjak (1989) studied meat-processing workers who were exposed to ambient temperatures between –40°C and –20°C for 3.5 $h \cdot day^{-1}$ and compared them to workers either exposed to less severe cold (4–8°C) or normal room temperature. No acute changes were observed in LH in any group, although workers chronically exposed to extreme cold had lower basal LH values. LH decreased over time during a 500-km ski-trek (6 weeks) in Greenland, reaching a nadir during the 4th week, and then increasing during the final 2 weeks (Johansen & Norman 1991).

Menstrual cycle status affects thermoregulatory responses in cold environments. During the luteal phase, when endogenous levels of estrogen and progesterone are high, shivering is lower at any given mean body temperature and there is also higher heat flux from the body (Gonzalez & Blanchard 1998). Hessemer and Brück (1985) found that the temperature onset for shivering was ~ 0.5°C higher in the luteal phase, paralleling the rise in core temperature observed during this phase. Likewise, Charkoudian and Johnson (1999) also demonstrated that the onset of cutaneous vasoconstriction began at a higher internal temperature during the luteal

phase. These studies demonstrate that reproductive hormones influence thermoregulatory effector responses in the cold. However, it is less clear what effect cold exposure has on estrogen and progesterone secretion. There are no studies describing how cold exposure impacts these reproductive hormones.

Summary

Information on the effect of exercise–cold stress on endocrine responses is limited. Because there have been relatively few studies completed and these have used a variety of experimental approaches, it is difficult to discern if the changes are due to the interaction of exercise–cold stress or if they are due to the independent effects of exercise or cold exposure. Future studies need to systematically examine the interaction of exercise duration, mode and intensity with cold exposure to determine endocrine responses and, more importantly, the role of the endocrine system in maintaining physiological homeostasis. Whether the changes in hormone levels as a result of cold exposure have important physiological or performance consequences is unknown. An example is the role of thyroid hormones during acute cold exposure. What happens if a person with hypothyroidism exercises in a cold environment? Are they worse off or does it not matter? This question cannot be answered at this point. The use of patients with clinical endocrine disease may further our understanding of the physiological role of specific hormones during exercise–cold stress.

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Chapter 34

Growth, Maturation and Hormonal Changes During Puberty: Influence of Sport Training

JAMES N. ROEMMICH

Introduction

The timing and tempo of pubertal maturation and somatic growth are under genetic control but modified by nutritional status and a host of hormones. Growth and maturation are slowed during chronic undernutrition due to alterations in the hormonal milieu including reduced sex steroid, insulin-like growth factor I (IGF) and thyroid hormone concentrations. Conversely, overnutrition results in early maturation and an increased growth velocity due to increased secretion of adrenal androgens, sex steroids and IGF-I. The influence of nutrition and endocrine function on growth and maturation have led many to wonder if sport training influences the rate of growth and maturation of young athletes. For instance, could the great exercise energy expenditures and limited nutritional intake of some athletes result in limited energy availability and lead to delayed puberty and slowed growth? This question has received great public interest due to portrayals of ever younger gymnasts and figure skaters training year round, sometimes far from home, to prepare their bodies and hone their skills with the best coaches. Another common question is whether sport training in the presence of adequate nutrition increases growth and accelerates the rate of pubertal maturation. An acute bout of exercise reliably increases the secretion of growth-related hormones and these increases in hormone secretion occur with each bout of training. Chronic training alters the basal secretion of growth-related hormones so that the target tissues such as muscle and bone are exposed to greater or smaller than normal concentrations of hormones throughout the day. This chapter reviews the processes of growth and maturation, the hormonal axes regulating growth and maturation, the influence of acute exercise and of sport training on the hormonal axes controlling growth and maturation, and the effects of exercise and sport training on the growth and maturation of youth.

Growth and maturation

Linear growth and sexual maturation are mainly controlled by the growth hormone–insulin-like growth factor I (GH–IGF-I) and the hypothalamic–pituitary–gonadal (HPG) axes. Many of the growth effects of GH, including long bone growth and anabolic effects on muscle, are mediated through IGF-I, which is synthesized locally by target tissues or the liver, the predominant source of circulating IGF-I. IGF-I also acts as a negative feedback signal to reduce GH secretion. Serum IGF-I concentrations decline during periods of inadequate energy intake resulting in an opening of the feedback loop and increased GH secretion, but low IGF-I production. The net result is relative GH resistance and slowed linear and somatic growth.

Prepubertal growth is almost exclusively dependent on GH, IGF-I and thyroid, which has a permissive effect on GH secretion. The marked acceleration in growth velocity at puberty results from the interaction of the GH–IGF-I and HPG axes with a continued permissive effect of thyroid hormone. In girls and boys, rising estrogen concentrations stimulate twofold increases in GH secretion that are paralleled by increased serum IGF-I concentrations (Veldhuis

et al. 1997, 2000; Roemmich *et al*. 1998; Mauras 2001). At low concentrations, sex steroids and GH synergistically stimulate skeletal growth, but at greater concentrations, estrogen induces epiphyseal fusion.

Leptin is a recently discovered hormone that assists in regulating long-term energy balance and reproductive function by signaling the size of the fat stores and energy availability to the feeding and reproductive centers of hypothalamus (Cunningham *et al*. 1999; Foster & Nagatani 1999). These dual roles allow leptin to link nutritional status with proper reproductive function in adults and to the onset of puberty in youth (Cunningham *et al*. 1999; Foster & Nagatani 1999). Anecdotal evidence for a role of leptin in human puberty includes leptin concentrations of boys and girls rising just before puberty (Blum *et al*. 1997; Clayton *et al*. 1997; Garcia-Mayor *et al*. 1997; Mantzoros *et al*. 1997) and an inverse relationship between leptin concentrations and age at menarche (Matkovic *et al*. 1997). Experiments of nature lend stronger evidence in that patients lacking leptin protein do not attain pubertal maturity until leptin replacement therapy (Farooqi *et al*. 1999).

Nutrition modifies growth and pubertal timing

The influence of nutritional status on pubertal timing is clearly demonstrated by the steady decline in age, but stable body weight at menarche from the mid-19th century to mid-20th century. The signals that reawaken the HPG axis at the onset of puberty and then keep it operative remain unclear. Presumably, a non-gonadal mediated mechanism determines that the energy stores are large enough to sustain rapid growth, maturation and reproduction. Likely candidates for this signal are the availability of metabolic fuels and hormonal signals including leptin, insulin and components of the GH–IGF-I axis. The influence of nutrition on the metabolic signals controlling growth and pubertal maturation has been extensively reviewed (Foster & Nagatani 1999; Rogol & Roemmich 2000; Moschos *et al*. 2002).

Acute exercise and hormone release

Most hormones involved in growth and maturation also play key roles in controlling macronutrient metabolism during the increased metabolic demands of exercise. Endocrine changes during exercise promote a catabolic environment to enhance fuel mobilization. Exercise stimulates GH, glucagon and cortisol secretion while secretion of the anabolic hormone insulin is reduced to allow for the mobilization of carbohydrates and fatty acids needed to meet the increased energy demands of physical activity. Exercise also stimulates testosterone and estrogen secretion which may influence the proportion of carbohydrate and lipid utilization (Braun & Horton 2001) and perhaps stimulate tissue repair post-exercise. The responses of key hormones involved in growth and maturation to acute exercise are summarized below.

Acute exercise and the GH–IGF-I axis

Acute aerobic exercise so reliably increases the serum GH concentration that it is used as a test to assess adequate GH secretion (Bouix *et al*. 1994; Marin *et al*. 1994; Ghigo *et al*. 1996). Resistance exercise protocols that emphasize a great number of repetitions and short rest periods also reliably increase GH secretion (Kraemer, W.J. *et al*. 1990, 1991; Häkkinen & Pakarinen 1995; Roemmich & Rogol 1997). Exercise influences on growth stimulation could be hypothesized to be maximized during puberty as exercise-induced GH secretion is greatest during the adolescent growth spurt. Increased concentrations of sex steroids, especially estrogen, play a major role in enhancing exercise-induced GH release during puberty (Bouix *et al*. 1994; Marin *et al*. 1994; Ghigo *et al*. 1996).

As the downstream mediator of GH action, IGF-I secretion would also be expected to reliably increase with exercise, but it does not. Reviews of both aerobic (Jenkins 1999) and resistance (Deschenes & Kraemer 2002) exercise have highlighted the mixed results regarding the IGF-I response to an exercise bout. IGF-I and GH concentrations may not both increase during exercise due to locally produced IGF-I in active target tissues, such as muscles acting through paracrine or autocrine rather than endocrine mechanisms (DeVol *et al*. 1990; Yan *et al*. 1993; Cappon *et al*. 1994).

Acute exercise stimulates the HPG axis

A short-term exercise bout in adult men increases serum testosterone concentrations in direct proportion to the exercise intensity (Hackney 1996). However, the evidence is contradictory regarding increases in the secretion of testosterone's upstream signal, luteinizing hormone (LH) suggesting that increased testosterone secretion during short-term exercise is not LH-mediated. Exercise-induced increases in testosterone concentration are short-lived and return to baseline about 40 min after exercise is stopped. In adults, testosterone concentrations begin to return to basal concentrations after about 60 min of exercise and may dip below basal concentrations before recovering over the next 24–72 h (Hackney 1996). The impact of acute exercise on the HPG axis of youth has not yet been widely studied and there are not enough data to draw conclusions. Maximal aerobic exercise failed to increase serum testosterone concentrations of boys regardless of stage of pubertal maturation (Fahey *et al*. 1979), while weight training in adolescent boys increases serum testosterone concentrations in some (Kraemer, W.J. *et al*. 1992; Fry *et al*. 1993) but not all (Pullinen *et al*. 2002) studies. Increased testosterone secretion in response to exercise may be dependent on training history as youth who trained for 2 or more years, regardless of age, have greater increases in serum testosterone concentrations compared to youth who have trained for shorter periods (Kraemer, W.J. *et al*. 1992; Fry *et al*. 1993).

In women, a bout of endurance exercise also produces a reliable but short-lived increase in serum testosterone (Keizer *et al*. 1987; Consitt *et al*. 2001) and estradiol concentrations (Jurkowski *et al*. 1978; Nicklas *et al*. 1989). The estradiol response is dependent on the exercise intensity and the menstrual cycle phase with greater estradiol secretion during the luteal phase than the follicular phase (Jurkowski *et al*. 1978; Nicklas *et al*. 1989). In contrast, resistance exercise training by women does not produce a reliable increase in serum testosterone concentrations (Kraemer, W.J. *et al*. 1993, 1998; Kraemer, R.R. *et al*. 1995), but does increase serum estradiol concentrations, especially during the luteal phase (Jurkowski *et al*. 1978; Nicklas *et al*. 1989). Very few studies have investigated the effects of aerobic or resistance exercise on the reproductive axis of girls. Increased serum concentrations of both estrogen and progesterone have been noted after submaximal exercise (Viru *et al*. 1998) and both submaximal (Viru *et al*. 1998) and maximal (Kraemer, R.R. *et al*. 2001) aerobic exercise increases serum testosterone concentrations of pubertal girls.

Acute exercise and leptin

In adults, a single bout of exercise does not reliably alter serum leptin concentrations immediately following exercise (Perusse *et al*. 1997; Racette *et al*. 1997; Weltman *et al*. 2000; Kraemer, R.R. *et al*. 2002), but may produce reductions 24–48 h after exercise (Essig *et al*. 2000; Olive & Miller 2001). Longer exercise bouts of 2 or more hours are more likely to reduce serum leptin concentrations (Duclos *et al*. 1999; Karamouzis *et al*. 2002), but the reduction appears to be more due to negative energy balance than the exercise bout per se (van Aggel-Leijssen *et al*. 1999; Hilton & Loucks 2000). There are not yet enough data to draw conclusions about the effects of an exercise bout on the leptin secretion of youth. A single study, performed in adolescent female distance runners, found that maximal exercise increased serum leptin concentrations (Kraemer, R.R. *et al*. 2001).

Exercise training and basal hormone concentrations

The endocrine systems of the body adapt to the repeated stimulation of exercise training by: (a) altering the strength of the exercise stimulus necessary to increase or decrease hormone secretion; (b) altering the tissue responsiveness to the hormone by altering the amount of unbound 'biologically active' hormone by increasing or decreasing the number of circulating binding proteins that bind the hormone and protect it from degradation but also render it biologically inactive, the number of cellular hormone receptors, or post-receptor function; and (c) working through neuroendocrine adaptations to alter basal and maximal hormone secretion. The following sections summarize exercise training effects on growth-related hormone axes.

Exercise training and the GH–IGF-I axis

The most well-controlled study of the influence of exercise training on resting GH secretion has shown that 1 year of endurance training 3 days per week above the lactate threshold and 3 days per week at the lactate threshold increased 24-h mean serum GH concentrations of women by 75%, but no change was observed in women who trained 6 days per week at the lactate threshold (Weltman *et al*. 1992). Other studies have reported direct relationships between aerobic fitness and both resting GH secretion (Weltman *et al*. 1992) and serum IGF-I concentrations (Poehlman & Copeland 1990) of adults. With a couple of exceptions (Borst *et al*. 2001; Marx *et al*. 2001), resistance training has little effect on resting GH secretion or serum IGF-I concentrations of young and older adults (Kraemer, W.J. *et al*. 1998, 1999; McCall *et al*. 1999; Häkkinen *et al*. 2001; Deschenes & Kraemer 2002).

Aerobic fitness is directly related to GH secretion in adolescent girls and boys (Eliakim *et al*. 1996, 1998b) as are serum IGF-I concentrations (Eliakim *et al*. 2001; Scheett *et al*. 2002) although not consistently (Eliakim *et al*. 1996, 1998a). While these results suggest that aerobic training upregulates the GH–IGF-I axis of youth, correlation does not prove causality. Prospective studies do not support a direct relationship between aerobic fitness and secretion of GH and IGF-I. Several short-term (5-weeks, 90 min·day^{-1} of discontinuous exercise, 5 days per week) training studies of youth that produced gains in aerobic fitness and anabolic effects on mid-thigh muscle produced no changes in GH secretion (Eliakim *et al*. 1996, 1998a). These training protocols also produced paradoxical reductions in serum IGF-I concentrations and alterations in growth hormone binding protein (GHBP) and IGF-I binding proteins reflecting a catabolic state (Eliakim *et al*. 1996, 1998a; Scheett *et al*. 2002). It is unclear how anabolic effects are seen in muscle tissue under a catabolic hormonal environment, or why a catabolic environment occurs, but this same exercise program increases proinflammatory cytokines that may suppress the GH–IGF-I axis (Scheett *et al*. 2002). Aerobic training also reduces IGF-I concentrations and alters IGF binding protein concentrations of some adults (Rosendal *et al*. 2002), while others have reported increases in total and free-IGF-I (Koziris *et al*. 1999). These catabolic alterations in the GH–IGF-I axis may be reversed sometime after 5 weeks of training as youth and adults who are more fit at the beginning of the training have the smallest increases in inflammatory cytokines (Scheett *et al*. 2002) and the quickest normalization of serum IGF-I concentrations as training continues (Rosendal *et al*. 2002).

Exercise training and the HPG axis

There has been a major increase in the number of women participating in competitive sports over the past 40 years. Enactment in the USA of Title IX of the Educational Amendments Act of 1972, opportunities to participate in Olympic distance running events, ice hockey and soccer, and the formation of highly publicized professional athletic teams have all led to a greater number of women engaging in strenuous exercise (Harber 2000; Loud & Micheli 2001). Many of these female athletes engage in great volumes of exercise while being placed under self-imposed and external pressures to maintain a lean physique to enhance performance and an esthetic appearance. Unfortunately, the resulting low energy availability can produce reproductive dysfunction in women ranging from luteal phase insufficiency to secondary or 'athletic' amenorrhea (Williams *et al*. 2001; De Souza *et al*. 2003; Loucks & Thuma 2003). The estimated prevalence of amenorrhea in athletes (5–66%) is much greater than in the general population (2–5%) (Yeager *et al*. 1993), and the prevalence of less symptomatic conditions such as luteal phase insufficiency is probably even greater.

Reproductive dysfunction can occur within 8 weeks of strenuous training (Bullen *et al*. 1985) and likely results from decreased activity of the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator resulting in suppressed LH pulse frequency and, ultimately, reduced estradiol secretion (Williams *et al*. 2001; De Souza *et al*. 2003; Loucks & Thuma 2003). There are numerous health issues resulting from reproductive dysfunction including reduced bone density, scoliosis and cardiovascular risks (Constantini & Warren 1994), and the constant pressure to limit energy intake can result in disordered eating (Sabatini 2001). Energy and nutrient deficiencies are very concerning in

young athletes as the immature reproductive axis may be less resilient to nutritional stress resulting in delayed menarche or primary amenorrhea (Warren 1980; Frisch *et al*. 1981; Constantini & Warren 1994; Roemmich & Rogol 1995) and perhaps lifelong alterations in reproductive function. Delayed menarche and nutritional deficiencies may also result in inadequate bone mineral accrual, increasing the risk of fractures and osteoporosis later in life (Sabatini 2001).

The impact of sport training on the male reproductive system has been much less studied. The data are inconsistent but abnormalities include normal or elevated LH concentrations with testosterone concentrations of 60–80% of control men, suggesting a modest central HPG impairment (Hackney 1996, 2001; Hackney *et al*. 2003). Recent evidence suggests a peripheral mechanism may also reduce testosterone secretion as endurance-trained men have a lower testicular responsiveness in the form of reduced testosterone secretion to GnRH stimulation than sedentary control men (Hackney *et al*. 2003), with oligospermia in some men (Hackney 1996, 2001). In contrast to women who can experience reproductive axis dysfunction within several months, reductions in serum testosterone are most prevalent in men who have been endurance training for several years (Hackney 1996, 2001). Women are more likely to limit energy availability by combining endurance exercise with dietary restriction, and the HPG axis of women may be more sensitive to energy availability due to the importance of adequate energy stores for pregnancy and lactation.

Few studies have examined the effect of endurance exercise training on the hypothalamic–pituitary–testicular (HPT) axis of pubertal male athletes. During a short 2-month sport season there were no changes in total or free testosterone concentrations of adolescent male runners (Rowland *et al*. 1987).

Exercise training and leptin

There has been much interest in the influence of sport training on serum leptin concentrations due to the importance of energy availability for maintaining reproductive function and leptin's role in signaling both energy stores (i.e. body fat) and energy availability (Dubuc *et al*. 1998; Wadden *et al*. 1998). The separate influence of body fat stores and energy balance on serum leptin concentrations and reproductive function is demonstrated in elite amenorrheic athletes who have lower serum leptin concentrations than elite cyclic women despite similar amounts of body fat. The unusually low serum leptin concentrations are explained by undernutrition in the amenorrheic athletes as their energy intake was only 73% of the cyclic athletes (Thong *et al*. 2000). The importance of undernutrition, independent of body fat, on serum leptin concentrations and menstrual dysfunction has also been found in recreationally active amenorrheic and cyclic women matched for body fat (Miller *et al*. 1998). These data suggest that exercise training per se does not alter serum leptin concentrations beyond training-induced alterations in body fat as previously reviewed (Kraemer, R.R. *et al*. 2002). Undernutrition produces an independent negative effect on serum leptin concentrations.

Sport training, growth and maturation

The data demonstrate that an acute bout of exercise and sport training alter the serum concentrations of hormones involved in growth and maturation, but most of the data have been collected in adults and much more research is required in children and adolescents. In reproductively mature adults who are no longer growing, sport training: (a) can increase basal GH secretion, which increases lipolysis and may help resist the accrual of body fat; and (b) can have detrimental effects on reproductive function when combined with dietary restriction. Although these data demonstrate the potential of sport training for altering serum concentrations of hormones involved in growth and maturation, they cannot be generalized to youth athletes. The following sections focus on the evidence, if any, supporting the stimulatory or inhibitory effects of sport training on the growth and pubertal maturation of youth athletes.

Non-weight control sports

There is some evidence that exercise may stimulate linear bone growth in well-nourished animal models. Increased bone loading, as would occur with

physical activity, increases long bone growth of adolescent rats (Simon 1978; Simon & Papierski 1982), and running accelerates growth in hamsters via increased GH secretion (Borer & Kaplan 1977; Borer *et al*. 1979, 1986; Conn *et al*. 1993). Moreover, during recovery from protein–energy malnutrition, physically active children aged 2–4 years grow more in length and lean body mass than less active children over a 6-week period (Torun & Viteri 1994). However, the animal studies may not model the effects of exercise training on the growth of humans and the clinical study of children may not generalize well to healthy boys and girls engaging in intensive sport training. Fortunately, there are large data sets of well-nourished youth involved in sport training that make these generalizations unnecessary.

Studies of healthy youth show that for the vast majority of boys and girls participating in sport such as track and field, baseball, basketball, volleyball, football and soccer, training consists of exercise energy expenditures of 837–1674 kJ (200–400 kcal) 3–5 days per week with no concerns placed on lowering body weight. Many of these athletes are slightly taller and more mature than their non-athletic peers (Malina 1994; Malina & Bielicki 1996; Malina *et al*. 1997). These results could be interpreted that exercise increases the rate of growth and maturation, but it is more likely that these are self-selected inherited traits because strength and power advantages caused by earlier maturation are major factors attracting children to sport (Malina 1994). This issue highlights the difficulty in separating the effects of exercise from genetically programmed growth as first preselection and later natural selection for a sport creates a non-random population that may skew the data about patterns of growth and maturation.

The effects of sport training on hypertrophic growth such as an increase in size of a muscle, bone or organ in response to a stimulus are clear and hypertrophic growth for some tissues can be greatest during childhood and adolescence. For instance, regular load-bearing exercise stimulates bone mineral accrual in both laboratory animals and humans (Kannus *et al*. 1996; Lanyon 1996; Smith & Gilligan 1996). Immature bone increases bone formation more than mature bone to a given amount of stress because the greater compliance of immature bone results in greater strains (Smith & Gilligan 1996). As such, female tennis players who start playing before menarche have a twofold greater forearm bone mineral content (BMC) than those who start after menarche (Kannus *et al*. 1995; Haapasalo *et al*. 1998).

Weight control sports

Some athletes, including wrestlers, gymnasts and some distance runners, combine great energy expenditures with dietary restraint to maintain a low body weight. This practice affords the athlete eligibility to compete in a weight class that may provide them with a competitive advantage, to maintain a high strength to body weight ratio, or to maintain a thin 'esthetic' body physique. These are the youth at greatest risk of slowed growth and pubertal maturation. However, even within this select group of athletes only a small percentage experience limited energy availability great enough to slow linear growth and pubertal maturation. The remainder of this review will focus on the growth and maturation of two of the most widely studied high-risk athletic groups, female gymnasts and male wrestlers.

GYMNASTS

Gymnasts gain a competitive advantage by developing muscular strength within a shorter, lighter and esthetic body. The performance of female gymnasts often decreases after the onset of puberty due to increases in height and fat mass and development of the secondary sexual characteristics. There is much concern that elite gymnasts may use sport training and energy restriction to maintain a slender physique and delay growth and puberty to remain competitive. The concern has become greater as ever-younger and leaner girls participate at the elite level of gymnastics. Gymnasts begin training around age 5 years, and by the age of 10 years elite gymnasts are training up to 36 $h \cdot wk^{-1}$, year round (Caine *et al*. 2001). From 1976 to 1992 the mean age of Olympic gymnasts decreased from 18 to 16 years, and, although the average height decreased 11% during this time, the mean weight decreased by 22% (Sabatini 2001).

Many studies have shown that gymnasts are

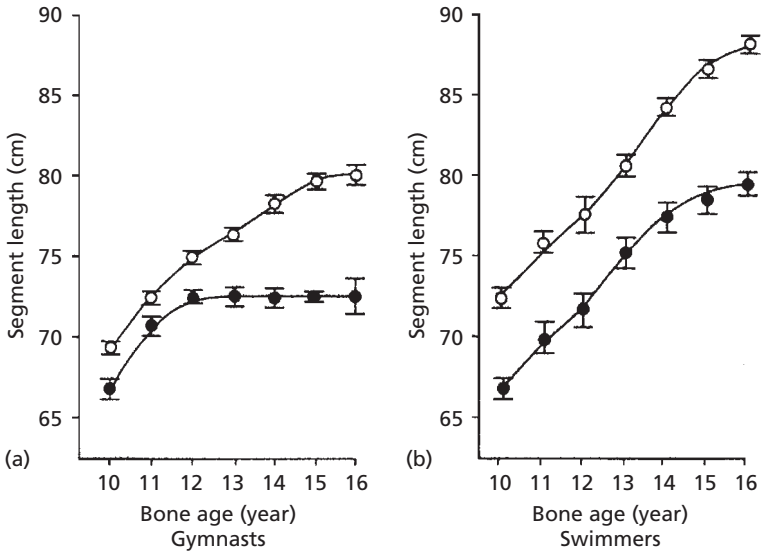

Fig. 34.1 Growth in sitting height (open circles) and leg length (closed circles) from bone age 10 years to bone age 16 years. Gymnasts are shown in (a) and swimmers in (b). Note the lack of increase in leg length after bone age 12 years in the gymnasts. (From Theintz *et al*. 1993.)

shorter than average and that height disparities increase with advancing age and the level of gymnastics competition (Caine *et al*. 2001). All measures of pubertal maturation, including bone age, development of the secondary sexual characteristics and age at menarche, are also delayed in most competitive gymnasts (Caine *et al*. 2001), which explains the greater differences from normal in the height of gymnasts with advancing age. The growth and maturation characteristics of gymnasts may be explained in part by self-selection and genetics as elite gymnasts are shorter than non-elite gymnasts before gymnastics training and are children of short parents who also had later than average puberty (Peltenburg *et al*. 1984; Theintz *et al*. 1989; Baxter-Jones *et al*. 1995). Whether gymnastics training reduces growth independent of self-selection and genetic influences on growth is unclear. Similar to menstrual dysfunction in adult athletes, slowed growth and maturation in girls may be more the result of undernutrition or psychological stress than gymnastics training. Dietary restriction of 80–90% of recommended amounts is very prevalent in gymnasts and associated with reduced height and delayed bone age (Caine *et al*. 2001). Olympic-level male gymnasts with adequate energy intake do not experience the same delayed puberty or hormonal alterations as their female counterparts (Weimann 2002), but this may also be due to differences in sensitivity of the sexes to nutritional insult.

However, there is some evidence that gymnastics training slows growth and pubertal maturation. A controversial study reported a delayed age at menarche, slowed linear growth, the lack of a normal growth spurt and reduced growth potential of gymnasts compare to swimmers (Theintz *et al*. 1993). Leg length velocity slowed at puberty with almost no growth after a bone age of 12 years (Fig. 34.1). The gymnasts also had significantly lower serum concentrations of estradiol and IGF-I than swimmers even when analyzed by bone age (Theintz 1994), suggesting a depression of both of the major axes involved in growth and maturation. The adult height of gymnasts can be shorter than expected based on mid-parental height (Lindholm *et al*. 1994), and growth spurts do not occur in some girls until the training load is reduced (Lindholm *et al*. 1994; Caine *et al*. 2001). Other studies have reported accelerated or catch-up growth after retiring from gymnastics competition (Bass *et al*. 2000; Caine *et al*. 2001), suggesting that growth was slowed when training.

WRESTLERS

Wrestling is made fairer by placing participants in weight classes so that a smaller wrestler does not have to compete against a larger wrestler. Wrestlers must weigh-in before competition to certify that their body weight is within limits of the weight

class. To gain an advantage by competing in a lower weight, wrestlers reduce their body weight through a combination of exercise, dietary restriction and dehydration. Typical wrestling training lasts 2–2.5 h·day^{-1}, 5–6 days per week. Adolescent wrestlers' nutrient intakes can consistently fall below 50% of the recommended amounts during a 3–4 month season (Fig. 34.2) (Roemmich & Sinning 1997b) resulting in reduced protein nutritional status (Fig. 34.3) (Horswill *et al*. 1990).

This undernutrition is accompanied by low testosterone and free-testosterone concentrations while serum LH concentrations are conserved (Roemmich & Sinning 1997a) (Fig. 34.4). In a well-nourished individual, low testosterone concentrations result in reduced testosterone negative feedback and elevated LH concentrations. The lack of increase in LH secretion in adolescent wrestlers suggests a central disruption of the HPG axis, most likely a reduction in GnRH secretion. Function of the GH–IGF-I axis was also altered with undernutrition in adolescent wrestlers (Roemmich & Sinning 1997a) (Fig. 34.5). A partial GH resistance occurs as indicated by late-season elevations in GH concentrations while IGF-I concentrations are reduced. Decreases in serum GHBP may also modulate serum GH concentrations. GHBP enhances the growth promoting effects of GH. When GHBP concentrations are reduced, more GH is released to continue growth at its genetically determined rate. Reductions in GHBP concentrations also signify a down-regulation of GH receptors, since GHBP is an index of GH tissue receptor number. Down-regulation would result in GH resistance and reduced growth, so that even with increased GH concentrations, growth would be slowed to conserve energy for critical body functions.

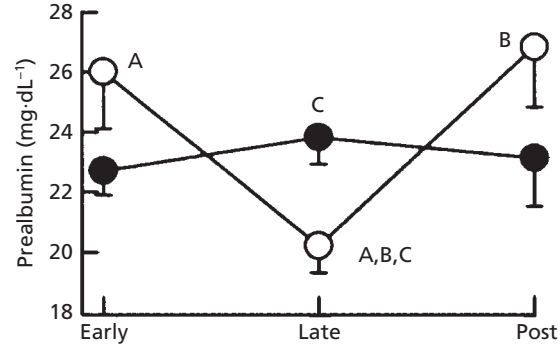

Fig. 34.3 Serum prealbumin concentration (mg·dL^{-1}) for the wrestler (open circles, n = 9) and control (filled circles, n = 7) groups. Like letters are significantly different. Values expressed as mean (± SE). For definitions see Fig. 34.2. (Redrawn from Roemmich & Sinning 1997b.)

Although these nutritional and hormonal alterations have the potential of altering the growth and maturation of wrestlers, over a sport season, the incremental growth in stature and rate of skeletal maturation of wrestlers is similar to that of controls during both the sport season and post-season (Fig. 34.6) (Roemmich & Sinning 1996, 1997b). However, hypertrophic growth of fat-free mass and other soft tissues are slowed during the sport season

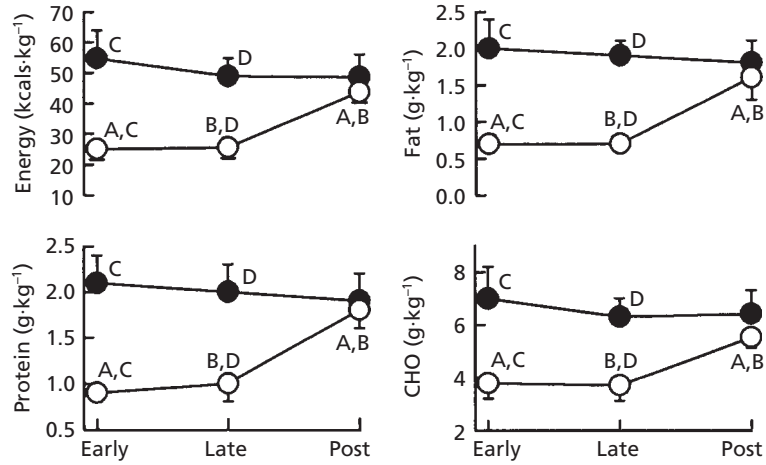

Fig. 34.2 Relative intake of energy (kcal·kg^{-1}·day^{-1}), protein (g·kg^{-1}·day^{-1}), fat (g·kg^{-1}·day^{-1}), and carbohydrate (CHO, g·kg^{-1}·day^{-1}) for wrestler (open circles, n = 9) and control (filled circles, n = 7) groups. All subjects were tested in mid-November, 1–2 weeks prior to the first wrestling match (Early), 3.5–4.0 months later (Late) depending on when the wrestler no longer qualified for tournament competition, and 3.5–4.0 months after the wrestling season ended (Post). Like letters are significantly different. Values expressed as mean (± SE). (Redrawn from Roemmich & Sinning 1997b.)

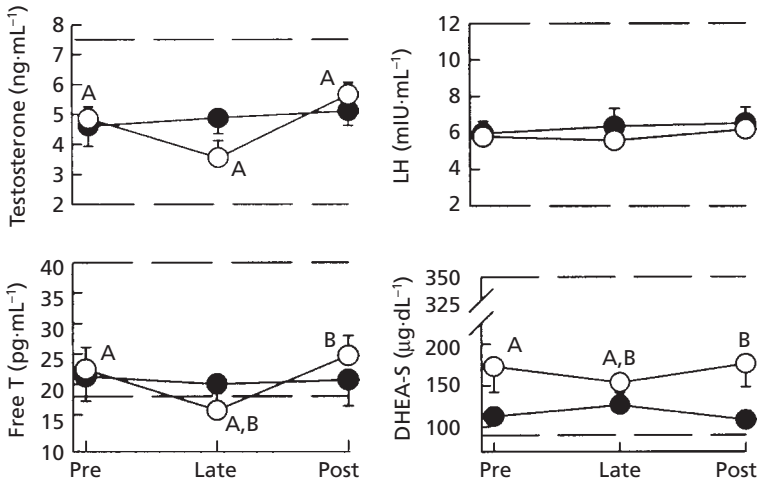

Fig. 34.4 Mean (± SE) concentrations of testosterone, free-testosterone (Free-T), luteinizing hormone (LH), and sex hormone binding globulin (SHBG) in the wrestler (open circles, n = 9) and control (filled circles, n = 7) groups during the pre and late portions of the wrestling season and during the post-season. Like letters are significantly different. Serum hormone concentrations are the mean of eight samples drawn every 20 min. Like letters indicate $p \leq 0.05$. Dashed lines indicate upper and lower normal range for each hormone concentration. For definitions see Fig. 34.2. (Redrawn from Roemmich & Sinning 1997a.)

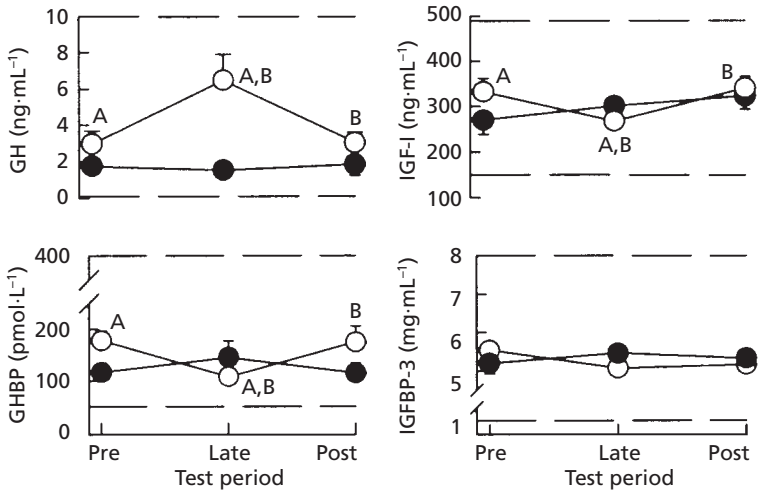

Fig. 34.5 Mean (± SE) concentrations growth hormone (GH), insulin-like growth factor I (IGF-I), growth hormone binding protein (GHBP), and insulin-like growth factor I binding protein-3 (IGFBP-3) in the wrestler (open circles, n = 9) and control (filled circles, n = 7) groups during the pre and late portions of the wrestling season and during the post-season. Serum hormone concentrations are the mean of eight samples drawn every 20 min. Like letters are significantly different and indicate $p \leq 0.05$. Dashed lines indicate upper and lower normal range for each hormone concentration. For definitions see Fig. 34.2. (Redrawn from Roemmich & Sinning 1997a.)

followed by catch-up growth in the post-season (Fig. 34.7) (Roemmich & Sinning 1996, 1997b).

Conclusion

Sport training produces acute and chronic alterations in serum hormone concentrations but for the vast majority it does not alter the rate of genetically programmed linear growth or pubertal development. Normal linear growth encompasses the third to 97th percentile for height and height velocity. Individual differences in pubertal timing may cause some athletes to temporarily track along a lower or higher percentile. Early maturing athletes will have an increased height percentile while late maturing athletes will have a decrease in height percentile. Although the average height for participants in some sports may be less than the 50th percentile, this is probably due to self-selection rather than sport training as their growth continues to track along the same relatively low percentile before and during training. Boys and girls with genetically determined delayed puberty and/or short stature would migrate towards gymnastics and wrestling because their smaller size will be a disadvantage in some sports, but an advantage in gymnastics and wrestling. Undernutrition caused by training and dietary restriction appears to produce a disruption

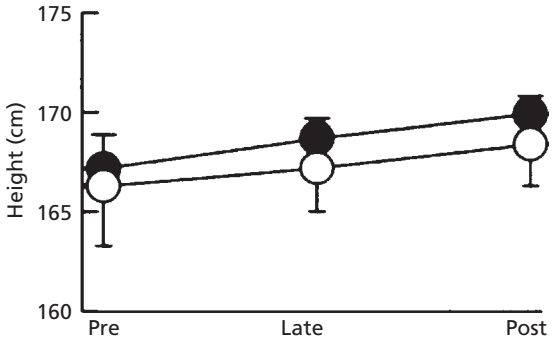

Fig. 34.6 Height for the wrestler (open circles, n = 9) and control (filled circles, n = 7) groups. There were no differences in height between the groups. For definitions see Fig. 34.2. (Redrawn from Roemmich & Sinning 1997b.)

of the HPG and a partial GH resistance in wrestlers and may also delay maturation of the reproductive axis of some elite gymnasts. The 3–4 month period of undernutrition does slow hypertrophic growth of lean tissue but is followed by long periods of adequate nutrition so the period of undernourishment is not long enough to slow linear growth or maturation or reduce the adult height of wrestlers. Elite female gymnasts who are training and limiting their dietary intake year round may be at greater risk for limiting their growth. Gymnastics training may slow growth, but more data are needed to confirm this result. The separate effect of gymnastics training and dietary restriction on the growth, maturation and neuroendocrine axes of gymnasts has not yet been adequately studied.

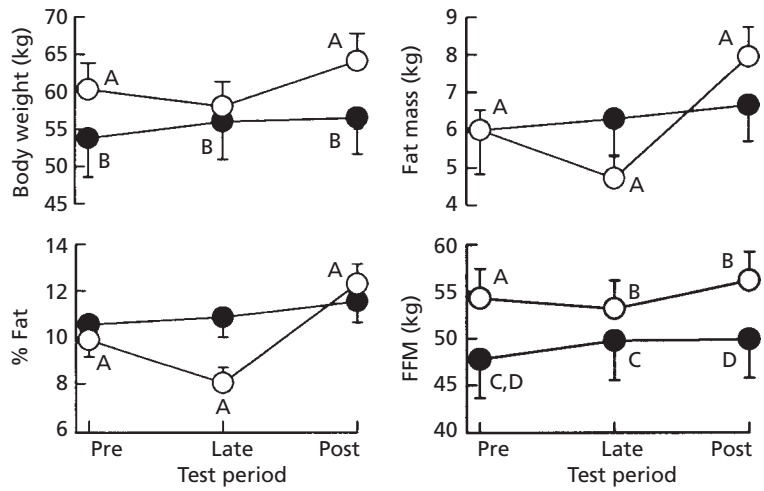

Fig. 34.7 Body weight, percentage body fat (% Fat), fat mass and fat free mass (FFM) for the wrestler (open circles, n = 9) and control (filled circles, n = 7) groups. Like letters are significantly different. Values expressed as mean (± SE). For definitions see Fig. 34.2. (Redrawn from Roemmich & Sinning 1997b.)

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Chapter 35

Effects of Testosterone and Related Androgens on Athletic Performance in Men

KARL E. FRIEDL

> In spite of quantitative differences in the gene products of tissues from males and females, there are few important sexual differences in the genome, with the exception of the male-specific Y chromosome. The major function of this chromosome is to determine sexual differentiation; in its absence, ovaries develop. It is, then, the secretory products of the gonads that are responsible for sexual dimorphism. . . . As a consequence of its widespread action, testosterone is the major hormone responsible for the sexual dimorphism of nonreproductive tissues.
>
> Bardin & Catterall 1981, p. 1285

Introduction

Although gender differences in body size and upper body strength may have been evident for centuries, the basis of these differences is still poorly understood. This ignorance hampers our ability to predict the effects of supplemental androgens administered to normal adult males for performance enhancement. Androgens administered to androgen-deficient men, prepubertal boys and normal women can produce marked effects on size and strength (Stanhope *et al*. 1988; Bhasin *et al*. 1997; Elbers *et al*. 1999), but it does not necessarily follow that an excess of androgens to normal men will provide more of the same effects along the same continuum. There is no specific disease of androgen excess in males, perhaps suggesting that more androgen does indeed produce more of the same biological effects (Can a man *be* over-virilized?) (Bardin *et al*. 1990). Alternatively, it could be reasonably expected that limiting mechanisms simply will not allow open-ended or cumulative effects to occur (e.g. changes in receptor density and binding activity, negative feedback loops and non-linear interactions with other hormones) (Glass & Vigersky 1980). In this case, observed effects from supraphysiological doses of androgens may contrast those that occur in nature, producing pharmacological effects that are very different than those associated with male characteristics (e.g. stroke risk, gynecomastia, osmoregulatory changes). For practical purposes, supraphysiological could be defined as a dose that increases circulating levels of testosterone above the normal range, or other androgen formulations with rough bioequivalence to these above-normal testosterone levels (even though measured testosterone may be markedly suppressed); in some regards, any administration of exogenous androgen is supraphysiological because it does not precisely mimic circadian fluctuations and other properties of the body's own hormonal secretions. In the absence of data, speculative and anecdotal self-experiments by athletes have led to widespread gym lore about androgen use, and non-users are left with concerns about unfair performance advantages. A greater understanding of androgen physiology can improve the scientific basis of training regimens for athletes and help define the full range of modifiable human performance. This chapter considers the available data on testosterone and related androgenic steroid effects on athletic performance and attempts to expand on earlier thoughtful reviews (Wright 1980; Haupt & Rovere 1984; Wilson 1988; Yesalis 2000).

Metabolism and action of testosterone and related androgenic steroids

The basic physiology of testosterone production and secretion is relatively well known, while the far-reaching effects of testosterone on cells throughout the body, as well as interactions with other anabolic hormones, are not as well understood (Bardin & Catterall 1981). Testosterone is a steroid hormone that is made from cholesterol through a series of enzyme-regulated steps in the Leydig cells of the testes of men, and in the adrenal cortex of men and women. The key regulator of the testicular secretion is luteinizing hormone (LH), a protein hormone from the pituitary gland. LH secretion, in turn, is regulated by gonadotropin-releasing hormone (GnRH), which is regulated in the hypothalamus. LH and GnRH secretion are controlled, in part, by circulating levels of testosterone in a negative feedback loop. If testosterone or a related androgenic compound is administered artificially, the system down-regulates, reducing the amount of testosterone synthesized by the testes. If higher than normal circulating levels are sustained by administration of androgen, the testes substantially reduce testosterone and sperm production, reducing the volume of the testes as the seminiferous tubules are depopulated of maturing germ cells (a readily reversible phenomenon). This noticeable reduction in testicular volume (Kiraly 1988; Friedl *et al*. 1991) is one of the androgen effects that make blinded studies in androgen-experienced athletes difficult to conduct.

Testosterone's effects on body tissues are far more complicated than its production and secretion. Testosterone is directly active in most tissues, acting through a specific androgen receptor. Additionally, in some tissues such as the prostate and hair follicles, testosterone is converted to 5α-dihydrotestosterone (DHT). DHT has greater potency than testosterone on androgen receptors, but is also more rapidly metabolized to biologically inactive 5α-androstanediols. In fat cells, where high concentrations of aromatase enzyme are present, some of the testosterone is converted to estrogen, with estrogen actually playing important roles in the mediation of apparent testosterone actions in some tissues such as intra-abdominal fat metabolism, regulation of key hepatic proteins, bone mineral metabolism and some brain effects (e.g. regulation of gonadotropin secretion). In muscle cells, testosterone appears to act directly on androgen receptors, which are present in much lower density than in more androgen-responsive tissues such as prostate, and then is excreted as a water-soluble conjugate, 3α-androstanediol glucuronide; muscle lacks the 5α-reductase to convert testosterone to DHT (Michel & Baulieu 1980; Hughes & Krieg 1988). Androgens, including testosterone and DHT, are bound to a variety of carrier proteins in circulation, most specifically to sex hormone binding globulin (SHBG), but also non-specifically to albumin. This constitutes a large circulating 'reserve' pool of immediately available steroids that has some protection against metabolism and excretion while bound, acting in a kinetic balance with receptors and competitive ligands that may vary between tissues, with only 1–2% unbound (or 'free') at any time. Testosterone activates the secretion of other potent anabolic hormones such as insulin-like growth factor I (IGF-I) and erythropoeitin, and potential benefits to athletic performance may accrue from these indirect effects of androgen. Localized effects of testosterone, including such interactions at the tissue levels, may be very important and are just beginning to be appreciated with the availability of modern research methods such as transgenic animal studies. Testosterone can also bind to other receptors such as glucocorticoids, in this case providing competitive inhibition of certain catabolic stress responses induced by cortisol; thus, part of the anabolic action of testosterone may actually be exerted through this crossover binding to modify catabolic responses (Hickson *et al*. 1984; Janne 1990).

Pharmacological compounds that have been developed to extend or modify the effect of testosterone build on some of these understood effects of androgens (Liddle & Burke 1960; Kruskemper 1968; Kochakian 1988). Thus, steroids that cannot be converted to estrogenic compounds or to DHT will exhibit characteristic effects different from those of testosterone, with reduced effects on bone or prostate, respectively (Sundaram *et al*. 1995). Some steroid-using athletes include aromatase inhibitors

in the drug regimen to block conversion to estrogens, or use estrogen receptor blockers to prevent estrogenic actions, in attempts to prevent gynecomastia and other side effects (Friedl & Yesalis 1989). Most androgenic-anabolic steroids have been developed to reduce the 'androgenic' effects on reproductive tissues such as prostate, in favor of 'anabolic' effects on muscle and bone; these would focus on steroid structures that cannot be readily reduced to DHT (Hershberger *et al*. 1953). Testosterone administered either orally or by injection promptly reaches circulation and is rapidly removed by the liver and metabolized to products that can be readily excreted in urine or bile. Modifications with 17-alkylation protect testosterone against this rapid elimination, making methyl testosterone an orally active androgen, but the metabolites produced in various tissues are 17-alkylated compounds that will exhibit different binding properties and biological effects. Adverse effects on the liver are one of the properties inherent in the orally active 17-alkylated androgens. Methandienone (Dianabol) is the most important orally active 17-alkylated androgen in the studies of athletic performance (Friedl 2000). This was the orally active drug of choice by steroid-using athletes in the 1960s and 1970s but has been since taken off the market by major drug manufacturers (Yesalis *et al*. 1988). Modifications of testosterone with an ester side chain also protects against rapid elimination, but because this side chain can be hydrolyzed to provide graded release of testosterone into circulation from deep intramuscular injection sites, the effects are different than those of the 17-alkylated compounds. The duration of action of these esterified androgens is roughly dependent on the length of the side chain; a relatively short side chain in testosterone undecanoate makes this a relatively short acting testosterone (usually used orally) (Schurmeyer *et al*. 1983), while high levels of testosterone can be maintained with testosterone enanthate (TE) or testosterone cypionate in weekly injections, and the very long chained testosterone buciclate maintains circulating levels for months (Behre & Nieschlag 1992). A compound that differs from testosterone only by the elimination of one methyl group at the 19 carbon position ('19-nortestosterone' or nandrolone) was developed to try to reduce androgenic side effects on prostate through its properties that prevent aromatization to estrogens and its metabolism to compounds that are weaker than DHT (e.g. 19-nordihydrotestosterone) (Hershberger *et al*. 1953; Sundaram *et al*. 1995). Nandrolone esters (e.g. nandrolone decanoate or 'Deca-Durabolin') and testosterone esters (e.g. TE) are the most commonly used androgenic preparations today, and these are the compounds that have been used in the majority of modern studies on performance effects. A wide variety of androgens are active in muscle, while requiring greater selectivity for some other tissues; for example, protection of bone against glucocorticoid effects requires an aromatizable androgen, while nandrolone compounds are effective in preserving muscle but not bone (Crawford *et al*. 2003). Any administration of exogenous steroid is nonphysiological, even at doses that restore mean circulating levels to 'normal', as there is a large fluctuation in normal circulating levels of testosterone during the day.

Dietary supplements that are legally marketed as 'steroid replacers' in the USA include a wide range of products (e.g. sterols, sapogenins, androgen metabolites, yohimbe bark, boron), none of which have any convincing scientific basis for performance enhancement in normal men. Some products have been tainted with banned substances that will give a positive drug test; others are simply bogus and even harmful (Friedl *et al*. 1992b). There is no over-the-counter drug that comes close to the actions of testosterone, or significantly stimulates its production or release in the body (King *et al*. 1999). If such an effective product surfaced, it would likely be added to the list of androgens and other substances banned by athletic federations.

Most recently, new 'designer' steroids have been detected in urinalyses from elite athletes (Catlin *et al*. 2002). Tetrahydrogestrinone is a compound related to gestrinone, a steroid used to treat endometriosis in women, and is the latest to turn up in drug testing in a wide variety of sports including track and field, football and boxing (Knight 2003). The novelty is not in some new marvelous mechanism of action, but rather in the fact that the compound was new to human use and therefore not

specifically tested for in routine drug screens. Other synthetic steroids have been administered in attempts to correct the abnormal profile of androgen metabolites that signal exogenous use of androgens (Aguilera *et al*. 2002). The use of new drugs with little or no evaluation of human safety should be of great concern to normal healthy athletes.

For purposes of this chapter, anabolic steroids are used to denote synthetic androgenic steroids other than the testosterone compounds, but are used somewhat interchangeably, as anabolic and androgenic properties are notional and have never been effectively separated (Kochakian 1988).

Is there a male athlete hypogonadal equivalent to athletic amenorrhea?

Environmental regulators of testosterone secretion

Female athletes have been commonly warned that reproductive cycles may cease with too much exercise, as part of a 'syndrome' involving exercise, amenorrhea and bone mineral loss. Extrapolations from the theory of an athletic amenorrhea in men suggest that with high volume physical training, testosterone will be suppressed in male athletes. Furthermore, it has been suggested that these athletes might benefit from 'replacement' androgen therapy. This interpretation may have started with military training studies in which marked reductions in testosterone were noted in healthy young men (Aakvaag *et al*. 1978). In normal men working hard with inadequate energy intake, testosterone can fall nearly to castrate levels. This has been shown in high school wrestlers during their wrestling season while they are energy-restricted (Strauss *et al*. 1985; Roemmich & Sinning 1997) and in elite soldiers during short- and long-term intensive training (Opstad 1992a; Friedl *et al*. 2000). Guezennec *et al*. (1994) demonstrated a testosterone decline with increasing energy deficit in French commandos. However, testosterone levels are rapidly restored to normal with refeeding, even in the face of other persistent stressors such as workloads exceeding 25 $MJ \cdot day^{-1}$ (6000 $kcal \cdot day^{-1}$) (Friedl *et al*. 2001). Thus, these large reductions, generally much larger than those observed in athletes in training, can be explained by inadequate energy intake and not as an exercise-specific effect (Friedl 1997). As it turns out, even the concept of a female athletic amenorrhea produced by high volume exercise may not be correct; earlier studies were confounded by under-reporting food intake. As observed in men, energy deficit, not exercise, appears to be the key stressor affecting reproductive cycles in women (Loucks & Thuma 2003; Loucks 2004).

Psychological factors also affect testosterone levels in healthy men. The thrill of victory may produce short-term increases in circulating testosterone levels (Ursine *et al*. 1978; Elias 1981), while anxiety produces reliable declines (Bourne *et al*. 1968; Kreuz *et al*. 1972; Francis 1981). Mood and motivation in human research is difficult to control or ethically manipulate. However, in one novel study of men in parachute training making a stressful jump out of a high tower, the initial jumps resulted in a marked stress effect with acute suppression of testosterone and elevation of cortisol ('distress'), while later experienced jumps led to acute elevation of testosterone and no change in cortisol ('eustress') (Ursine *et al*. 1978). There is a large body of literature on the effects of defeat and chronic stress on testosterone suppression in animals that suggests this is a key regulator of normal testosterone in mammalian species (Blanchard *et al*. 1993). In humans, chronic anxiety appears to have a prominent suppressive effect on testosterone and may even provide a more reliable indicator of a noxious stress than traditional measures of the hypothalamic–pituitary–adrenal axis such as cortisol (Kreuz *et al*. 1972; Francis 1981; Hellhammer *et al*. 1985).

Effects of resistance and endurance exercise on testosterone

Exercise itself does not appear to be an important regulator of testosterone secretion through either primary (actions at the testes) or higher levels (actions at the pituitary or hypothalamus), although little is known about the neurobiology of exercise (Rogol *et al*. 1984), or any direct signals from exercising muscle that may affect the gonadal axis. Endurance athletes have been variously reported

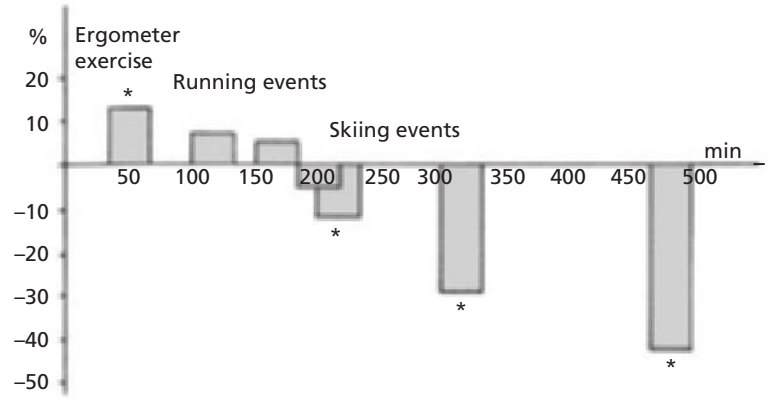

Fig. 35.1 Apparent relationship between duration of exercise and serum testosterone from various studies, suggesting a role for a progressive metabolic stimulus such as declining glucose availability on regulation of testosterone secretion. (From Viru 1985.)

to have modest reductions in resting testosterone levels (Wheeler *et al*. 1984; De Souza *et al*. 1994), or no differences (Bagatell & Bremner 1990; Lucia *et al*. 1996), compared to sedentary controls. Male runners reducing their weekly mileage from 81 km·wk^{-1} to 24 km·wk^{-1} demonstrated no change in low, normal testosterone levels, even though creatine kinase levels were reduced by half, reflecting a reduction in muscular strain (Houmard *et al*. 1994). The biological significance of this altered status to low-normal circulating levels observed in some studies is unknown.

Exercise can acutely increase or decrease circulating testosterone, depending on the mode and intensity of exercise (Schmid *et al*. 1982; Viru 1985). Increases occur during and after relatively short, high intensity work such as resistance training or sprint events, while declines are associated with increasing duration endurance events and are especially noted in marathon and ultra-running events. Endurance exercise has been reported to decrease testosterone by as much as half, even after appropriate comparison to same time-of-day baseline values (Dessypris *et al*. 1976; Morville *et al*. 1979; Kuoppasalmi *et al*. 1980; Schurmeyer *et al*. 1984). These effects observed during or shortly after the exercise period, may persist into the next day or two after cessation of exercise. The physiological consequences of this short-term suppression are uncertain; this decline is comparable to the changes observed daily in normal men, and the observed reductions still do not fall into the range of a clinical hypogonadism that would normally justify treatment. Mechanisms for this change with prolonged exercise have been variously postulated and investigated. Catecholamine effects on testicular blood flow (Collu *et al*. 1984) and cortisol suppression of testosterone production (Bambino & Hsueh 1981) do not seem to occur at physiological levels (Sapolsky 1985, 1986). Increased clearance rates of testosterone by exercising muscle (e.g. increased excretion of 3α-androstenedione-glucuronide) appears to occur (Ponjee *et al*. 1994), but could be construed as an increased biological action reflected in greater turnover at the muscle target site, rather than a deficiency state. It may be that this transient decline in testosterone that appears to increase with duration of exercise (Fig. 35.1) is explained simply by a within-exercise energy deficit, highlighting the sensitivity of GnRH to low glucose levels in the hypothalamus (Opstad 1992b; Loucks 2004). A similar effect of a progressive 'starvation' profile has been suggested for changes in thyroid hormone levels during prolonged exercise (O'Connell *et al*. 1979). Some extreme endurance athletes recognize the need for liquid carbohydrate replenishment during their exercise (Saris *et al*. 1989), although whether or not this better sustains testosterone levels and muscle mass has not been studied.

In contrast to prolonged endurance exercise, resistance and sprint exercise usually produces short-term elevation in testosterone concentration (Sutton *et al*. 1973; Cumming *et al*. 1986; Kraemer *et al*. 1991), or no change observed (Guezennec *et al*.

1986). Several mechanisms would be reasonably expected to temporarily increase serum concentrations. Liver blood flow markedly declines with increasing intensity of exercise (Rowell *et al*. 1964) and would reduce hepatic clearance and temporarily increase serum concentrations. Fluid shifts with movement out of the vascular space during exercise (Wilkerson *et al*. 1980) would increase the concentration of all protein-bound hormones such as testosterone, which cannot move with the fluid. Dopamine and prolactin may regulate testosterone release, but in one study, testosterone changes were unaffected when the exercise-induced rise in prolactin was blocked by L-dopa (Jezova-Repcekova *et al*. 1982). Sympathetic activity may also play a role in exercise-induced testosterone increase (Jezova & Vigas 1981).

Effects of endogenous testosterone changes on performance

Temporary exercise-induced declines in testosterone may not have much effect on body composition, as much more profound reductions in testosterone lasting weeks have relatively small effects. Artificial hypogonadism induced for 10 weeks in healthy young men who were not exercising produced a 2 kg shift in lean-to-fat tissue balance (Mauras *et al*. 1998). In healthy young soldiers who were underfed while working hard, individual differences in the level of testosterone suppression was a relatively small contributory factor in the proportion of lean tissue lost during weight loss over 8 weeks, even though the association was statistically significant (Friedl 1997). This is supported by findings in animal studies, where fasting and exercise caused smaller testosterone reductions in endurance-trained animals and this difference was also associated with higher lipolysis and decreased glyogenolysis, possibly protecting muscle protein (Guezennec *et al*. 1984b).

Acute increases in testosterone observed with resistance exercise may be enough to trigger tissue receptor-level changes or other anabolic hormones, but this remains unknown. In training studies conducted by Kraemer *et al*. (1991), a comparison of bodybuilder to power lifter routines with differences in repetitions and duration of rest periods between sets demonstrated large differences in growth hormone responses, but a minimal difference in the magnitude of testosterone increases in either routine. One can only speculate on the significance of brief testosterone changes in either direction to synergistic actions with other musculotrophic hormones such as local tissue activity of IGF-I, including in the final determination of muscle hypertrophy under the influence of strength demands for strength athletes and the slimming down of muscle mass that would be advantageous for distance runners.

Effects on muscle mass: how big can humans get?

Increases in total body weight

Androgen supplementation to normal men increases body weight and amino acid incorporation into muscle protein. The precise nature of the observed weight gain, how much can be added with continuous androgen administration, and how much will remain after steroid cessation is far from clear. In general, humans increase or lose lean and fat mass in roughly the same proportions as they move up and down the scale of energy balance, with approximately two-thirds of the changes in fat mass. Forbes has plotted these trends on the basis of data from overfeeding and underfeeding studies (Fig. 35.2) (Forbes 1993). He suggests that supplementation with anabolic hormones moves individuals off the normal trajectory, with a substantial increase in the lean component and even a reduction in the fat component. In other words, androgen supplementation has been suggested to provide a nutrient partitioning effect similar to the class of β-adrenergic agonist drugs that have been developed for animal food production (e.g. clenbuterol) that favor a specific increase in the lean mass component. Forbes was one of the first to illustrate this principle in a study with high-dose androgen administration to normal men (see Fig. 35.3, below) (Forbes *et al*. 1992). However, all studies to-date, including this one, leave nagging questions about the role of dietary intakes, training effects, and the

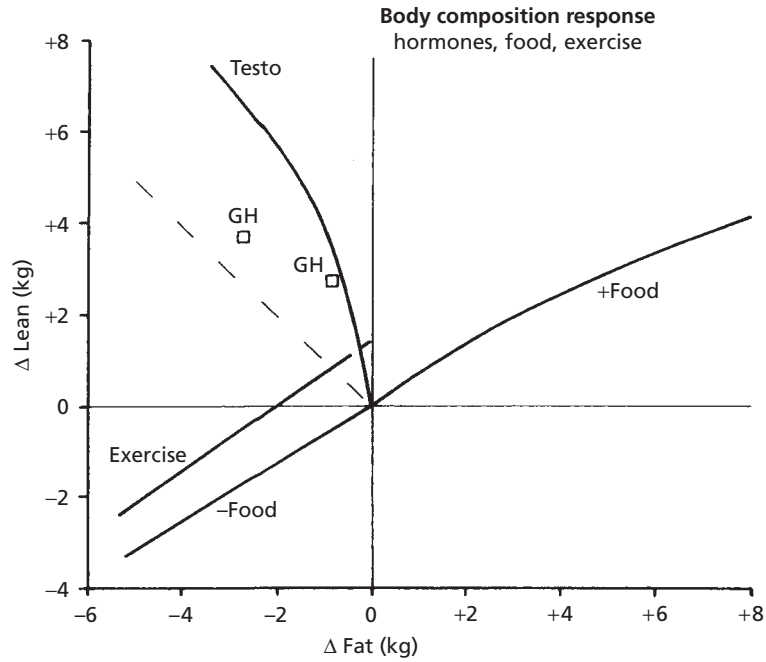

Fig. 35.2 Conceptual relationship between fat and lean components during changes in body weight and the effect of superimposed anabolic hormone administration. GH, growth hormone. (From Forbes 1993.)

validity of the assumptions underlying each of the methods used to estimate these changes.

Weight gain is a well-known side effect of androgens used in clinical practice and in male contraception trials using supraphysiological doses of injectable testosterone esters (typically 200 mg·wk^{-1}) (World Health Organization Task Force 1990; Young *et al*. 1993). A typical period of 6 weeks or longer of continuous testosterone ester administration to normal adult males provides about 3–5 kg of weight gain within the first few weeks (Mauss *et al*. 1975; Friedl *et al*. 1990, 1991; Welle *et al*. 1992; Young *et al*. 1993; Pope *et al*. 2000). Orally active androgens have the same effects. A large series of studies in the early 1970s with methandione (Dianabol) showed consistent increases of about 2 kg in men given at least 10 mg·day^{-1} for 3–4 week periods, regardless of physical training status (Friedl 2000). It is not clear that this weight gain effect is either dose- or time-dependent. In the male contraceptive studies that have given androgen (testosterone enanthate, TE) at a dose of 200 mg·wk^{-1} for more than a year, the individuals do not continue to gain weight, despite the sustained doubling or tripling of circulating testosterone levels with this dosing regimen. There were no effects in normal men at typical doses used in replacement therapy for hypogonadal men (e.g. 100 mg·wk^{-1} of testosterone cypionate or nandrolone decanoate, ND) (Crist *et al*. 1983), but doubling the dose (ND, 200 mg·wk^{-1} for 8 weeks) produced significant increases in weight (Hartgens *et al*. 2001). In another study, androgen doses that produce 'replacement' levels in normal men (TE, 100 mg·wk^{-1}) did not change body weight, while high levels of TE (300 mg·wk^{-1}) and two doses of ND (100 mg·wk^{-1} and 300 mg·wk^{-1}) produced significant weight gain (Friedl *et al*. 1991). A study that was twice as long with comparable weekly dosing (TE 280 mg·wk^{-1}) did not cause twice as much weight gain (Friedl *et al*. 1990). Bhasin *et al*. (1996) doubled the weekly dose of testosterone (TE, 600 mg·wk^{-1} for 10 weeks) and also observed a similar final increase of about 3.5 kg, while in the group of men who included exercise with the same high dose, this weight increase nearly doubled. Pope *et al*. (2000) found an increase of 3.0 kg following a typical bodybuilder regimen of increasing doses of testosterone cypionate from 150 mg·wk^{-1} up to 600 mg·wk^{-1} over 6 weeks. (They reported all of this weight gain as

lean mass on the basis of skinfold thicknesses that showed little change with androgen.) Although Forbes has suggested a dose–response relationship to lifetime exposure to androgens (Forbes 1985), these data suggest that supraphysiological doses of androgens provoke a specific weight increase as a threshold effect, after which mechanisms adjust and prevent further gains. If this were not true, one would expect all normal men to continue to gain weight from cumulative androgen exposure after the initial pubertal surge in testosterone, instead of reaching stable weights in the early 20s without further dramatic gains.

These increases in weight observed in controlled studies and clinical observations of high-dose androgen administration to normal men reflect only a portion of the apparent weight differences that have been estimated for anabolic steroid-using bodybuilders. In dedicated bodybuilders, Kouri *et al*. (1995b) found a difference in weight between steroids users and non-users of nearly 10 kg, with comparable estimates of adiposity between the groups (~ 12.5% body fat). A comparison of the data for 20 presteroid era Mr. America winners (1939–1959) to a group of popular modern bodybuilders indicated an estimated difference of 20 kg of mass, presumably lean mass. Changes in nutrition and health care over the past century have increased the lean mass of typical young US soldiers by nearly 10 kg (Friedl 2004). Thus, it would be reasonable to ascribe some of the weight gain in bodybuilders since the 1940s and 1950s to a combination of this improved nutrition, as well as exercise science techniques; however, Kouri's data provide at least a rough estimate of the upper limit of the mean gain that might be attributable to androgen use. The difference of 3–5 kg gains in controlled trials and 10 kg seen in androgen-using bodybuilders may reflect an effect of repeated cycles of use, as well as training and dietary interactions.

Characterizing changes in body composition

The nature of androgen-induced weight gain has never been adequately characterized. A key problem is that most studies have measured body composition using only one technique, with each technique dependent on certain assumptions that may not hold true with anabolic steroid administration. Although the combined error of multiple techniques in a multicompartment model of body composition is generally smaller than the biological variation in body compartments measured (Friedl *et al*. 1992a), multicompartment models may also not resolve the question if steroid-induced changes confound the assumptions of these methods. For example, if a portion of the androgen response includes an increase in total body water relative to the total lean mass, as has been suggested but never properly tested (Freed *et al*. 1975; Hervey *et al*. 1976; Wilson 1988), this could produce significant errors in the estimated changes determined by each of the principal body composition techniques employed, by violating the assumptions about the composition of the lean compartment for each method. Underwater weighing assumes 1.1 $g \cdot cm^{-3}$ density of lean tissue, dual energy X-ray absorptiometry (DEA) assumes an attenuation coefficient based on normally hydrated soft tissue, potassium-40 depends on a fixed distribution in the lean compartment (66.6 $mmol \cdot kg^{-1}$), and deuterium dilution assumes a fixed hydration level (usually 72%) of the lean mass. These might not be trivial errors. In semi-starvation of normal men, where hydration of the lean mass increases in a subclinical edema, DEA measures 3.5 kg more tissue than is actually present, based on simple scale weights (Friedl *et al*. 1994). Although speculative, a change in the opposite direction with rapid increase in body mass produced by androgens could also be accompanied by increased water retention. If this is the case, each of the body composition methods would be expected to incorrectly estimate the changes in the lean mass component of weight.

Hervey attempted to resolve the question in his studies of high-dose methandienone (100 $mg \cdot day^{-1}$ for 6 weeks). Total body potassium measurements predicted that an average weight increase of 3.3 kg was composed of 6.3 kg lean mass gain and 2.5 kg fat loss. However, body density measured by underwater weighing predicted 2.4 kg lean mass gain and 0.9 kg fat gain, and skinfold thicknesses did not change (Hervey 1976). A second study with the same dosing produced comparable average

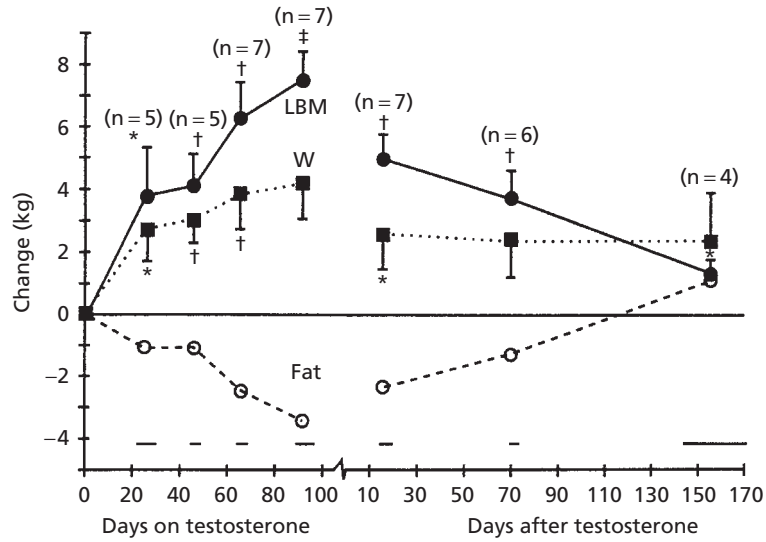

Fig. 35.3 Changes in body weight and lean and fat mass components in healthy young men administered 3 mg·kg^{-1}·wk^{-1} testosterone enanthate for 12 weeks and following cessation of drug administration. LBM, lean body mass; W, weight. (From Forbes *et al*. 1992.)

weight gains of 3.6 kg and similar changes in the total body potassium (Hervey *et al*. 1981). This time, total body nitrogen was also measured by neutron activation analysis, with an estimate that if the increased body weight reflected new muscle, body nitrogen should increase by about 100 g. The measured increase was more than double this value, raising new questions about the nature of the increase.

Forbes *et al*. (1992) reported on a typical regimen of approximately 200 mg·wk^{-1} of TE for 12 weeks, showing an average weight gain of 4.1 kg (Fig. 35.3). Based on potassium-40 measurements, he estimated an increase in the lean mass component of 7.5 kg by the end of 12 weeks of drug administration. This was corroborated by urinary creatinine excretion that increased by an average 358 mg·day^{-1} (on a meat-free diet), corresponding to an estimated lean mass increase of 8.6 kg.

There is an apparent rapid loss from the lean mass compartment following androgen cessation. In this same study, Forbes *et al*. (1992) reported a prompt decline in weight to about 2.5 kg within the first 10 days following steroid cessation, and this new weight was maintained over the next 6 months (Fig. 35.3). The estimated lean mass declined to about half of the peak gain by 2 months following steroid cessation, and further declined over 6 months to just over 1 kg from the initial baseline. This was balanced at stable weight by an assumed regain of fat weight following an initial average loss of 3.4 kg. The loss of at least some of the weight gain following steroid cessation is comparable to the observations in normal men in whom testosterone is artificially suppressed. Normal men artificially reduced to prepubertal levels of circulating testosterone for 10 weeks had a 2 kg loss of lean mass, with a comparable increase in fat mass, as measured by DEA (Mauras *et al*. 1998). This suggests a labile component of lean mass that has some dependence on threshold changes in androgen levels.

Several studies have used imaging or muscle biopsies to conduct morphological analyses on the cross-sectional area of muscle and muscle fibers. Histological analyses of muscle biopsies from the vastus lateralis of normal subjects following administration of TE 200 mg·wk^{-1} for 6 weeks was not able to demonstrate significant increases in muscle fiber diameter (Griggs *et al*. 1989), while two other studies using athletes found significant increases in muscle fiber area of this muscle (Alen *et al*. 1984; Kuipers *et al*. 1993). A more recent study found that TE 300 and 600 mg·wk^{-1} for 20 weeks in normal men with endogenous androgen suppression significantly increased the cross-sectional area of muscle fibers and myonuclear number in biopsies of the vastus lateralis, indicating an effect of androgen on muscle

hypertrophy; there was no significant change in the proportion of type I and type II fibers (Sinha-Hikim *et al*. 2002). This same study demonstrated dose-dependent increases in vastus muscle volume measured by magnetic resonance imaging (MRI), and an apparent dose-dependent increase in total lean mass at 300 and 600 mg·wk^{-1} TE. In their earlier study, Bhasin *et al*. (1996) found significant increases in MRI-measured muscle cross-sections of both the triceps and quadriceps of the men receiving testosterone, whether or not they included exercise. However, no change was found for the exercise alone group, which supports the overall effect of androgens on muscle mass increase. New state-of-the-art techniques to quantify skeletal muscle should be of great value in terms of ease and precision for future studies (Gallagher *et al*. 1999).

Androgens increase muscle protein synthesis, but do not typically cause an increase in total body protein synthesis. Griggs *et al*. (1989) studied healthy men administered approximately 200 mg·wk^{-1} TE for 12 wks and found typical increases in lean mass based on potassium-40 and creatinine excretion, accompanied by a 27% mean increase in muscle protein synthesis rates. The observed increases are therefore presumed to be a shift in the balance between synthesis and degradation rates, with an overall increase in turnover rates. Presumably, the effect is mediated directly through androgen receptors in the muscle, but this mechanism has not been well characterized, and there appear to be major differences in the androgen responsiveness of different muscles (Gustafsson *et al*. 1984; Hughes & Krieg 1988; Antonio *et al*. 1999). New studies of androgen-receptor signaling pathways in muscle are beginning to identify an inverse relationship between new muscle cell development and adipocyte development that appear to link androgen effects on fat and muscle shifts (Lee 2002), as has been previously suggested for steroid hormone actions in bone in the balance of osteoblasts and adipocytes. Other transgenic studies that use targeted overexpression of androgen receptor (Wiren *et al*. 2003) and muscle-specific IGF-I (Paul & Rosenthal 2002) in muscularly overdeveloped 'mighty mouse' animal models are likely to produce near-term breakthroughs in our understanding of bone, muscle and fat regulation by androgens, as well as the essential role of estrogens in these effects.

Studies of regional fat tissue metabolism suggest that androgens reduce triglyceride accumulation in intra-abdominal fat, but do not have this effect on subcutaneous fat (Marin 1995; Marin *et al*. 1996), although testosterone treatment produces an increase in visceral fat in women (Elbers *et al*. 1997). An increase in lean mass might further influence body composition by changing energy requirements, but there is no apparent androgen-specific increase in energy metabolism. Welle *et al*. (1992) found a 7% increase in basal metabolic rate with TE (200 mg·wk^{-1} for 12 weeks), and this increase could be completely accounted for by the increase in skeletal muscle mass. An informative study of the body composition changes was conducted using the 'Leydig clamp' model of Bhasin and his colleagues, with suppressed endogenous testosterone combined with sub- and supraphysiological doses of testosterone enanthate (25–600 mg·wk^{-1} for 20 weeks) (Bhasin *et al*. 2001). Changes in lean men were assessed by DEA, including regional lean and fat estimates, and by multiple MRI slices of the thigh and abdomen (Woodhouse *et al*. 2004). The DEA data indicate increases in truncal and extremity lean mass with high doses, and increases in fat mass with subphysiological doses. MRI scans indicated increases in adipose tissue volumes in all regions assessed (intra- and subcutaneous abdominal, and intermuscular and subcutaneous thigh) at low doses, with less consistent reductions in adipose tissue volume at supraphysiological doses. These data provide confirmation of androgen-induced increases in lean components of body composition and decreases in fat mass.

Mechanisms of performance enhancement

Are observed strength gains only from increased muscle mass?

An increase in muscle size is associated with increases in strength, since maximal force production in a muscle is directly proportional to the cross-sectional muscle area. It has been suggested that androgens produce their main effects simply

through the increase in muscle size, without other changes in neuromuscular performance (Schroeder *et al*. 2003). There are other possible explanations for androgen-induced strength gains in athletes, including neuromuscular adaptations and muscle biochemical and physiological alterations, but these remain largely undefined. One of the first published strength studies in normal men involved four medical students given 50 mg·day^{-1} methyl testosterone along with creatine, and tested for changes in grip strength in a single-blind, crossover study with 3 weeks of steroid use (Samuels *et al*. 1942). No improvements in performance were observed. Among the difficulties in objectively assessing the direct effects of androgens on muscular strength are the variability inherent in strength tests, establishing appropriate controls for both individual experience and current training status, blinding the subjects to their treatment (because of obvious changes such as testicular volume), and the potentially important interactions with other factors that may also be influenced by androgens, such as motivation and training during the study.

Earlier studies with methandienone (10 mg·day^{-1}, or more, for 3–4 weeks) produced significant improvements of approximately 15% in one repetition maximum (1 RM) bench press performance, including some blinded tests and a mix of trained and untrained subjects, as previously reviewed (Haupt & Rovere 1984; Friedl 2000). Methandienone was quite consistent in increasing strength performance, although considerable discussion about the interactions with exercise has never been resolved (e.g. Freed *et al*. 1975; Hervey *et al*. 1981). With careful blinding, including a testosterone 'replacement' comparison dose group of TE (100 mg·day^{-1}), a high dose of TE (300 mg·day^{-1} for 6 weeks) produced significant increases in elbow flexion and knee extension tests (Friedl *et al*. 1991). Despite equivalent gains in body weight in ND treatment groups 100 and 300 mg·day^{-1}, there were no significant increases in strength. An increase in hip adduction was the only muscle strength measure that changed in untrained men receiving TE (200 mg·wk^{-1} for 24 weeks) (Young *et al*. 1993). Bhasin *et al*. (1996) measured an increase in 1 RM bench press with TE (600 mg·wk^{-1}) and demonstrated that steroid and exercise each produced significant gains that were additive in the steroid and exercise group (Fig. 35.4). In Bhasin's more recent dose–response study, TE (300 and 600 mg·wk^{-1}) significantly increased 1 RM leg press (average increases of ~ 75 kg) and leg power (~ 40–50 W increase), while no changes were observed at replacement or lower doses (Bhasin *et al*. 2001). This corresponded to increases in thigh volume and muscle fiber size in these two groups. These strength changes did not appear to be dose-dependent. No changes were observed in leg press repetitions to failure ('fatigability') (Storer *et al*. 2003).

Separate from the effects of muscle cross-sectional area, effects on psychomotor enhancement have been suggested. Methandienone was reported to significantly shorten patellar reflex time in weight lifters (Ariel & Saville 1972), but an intensive study of a small group of elite steroid-using Finnish strength athletes showed no changes in psychomotor or motor speed tests compared to a control group of athletes (Era *et al*. 1988). Both groups improved physical performance during 24 weeks of training, without an apparent androgen effect (Alen *et al*. 1984).

Aggression and motivation

Testosterone has become synonymous with aggressive power. One of the most difficult aspects of studying androgen performance effects is ensuring that the subjects are blinded to the treatment, in part because many subjects perceive a change in their mental outlook when they are receiving high-dose androgens (Freed *et al*. 1975; Wright 1980). The change in mental status has been variously described as an increase in feelings of energy, a compulsive focus on winning their event, and complete disinhibition of aggressiveness, even to the point of murderous rage. Pope has reported extensively on the psychological effects of androgen supplementation for over a decade (e.g. Pope & Katz 1988, 1994). Pope's research team found significant neuropsychological effects in a thoughtful study designed to mimic the increase in androgen dose employed by many bodybuilders by stepping the weekly dose up from 150 mg·wk^{-1} to 300 mg·wk^{-1} to 600 mg·wk^{-1} (Kouri *et al*. 1995a; Pope *et al*. 2000). In this, and other studies reviewed by Pope and our own researchers (e.g. Hannan *et al*. 1991), individual manic or

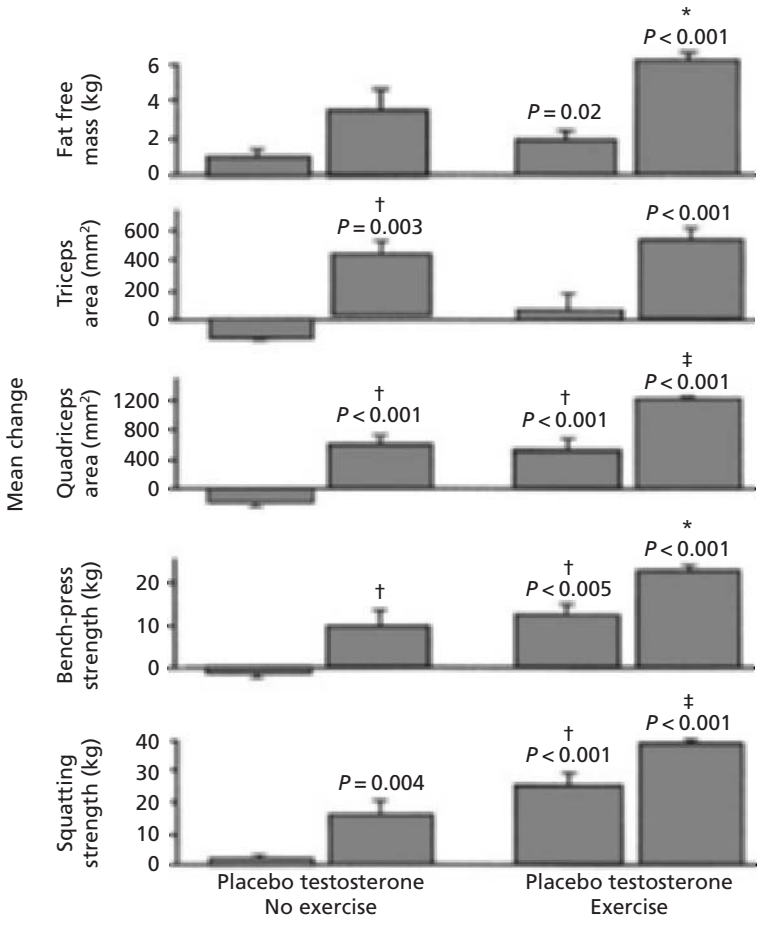

Fig. 35.4 Changes in body composition and strength measures in healthy young men administered placebo or 600 mg·wk^{-1} testosterone enanthate for 20 weeks with or without an exercise program. (From Bhasin *et al*. 1996.)

hypomanic reactions have been observed, supporting the anecdotal case reports on manic behavior in androgen users. Pope *et al*. (2000) found significant responses in a specially designed test, the Point Subtraction Aggression Paradigm (PSAP), as well as the Young Mania Rating Scale and daily diary scores. The PSAP challenge test was particularly novel and appropriate in this study, probing individuals for a low threshold of aggressive response. A better understanding of who is at risk for the idiosyncratic reactions is vitally important to future human studies. Conceivably, the action is based on crossover activity to corticosteroid receptors (Janne 1990); psychotic episodes with high-dose corticosteroids have been well documented (Lewis & Smith 1983).

It is clear that almost irrational attitudes of invincibility provide a performance advantage, allowing athletes to withstand psychological fatigue and discomfort to persist and win. However, neurophysiologists have not completed the androgen connection between the behaviors, the neural circuits, and the biochemistry. Hannan *et al*. (1991) found an increase in dopamine metabolites in peripheral circulation of men administered high-dose androgens, suggesting the possibility of an interaction with the dopaminergic system, which has been implicated in a range of behaviors from highly focused, goal-directed behavior to psychosis. Indeed, androgens have been since demonstrated to increase HVA/DA ratios in the striatum of rats given one of four different androgens; methan-

dienone specifically increased dopamine synthesis (Thiblin *et al*. 1999). Hannan *et al*. (1990) had also shown in animal studies that stereotypic cage-exercising behavior was increased by androgen administration. Exercise effects on brain dopamine activity have been suggested to play a role in delaying exhaustion (Chaouloff *et al*. 1987) and might be interactive with androgens.

What do androgens do for endurance performance?

Androgens may provide endurance advantages through other non-psychological effects, including both energy availability and the oxygen-carrying capacity of the blood. These could be beneficial to performance of endurance athletes such as distance runners and cyclists. Earlier studies examined the effects of androgen supplementation on running and swimming performance. In an article that received high visibility in *Science*, Johnson and O'Shea (1969) reported that methandienone (10 $mg \cdot day^{-1}$ for 3 weeks) increased maximal oxygen uptake by 15%, and significantly more than the gain in non-blinded control training partners. The same drug regimen failed to improve swim performance over that of a control group (O'Shea 1970), as did administration of oxandrolone (10 $mg \cdot day^{-1}$ for 6 weeks) (O'Shea & Winkler 1970). Johnson *et al*. (1972) attempted to replicate the initial study reported in *Science*, measuring aerobic capacity after the same drug regimen of methandienone (10 $mg \cdot day^{-1}$ for 3 weeks) or stanazolol (6 $mg \cdot day^{-1}$ for 3 weeks), and a running training program, but found no change in maximal oxygen uptake or mile run time. Johnson speculated that the discrepancy in results could be explained by androgen-induced leg strength improvements that may have produced specific improvements in the bike test used in the original study, rather than a real increase in aerobic capacity. No further tests of androgens on aerobic performance in normal men have been reported.

There are plausible mechanisms to suggest androgen benefits to endurance performance. Androgens boost red blood cell production through stimulation of erythropoeitin synthesis and secretion, direct stimulation of bone marrow hematopoiesis, and stimulation of iron incorporation into red blood cells. While androgen treatment has provided significant increases in hematocrit and hemoglobin in anemic patients (Neff *et al*. 1981), studies of high-dose androgen administration to normal men have not typically reported further increases (Mauss *et al*. 1975; Alen 1985; Kiraly 1988). However, Palacios *et al*. (1983) found a significant increase in red cell counts in normal men administered TE (200 $mg \cdot wk^{-1}$ for 16 weeks), with modest increases in hematocrit and hemoglobin. Although rare, polycythemia has been reported in hypogonadal patients treated with androgens, usually in older patients. There may also be increases in blood volume (Wilson 1988), although this has not been verified in athletes. Thus, it is unclear that androgens boost hematological parameters in normal men, but if they did, there could be clear benefits to endurance performance. Erythrocyte infusions into normal men can substantially increase maximal oxygen uptake (Muza *et al*. 1987; Sawka *et al*. 1987). The downside is an increased risk of thromoembolytic injury such as stroke, especially with a potentiating effect of dehydration during intensive performance (Sawka *et al*. 1996). This is comparable to the risks that may be encountered by cyclists and other athletes using exogenous erythropoeitin for performance enhancement (Adamson & Vapnek 1991).

Effects on the heart muscle and increases in stroke volume would theoretically also benefit endurance athletes. While the effects of androgens on left ventricular hypertrophy have been observed in strength athletes (e.g. McKillop *et al*. 1986), this has not been studied in endurance athletes, where steroid use appears to be less prevalent or far more covert.

It remains to be determined if androgens enhance lipolysis and energy availability for endurance performance. In one of the few studies to examine metabolic advantages, Guezennec *et al*. (1984a) tested endurance and metabolism of rats with elevated androgen levels during treadmill running for 7 h, but found no significant effects of testosterone other than a preservation of some of the glycogen stores. Other metabolic effects are potentially mediated through hormones such as IGF-I (Hobbs *et al*. 1993). Testosterone increases circulating IGF-I

levels, while nandrolone, which does not produce the same metabolites including estrogens, had no effect on total IGF-I levels. This suggests that there are differences in some of the biological actions of various synthetic anabolic steroids, determined by the receptor-binding affinities of the compounds and their metabolites. Androgens act to modify glucose disposal in normal men (300 mg·wk^{-1} ND or TE) through non-insulin mechanisms (Hobbs *et al*. 1996).

Other hormonal responses to exercise, such as the acute rise in circulating growth hormone that occurs so dramatically in response to a serious strength athlete routine (more repetitions and short rests) compared to a body builder routine (higher weight and longer rests) (Kraemer *et al*. 1991), may also account for some of the effects currently ascribed to anabolic steroids. Growth hormone responds to acute metabolic signals, and like testosterone, specifically stimulates muscle protein and bone mineral accretion, and stimulates the release of IGF-I (Hindmarsh *et al*. 1997; Lee *et al*. 1997). It will be important to understand these interactions in the hormonal responses to exercise in future training studies in order to develop a systematic approach to science-based nutrition and training guidance.

Eunuchs may live longer, but do androgen-using athletes die sooner?

The health risks associated with androgen supplementation to normal men have been extensively reviewed and discussed elsewhere (e.g. Friedl 1990, 2000). In summary, the primary risks are a reversible reduction in sperm count and an accompanying testicular atrophy; hepatic adenoma that may be benign but may also result in hemorrhage and death; an adverse serum lipid profile with marked suppression of high-density lipoprotein (HDL) -cholesterol and unknown heart disease risk outcomes; changes in impulsivity and aggressiveness; skin changes including hair loss, oily skin, and acne; gynecomastia; and weight gain. Testosterone also plays a permissive role in prostate cancer, but a causative role remains to be demonstrated. From case reports of athletes using androgenic steroids, the majority of serious adverse health consequences have been reported for bodybuilders. For example, in one review, 20 of the 28 case reports and most of the deaths involved bodybuilders (Friedl 2000).

Health benefits appear to accrue in replacement therapy in aging men, similar to hormone replacement therapy in women. These benefits include maintaining muscle mass and strength, which may be important in preventing injuries; maintenance of bone mass and prevention of male osteoporosis; antidepressant effects; and important effects on libido and motivation. Studies in the past decade indicate that androgens can increase glucose disposal separately from insulin-mediated mechanisms (Hobbs *et al*. 1996), as well as reduce intra-abdominal fat (Marin *et al*. 1995), both of which may be important in preventing type 2 diabetes. Reduction in fibrinolytic activity (Fearnley & Chakrabarti 1962) may reduce certain types of cardiovascular risks, perhaps countering effects such as the reduction in HDL-cholesterol, but conceivably increase hemorrhagic stroke risk. Although there is no defined disease of androgen excess in men, perhaps the effects of androgens are so ubiquitous that a wide variety of adverse health outcomes ranging from prostate cancer to heart disease are all diseases of androgen excess, just as being male is associated with shorter average lifespan. As a corollary of this, athletes that use androgenic steroids may be further trading lifespan for poorly defined performance advantages. Although eunuchs may have a longer average lifespan than normally virilized men (Hamilton 1948), it is unknown if the reverse is true. Epidemiological studies of retired athletes have been proposed but are fraught with complications, particularly in accurately ascertaining androgen administration histories and controlling for differences such as risk-taking behaviors in steroid users compared to non-steroid users.

Concern about the current increase in testosterone supplementation of older men at least has a tradeoff in disease prevention by reducing osteoporotic fractures and sarcopenia (that increases musculoskeletal injury risks). Replacement doses have been administered to many older men (> 45 years), and prescriptions for testosterone-replacement therapy have skyrocketed in the US in the past 5 years, providing a new cohort of men for studies

that are likely to provide new insights into the health consequences of androgen supplementation. There is also a growing database of experience with androgen administration to normal men from male contraceptive studies around the world. Other data on high-dose supplementation is emerging from efforts to sustain lean mass in muscular dystrophy and acquired immunodeficiency syndrome (AIDS) patients.

Conclusions

Androgenic steroids are banned at various levels of athletic competition, initially because of concerns about an uneven playing field, but then also out of concern for the health risks to the athletes. The greatest advantage is in bodybuilding in which huge muscle development is the goal, and the majority of case reports of serious health outcomes have centered on these athletes who are more likely to use very high doses that exceed levels that can be ethically studied. Other athletes appear to derive benefits from androgens for strength, endurance and overall psychological advantage. Prediction of the specific and individual effects of androgens is still uncertain. All of these compounds appear to have some positive musculotrophic effect, but the nature of this effect is still not well understood and almost certainly involves complex interaction with other hormones at the tissue level. Management of diet and psychological stress are well-known factors in peak athletic performance, and it appears that endogenous testosterone levels can be markedly affected by these factors, making it a useful indicator of training status for males. The current thinking is that androgens may be useful in maintaining health and well-being in aging men as replacement therapy to prevent sarcopenia and osteoporosis. Supraphysiological doses that double or triple circulating levels have been tested in healthy young men for male contraception without remarkable effects other than modest weight gain. Higher doses, particularly when combined with exercise, may produce substantial gains in muscle mass and strength. Anecdotal reports of the use of extraordinarily high levels of androgenic steroids that extend well beyond the observations in controlled studies, involve primarily body builders and are associated with the majority of case reports of serious adverse health effects.

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Chapter 36

Growth Hormone and Sport

JENNIFER D. WALLACE AND ROSS C. CUNEO

Introduction

Evidence of growth hormone doping in sport

Synthetic human growth hormone (hGH), produced by recombinant DNA technology (recombinant human growth hormone, rhGH), has been available for clinical use since the late 1980s. It is widely available on the black market, with the *Underground Steroid Handbook* promoting the perceived benefits to athletes. In recent years in Australia and the UK, rhGH has been stolen from hospital pharmacies and a pharmaceutical company warehouse, much of it believed to be destined for use by sportspersons. The discovery in 1998 of the possession of vials of rhGH by Chinese swimmers attending the World Swimming Championships in Perth, Australia, and cycling teams in the Tour de France in Europe, has confirmed suspicions that hGH is in use at the highest levels of sporting competition.

Rationale for growth hormone abuse by athletes: a brief introduction

The abuse of rhGH to enhance performance proceeds despite a lack of controlled studies to objectively confirm or refute potential benefit to athletes. What then might be the reasons for athletes choosing to abuse growth hormone (GH)? In an attempt to gain insight into the reasons athletes and coaches might promote this activity the biological effects of GH will be covered in detail below, but a brief overview is now provided. The use of rhGH to promote linear growth in short and GH-deficient (GHD) children is well documented. Abuse of rhGH to augment height in normal children may theoretically enhance performance in sports such as basketball or swimming where height is advantageous. The physiological role and actions of GH in adult life has been established from studies of patients with GHD and in catabolic illnesses. GH is anabolic and can increase muscle mass, suggesting its use to build muscle or as a reparative agent to sustain heavy training. The ability of GH treatment to reduce body fat has made it attractive to athletes who wish to reduce body fat and improve their power to weight ratio. This lipolytic effect of GH is also potentially attractive to the endurance athlete, as an agent to release fatty acids into the bloodstream, as a source of energy, and hence as a means of preserving glucose, in long duration events such as the marathon. GH has also been shown to have effects on the heart, thermoregulation and blood volume which could be interpreted as potentially performance enhancing. The ability of GH to stimulate endorphins, a natural analgesic, and to have positive effects on mood, may also allow athletes to train more effectively. GH treatment in GHD adults has been shown to increase aerobic exercise capacity ($\dot{V}o_{2max}$) by up to 20%, even without training. However it must be emphasized that these improvements are the results of normalizing low GH levels in deficient adults, and extrapolation of these results to GH treatment in normal individuals is not established. As an example, GH treatment appears to increase muscle strength in GHD adults, but acromegalic patients who have chronically elevated

GH levels have increased muscle size but with reduced strength compared to normal adults. GH administration to weightlifters does not appear to enhance the ability to perform resistance work.

Potential side effects of growth hormone doping

Physiological levels of GH, as used in adults treated for GHD, merely replace endogenous production in a doping athlete. It is therefore necessary for athletes to take supra-physiological doses of GH to achieve an effect. The main adverse effects of short-term GH exposure are fluid retention and arthralgias. However, if athletes wish to reproduce the effects of GH exposure they might be tempted to expose themselves to high levels of GH for an extended period of time, with the attendant risks of serious, often irreversible, adverse effects. Nature has provided us with a model of GH doping in a condition of pathological GH excess. When this condition presents in childhood, before bone fusion has occurred, it is known as gigantism, as it accelerates bone growth, producing abnormally tall individuals. If the condition occurs in adults it is called acromegaly. GH excess in adults does not influence height, but has irreversible effects on bone growth, resulting in big hands and feet, a prominent forehead and jaw with splayed teeth, accelerated degenerative arthritis and enlargement and deformity of the vertebrae. Soft tissues are also affected, eventually resulting in the obvious features of a large nose and lips. Internal organs enlarge, leading to multisystem pathology, and a range of disorders such as high blood pressure, sleep apnoea, diabetes mellitus and a range of cancers (especially colon cancer) are common results of chronic pathological elevation of GH.

Development of a test for growth hormone abuse in sport

While details of current progress will be presented later in this chapter, some introductory comments are required. Most of the agents on the list of banned drugs in sport are foreign or exogenous substances. In most cases, simple detection of the presence of such a substance, in blood or urine, constitutes a positive test. Unique problems exist for endogenous *proteins* like GH and erythropoietin.

GH as a marker of GH abuse. There are two main reasons why it is unlikely that the detection of GH abuse could be achieved by solely measuring GH in serum or urine. Firstly, GH is produced in a pulsatile manner and is stimulated by a number of factors including exercise; an elevated level of serum GH could possibly indicate doping, but it could equally be the result of a naturally occurring pulse or a response to stress or exercise. Secondly, GH used in doping is produced by recombinant DNA technology, and is identical to the major circulating variant of GH produced by the human pituitary gland. It is therefore impossible to distinguish exogenous from endogenous GH. Manufacturers of rhGH are not keen to chemically label rhGH, as the process would involve considerable expense, and necessitate clinical trials to demonstrate that labeling does not change the biological activity of the product. Furthermore, labeling of GH would also not result in detection of GH from unlicensed sources. From these considerations, strategies for a test for GH abuse have evolved using naturally occurring markers of GH action in the human body. A detailed examination of the biological actions of GH, as they potentially apply to sporting performance and to the selection of markers of GH abuse, is therefore now presented.

Biological actions of growth hormone: rationale for the use of growth hormone by athletes

The biological actions of GH have been clearly demonstrated in response to administration of rhGH to children with short stature, adults with GHD, adults and children suffering from a number of catabolic states, and in disease states where circulating GH levels are chronically high due to secretion from a pituitary tumor (gigantism or acromegaly). From these clinical data, it is evident that GH has growth promoting effects on long bones, is anabolic, lipolytic, has multiple effects on carbohydrate metabolism and is important for fluid homeostasis. In

addition it appears to have beneficial effects on physical strength and endurance, on thermoregulation, cardiovascular health, psychological well-being and on the immune system. We aim to present a detailed mechanistic examination of the effects of GH as demonstrated by clinical conditions, and highlight the potential risks and benefits that sportspersons may, theoretically, experience should hGH be abused.

Bone growth

Reduced linear growth of long bones, short stature and reduced bone density are evident in children with GHD (Shore *et al*. 1980). In contrast, GH hypersecretion in children (gigantism) results in abnormally tall individuals as a result of linear overgrowth of long bones with unfused epiphyses. The capacity of GH administration to stimulate long bone growth, in children with GHD, and in children with short stature due to other causes, is well documented (Root *et al*. 1998), and is a recognized treatment to augment adult height in children with GHD world wide. GH abnormalities of adult onset would not be expected to affect height, as prior fusion of bone epiphyses would preclude changes in long bone length. Chronic GH hypersecretion in adults (acromegaly), results in several irreversible effects on bone, such as overgrowth of the jaw bone, and an increase in the size of bones in the hands and feet. GH treatment in GHD adults has been shown to increase bone turnover acutely. This results in a biphasic effect on bone mineral content and bone mineral density, with an initial fall followed by a return to baseline levels and increases above baseline levels (Vandeweghe *et al*. 1993; Ohlsson *et al*. 1998).

The theoretical benefits of GH use, in terms of bone metabolism in sportspersons, is twofold. GH may result in increased height, if treatment were started prior to epiphyseal fusion, to benefit height related sporting performance. In adult athletes, GH may theoretically increase bone strength, thereby minimizing stress fractures or ligamentous/tendous bone-insertion fractures. The basis for such expectations has not been subjected to scientific study.

Body composition

The anabolic and lipolytic effects of GH are clearly evident in the deranged body composition of children and adults with GHD. In addition to a reduction in bone mass, childhood GHD is characterized by subnormal lean body mass and obesity, and treatment with GH results in increases in bone and muscle mass and a decrease in adipose tissue (Collip *et al*. 1973; De Boer *et al*. 1992). Discontinuation of GH treatment when children reach adulthood has been shown to result in a reduction in lean body mass and increase in fat mass within 12 months (Rutherford *et al*. 1989; Juul *et al*. 1998).

The GHD syndrome in adults has only been recognized within the last decade. The first randomized controlled studies of GH replacement to adults were published in 1989 (Rahkila *et al*. 1989; Salomon *et al*. 1989). Jørgensen and colleagues (Rahkila *et al*. 1989) studied 22 GHD adults in a randomized, double-blind placebo-controlled cross-over study for 4 months with a daily GH dose of 2 $IU \cdot m^2$ ($\cong$ 600 $\mu g \cdot m^2$ or 15 $\mu g \cdot kg^{-1} \cdot day^{-1}$). Treatment resulted in no change in body weight, with an increase in the mean muscle volume of the thigh and a corresponding decrease in mean thigh adipose tissue as assessed by computerized tomography (CT). Subscapular skinfold thickness was also significantly reduced with GH treatment. Salomon *et al*. (1989) performed a randomized, double-blind placebo-controlled trial in 24 adults for 6 months using a GH dose of dose 0.07 $IU \cdot kg^{-1}$ of body weight per day ($\cong$ 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and also showed no effect on body weight and a mean increase in lean body mass of 5.5 kg and a corresponding decrease in fat mass of 5.7 kg, as assessed by measurement of total body potassium (TBK) counting. The criticisms of these studies have been the small number of subjects studied and the high GH doses used. A review by Carroll *et al*. (1998) summarizes numerous studies which support the ability of GH to increase lean body mass and decrease body fat, especially abdominal fat, using a variety of GH doses and a large range of techniques to analyse body composition. A large multicentered, randomized, double-blind, placebo-controlled study of GH administration to 166 GHD adult patients, using a much smaller dose than the early studies,

and employing a range of body composition techniques, has recently confirmed the ability of GH treatment to increase lean and decrease fat tissue in this patient population (Cuneo *et al*. 1998).

Excessive GH secretion results in increased lean body mass and reduced body fat. A study of 150 acromegalic subjects in Sweden revealed that they had increased lean body mass and reduced fat mass when compared with 476 normal subjects (Bengtsson *et al*. 1989). Body composition was assessed by the four-compartment model using assessment of TBK and body water. TBK was initially measured by a potassium radioisotope (^{42}KCL) dilution technique and later by whole body potassium counting, and total body water was measured using the tritiated water dilution technique. Body cell mass as estimated by TBK showed a mean increase above normal of 2.7 and 2.2 kg in acromegalic male and female subjects respectively, and fat mass was shown to be lower than predicted with men and women having a mean fat mass 7.8 and 5.6 kg below normal. The same group demonstrated that successful treatment of acromegaly does not normalize body composition, but results in persistent elevation of body cell mass, a reduction in total body water and an increase in body fat (Bengtsson *et al*. 1989b). This may encourage athletes into believing that doping with supra-physiological levels of GH may result in increases in lean body mass which can be maintained after doping has ceased. It has been suggested however that 'cured' acromegalic subjects with low serum GH may have abnormal 24-h secretory profiles which possibly contribute to maintenance of lean body mass. A more recent study of body composition in acromegalics (O'Sullivan *et al*. 1994) using dual X-ray absorptiometry and sodium dilution, has confirmed that acromegalics are less fat than normal controls and increase their fat mass following successful treatment. They demonstrated that fat free soft tissue mass (FFSTM) was elevated by acromegaly, but that this increase was due to increased body water and not increased body cell mass, perhaps suggesting a lack of anabolic effect of GH on acromegalic muscle. In contrast to the Bengtsson study, successful treatment of acromegaly resulted in a reduction in FFSTM in this study, which furthermore was shown to represent a lack of effect on body cell mass with reduction in FFSTM being explained by the reduction in body water.

Limited data is available on the effect of GH administration on body composition in normal subjects and athletes. Crist *et al*. (1988) performed a double-blind placebo-controlled study of GH administration to eight subjects who acted as their own controls. This study demonstrated body composition changes in highly conditioned adults given exogenous GH (30–50 $\mu g \cdot kg^{-1}$ three times per week), during 6 weeks of progressive resistance training. Using hydrodensitometry an increase in fat free mass (FFM) of 4% and a decrease in body fat of 4% was demonstrated. Hydrodensitometry was also used by Yarasheski *et al*. (1992) in a placebo-controlled study of GH treatment in young men undertaking heavy resistance exercise. Seven subjects were treated with GH ($40\ \mu g \cdot kg^{-1} \cdot day^{-1}$) during the 12-week training program. GH treatment resulted in a greater increase in FFM than placebo treatment in subjects undertaking identical exercise. Similar results were found when GH was administered to older men during resistance training. GH administration with a training program resulted in greater increases in FFM than training alone: 23 healthy male sedentary subjects, with a mean age of 67.1 years, took part in a 16-week program of progressive resistance training after being randomized to receive GH or placebo. Eight subjects received GH at a dose of $12.5–24\ \mu g \cdot kg^{-1} \cdot day^{-1}$, which resulted in an 8% increase (4.8 kg) in mean FFM as assessed by hydrodensitometry (Yarasheski *et al*. 1995). An additional five subjects were unable to complete the study due to side effects related to fluid retention. Lack of change in vastus lateralis muscle protein synthesis and strength, along with changes in body water that contributed to the increased FFM with GH treatment suggest that the addition of GH to resistance training did not contribute to an increase in muscle tissue. Deyssig *et al*. (1993) was unable to show any effect of GH on body composition in highly trained athletes with low-fat mass in a placebo-controlled study of GH for 6 weeks at a dose of $0.09\ U \cdot kg^{-1} \cdot day^{-1}$ ($\cong 30\ \mu g \cdot kg^{-1} \cdot day^{-1}$). Measurements of fat and lean mass were however estimated only by skinfold

measurement which may have been a relatively insensitive technique.

Athletes may interpret the changes in body composition demonstrated by these studies to indicate that doping with GH could potentially result in similar body composition changes resulting in increased lean body mass without training and decreased fat mass. This would be an attractive proposition to those athletes striving to improve their power to weight ratio, or to body builders who aim to develop maximum muscle mass with minimal subcutaneous body fat.

Strength and endurance exercise

Details of skeletal muscle function have been assessed most carefully in the quadriceps group. Before treatment, adults with GHD had significantly reduced isometric quadriceps force compared to carefully matched, normal individuals (Cuneo *et al*. 1990). Most of this deficit could be explained by reduced muscle cross-sectional area (Cuneo *et al*. 1990; Janssen *et al*. 1999). When force was expressed per muscle cross-sectional area, an additional small deficit was still evident, particularly in males; possible explanations include differences in training, muscle fiber or myofibrillar density, or neuromuscular excitation. Normal muscle histology and CT density of quadriceps muscle (Cuneo *et al*. 1992) suggest that muscle fiber density was not at fault. Rutherford *et al*. (1991) performed an uncontrolled study of GHD children during 1 year after the cessation of rhGH treatment when final height was attained. They found significant reductions in quadriceps cross-sectional area and force (with a trend toward reduced muscle-fiber areas on biopsy) over the year. Isometric force in muscle groups other than the quadriceps have shown essentially no difference from a normal population (Cuneo *et al*. 1990). Respiratory muscle function may also be reduced in adults with GHD (Merola *et al*. 1996).

Short-term controlled studies of rhGH treatment (4–6 months) (Rahkila *et al*. 1989; Cuneo *et al*. 1991a; Woodhouse *et al*. 1999), have not been able to demonstrate a statistically significant increase in quadriceps force despite clear increases in thigh muscle cross-sectional area. Longer-term, uncontrolled studies have shown a progressive increase in quadriceps isometric force toward normal over 12–36 months (Jørgensen, J.O.L. *et al*. 1990b; Rahkila *et al*. 1994; Johannsson *et al*. 1997; Janssen *et al*. 1999). Proximal, limb-girdle muscle force has been shown to increase significantly in adults with GHD after rhGH treatment (Cuneo *et al*. 1991a). A study of the effects of 5 years of GH therapy on 109 GHD adult subjects in a single center, prospective, open label study has demonstrated normalization of isometric and isokinetic knee flexor and extensor strength and an improvement but lack of normalization of hand grip strength (Svensson *et al*. 2003).

Histology of vastus lateralis taken by needle biopsy from adults with GHD has shown normal fiber type proportions and areas (Whitehead *et al*. 1989; Cuneo *et al*. 1992). No features of Cushingoid myopathy were evident. Differences in fiber type function have not been assessed in detail, but significant correlations between type I relative fiber area and maximal aerobic performance and between type II relative fiber area and maximal force generation (Whitehead *et al*. 1989) suggest that individual muscle fiber-type function is qualitatively normal. Following treatment, vastus lateralis histology either remained unchanged (Whitehead *et al*. 1989; Cuneo *et al*. 1992), or showed an increase in type I and type II mean fiber areas (Woodhouse *et al*. 1999). The reason for the difference is unclear, but methodological limitations of this technique are well known. No features of an acromegalic myopathy were seen following GH replacement.

In conclusion, GHD appears to cause a reduction in skeletal muscle mass, not function, which results in reduced muscle force generation which can be overcome in some but not all muscle groups with GH treatment over a period of several years.

In untreated adults with GHD, maximal oxygen uptake ($\dot{V}O_{2max}$) measured during cycle ergometry has been shown to be significantly reduced (mean of 72–82% of that predicted from age, sex and height) (Cuneo *et al*. 1991b; Whitehead *et al*. 1992). Power output is also likely to be reduced (Rahkila *et al*. 1989; Cuneo *et al*. 1991b; Whitehead *et al*. 1992). Mean maximal heart rates of 90% and respiratory exchange ratio (*RER*) greater than 1.0 suggest that maximal or near maximal subjective effort had been

obtained (Cuneo *et al*. 1991b). GHD may have a negative chronotropic effect, demonstrated in some studies (Rahkila *et al*. 1989) but not others (Cuneo *et al*. 1991b; Nass *et al*. 1995). Following rhGH treatment, maximal exercise performance (maximal oxygen uptake and maximal power output) improved markedly (mean changes 20–30%) (Rahkila *et al*. 1989; Cuneo *et al*. 1991b; Whitehead *et al*. 1992; Nass *et al*. 1995). Submaximal exercise capacity, measured as anaerobic threshold, also improved suggesting that activities performed during sedentary and strenuous daily life would be accomplished with less metabolic stress (Cuneo *et al*. 1991b; Nass *et al*. 1995). Some studies have controlled physical activity and still demonstrated beneficial effects (Salomon *et al*. 1989; Cuneo *et al*. 1991b). One other study did not demonstrate a statistically significant increase in maximal exercise capacity, but anaerobic threshold did increase, and this appeared to be a major contributor to the subjective sensation of fatigue experienced by these patients (Woodhouse *et al*. 1999). Explanations for improved aerobic performance may relate to: (a) increased oxygen pulse (Cuneo *et al*. 1991b, Nass *et al*. 1995) and increased cardiac output (Cuocolo *et al*. 1996); (b) increased lean body mass and skeletal muscle mass (Cuneo *et al*. 1991b); (c) altered fuel utilization (see below); and (d) altered thermal homeostasis (see below). Additionally, adults with childhood-onset GHD have reduced lung volumes and respiratory muscle force generation (Merola *et al*. 1996), whereas adult-onset patients only have a problem with the latter (Cuneo *et al*. 1991b). In acromegaly, maximal aerobic capacity appears to be impaired in the untreated state, and to improve only in patients who have an improvement in cardiac output with normalization of serum GH and insulin-like growth factor I (IGF-I) levels in response to treatment (Colao *et al*. 2000). Fatigue and impaired ventilatory threshold in acromegaly can be normalized with successful reduction of IGF-I (Thomas *et al*. 2002).

Therefore, substantial abnormalities of physical performance, likely to impinge on daily activities, result from GHD, and quite dramatic improvements in clinically meaningful aerobic performance result from rhGH replacement. In contrast to the situation in GHD subjects, patients with acromegaly have a proximal myopathy. The reason for this lack of function despite apparent muscular hypertrophy is obscure but may relate to histological features characterized by cellular infiltrates and type II fiber atrophy, type I fiber hypertrophy and connective tissue depositions (Nabarro 1987), compression or other neuropathic processes, or other undefined factors.

Athletes may consider rhGH abuse to augment strength and/or endurance performance but controlled data are lacking or discouraging.

Metabolic effects of growth hormone: protein anabolism

In order to explore the mechanism responsible for the increases in lean body mass in GHD adults treated with GH, studies of protein turnover have been conducted in this patient population (Russell-Jones *et al*. 1993). Whole body isotopic leucine turnover studies were performed using L-1-^{13}C-leucine to measure protein degradation (leucine Ra), and protein synthesis (non-oxidative leucine Rd). Whole body protein turnover was measured in 18 severely GHD adults, in a double-blind, placebo-controlled trial of GH/placebo at a dose of 0.018 $U \cdot kg^{-1} \cdot day^{-1}$ for 1 month, followed by 0.036 $U \cdot kg^{-1} \cdot day^{-1}$ ($\cong 12\ \mu g \cdot kg^{-1} \cdot day^{-1}$) for 1 month. Results indicated that the increase in lean body mass resulting from GH treatment in GHD adults is due to an increase in protein synthesis with no change in protein degradation when results are expressed in terms of lean body mass. The potent anabolic effect of GH on whole body protein synthesis has also been clearly demonstrated in normal human subjects in response to GH administration (Horber & Haymond 1990). Leucine turnover studies in the normal population were mechanistically consistent with those obtained from GHD subjects, with GH administration resulting in an increase in protein synthesis and no effect on proteolysis.

A number of therapeutic uses of GH as an anabolic agent have been explored. Hypocaloric feeding induces catabolism in normal subjects reflected by a loss of total body nitrogen. The capacity of GH to prevent nitrogen loss in normal subjects with hypocaloric feeding was demonstrated (Manson & Wilmore 1986). Similarly GH administration has

been shown to conserve nitrogen in obese subjects during short-term dietary restriction (Snyder *et al*. 1988), where 20 obese subjects were studied in a placebo-controlled trial of GH administration during a 12-week restricted-energy intake of 24 kcal·kg (100 kJ·kg) ideal body weight (IBW)$^{-1}$. Recombinant methionyl GH was injected intramuscularly every other day at a dose of 100 μg·kg IBW^{-1}. The nitrogen-sparing effects of GH were only evident for the first 5 weeks of the study. The authors attribute the lack of enduring GH effect on nitrogen retention to development of insulin-like growth factor I (IGF-I) resistance.

The protein anabolic property of GH has been utilized in a number of catabolic states. Surgically produced acute stress can result in exaggerated protein catabolism, and GH treatment following surgery has been shown to preserve body cell mass and promote wound healing, and to accelerate wound healing in severely burned children (Wilmore & Catabolic 1991). Experimental wounds heal with greater strength following GH treatment than with placebo (Jørgensen, P.H. & Andreassen 1988; Belcher & Ellis 1990). It has recently been shown that intratendonous injection of IGF-I results in improved healing of experimentally induced flexor tendonitis in horses (Dahlgren *et al*. 2002). We have explored the ability of GH to overcome protein catabolism in chronic liver disease (Wallace *et al*. 1996). GH treatment in this patient population was shown to result in a significant increase in lean tissue mass as assessed by TBK and bioimpedance analysis. The increase in TBK, the main intracellular ion, was similar in magnitude to that observed in treatment of GHD adults (Cuneo *et al*. 1991a).

Due to the ethical constraints of administering GH to athletes the effect of GH administration to athletes on protein turnover has not been extensively investigated. Young, untrained men were treated with GH in a 12-week placebo-controlled study of resistance exercise (Yarasheski *et al*. 1992). Turnover studies with ^{15}N glycine and ^{13}C leucine showed that training supplemented with GH resulted in increased rates of whole body protein synthesis and greater whole body protein balance. The *in vivo* fractional incorporation rate of leucine into protein was assessed with biopsy of the quadriceps muscle. Muscle protein synthesis rate was shown to be unchanged by GH treatment, and a corresponding lack of change in muscle strength was evident. The authors have concluded that increases in total body protein occur when training is accompanied by GH treatment, but that extracellular protein and not muscle mass accounts for this change. In contrast, experienced weightlifters were given GH for 14 days at a dose of 40 μg·kg^{-1}·day^{-1}, and ^{13}C leucine turnover studies were performed before and after the treatment phase (Yarasheski *et al*. 1993). The findings were that such short-term GH treatment did not increase muscle protein synthesis in experienced weightlifters. Whole body protein breakdown was also not affected by GH treatment. This was, however, a very small study in seven subjects and did not include a control group.

Exercise can therefore be considered as a model for catabolic stress in humans. The ability of GH to overcome catabolism in many disease states, and to enhance wound healing, may lead some athletes to believe that the anabolic properties of GH may enable them to train harder with less protein breakdown and to enhance recovery from heavy training. The available experimental data do not however support such a concept. Empirical studies of wound healing in athletes have not as yet been undertaken.

Nitrogen retention has been clearly documented following GH treatment in GHD (Henneman *et al*. 1960), in the long-term resulting in the previously mentioned changes in body composition. Protein turnover studies show a direct effect of GH and IGF-I on protein synthesis (Russell-Jones *et al*. 1993; Umpleby & Russell Jones 1996; Salomon *et al*. 1997), while insulin's anabolic action is mediated via a reduction in protein breakdown (Umpleby & Sönksen 1985; Umpleby *et al*. 1986). Thus GH, IGF-I and insulin appear to act as an 'anabolic team'. Fasting hyperinsulinaemia, generated by GH's insulin antagonistic and insulinotropic (Davidson 1987; Press 1988; Fowelin *et al*. 1993; Jørgensen, J.O.L. *et al*. 1993; O'Neal *et al*. 1994) actions, may contribute to the anabolic effect of GH.

Metabolic effects of growth hormone: lipolysis and lipid oxidation

Clinical evidence of the lipolytic action of GH is provided by the ability of GH treatment to decrease the

excessive levels of adipose tissue in GHD adults, and for the reverse to result from successful treatment of acromegaly. Direct evidence of the role of GH in lipolysis has been demonstrated in many *in vitro* and *in vivo* studies. Harant *et al*. (1994) exposed fat cells from five GHD adults to GH and demonstrated a lipolytic effect as evidenced by glycerol appearance in medium. In an uncontrolled study, they showed that 6 months of GH treatment (0.125 $IU \cdot kg^{-1} \cdot wk^{-1}$ for 1 month and 0.25 $IU \cdot kg^{-1} \cdot wk^{-1}$ [$\cong 12\ \mu g \cdot kg^{-1} \cdot day^{-1}$] for the subsequent 5 months) further increased the lipolytic activity in fat cells from these patients.

A number of studies have demonstrated the ability of GH administration to increase circulating free fatty acid (FFA) levels in normal human subjects (Davidson 1987; Press 1988). Cheng and Kalant (1970) studied 10 adult human subjects and demonstrated that 5 mg of intravenous GH was able to double serum FFA levels approximately 5.5 h later. Subcutaneous adipose tissue, from 21 normal subjects, nine of which were undergoing abdominal surgery for non-malignant conditions, showed an increase in lipolysis, measured as glycerol release, in response to GH (Ottosson *et al*. 2000). Recently, the effects of GH replacement in adults with GHD on exercise-induced changes in lipid metabolism have been examined. Gibney *et al*. (2003) performed a randomized GH withdrawal study in replaced GHD adults. Studies of both rest and exercise, after 3 months of GH cessation, showed that GH increased the rate of appearance (Ra) of both glycerol and FFAs and the disappearance rate (Rd) of FFA. Glucose Ra is index of lipolysis from adipose tissues since glycerol is not reincorporated into triglycerides and FFA Ra is the parallel change to glycerol Ra suggesting little FFA re-esterification. The increased FFA Rd may result from either increased FFA availability or lipid oxidation. This small study suggests GH is an important regulator of intermediary metabolism during exercise. GH excess is also associated with an increase in lipid oxidation (O'Sullivan *et al*. 1995).

Adipose tissue has been shown to express GH but not IGF receptors and the lipolytic effect of GH is therefore thought to be a direct action of GH on adipose GH receptors (GHRs). In order to elucidate the mechanism for lipolysis with GH administration Hussain *et al*. (1994) administered either GH, IGF-I or a combination of GH and IGF-I to eight GHD adult subjects. Circulating FFA levels were similarly elevated with GH or IGF-I and an increase in lipid oxidation occurred with both treatments. Combination therapy resulted in a synergistic effect. The direct effect of GH on adipose tissue is thought to be achieved by its ability to enhance the reactivity of hormone-sensitive lipase to lipolytic hormones (Hussain *et al*. 1994). Hussain proposes that the lipolytic effect of IGF-I is indirect, and is a result of the ability of IGF-I to inhibit insulin secretion 'therefore releasing the brakes on lipolysis' (Hussain *et al*. 1994).

The demonstrated ability of GH to increase lipolysis thereby increasing circulating FFA levels, and also to subsequently increase lipid oxidation may make it attractive to athletes performing extreme endurance activities, such as marathon running, as it could potentially provide a source of circulating fuel and at the same time protect carbohydrate stores.

Metabolic effects of growth hormone: carbohydrate metabolism

GH has traditionally been seen as insulin-antagonistic, counteracting the actions of insulin to restrain hepatic glucose production and promote peripheral glucose disposal (Davidson 1987; Press 1988). Indeed patients with acromegaly have a high prevalence of carbohydrate intolerance and diabetes mellitus. The situation with GHD and more subtle degrees of GH replacement is, however, far more complex and the implications for athletic performance difficult to assess.

Insulin sensitivity is a feature of GHD in infancy and childhood where fasting hypoglycemia causes clinical concern. In adults with GHD, however, a spectrum of insulin *insensitivity* has been described, ranging from normal to insulin resistant, where the differences have been attributed to the degree of global and especially visceral adiposity (Salomon *et al*. 1994; Hew *et al*. 1996). Alford *et al*. (1999) have described severe insulin resistance in adults with untreated GHD, in association with visceral adiposity and low intramuscular glycogen concentrations. Statistically, the severity of the defect correlated with duration of GHD, degree of hypertriglyceridemia and hyperinsulinemia. While the increases in

serum insulin and glucose tend to return toward baseline after several months (Salomon *et al*. 1989, 1997; Bengtsson *et al*. 1993; Cuneo *et al*. 1998), more formal measurements of insulin action suggest either normalization of (Fowelin *et al*. 1993) or persistence of the resistant state (Alford *et al*. 1999; Bramnert *et al*. 2003) following 6–24 months of GH replacement. The data favoring an amelioration of insulin resistance has been attributed to reductions in visceral adiposity and increases in lean mass (Fowelin *et al*. 1993; Christopher *et al*. 1998), and discrepancies may relate to GH dose, duration, other pituitary hormone replacement therapies (notably glucocorticoids) and the persistence of hyperinsulinemia (Alford *et al*. 1999).

GH clearly affects fuel utilization. Acutely, GH replacement in patients with GHD or short-term treatment in normal individuals alters substrate availability (Jørgensen, J.O.L. *et al*. 1990a; Vahl *et al*. 1997), increases lipolysis and lipid oxidation (see above) and reduces carbohydrate oxidation (Moller *et al*. 1990), probably by means of substrate competition. In contrast to these acute effects of essentially transient pulses of GH, patients with acromegaly, who have chronically elevated GH serum concentrations, have been shown to have hyperglycemia and hyperinsulinemia, resistance to the action of exogenous insulin and tendencies to increased carbohydrate oxidation, increased non-oxidative glucose turnover and to have exaggerated amounts of intramuscular glycogen. Attempting to predict what this means for athletic performance is difficult, but potentially long-duration aerobic exercise may benefit from the greater stores of glycogen with fat being used as a fuel initially, but this is entirely speculative. Recently, the effects of brief, supra-physiological doses of GH administered to competitive cyclists have been reported (Lange, K.H. *et al*. 2002). In a double-blind, cross-over designed study, an exaggerated lactate response to intense, aerobic exercise has been described, and some individuals appeared to be unable to complete the exercise following GH administration. This appears to be the first controlled assessment of the effects of GH on aerobic performance in athletes, and it does not support the theory that GH may be beneficial, but the data set is small and requires further assessment.

Growth hormone, cholesterol metabolism and vascular health

GH appears to have a bi-modal pattern of effects on vascular health. In early reports of patients with GHD, mostly involving children or young adults, GHD resulted in increased triglyceride and cholesterol concentrations (predominantly low-density lipoprotein [LDL] cholesterol) of a mild degree in 20–50% of cases (Merimee *et al*. 1972; Winter *et al*. 1979; Blackett *et al*. 1982; LaFranchi *et al*. 1985). In adults with GHD, mild increases in LDL- and total cholesterol levels have been reported in 40–50% of patients compared to age, weight and sex-matched controls (Libber *et al*. 1990; Cuneo *et al*. 1993). These same studies also reported a significant prevalence of hypertriglyceridemia, but differed in reporting increased or reduced HDL-cholesterol concentrations. Recent turnover studies have shown increased Apo B-100 synthesis and decreased clearance in adults with GHD, providing further evidence for an atherogenic state in this syndrome (Cummings *et al*. 1997). GH treatment improves many of these abnormalities (Cuneo *et al*. 1993, 1998; Rosén *et al*. 1994; Beshyah *et al*. 1995; O'Neal *et al*. 1996). An 18-month open label study of 40 subjects showed that the most notable changes in decreasing total/high-density lipoprotein (HDL) cholesterol ratio and increasing HDL cholesterol were evident towards the end of the observation period (Beshyah *et al*. 1995), suggesting that the cholesterol changes are likely to persist. Indeed, 10-year follow-up studies have confirmed GH replacement in GHD adults maintains a favorable lipid profile (Gibney *et al*. 1999). There are complex mechanisms thought to explain the GH effect on cholesterol metabolism, one of the main being an effect to up-regulate the hepatic LDL-receptor expression (Rudling *et al*. 1992), thereby increasing LDL-cholesterol clearance and reducing hepatic cholesterol synthesis.

The long-term follow-up of adults with hypopituitarism is characterized by increased total and cardiovascular mortality rates (Rosén & Bengtsson 1990; Wüster *et al*. 1991; Bulow *et al*. 1997). These studies all assume that hypopituitary patients on conventional hormone replacement have GHD and optimal hormone replacement. More direct studies

however confirm an increased prevalence of atherosclerotic plaques in both adults and children with GHD (Markussis *et al*. 1992; Capaldo *et al*. 1997). It is assumed that the dyslipidemia and visceral adiposity account for much of the observed excess mortality, but non-physiological replacement with other pituitary hormones (Al-Shoumer *et al*. 1995) and altered coagulation or fibrinolysis may also contribute (Beshyah *et al*. 1993; Johansson *et al*. 1994, 1996). Some data suggest the latter improves with rhGH replacement (Cuneo 1998). A recent study of 10 years of rhGH treatment has shown a reduction in the prevalence of abnormal arterial compliance was in GHD patients receiving long-term treatment compared to those who did not (Gibney *et al*. 1999). While this is a very small study with some selection biases, it represents the first direct clues that long-term mortality may be reduced with rhGH replacement. In acromegaly, vascular mortality is one of the leading causes of death related to that condition, and appears predominantly related to hypertension, cardiomyopathy, obstructive sleep apnoea and diabetes mellitus.

Cardiovascular effects of growth hormone

GH appears to be important for normal cardiovascular health and may contribute to the increased cardiovascular morbidity and mortality in conditions of both GHD and GH excess. Hypopituitary patients, replaced with other pituitary hormones but not GH, have greater cardiovascular risk factors than normal healthy controls as demonstrated by high body mass index and plasma triglycerides, low plasma HDL cholesterol and a higher incidence of hypertension (Rosen *et al*. 1993). Death from cardiovascular disease in similar hypopituitary adults is more common than in the normal population, particularly as a result of cerebrovascular disease. GH excess has also been shown to result in adverse cardiovascular effects. Chronic supra-physiological levels of GH in acromegaly result in an increased prevalence of cardiovascular disease and cardiovascular related deaths in these patients, particularly from a hypertrophic cardiomyopathy (Carroll *et al*. 1998).

The opportunity to study acromegalic and GHD subjects provides us with natural models with which to examine the cardiac effects of chronically augmented or decreased GH activity. Fazio *et al*. (1997) have characterized some cardiac structural and functional consequences of chronically altered GH secretion. This group studied 30 adult acromegalic subjects and 25 adults with childhood-onset GHD. Two groups of 30 normal controls were evaluated as control subjects. Echocardiography of acromegalic and GHD hearts revealed an association with chronic circulating serum GH concentrations and left ventricular wall thickness (LVWT) and associated total left ventricular mass (LVM), with acromegalics having increased and GHD subjects decreased LVM. This suggests an anabolic effect of GH on cardiac tissue. Evidence is available for both direct anabolic effects of GH and IGF-I on cardiac tissue (Saccá *et al*. 1994). Cardiac wall stress was shown to be decreased in the high GH state and increased in the low GH state. An uncontrolled study of 12 months of GH treatment in 12 GHD subjects, with doses of rhGH ranging from 0.025 to 0.050 $IU \cdot kg^{-1} \cdot day^{-1}$ ($\cong 9–18\ \mu g \cdot kg^{-1} \cdot day^{-1}$) resulted in an increase in LVM secondary to an increase in LVWT, and a consequent reduction in cardiac wall stress (Fazio *et al*. 1997). By demonstrating that GH is able to favorably affect cardiac structural and functional deficits evident in GHD, and by characterizing similar but exaggerated effects in acromegaly, the authors propose to have demonstrated that circulating GH is implicated in a novel mechanism of cardiac wall stress regulation in humans. In GHD adults, Cuneo *et al*. (1991c) and Caidahl *et al*. (1994) showed that GH treatment increased LVM (but not wall thickness), and increased stroke volume. Caidahl *et al*. (1994) also demonstrated an increase in heart rate and cardiac output, and a decrease in total peripheral resistance. The effect on peripheral vascular resistance (*PVR*) is consistent with known renal/peripheral vascular effects of GH to be discussed below. GH replacement has been shown to stimulate and normalize nitric oxide production in adults with GHD, which would be expected to result in marked arterial vasodilation and reduce *PVR* (Böger *et al*. 1996).

GH administration to normal subjects. The first study of the short-term cardiac effects of GH administration

to normal men was published by Thuesen *et al*. (1988). Eleven healthy male subjects with a mean age of 31 ± 5 years were studied before and after 7 days of GH administration and 7 days after treatment cessation. Large doses of GH were administered subcutaneously (3.2 IU in the morning and 6.4 IU in the evening; total ≅ 3.2 mg). GH treatment resulted in a significant increase in resting heart rate but did not affect systolic or diastolic blood pressure. Echocardiography showed no changes in heart wall thickness, but end-systolic diameters decreased, circumferential shortening velocity increased, stroke volume was unchanged, but cardiac output increased. The authors suggested that GH induced positive chronotropic and inotrophic effects. While *PVR* was not measured in this study, the authors also suggested that the apparent increased myocardial contractility may be secondary to an increase of peripheral blood flow.

A recent study (Cittadini *et al*. 2002) has shown that supra-physiological doses of GH induce rapid changes in cardiac morphology and function. Thirty healthy male and 30 female volunteers were studied in a double-blind, randomized, placebo-controlled study of GH administration. Subjects were classified as fit on the basis of two training sessions per week for 1 year (nature, intensity and duration of exercise not defined). Subjects were assigned to receive daily subcutaneous injections of GH at a dose of either 30 or 60 $\mu g \cdot kg^{-1} \cdot day^{-1}$ or placebo for 28 days with the first 7 days at a dose corresponding to 50% of the target dose. Cardiovascular effects of GH were dose-dependent with the higher dose of GH showing a 12% increase in LVM and a 10% increase in LVWT which was concentric in nature. The lower, but still supra-physiological dose of GH showed statistically non-significant changes similar in direction but not in magnitude. No change in ejection phase indices were apparent with either dose of GH. Cardiac index, measured at rest but still closely related to measures of exercise performance such as maximal oxygen consumption, was increased by 11% with the higher GH dose, and was associated with a corresponding fall in systemic vascular resistance. In the absence of exercise performance measures in this study, the authors were cautious in suggesting that such findings might increase exercise capacity, placing more emphasis on the known detrimental effects of GH excess in acromegaly that causes left ventricle (LV) hypertrophy, and cardiac failure. The fact that GH increased LV mass in only 4 weeks suggested that detrimental effects of GH on the myocardium may occur rapidly. They speculated that concurrent use of anabolic steroids and GH may magnify the detrimental myocardial effects of each.

Renal effects of growth hormone

GH has a number of demonstrated effects on kidney structure and function. In addition to the expected anabolic effects, GH also influences renal hemodynamics, body fluid homeostasis, renal acid-base homeostasis and possibly even contributes to glucose homeostasis.

Anabolic effects. The anabolic effect of GH on kidney tissue is evident in the renal hypertrophy resulting from chronic exposure of to high levels of circulating serum GH in the condition of acromegaly (Kopple & Hirschberg 1990). It is difficult to hypothesize how this could impact positively on exercise performance, and the renal effects of long-term GH doping would most likely be detrimental as it has been suggested that chronic exposure to high levels of circulating GH might predispose to glomerulosclerosis (Yang, C.-W. *et al*. 1993) and possibly even renal failure (Kopple & Hirschberg 1990).

Hemodynamic effects. The effects of GH on renal hemodynamics are well documented. GH treatment increases renal plasma flow (*RPF*) and glomerular filtration rate (*GFR*) in the GHD adult (Hirschberg & Kopple 1988; Hirschberg *et al*. 1989; Caidahl *et al*. 1994). Chronic GH excess in acromegaly results in elevated *RPF* and *GFR* which is reduced post hypophysecotomy (see Kopple & Hirschberg 1990 for review). GH administration in normal subjects also results in an increase in *RPF* and *GFR* and a reduction in renal vascular resistance (*RVR*) (Christiansen *et al*. 1981; Kopple & Hirschberg 1990). For example, Christiansen *et al*. (1981) administered GH to seven normal male subjects twice a day for 1 week, at doses of 2 and 4 IU morning and even-

ing, resulting in an increase in *RPF* from 554 to 601 $mL \cdot min^{-1} \times 1.73\ m^2$ and *GFR* from 114 to 125 $mL \cdot min^{-1} \times 1.73\ m^2$. GH has no acute effect on the renal hemodynamics or filtration within 2 h of administration, but *RPF* and *GFR* increase and renal vascular resistance (*RVR*) decreases within 24 h, paralleling changes in serum IGF-I (Hirschberg *et al*. 1989; Kopple & Hirschberg 1990). Guler *et al*. (1989a, 1989b) have demonstrated that IGF-I administration also increases *RPF* and *GFR* and decreases *RVR* resistance in normal human subjects. Therefore, the hemodynamic effects of GH are now thought to be largely mediated by IGF-I, and that IGF-I acts as a potent vasodilator, an effect mediated by nitric oxide (Schini-Kerth 1999). The potential impact of GH induced changes in renal hemodynamics on exercise performance are unknown, but the increase in *RPF* probably reflects the reduction in systemic vascular resistance and increased cardiac output.

Renal gluconeogenesis. GH may contribute to enhanced fuel homeostasis during endurance exercise. GH-induced increases in renal gluconeogenesis, taking up lactic acid and converting it to glucose for export to the working muscle, may be significant during prolonged work (Poortmans 1977), but experimental demonstration of this hypothesis is yet to be shown.

Fluid homeostasis. GH administration results in sodium retention with subsequent fluid retention. This antinatriuretic effect of GH is apparent from a number of clinical observations. GHD adults have reduced extracellular water and plasma volume which is increased with GH treatment (Møller *et al*. 1996). The chronic GH excess of acromegaly results in a corresponding increase in total body water and blood volume (Ikkos *et al*. 1954; Bengtsson *et al*. 1989b). Fluid retention is a well-documented side effect of GH treatment, and can cause oedema, weight gain, carpal tunnel syndrome and hypertension when given in supra-physiological doses to GHD adults (Jørgensen, J.O.L. 1991). Fluid retention with GH treatment is dose dependent and transitory when physiological replacement is achieved in these GHD adults. Subjects with chronic liver disease develop GH resistance as far as the anabolic effects of GH are concerned, but appear to remain sensitive to the antinatriuretic effects of GH (Wallace & Cuneo 1995).

Effects of GH administration on sodium and fluid homeostasis in normal subjects. To demonstrate the antinatriuretic effect of GH on normal subjects, and to explore potential mechanisms for this effect, Ho, K.Y. and Weissberger (1990) administered GH to six male subjects aged between 21 and 27 years at a dose of 0.2 $IU \cdot kg^{-1} \cdot day^{-1}$ ($\cong 70\ \mu g \cdot kg^{-1} \cdot day^{-1}$) for 5 days. This short-term supra-physiological treatment resulted in a mean weight gain of 1.1 kg, and reduced mean 24-h urinary sodium excretion from 197 to 38 mmol. Urine output was significantly reduced from 1652 to 848 $mL \cdot day^{-1}$. These changes were associated with activation of the renin–angiotensin system (RAS). Plasma-renin activity increased from 730 to 3608 fmol angiotensin-$I \cdot L^{-1} \cdot s^{-1}$ and aldosterone increased from 52 to 402 $pg \cdot mL^{-1}$. GH has also been shown to increase plasma angiotensinogen concentrations and angiotensin II receptor density in the both the kidney and adrenal of dwarf rats (Wyse *et al*. 1993), providing additional points of influence of GH and the RAS.

This data strongly supports the role of the RAS in GH induced effects on fluid homeostasis. It is however proposed that a number of other mechanisms are also possible (Ho, K.Y. & Kelly 1991), as the fluid retention associated with chronic excess in acromegaly is not associated with activation of the RAS. A number of experimental studies in animals suggest that GH may have a direct effect on renal tubular absorption of sodium independent of other effects on the RAS (Stein *et al*. 1952; Karlberg & Ottosson 1982).

Atrial natriuretic peptide. Atrial natriuretic peptide (ANP) is secreted by the heart usually in response to activation of cardiac stretch receptors in response to increased plasma volume. An increase in serum ANP stimulates renal tubular sodium excretion. Short-term GH administration to GHD subjects (7 days) has no effect on blood levels of ANP (Hoffman *et al*. 1996); however, long-term treatment (6 months), which normalizes circulating IGF-I

levels, has been shown to normalize ANP with no change in angiotensin II (Ekman *et al*. 2002). High levels of GH administered to normal healthy adults for 2 weeks results in a marked reduction in circulating ANP levels, which is associated with an expansion in extracellular fluid (ECF) volume but no change in plasma volume (Møller *et al*. 1991). The lack of change in plasma volume would perhaps explain the unexpected fall in ANP in the presence of expanded ECF as changes in ECF alone would not be expected to activate cardiac stretch receptors. Møller *et al*. (1991) propose that the reduction in ANP in response to GH may be due to stimulation of ANP release from the atrias and a subsequent fall in atrial pressure. This would be consistent with reports of GH-induced increased myocardial contractility in normal humans (Thuesen *et al*. 1988).

Renal acid-base metabolism. The role of GH in the regulation of renal acid-base metabolism has been demonstrated in both animals and man. Welbourne & Cronin (1991) studied isolated, perfused kidneys from hypophysectomized rats which exhibited a marked reduction in net acid secretion in response to GH but not IGF-I or insulin. GH was able to accelerate tubular H^+ secretion in kidneys from both hypophysectomized and intact rats, and to enhanced urinary acidification (Welbourne & Cronin 1991). GH has also been shown to directly affect ammoniagenesis in the isolated renal proximal tubules of dogs (Chobanian *et al*. 1992). The authors suggest a role for hGH in the regulation of acid-base metabolism under physiological conditions in which increased net acid excretion is important. Homeostasis of acid-base metabolism during acute exercise is clearly such a scenario. GH has been implicated in systemic and renal acid-base homeostasis in adult humans. Sicuro *et al*. (1998) showed that GH administration to normal subjects was able to partially correct experimentally induced chronic metabolic acidosis by raising serum bicarbonate levels via an increase in net acid excretion. Acidosis contributes to fatigue in many types of exercise, and ingestion of sodium bicarbonate has been demonstrated to prolong exercise time at maximal power output (Jones *et al*. 1977). Vomiting and diarrhea are potential side effects of ingesting large doses of sodium bicarbonate, so it is possible that GH could be used as an alternative means to alter blood pH and enhance exercise time at maximal power output without the adverse effects of oral bicarbonate ingestion.

Thermoregulation

Sweating is one of the most important thermoregulatory homeostatic mechanism for the exercising athlete, and a number of factors suggest that human sweat gland function is influenced by the GH–IGF-I axis. GHD is associated with reduced sweating capacity (Sneppen *et al*. 2000). Patients born with a GHR defect resulting in severely stunted growth (Laron syndrome) have severely impaired sweat secretion rates (Main *et al*. 1993). Hypohydrosis in GHD poses thermoregulatory difficulties during exercise due to a predisposition to hyperthermia (Juul *et al*. 1995). In contrast, acromegaly is characterized by hyperhydrosis, an excessive activation of sweating mechanisms (Sneppen *et al*. 2000). Successful reduction of excessive sweating in acromegalics has been repeatedly achieved in studies using the long-acting somatostatin analog octreotide (Stewart *et al*. 1995; Davies *et al*. 1998).

GHRs have been localized in human skin and cultured skin fibroblasts (Oakes *et al*. 1992). In that study GHR were demonstrated on eccrine sweat glands using a monoclonal antibody. Lange, M. *et al*. (2001) demonstrated that untreated GHD adults have thin skin and a reduction in the area of eccrine sweat gland glomeruli compared to normal subjects, whereas the area of the sebaceous glands appeared normal. Long-term GH treatment resulted in normalization of eccrine sweat gland glomeruli area, improvment of the very low sweat secretory rate but did not appear to fully return skin thickness to normal in childhood onset GHD. The research groups of both Lange and Hassan (Hasan *et al*. 2001; Lange, M. *et al*. 2001) have examined skin biopsies from GHD adults and showed reductions in acetylcholinesterase (AchE) and vasoactive intestinal polypeptide (VIP) in the nerves supplying sweat glands, and showed that GH treatment increased staining for AchE and VIP in the sudomotor nerves and restored sweat rates. They also report that acromegalic subjects have larger sweat gland acini and greater density of innervation to sweat glands

and propose that this suggests a trophic effect of GH on sweat gland epithelium and/or associated nerves.

It is possible that GH doping could enhance thermoregulation in competition conducted in extreme heat via a number of mechanisms: by reducing fat mass and therefore insulation, by mechanisms associated with increased blood flow via vasodilatory effects, by direct effects on sweat gland morphology, or by influencing sudomotor synapse constituents. Excessive GH-induced sweating could theoretically compromize fluid homeostasis during prolonged exercise in the heat.

Growth hormone and the immune system

All elements of the GH–IGF axis are represented in the immune system. GH, GHRs, IGFs and their receptors and binding proteins are expressed in the thymus, lymph nodes and peripheral blood lymphocytes of both children and adults (Yang, Y. *et al*. 1999). This suggests that GH and IGF-I may have both an endocrine and an autocrine/paracrine effect on the immune system. The thymus gland has been described as a target organ for GH and the effects of both pituitary-derived and extra-pituitary GH are summarized by Savino *et al*. (2002). Known actions of GH include enhancement of thymic microenvironmental cell-derived secretory products such as cytokines and thymic hormones, increased thymic epithelial cell (TEC) proliferation *in vitro*, synergism with anti-CD3 to stimulate thymocyte proliferation, influence on thymocyte traffic, and enhancement of human T-cell progenitor engraftment into the thymus.

A number of animal studies have shown that treatment with GH reduces susceptibility to infection. For example, a controlled study of rhGH administration to experimentally burned mice showed the impaired immune response in these animals, as reflected by a decreased interferon-γ response of splenic mononuclear cells, was overcome with GH treatment. GH treatment has been shown to stimulate the appearance of cytostatic macrophages and to result in an increased survival rate in these burned mice infected with herpes symplex virus type I (Takagi *et al*. 1997). GH administration to cattle has been shown to be protective against bacterial but not parasitic infection. The authors suggest that this effect may relate to the inhibition of tumor necrosis factor by GH (Sartin *et al*. 1998).

In humans, GH appears to have some clinically relevant effects on immunity. Both GHD children and adults have been shown to have abnormalities in immune function variables. Both the concentration and proportion of natural killer (NK) cells and the stimulated NK-cell activities were reduced in adult GHD patients compared to controls. GH treatment did not, however, appear to alter this situation, and these patients do not appear to be more susceptible to infection than the non-GHD population (Sneppen *et al*. 2002). We are unaware of literature suggesting that patients with acromegaly have altered immunity. In contrast, supra-physiological doses of rhGH in severely ill humans in intensive care has been shown to increase mortality as a result of infection (Takala, J. *et al*. 1999).

While effects on the immune system within the physiological range of GH actions appears minimal, this does not rule out the possibility that GH administration may protect against the immunosuppression of stress. GH is considered to have an important role in the immune system with reviewers of the available literature suggesting that GH and IGF-I, along with prolactin and thyroid hormones, are critical immunoregulatory factors. Mice knockout models for the primary hormones or their receptors suggest that, although these hormones may not be required for lymphocyte development or antigen responsiveness, they most likely play a mayor role in counteracting the effects of stress-mediated negative immunoregulatory factors, such as glucocorticoids (Jeay *et al*. 2002). GH and/or IGF-I may therefore ensure immune system homeostasis and reduce the susceptibility to stress-induced disease (Dorshkind & Horseman 2001).

Prolonged periods of intense exercise training in human adults decrease neutrophil function, serum and salivary immunoglobulin concentrations, NK cell numbers and possibly cytotoxic activity in peripheral blood, and an increase in the incidence of upper respiratory tract infections (URTIs) has been documented in athletes (Nass *et al*. 1995; Dahlgren *et al*. 2002). The immunoprotective value of GH administration to animals discussed above may lead athletes to conclude that GH doping may

protect them from URTI and subsequent training down-time.

Hematological

Chronic GH exposure in acromegaly results in increased total blood volume, plasma volume and red blood cell volume, all of which are reduced with treatment (Henneman *et al*. 1960; Merola *et al*. 1996). This mild hematinic effect may potentially benefit aerobic-based sporting performance.

Growth hormone and central nervous system effects

GH and IGF receptors are distributed widely throughout the human central nervous system (CNS) (Johansson *et al*. 1995; Nyberg & Burman 1996), suggesting a role for GH and IGF-I in the CNS. Adults suffering from GHD report a lower than normal quality of life when responding to self-rating questionnaires even when compared with subjects suffering other chronic illnesses. Psycholoical deficits such as depression, mental fatigue, low self esteem and reduced levels of life fulfilment are seen to significantly contribute to the reduced quality of life (Wallymahmed *et al*. 1999). GH treatment of GHD adults results in significant improvements in perception of energy level and mood in these patients (McGauley *et al*. 1990; Carroll *et al*. 1998; Cuneo *et al*. 1998). GHD adults are also reported to suffer from cognitive deficits such as impaired memory and lack of concentration which can be improved with GH treatment (Burman & Deijen 1998). It has been argued that the effects of GH treatment on psychological parameters are merely secondary to increases in lean body mass, decreases in body fat and enhanced exercise performance, but the changes in mood and cognition often appear well before any measurable changes in physical status, which lead researchers to suspect that GH may have direct effects on CNS function.

GH has central effects on major monoamine metabolites and neuropeptides involved in the control of attention and mood. Johansson *et al*. (1995) showed that 1 month of GH treatment in GHD adults resulted in decreases in homovallinic acid (HVA) and vasoactive intestinal peptide and increases in GH, IGF-I, insulin-like growth factor binding protein-3 (IGFBP-3) and β-endorphin concentrations in cerebrospinal fluid. This was a significant study as it demonstrated that GH could cross the blood brain barrier. High levels of HVA have been observed in psychiatric patients with clinical depression, and successful treatment of depression with antidepressant drugs is associated with a reduction in cerebrospinal fluid (CSF) levels of HVA. This strongly suggests that improvements in mood with GH treatment are related to effects of GH and/or additional components of the GH–IGF axis such as IGF-I and IGFBP-3 on the CNS. In a cross-over, placebo-controlled, 9-month treatment study, Burman *et al*. (1996) also showed that CSF concentrations of monoamine metabolites and neuropeptides involved in the control of attention and mood were abnormal in GHD patients and normalized with GH treatment. The dopamine metabolite HVA significantly decreased with GH treatment and the excitatory amino acid aspartate increased with 9 months of GH treatment. They confirm the previous findings that GH can cross the blood–brain barrier. The CSF levels of HVA and aspartate were shown to be similar between GHD adults and psychiatric patients suffering with severe depression, with the change in these neurotransmitters in GHD subjects following GH treatment being equivalent in magnitude to the changes achieved with antidepressent medication in psychiatric patients. Unlike Johansson *et al*. (1995) they did not observe a change in CSF levels of β-endorphin with GH administration. This lack of change in β-endorphin may be due to differences in GH dose or assay used or may merely mask a an initial increase in β-endorphin with short-term treatment and an equalibration with chronic treatment An elevation of β-endorphins and have been shown to following exercise and are associated with a post-exercise increase in positive mood (Harte *et al*. 1995).

Heavy training in athletes has been associated with negative changes in psychological factors such as depression, anger, global mood disturbance and a reduction in general well-being (Morgan *et al*. 1988). It is possible that overtraining contributes to changes in GH–IGF axis as reductions in IGF-I have

been noted with exhaustive exercise (Jahreis *et al*. 1991; Tigranian *et al*. 1992). We speculate that GH doping may allow an athlete to undergo harder training without negative changes in mood by perhaps normalizing levels of monoamine metabolites and neuropeptides and thereby preventing psychological disruptions which could lead to negative impacts on concentration and motivation.

Summary of theoretical beneficial (and detrimental) effects of growth hormone abuse by athletes

Table 36.1 outlines the main potential benefits that can be extrapolated from the literature regarding GH effects in adult humans. It must be emphasized that most of these conclusions remain speculative and await appropriate scientific scrutiny. Table 36.2 outlines potential adverse effects of GH abuse in athletes.

Development of a test for growth hormone abuse in elite sport: the physiological lessons

Given that GH is a banned substance under international doping control regulations, the next question might be how might one go about constructing such a test to detect GH abuse.

Potential markers of growth hormone doping

Measuring GH in blood or urine is unlikely to represent a useful test of GH abuse as manufactured and native hGH molecules are identical, and normal secretion of GH is pulsatile and occurrs naturally in response to a number of stimuli including exercise. Studies of rhGH treatment in clinical conditions (adult GHD, sepsis, burns, catabolic states and liver disease) and of patients with over-secretion of GH from a pituitary tumor has enabled us to define a number of blood-borne substances that change in response to GH administration. These 'markers of GH action' include: (a) components of the GH–IGF axis; (b) markers of bone and collagen turnover; and (c) molecular isoforms of GH. GH-responsive elements of the GH and IGF axis include GHBP, IGF-I and a number of IGFBPs, a number of which are used clinically to diagnose acromegaly. A detailed review of the regulation of the GH–IGF axis is beyond the scope of this article, but the reader is referred Cuneo *et al*. (2001). Markers of bone and collagen turnover were chosen given the prolonged

Table 36.1 Hypothetical reasons why athletes might abuse growth hormone: potential but unproven.

Effect	Sporting advantage
Bone	Height increase
	Increased bone turnover and trauma repair
Anabolic	Increased lean : fat ratio
	? Increased muscle or connective tissue
	Increased injury recovery
Cardiac	Inotropic and chronotropic increases in cardiac output
	Vasodilatory enhancement of peripheral perfusion
Lipolytic	Increased lean : fat ratio
	Sparing carbohydrate oxidation in endurance event
Carbohydrate metabolism	? Increased muscle glycogen content
	? Increased gluconeogenesis
Antinatriuretic/renal	Preservation of sodium and fluid homeostasis
	Enhanced acid excretion
Hematological	Increased red cell production
Sweat gland	Increased sweat-mediated heat dissipation
Vascular	Increased cutaneous vasodilation-mediated heat dissipation
Immunological	Improved immunity in training-mediated immune stress
Central nervous system	Enhanced mood/motivation

Acral enlargement	Permanent facial and peripheral bony disfigurement
	Obstructive sleep apnoea
Accelerated osteoarthritis	Permanent articular cartilage damage
Endocrine	Diabetes mellitus and carbohydrate intolerance
	Goitre
Antinatriuresis/vascular	Hypertension
	Acromegalic cardiomyopathy
Muscles	Acromegalic myopathy
Neurological	Acromegalic peripheral neuropathy
Malignancy	Increased incidence of malignancies, particularly colonic
Cutaneous	Hyperhydrosis, papillomata

Table 36.2 Potential adverse effects of growth hormone (GH) abuse in athletes.

stimulation of both bone resorption and formation that follow rhGH administration; for example, such markers stay elevated for months in adults with GHD after cessation of rhGH (Ohlsson *et al*. 1998). The third area of investigation, molecular isoforms of GH, probably warrants additional background discussion for the reader's benefit.

Molecular isoforms of growth hormone

hGH is produced in the anterior pituitary gland, and circulates in a variety of forms. Two genes for GH can be found on the long arm of chromosome 17: hGH-N or 'normal GH' and hGH-V, a 'variant' which is found to circulate during pregnancy. The main expression product of GH-N is a 191-amino acid polypeptide of molecular weight 22 kDa. Different species of GH can be generated by either: (a) alternate splicing of mRNA; (b) post-translational modification; (c) heterodimeric, homodimeric, or polymeric binding; and (d) proteolytic cleavage.

A number of alternatively spliced transcripts of the *hGH-N* gene have been described, with an excision within exon 3 results in a 20 kDa form of GH missing residues 32–46. Messenger RNAs have also been found which predict a 17.5 kDa GH variant in which the whole of exon 3 is skipped (Lecomte *et al*. 1987) and a 27 kDa GH variant which includes intron 4 (Hampson & Rottman 1987). Although GH variants of these sizes have been found in human serum, it is not yet known if they are products of alternative mRNA-splicing, or if they represent post-translational modifications. A number of size variants of GH are due to the ability of 22 kDa and 20 kDa GH to form both homo and hetero dimers and polymers up to pentameric GH. Further size variants can be attributed to the binding of GH with GHBPs. Two main binding proteins are known to circulate: a high affinity binding protein which is the extracellular portion of the GHR (Leung *et al*. 1987), and a larger, low-affinity binding protein consisting of transformed α_2-macroglobulin. The high-affinity binding protein strongly binds 22 kDa GH and shows minimal binding to 20 kDa GH. The low-affinity binding protein weakly binds 22 kDa GH and binds the 20 kDa variant with equal or slightly higher affinity (Baumann 1991). Glycosylation is also thought to result in a number of size variants of GH: 12 kDa, 27 kDa (Lewis *et al*. 1994), 24 kDa (Haro *et al*. 1996). Proteolytic cleavage between residues 43 and 44 of 22 kDa GH is thought to be responsible for the 5 kDa GH_{1-43} and 17 kDa GH_{44-191} forms of circulating GH. Degradation of GH in peripheral tissues has been suggested as the explanation for circulating forms such as 12, 16 and 30 kDa (see Bauman 1991 for detailed review). Other post-translational modifications occur which result in charge variants of GH, the most common of which are acetylation and deamidation. Circulating GH is thought to consist of predominantly 22 kDa GH (72%) and to a lesser extent the 20 kDa variant (20%) (Baumann *et al*. 1985), with other GH variants only occurring in very low concentrations. However, there is some data to suggest that the main circulating variant in some normal individuals may in fact be the 17 kDa GH_{44-191} variant (Warner *et al*. 1993; Sinha & Jacobsen 1994).

Biological actions of growth hormone isoforms

Twenty-two kilodalton GH has been shown to be responsible for a wide range of biological actions. It has been suggested that these often contradictory actions of GH could possibly be explained by the action of individual GH variants. Purification of 5, 17 and 20 kDa GH has allowed determination of their biological effects. Five kilodalton GH has been shown to be insulin potentiating and to overcome insulin resistance in yellow obese mice, and 17 kDa GH proves to be a highly diabetogenic fragment when injected into dogs. Twenty kilodalton GH binds to GHRs (Wada *et al*. 1997, 1998), and when injected into humans was shown to suppress serum 22 kDa GH levels and to elevate serum FFA and IGF-I (Hashimoto *et al*. 2000). This suggests that it has lipolytic and growth promoting actions. The authors suggest that this study demonstrates that 'regulation of 20 kDa-hGH secretion is physiologically the same as that of 22 kDa-hGH and that 20 K-hGH regulates hGH through GH-induced negative feedback' (Hashimoto *et al*. 2000, p. 601).

History of involvement with the GH-2000 project

In 1993 a review of GH and exercise (Cuneo & Wallace 1994) was commissioned by Peter Sonksen, in his capacity as a member of the Medical Sub-Committee of the International Olympic Committee (IOC). Subsequently, the IOC and the European Union funded research to develop a GH dope-detection strategy, to be conducted by members of the GH-2000 group, centered mainly in St. Thomas's Hospital, London (Professor Peter Sonksen), Aarhus Hospital, Denmark (Professor Jens S. Christiansen), Sahlgrenska Hospital, Sweden (Professor Bengt-Åke Bengtsson) and Naples Hospital, Italy (Professor Luigi Sacca), including scientists from Germany, Switzerland and Australia.

THE WASHOUT STUDY

Our main contribution to this program was 'The Washout Study', which aimed to define: (a) the markers most indicative of hGH administration; (b) their response to acute exercise (alone and combined with hGH administration); and (c) the disappearance kinetics of these markers following the cessation of hGH. Seventeen males, aged 18–40 years and body mass index (BMI) 23.6 ± 0.6, with a high level of habitual aerobic activity and aerobic fitness ($\dot{V}o_{2max}$ 56.0 ± 1.2 mL·kg^{-1}·min^{-1}) were studied on seven occasions. Following screening (visit 1), subjects were randomized to either rest or exercise studies in a cross-over design with the rest study controlling for postural effects. Subjects were then re-randomized to a double-blind, placebo-controlled, parallel study of rhGH or identical placebo treatment at a dose of 0.15 IU·kg^{-1}·day^{-1} for 7 days. Treatment was self-administered by subcutaneous abdominal injection at 20:00 h; the 7th dose was given 3 h prior to the post-treatment visit (visit 4). The exercise protocol was repeated on visit 4, and following rhGH withdrawal at visits 5, 6 and 7, which were performed 24, 48 and 96 h after the post-treatment visit (visit 4). All submaximal exercise tests used an identical protocol, consisting of three consecutive stages: stage 1 was 5 min at 1 W·kg^{-1}; stage 2 was 5 min at 2 W·kg^{-1}; and stage 3 was 20 min at 65% of the workload achieved at the predetermined $\dot{V}o_{2max}$ (corresponding to approximately 80% $\dot{V}o_{2max}$).

Acute exercise induced transient equimolar increases of approximately 20% for serum total IGF-I, IGFBP-3 and acid-labile subunit (ALS), exceeding the effect of hemoconcentration (see Mottram 1999 and Figs 36.1 & 36.2).

This suggests to us a novel mechanism for the regulation of the IGF ternary complex. These results confirm findings by Bang *et al*. (1990) where serum IGF-I increased by 26%, but the study was conducted in a very small number of subjects (three men and three women) with no rest control, with subjects cycling for 30 min at 60% of $\dot{V}o_{2max}$. Studies conducted since the design of our study, using a comparable exercise intensity, have also confirmed the response of IGF-I to acute exercise. Ten minutes of cycle ergometry at 72% of $\dot{V}o_{2max}$ in 11 young active adult subjects (10 male and one female), resulted in a 14% increase in serum IGF-I, which peaked at the end of exercise, and was not statistically different to baseline levels 20 min following exercise (Cappon *et al*. 1994). This study however

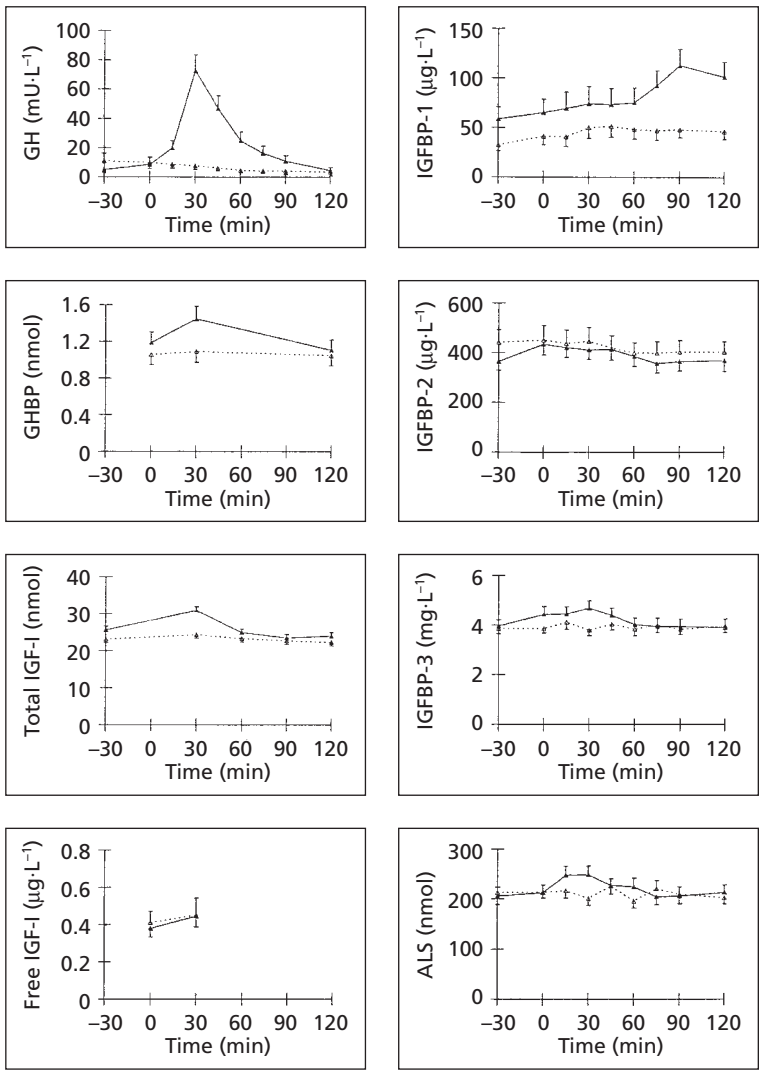

Fig. 36.1 The effect of acute exercise on the growth hormone–insulin-like growth factor (GH–IGF) axis. Subjects (n = 17) underwent random sequence exercise study (closed triangles; semi-recumbency from $t = -30$ until 0 min; exercise from $t = 0$ min with 10 min two-stage warm-up and 20 min at 65% of the workload at $\dot{V}o_{2max}$ [oxygen consumption approximately 80% $\dot{V}o_{2max}$]; semi-recumbency from $t = 30$ to 120 min) or rest study (open triangles; upright posture during equivalent 'exercise' period indicated by box). ALS, acid-labile subunit; GH, growth hormone; GHBP, growth hormone binding protein; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein. (Mottram 1999.)

did not have a rest control as part of the design. Similar results were presented by Schwartz *et al*. (1996) using an identical exercise protocol, in 10 active young male subjects, showing a mean increase in serum IGF-I of 13.3% at the end of exercise, with IGF-I returning to baseline after 10-min rest. This paper also shows that the IGF-I response to exercise is dependent upon the intensity of exercise and is GH independent, as exercise at approximately 46% of $\dot{V}o_{2max}$ was unable to stimulate GH release but still elicited an mean IGF-I response of 7.7%.

A unique aspect of our study was the measurement of serum free IGF-I in response to acute exercise in athletes, which was only assayed at the beginning and end of exercise, due to the labor intensity and expense of performing the assay. The circulating concentration of free IGF-I did not change with acute exercise, indicating that the increase in total serum IGF-I was due to an increase in IGF-I bound to IGF binding proteins and ALS. A detailed discussion of the IGF-I/IGFBP-3/ALS ternary complex will be expanded below. A sudden major increase in free IGF-I during exercise would

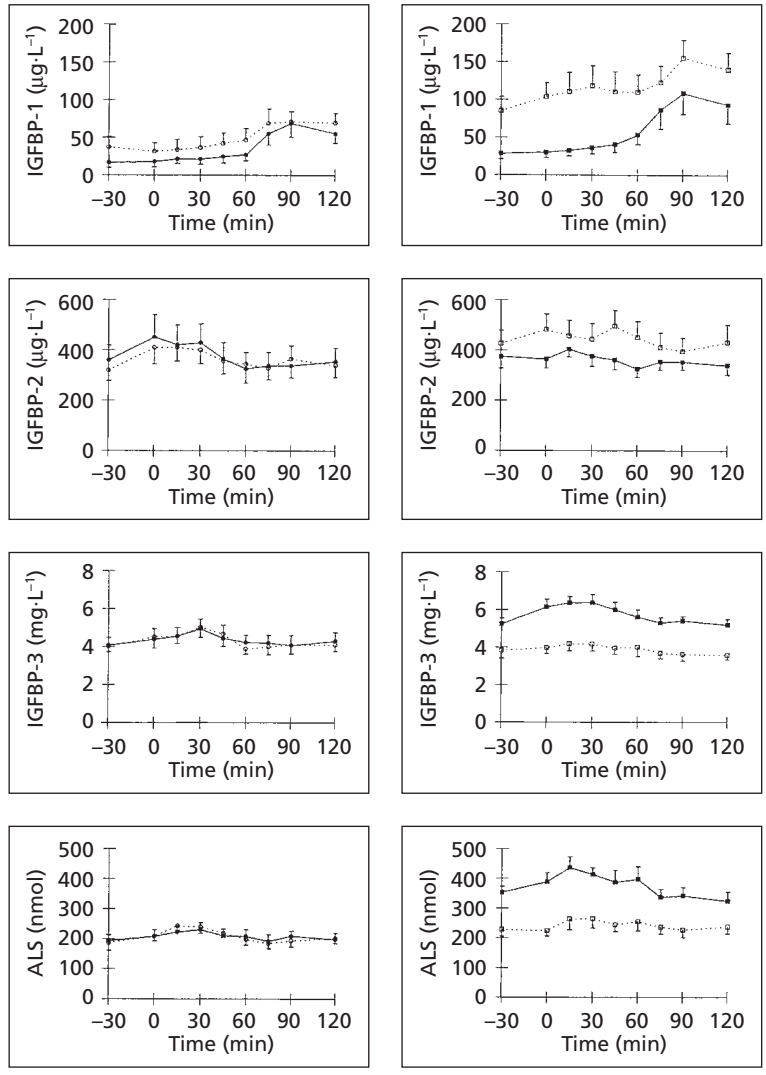

Fig. 36.2 The effect of recombinant human growth hormone (rhGH) treatment on insulin-like growth factor binding protein (IGFBP) -1, -2 and -3 and acid-labile subunit (ALS). Subjects underwent identical exercise tests before (open symbols) or after (closed symbols) randomization to treatment with placebo (circles; left panel) or rhGH (0.15 $IU.kg^{-1}.day^{-1}$; squares; right panel) for 7 days.

be counterproductive as it has potent insulin-like effects and would be expected to negatively affect both glucose and fat supply. If there is a requirement for additional IGF-I during acute exercise it would therefore make physiological sense for it to be delivered in a bound but releasable form.

There are a number of potential functions of an increase in bound IGF-I during acute exercise. It is possible that free IGF-I is metabolically active during acute exercise. The lack of change in concentration of free IGF-I during acute exercise may mask an increase in the disappearance rate, or turnover, of free IGF-I. If this is the case, we could hypothesize that the appearance of a bound pool of IGF-I, during acute exercise, could act as an acute reservoir of IGF-I. IGF-I has been shown to be stable throughout the day and is therefore probably tightly regulated. The appearance of an additional pool in the face of a metabolic challenge could serve to maintain equilibrium of both free and bound concentrations of serum IGF-I, while providing the additional IGF-I for short-term demands. Alternatively, exercise could possibly signal the release of IGF-I bound to binding proteins which could then be immediately

directed to target tissues, with the rising bound levels in the blood merely reflecting the residue from the acute influx. It is also possible that the additional IGF-I appearing in the circulation in response to exercise only becomes metabolized at the end of exercise. It may remain bound to its constitutive binding proteins in circulation, distributed to target tissues or released into the circulation as free IGF-I. We did not measure free IGF-I following exercise and are therefore unable to report on the post-exercise relationship between bound and free IGF-I.

The origin of exercise induced IGF-I is unknown. Circulating IGF-I is thought to be largely hepatic in origin, secreted in response to GH, but may also arise due to leakage or perhaps active secretion from non-hepatic tissues. The time course of GH stimulated hepatic IGF-I secretion is not rapid, so if the liver is the source of the rapid increase in serum IGF-I in response to exercise it is not likely to be GH-related. Electrical nervous stimulation of an isolated perfused cat limb results in increased IGF-I in the perfusate, suggesting that exercise could possibly result in secretion of IGF-I from tissues other than the liver (Sara *et al*. 1982), and in human studies the exercising limb has been shown to be a source of IGF-I (Brahm *et al*. 1997b). Therefore local IGF-I mediated effects seem feasible as a result of these exercise-induced changes in IGFs.

The biological actions of increased circulating IGF-I in response to acute exercise are currently unknown. If we consider the tissue distribution of IGF-I receptors, and results of acute infusion of recombinant human IGF-I into human subjects, we are able to speculate on a number of possible metabolic effects which may be facilitated by exercise-induced IGF-I. The two metabolic fuel reservoirs of the body, namely the liver and adipose tissue lack functional receptors for IGF-I and are probably therefore not the main targets of exercise-related serum IGF-I increases. The pancreas and skeletal muscle on the other hand are rich in IGF-I receptors and would therefore be logical targets for acute changes in IGF-I. Acute exercise is characterized by a fall in circulating insulin. It is possible that the acute increase in IGF-I and subsequent action via pancreatic IGF-I receptors may contribute to the exercise-associated fall in insulin. When the isolated perfused rat pancreas is exposed to IGF-I, insulin secretion is suppressed (Leahy & Vandekerkhove 1990), and acute infusion of IGF-I in humans is able to lower insulin even in the face of an euglycemic clamp (Boulware *et al*. 1992). It is possible, therefore, that a small acute rise in IGF-I with exercise could facilitate energy supply in the form of glucose and fat by reducing the exposure of hepatocytes and adipocytes to circulating insulin, which would favor uptake and not release by these tissues. Having an influx of IGFBP-3 and ALS with the exercise-induced IGF-I would be expected to avoid high concentrations of free IGF-I which could then act via hepatic and adipocyte insulin receptors to influence uptake and net release of these exercise fuels. IGF-I is also thought to have a protein sparing effect by means of inhibiting proteolysis. An acute increase in IGF-I in response to exercise could therefore serve the function of reducing potential protein catabolic effects. A number of studies have demonstrated that IGF-I administration leads to reduced protein degradation. For example, leucine oxidation was shown to decrease with IGF-I administration to male subjects (Nussey *et al*. 1994), and the appearance of branched chain amino acids was markedly decreased after administration of IGF-I to male and female subjects (Boulware *et al*. 1992).

The appearance of IGF-I in response to acute exercise may have a physiological role in glucose transport into working muscles. Peripheral glucose disposal is increased during acute exercise. It is possible that the enhanced uptake of glucose by muscle during exercise is facilitated by an IGF-I mediated increase in muscle blood flow. The potent arterial vasodilatory properties of IGF-I were demonstrated by infusing IGF-I into the brachial artery of human nine male subjects and producing a rapid increase in forearm blood flow within 5 min of the infusion. This effect was attributed to IGF-I-induced nitric oxide synthesis, as blockade of nitric oxide synthesis with mononetyyl-L-arginine abolished the increase in blood flow to IGF-I (Kiowski *et al*. 1994). These results have been confirmed in a larger study by Fryburg (1996). Napoli *et al*. (2003) have demonstrated that GH infusion into the brachial artery acutely stimulates endothelium dependent vasodilation and the related nitric oxide pathway. They

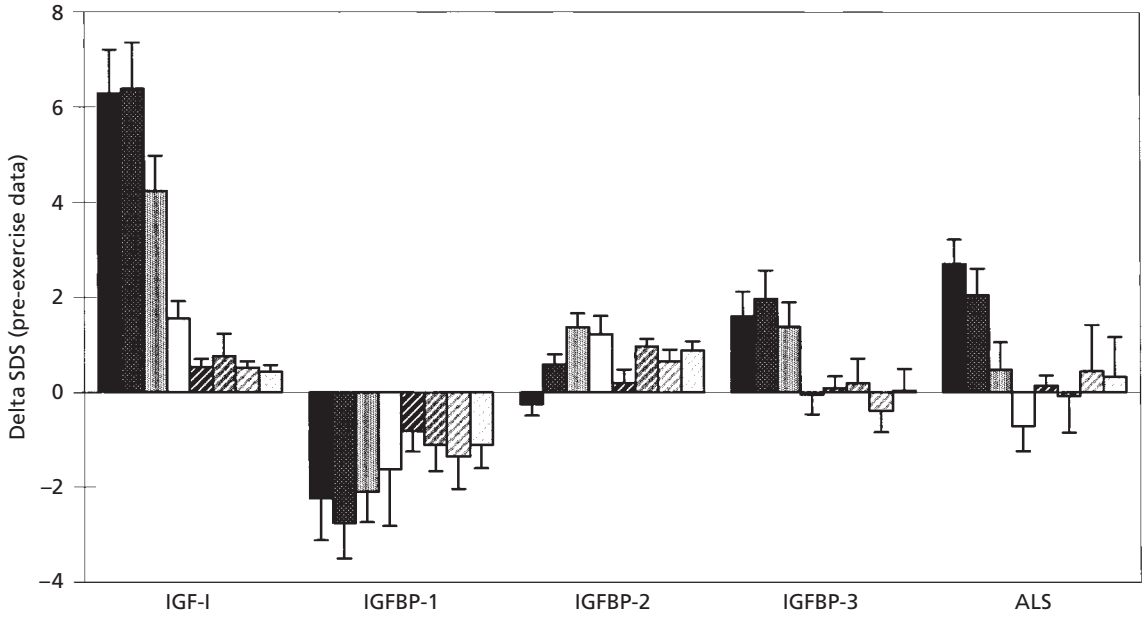

Fig. 36.3 The relative responses to growth hormone (GH) treatment. Data has been transformed into standard deviation scores (SDS) using mean and standard deviation from the pretreatment, pre-exercise data for the entire study group. Results report the differences in SDS (mean ± SEM) from pretreatment visit to visits 4, 5, 6 and 7 (3, 24, 51 and 99 h after the last dose of recombinant human growth hormone [rhGH], respectively) in the GH group (black, dark gray, mid-gray and light gray, respectively) and placebo group (black, dark gray, mid-gray and light-gray oblique stripes, respectively).

show that forearm IGF-I levels did not change during the infusion and suggest that the vasodilatory effect of GH is independent of circulating IGF-I.

Subcutaneous infusion of IGF-I, in humans, has been shown to affect capillary permeability in skin and retina (Franzeck *et al*. 1995a, 1995b). It is possible that this effect could be activated post-exercise in vascualture other than that associated with the skin and eyes, and could be a potential mechanism involved in the facilitation of transport of IGF-I binary complexes to target tissues.

IGFBP-1 showed a prominent post-exercise increment of 50%, perhaps minimizing post-exercise hypoglycemia. Acute exercise did not change IGFBP-2 and free IGF-I. Treatment with rhGH resulted in marked increases in free and total IGF-I, IGFBP-3 and ALS, and minor reductions in IGFBP-1 and -2, with all of these hGH-induced changes disappeared over 4 days. The total hGH response to acute exercise was abolished for several days in many individuals following rhGH treatment, presumably as a result of IGF-mediated negative feedback. Therefore IGF-I, and perhaps several IGFBPs, may be suitable markers of hGH abuse, given their diurnal stability and limited response to acute exercise.

GH-IGF axis, relative responses (Fig. 36.3). Data for individual analytes were converted into standard deviation scores (SDS), using the means and standard deviations of resting, pretreatment values as reference data. The following changes in response to 7 days of rhGH administration in the GH group were observed: IGF-I +6.3 ± 0.9, IGFBP-1 –2.2 ± 0.9, IGFBP-2 –0.3 ± 0.2, IGFBP-3 +1.6 ± 0.5 and ALS +2.7 ± 0.5 SDS, compared to respective changes in the placebo group of +0.5 ± 0.2, –0.8 ± 0.4, +0.2 ± 0.3, +0.1 ± 0.3 and +0.1 ± 0.2 SDS (Fig. 36.3). Using the lack of overlap of individual data points to separate the groups, serum IGF-I allowed correct identification of rhGH-treated individuals at visit 4 in 87.5% and 100% of cases for pre- and post-exercise time

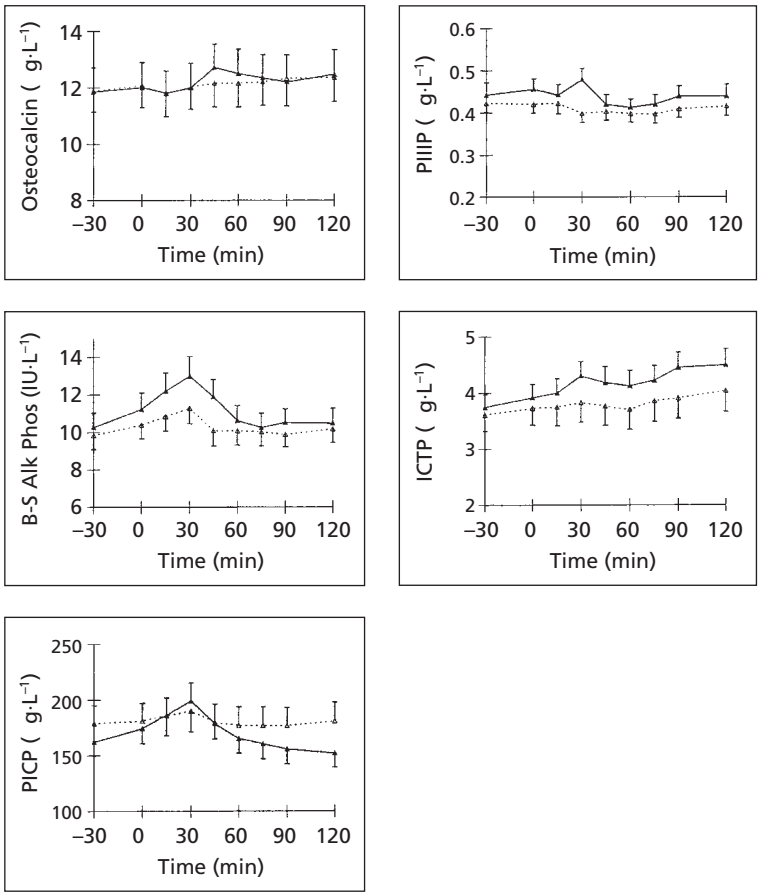

Fig. 36.4 The effect of acute endurance-type exercise on markers of bone and soft-tissue collagen turnover. Subjects (n = 17) underwent random sequence exercise study (semi-recumbency from $t = -30$ until 0 min; exercise from $t = 0$ min with 10-min two-stage warm-up and 20 min at 65% of the workload at $\dot{V}O_{2max}$; semi-recumbency from $t = 30$ to 120 min; solid symbols and continuous line) or rest study (upright posture during equivalent 'exercise' period indicated by box; open symbols and dotted line). B-S Alk Phos, bone-specific alkaline phosphatase; ICTP, carboxyterminal cross-linked telopeptide of type I collagen; PICP, carboxyterminal propeptide of type I procollagen; PIIIP, procollagen III N-terminal extension peptide.

points, respectively and in 75% and 87.5% of cases at visit 5 (27 h after the last dose of rhGH). Similarly, serum ALS allowed correct identification in 100% and 87.5% for pre- and post-exercise time points, and 100% and 50% at visit 5. Serum IGFBP-3 was less sensitive at each visit.

Acute exercise transiently increased serum bone-specific alkaline phosphatase (BS-ALP; +16.1%), carboxyterminal propeptide of type I procollagen (PICP; +14.1%), procollagen III N-terminal extension peptide (PIIIP; +5.0%), carboxyterminal cross-linked telopeptide of type I collagen (ICTP; +9.7%), without change in osteocalcin (Fig. 36.4) (Karila *et al*. 1999). These changes clearly show a transient increase in bone turnover following endurance exercise with limited weight bearing. Exogenous rhGH treatment increased serum osteocalcin, PICP, PIIIP and ICTP, most impressively in the latter two. Disappearance half-times following cessation of rhGH for pre- and post-exercise markers ranged from 248 to 770 h. Therefore, markers of bone and collagen turnover may be suitable markers of hGH use. Endurance and resistance type exercise, recovery post-exercise and differences in training must be considered when interpreting PIIIP and bone markers (Takala, T.E. *et al*. 1986, 1989; Virtanen *et al*. 1993; Brahm *et al*. 1996, 1997a, 1997c). Given that PIIIP was perhaps the most promising 'bone' marker to detect recent GH abuse, particular concern must be expressed since this is a marker of collagen turnover, and ligamentous injury may be considered a case for a 'false positive test'.

To demonstrate the relative magnitude of response to acute exercise of all forms of GH measured,

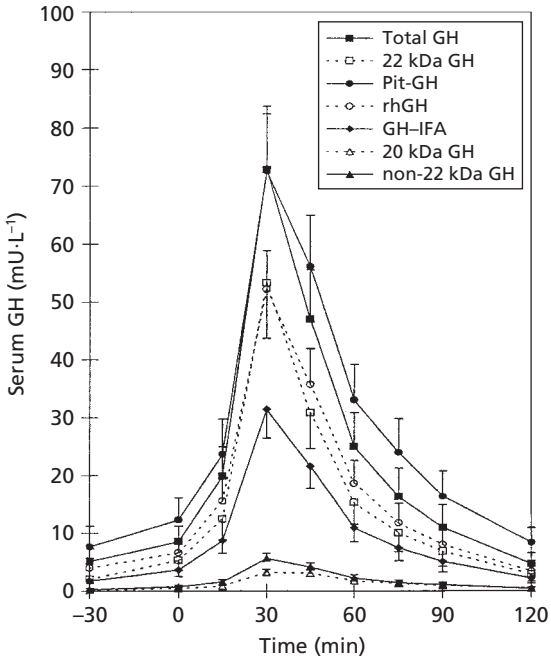

Fig. 36.5 The relative magnitude, of responses of different molecular isoforms of growth hormone (GH), to acute endurance-type exercise. GH–IFA, immunofunctional assay for growth hormone; Pit-GH, pituitary growth hormone; rhGH, recombinant human growth hormone. Data presented as mean ± SEM.

serum concentrations have been converted into SI units and presented together in Fig. 36.5.

The human pituitary produces several species of hGH, the most abundant being 22 kDa, with smaller amounts of 20, 17, and 5 kDa monomeric isoforms and oligomeric forms (Baumann 1991). Suppression of the non-22 kDa isoforms following exogenous 22 kDa rhGH administration would be expected on the basis of IGF-I mediated negative feedback. In collaboration with Swedish and German colleagues (Boguszewski *et al*. 1996; Wu *et al*. 1999), we have shown that acute endurance-type exercise increased both 22 and non-22 kDa isoforms (Fig. 36.5). The relative proportion of the latter increased from approximately 3% to 9% across the exercise protocol. Treatment with rhGH suppressed the exercise-induced responses, with an increase in the ratio of 22 kDa/total hGH and a decrease in non-22 kDa/total GH ratios (Fig. 36.6). Distinction between treatment groups was possible in most cases, but the effect may only be detectable for 24 h. Therefore, molecular isoforms of hGH may be suitable markers of very recent rhGH administration. In our study, urinary GH measurement was of limited use in detecting rhGH administration (Wallace *et al*. unpublished data).

Progress after The Washout Study: GH-2000 and others

One of the main sets of markers of GH action in athletes revealed by The Washout Study was the IGF system. Dall *et al*. (2000) extended these concepts in a study that recruited 50 male and 49 female subjects aged 18–40 years. Subjects were healthy but not athletic, given the entry requirement of two exercise sessions per week for 1 year. In a double-blind, placebo-controlled, parallel dose finding study rhGH (0.1 and 0.2 $IU \cdot kg^{-1} \cdot day^{-1}$) was administered subcutaneously each evening. Unfortunately 33% and 20% of women in the high- and low-dose treatment groups, respectively, used oral contraceptives. This observation is important in interpreting the serum IGF-I responses, since oral estrogens inhibit hepatic IGF-I generation (Weissberger *et al*. 1991). Serum IGF-I clearly increased with rhGH, with greater increases in men than women (Ho, K.K. & Weissberger 1992; Burman *et al*. 1997), and in the latter group a treatment effect was only seen in the higher dose group. The mean concentrations are not reported. Differentiation between the placebo and other groups was reported, using 4 SD above the whole group baseline data at 21 or 28 days of treatment as 50% and 33% in the high-dose female, and 7% and 23% in the low-dose female group. In men, corresponding figures were 86% and 64% in the high-dose and 73% and 60% in the low-dose group. Differentiation persisted after cessation of rhGH for 2 days in 13% and 6% in high- and low-dose female groups and in 20% and 33% in high- and low-dose male groups. No differentiation was possible 5 days after the last dose. Serum IGFBP-3 and ALS were much less discriminatory between groups, but the data was not presented. The IGF-I/IGFBP-3 ratio showed an interesting gender difference, with an apparent ceiling effect at 0.1 $IU \cdot kg^{-1} \cdot day^{-1}$ in the

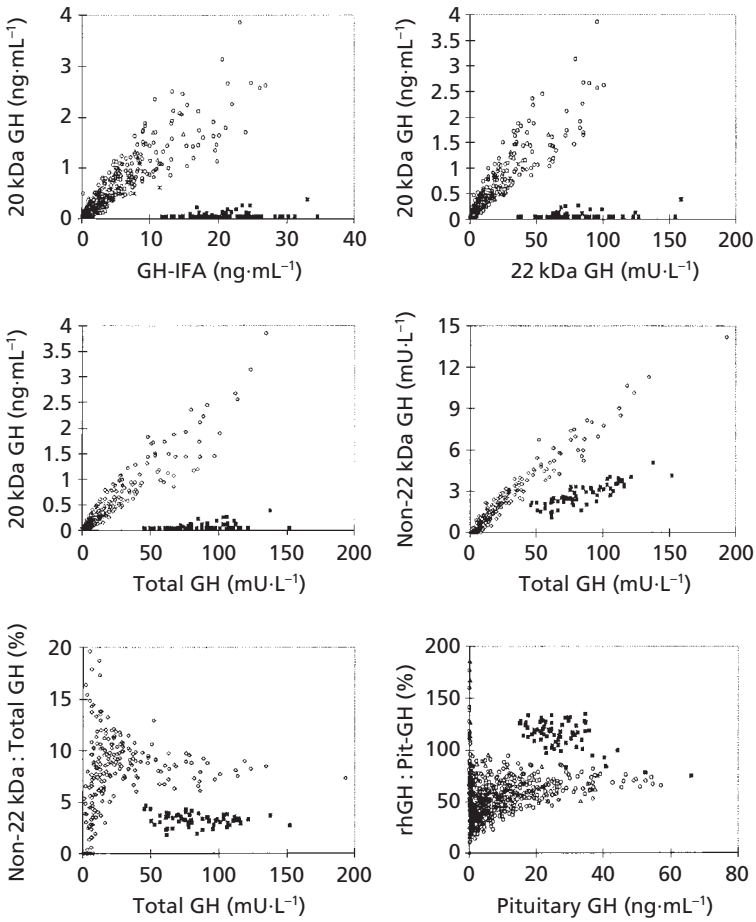

Fig. 36.6 Regression analysis of non-22 kDa growth hormone (GH) assays against total GH. Data represent 'normal' or untreated individuals (i.e. data at all time points from the placebo group from rest and exercise studies from visits 2–7 and recombinant human growth hormone [rhGH] groups from before intervention for visits 2 and 3; open circles). The rhGH group at visit 4 (i.e. 3 h after the last dose; solid squares), visit 5 (open triangles), visit 6 (open diamonds) and visit 7 (double crosses) are shown separately. Pit-GH, pituitary growth hormone.

males but a more progressive dose response effect in females. The authors considered this to be a good biological marker of GH status in other conditions (e.g. acromegaly), noted that it remained elevated for 2 weeks after ceasing rhGH in the current study, but did not present this data in terms of being able to discriminate between treated and untreated individuals. These data demonstrate that serum IGF-I (and perhaps IGF-I/IGFBP-3 ratio) may be a suitable marker to detect rhGH abuse in a cross-sectional fashion, using a single time point sample in an athlete compared to a population data set of comparable athletes.

This same study examined the stability of the IGF system in the placebo groups. The mean intra-individual coefficient of variation (CV) in the combined male and female placebo groups was between 10% and 15% for IGF-I, IGFBP-3 and ALS over seven time points across 84 days. These data may contribute to a detection system based on repeated measurements in elite athletes, where starting or stopping hGH abuse may be detected as a change in excess of specific biological variability in an individual athlete. To date, no data on the variation of such markers in truly elite athletes has been published to validate such an approach. Additional confounders that may reasonably be expected to contribute to day-to-day or month-to-month variability in IGF-I may include acute exercise (Cuneo & Wallace 1994), training phases (Hagberg *et al*. 1982; Jahreis *et al*. 1991; Tigranian *et al*. 1992), oral estrogens (Weissberger *et al*. 1991), pregnancy, anabolic steroid use (Karila *et al*. 1999), illness or injury and season.

Markers of bone turnover as potential agents to

detect hGH abuse were examined in the same cohort of healthy active adults. Markers of bone formation (osteocalcin, and PICP), bone resorption (ICTP) and extra-osseous collagen (PIIIP) were examined. Stability of all markers in the placebo groups was documented (CVs between 21% and 30%), but was somewhat higher than for the IGF axis markers. Examination of diurnal effects was not part of the study design, a potentially important consideration as discussed by Neilsen *et al*. (1990). Menstrual cycle was said to not influence these markers, but the statistical method to establish this conclusion was not presented. All four markers increased with rhGH treatment, with some important marker-specific differences. For example, men had greater increments in PIIIP and ICTP, again reflecting the lower responses in women as noted for the IGF axis. There was a dose-dependent effect with extra-osseous collagen marker (PIIIP) and the bone resorption marker (ICTP) having a clearly greater response with the higher rhGH dose. Time-specific responses were noted with more prolonged effects after withdrawal for osteocalcin and PIIIP. In general, bone and collagen markers remained elevated for longer periods after withdrawal of rhGH treatment, some up to 56 days. The results were presented in a different fashion to the IGF marker publication, using a discriminant analysis to assess the utility of a single marker and combinations of markers to differentiate between the placebo and the treated group. False positive rates, an unacceptable situation where the test might falsely accuse a 'clean' athlete of abusing rhGH, were lowest after 4 weeks of rhGH (between 2.7% and 12.8%). Sensitivity was 84–87% at the best time point, although the level of discrimination in terms of standard deviations was not presented.

GH-2000 also examined the effect of maximal exercise in elite athletes on the above markers of the IGF axis and markers of bone and collagen turnover (Ehrnborg *et al*. 2003). While the GH response to acute exercise has been well described, the effect of maximal exercise on most of these serum markers was unknown, certainly for the elite athlete. They studied 117 elite athletes spanning endurance sports (long-distance cyclists and cross-country skiers), power sports (weightlifters and sprint cyclists) and a mixture of other skill-based sports (alpine skiing, football, rowing, etc.). Accordingly, the nature and duration of the maximal tests were very different between sporting disciplines, and the majority of tests were not discipline specific (all skiers did treadmill tests, and tennis players, footballers, swimmers, etc., performed cycle ergometry tests). The protocols employed would not appear to mimic competition. No resting control data were collected. Statistically significant increases in IGF-I, IGFBP-3, ALS, ICTP and PIIIP were demonstrated, peaking at the end of exercise and declining to baseline values over the subsequent 30–60 min. These results were almost identical to The Washout Study. The limitations of this study include the pooling of vastly different sports, body types and stimuli applied, which appears to have resulted in very non-normally distributed data given the non-parametric analysis strategy used. While efforts to determine covariates of some of these values has been undertaken, sporting discipline or nature of the physical stimulus applied was not considered. The authors then present 10–90 percentile concentrations for these markers, but realistically many more observations would be required to consider such data as normative and defining of an abnormal value in a rhGH-abusing athlete. An important practical consideration arising from this and earlier studies is the recommendation to delay blood sampling from athletes until at least 30 min after the completion of exhaustive competition. This should be borne in mind when considering the large scale cross-sectional studies of markers in elite athletes, where this criterion has not been uniformly applied.

Cross-sectional normal values for such markers have recently been reported in abstract form by a GH-detection study group based in Australia (Howe *et al*. 2003). The aim was to provide reference data against which an athlete could be judged to either be normal or to be influenced by rhGH doping. As expected, widely varying results are seen between differing sporting disciplines, different races and different body morphologies. This wide variability persists after statistical correction for a number of morphological variables, suggesting that the use of a single serum sample of IGF-I (or related IGFBPs) will have low sensitivity. In addition, the timing of venesection after competition has not been considered in the study design,

contributing to a component of the variability, further weakening the robustness of such a detection strategy.

Interestingly, the effects of GH administration on high intensity aerobic exercise and metabolism has recently been published (Lange, K.H. *et al*. 2002). The authors studied seven cycle-trained males (mean $\dot{V}o_{2max}$ 65 ± 1 mL·kg^{-1}·min^{-1}) in a randomized, double-blind, placebo-controlled, cross-over study to assess the effect of a large, single, subcutaneous dose of rhGH (2.5 mg) 2 h prior to exercise. They found that two subjects could not complete the 90-min exercise protocol (staged at a workload that elicited 65% and 75% of a predetermined $\dot{V}o_{2max}$) during the rhGH day but could do so on the placebo day. While the significance of this finding is perhaps open to question, there were clear rhGH-induced increases in lipolytic responses (increased glycerol and FFA) and, surprisingly, in lactate. Unpublished results from The Washout Study corroborate these metabolic findings. The simple interpretation is that GH indices a lypolytic response and that this may result in a competition for the type of fuel used during exercise, favoring lipid substrates as opposed to carbohydrate sources. This would be consistent with the apparent decline in endurance capacity, since lipid oxidation requires more oxygen than does carbohydrate.

These studies confirmed the findings of The Washout Study, allow the confident selection of suitable markers and go a long way toward providing suitable reference data against which an athlete's results could be compared. Questions remain regarding: (a) the exact form of the testing strategy (in- and out-of-competition testing); (b) which markers are suitable in non-Caucasian athletes; (c) the statistical certainty required to label an athlete as having used hGH given the indirect nature of the tests; and (d) the acceptability of blood sampling as opposed to urine sampling. Blood sampling has been permitted at the Lillehammer and Nagano Winter Olympics, and it is believed that many athletes may prefer blood sampling to the invasion of privacy involved in urine collection.

Alternative testing methods. Mass spectrometry of proteins and stable isotope ratio assessment of the differences between endogenous and exogenous proteins are being explored but as yet do not appear practical for widespread use as a test.

The social implications. Side effects of performance enhancing drugs appear not to influence elite athletes. This apparent endorsement filters down to drive the use of performance-enhancing drugs in school-age athletes. We should endeavor to educate our future athletes and put in place suitable tests to deter widespread use of these agents.

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Chapter 37

Endocrinology of Overtraining

ANDREW C. FRY, JUERGEN M. STEINACKER AND ROMAIN MEEUSEN

Introduction

The pursuit of elite athletic performance often requires athletes to 'push themselves to the limit'. Any coach or athlete understands that to attain the greatest performance possible, athletes must routinely push themselves physically and mentally into new levels of performance. Such a tactic is often called overload, and although it is necessary for optimal athletic performance, it can also be quite problematic. In particular, what happens when the athlete goes 'a little too far' in their training and either becomes stagnant or, of even greater concern, begins to perform poorly. This training scenario is often called overtraining, and is of great interest to not only the athlete and coach, but also has implications for occupational and military settings.

Biological stress. The method in which biological systems deal with stress was eloquently described by the landmark work of Hans Selye (1956) as he presented the general adaptation syndrome (GAS; Fig. 37.1). Although originally applied to typical biological scenarios, the GAS nicely describes the process that an athlete uses to respond and adapt to training stresses, both the short-term immediate stresses from a single training session, as well as the long-term stresses from the chronic training program. Upon initiating a vigorous training session, the athlete begins the alarm phase where the body responds to the stresses applied. On completion of the session, the athlete ideally enters the resistance stage where he/she counters the biological responses to the training insult. Eventually, the athlete enters the adaptation stage where they adapt

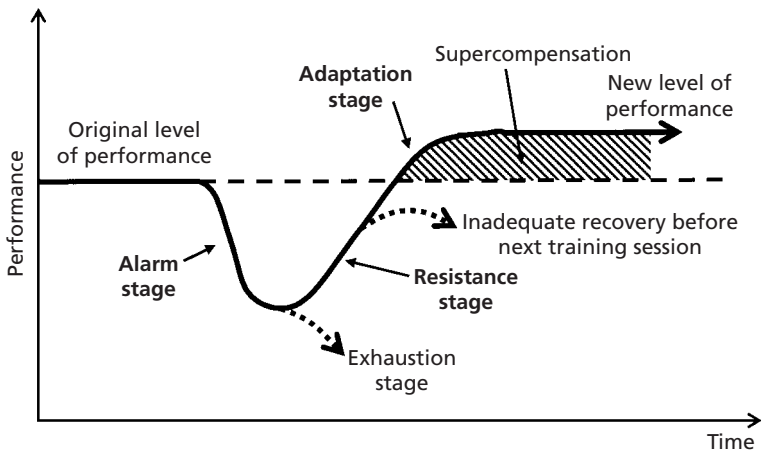

Fig. 37.1 The general adaptation syndrome (GAS) as applied to athletic training and performance. While the exhaustion stage as illustrated may be analogous to overtraining, it is more likely that the line representing inadequate recovery may be more indicative of the subtle onset of overreaching and overtraining often encountered in the sport setting. (Modified from Selye 1956.)

to the original stress and move to a higher level of biological functioning. In sporting terms, the athlete performs better. The problem of overtraining arises when the individual is unable to properly respond to the alarm phase, eventually reaching the exhaustion stage. In Selye's original model, the exhaustion stage results in the death of the system. In the sporting world, this results in performance decrements, and the 'death' of any hope of optimal performance.

Overtraining defined. Perhaps the most critical step when discussing overtraining, is providing an operational definition of this and related terms. For the purposes of this chapter, we will define overtraining as 'the accumulation of training and non-training stress resulting in long-term (i.e. several weeks or months or longer) decrement of performance capacity' (Fry, A.C. 1998; Kreider *et al*. 1998). A less deleterious version of overtraining is often refered to as overreaching, which is 'the accumulation of training and non-training stress resulting in short-term (i.e. several days) decrement of performance capacity' (Fry, A.C. 1998; Kreider *et al*. 1998). Although many different terms have been applied to this phenomenon, this chapter will use these definitions when discussing both the short- and long-term conditions. Perhaps the most important aspect of these definitions is the requirement for performance decrements. Although stress may be manifested in may ways, this chapter will focus on the training program-induced stresses (i.e. increased training volume and/or intensity). It should also be noted that 'overreaching' and 'overtraining' refer to the actual stimulus, and the term 'overtraining syndrome' refers to the resulting condition. As the overtraining literature is reviewed, it will become readily apparent that much of the information must be extrapolated from research on overreaching and stressful training phases. This is certainly due to the difficulty in observing this phenomenon in a controlled environment. Additionally, the authors would like to point out that actual long-term performance decrements due to overtraining do not occur as often as many might think. What many athletes and coaches call overtraining is actually overreaching, and recovery is relatively easy.

Symptoms of overtraining. Perhaps the overarching question when examining the topic of overtraining is how can it be detected before it becomes a chronic problem? Unfortunately, experts on the topic acknowledge that no specific, simple, and reliable parameters are known to diagnose overreaching and overtraining in the earliest stage (Kuipers 1998). Regardless, overtraining research has suggested many possible markers of an impending or existing overtrained state. Over 15 years ago, an excellent review of the topic suggested over 80 possible major symptoms in addition to performance decrements (physiological = 40 symptoms, psychological and information processing = 12, immunological = 14 and biochemical = 18) (Fry, R.W. *et al*. 1991). Subsequent years of study have only added to the list. Of particular interest to the present chapter are the numerous endocrine and neuroendocrine symptoms and responses to overtraining.

Physiological mechanisms. Obviously, any condition as complex as overtraining, is influenced by numerous physiological systems. Previous reviews have addressed the contributions of neuromuscular control (Lehmann *et al*. 1999b), tissue trauma (Smith 2004), the immune response (Robson 2003), psychological (Morgan *et al*. 1987; O'Connor 1997) and clinical depression (Armstrong & Van Heest 2002), to name a few. It has also been suggested that women may be more susceptible to overtraining (Shephard 2000), and research efforts are beginning to address the issue of gender (O'Connor *et al*. 1989; Uusitalo *et al*. 1998).

The central fatigue hypothesis has also been presented as a possible contributor to overtraining (Newsholme *et al*. 1992). This theory is based on the premise that the exercise-induced increase in circulating concentrations of the amino acid tryptophan ultimately result in elevated levels of serotonin (5-hydroxytryptamine, 5-HT) in the the brain. The neurotransmitter serotonin has been associated with fatigue-like conditions, and could conceivably contribute to an overtraining syndrome. It has been suggested that dietary supplementation with the branched chain amino acids (BCAA; leucine, isoleucine, valine) would minimize central uptake of tryptophan, resulting in an avoidance of

performance impairment. It should be noted, however, that thus far much of the research results are not supportive concerning the link between BCAA supplementation, 5-HT and performance decrements due to overtraining (Fry, A.C. *et al*. 1993; Meeusen *et al*. 1996).

Endocrine-related mechanisms. Perhaps the most studied overtraining-related physiological system is the endocrine and neuroendocrine system. In 1986, Adlercreutz and colleagues reported the relationship between overtraining and the anabolic hormone testosterone, the catabolic hormone cortisol and their ratio (testosterone/cortisol, T/C, and free testosterone/cortisol, FT/C) (Adlercreutz *et al*. 1986). From an endocrinologist's perspective, their suggestion that these ratios were indicative of the anabolic–catabolic status of the body was probably an oversimplification. However, these hormones and their ratios have repeatedly been shown to alter in response to physical stresses such as overreaching and overtraining (Häkkinen *et al*. 1987; Fry, A.C. *et al*. 1993). Closer examination of the hypothalamic–pituitary regulation of these hormones during overtraining has suggested the potential role of this axis on regulating peripheral concentrations of the stress-related hormones (Barron *et al*. 1985; Wittert *et al*. 1996; Urhausen *et al*. 1998).

Eventually, an important role of the autonomic nervous system (ANS) in an overtraining syndrome was suggested (Stone *et al*. 1991; Lehmann *et al*. 1998a). Two types of ANS-based overtraining have been termed the sympathetic and the parasympathetic overtraining syndromes (Stone *et al*. 1991; Lehmann *et al*. 1998a). It has been suggested that the sympathetic syndrome occurs prior to the parasympathetic syndrome. The sympathetic type is associated with a predominance of sympathetic nervous system activity, while the parasympathetic type is associated with a predominance of parasympathetic nervous system activity. As the individual attempts to respond to the stressful training, sympathetic activity is elevated in an attempt to cope. As the stress progresses, the sympathetic system becomes exhausted, resulting in parasympathetic predominance. Eventually, central mechanisms (i.e. brain neurotransmitters) contributing to this condition have been suggested (Meeusen 1999). Some of the recent advances in assessment of these types of overtraining syndromes have been based on the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes, as well as sympathetic activity (Meeusen *et al*. 2004; Steinacker *et al*. 2004). Detailed examination of these topics will be provided later in this chapter.

Not all sports are alike. Much of the overtraining-related research has focused on endurance types of sports and activities (Kuipers & Keizer 1988; Fry, R.W. *et al*. 1991; Lehmann *et al*. 1993a, 1998b; Keizer 1998; Urhausen *et al*. 1998). However, it was pointed out by van Borselen *et al*. (1992) that overtraining with heavy resistance exercise was likely to present very different responses and symptoms. Later reviews of subsequent resistance exercise overtraining research have reinforced this belief (Fry & Kraemer 1997; Fry, A.C. 1998 & 1999; Stone & Fry 1998; Fry, A.C. *et al*. 2001). More recently, it is becoming apparent is that overtraining for many sports involve considerable contributions from a combination of aerobic training, resistance exercise, and sport-specific conditioning (Chicharro *et al*. 1998; Gorostiaga *et al* 1999; Murlasits *et al*. 1999; Kraemer *et al*. 2004; Maso *et al*. 2004). As such, the characteristics of overtraining, and the resulting overtraining syndrome, are extremely dependent on the physiological characteristics of the specific sport. Therefore, this chapter will address the topic of overtraining based on the type of activities being studied.

Overtraining in endurance sports

Endurance sports and activities defined. A sport which involves continuous activity over a longer time requires a physical capability defined as endurance. Endurance can be defined as the maximum duration for which an exercise intensity can be sustained; the relation between intensity or velocity and maximum time can be described by a typical logarithmic power–duration curve. Endurance training shifts this curve up so that an exercise intensity can be sustained with longer duration and a given time frame with higher intensity. Endurance activities

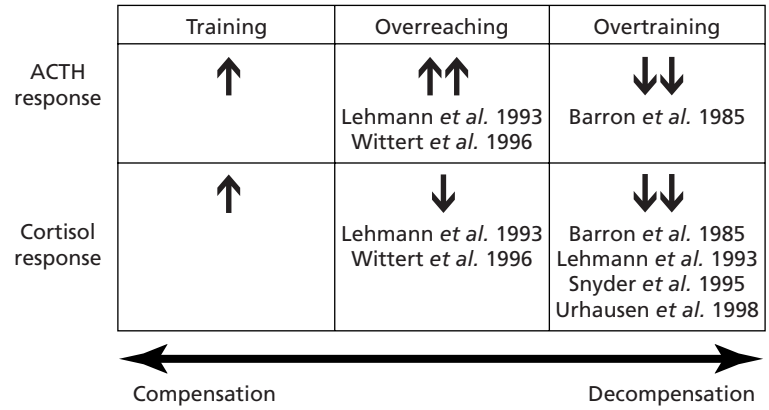

Fig. 37.2 Response of the hypothalamic–pituitary–adrenal (HPA) axis during normal training, overreaching and overtraining. When the body is able to adequately react to to the metabolic stresses of training, adrenocorticotropic hormone (ACTH) and cortisol are capable of responding (compensation). During overreaching, the cortisol response is attenuated even though the ACTH signal is augmented. During overtraining, both the ACTH and cortisol responses are blunted or missing (decompensation). (Modified from Lehmann *et al*. 1999b; Steinacker *et al*. 2004.)

can be classified according to the maximum duration in short-type (up to 4 min), middle-type (up to 15 min) and long-type endurance (longer than 15 min). Many activities have endurance components such as in ball games in which endurance exercise with lower intensity is combined with the sports specific exercise pattern.

Endurance exercises can be also defined by metabolic activity which involves mainly aerobic pathways—Krebs cycle and respiratory chain—and by the compartment—the mitochondria. The predominant substrate metabolism is fat and fat oxidation, but at higher exercise intensities, or with lower fitness, carbohydrates are also oxidized; for example, glucose and glycogen stores can be depleted (Baldwin *et al*. 1975; Richter *et al*. 2001).

Training often consists of repetitive phases of normal training, high training load phases, overload training phases, overreaching and recovery. During a training program, training load (defined by intensity, duration and frequency of exercise) varies and should gradually increase in response to the training-induced adaptation of various physical systems and in performance. This increase in training load is necessary to ensure further responses to a training program.

Coaches often organize training in phases in which training loads reach the athletes' capacities, followed by phases in which regeneration processes should predominate training. Such training cycles permit adjustment of training loads at high, sustainable levels for a short time, and are relatively safe (Steinacker *et al*. 1998). The process is called a supercompensation cycle (Fig. 37.2) and is also referred to as reaching. The exhaustion and fatigue in the high load training phases should elicit corresponding cellular stresses, and should consequently raise performance in the recovery phases as an adaptation to the training overload (Lehmann *et al*. 1997b). In such training cycles, fatigue is a common reaction in high load training phases and can be labeled as short-type overtraining (Lehmann *et al*. 1993a). This process can be described as a prolonged or accumulated acute stress response, and can be compensated by adequate recovery (Lehmann *et al*. 1997b).

The first and classical training experiments were designed to clearly distinguish the effects of volume and intensity, keeping in mind that there is a close relationship between exercise duration, intensity and substrate availability. The training group studied by Lehmann and colleagues involved experienced middle- and long-distance runners who performed 2 consecutive years of controlled training. Each year involved 4 weeks of standardized normal training, followed by: (i) an unaccustomed ~ 35% increase in training volume (ITV) for 4 weeks; or (ii) by a ~ 51% increase in training intensity (ITI) for 4 weeks (Lehmann *et al*. 1991). In a further study, Lehmann *et al*. (1993b) examined the effect of 6 weeks of endurance training versus interval training and 3

weeks of recovery in recreational athletes on serum hormone levels and pituitary function (endurance-intensity training, EIT).

Increases in endurance training volume. In ITV, classical symptoms of overtraining occurred such as fatigue, neuromuscular dysfunction and performance incompetence. At rest and during submaximal exercise, plasma levels of lactate, glucose, glycerol, free fatty acids and amino acids decreased. The nocturnal excretion of catecholamines decreased (Lehmann *et al*. 1991, 1992a). In the endurance arm of EIT (Lehmann *et al*. 1993b), adrenocorticotropic hormone (ACTH) was increased, prolactin, thyroid-stimulating hormone (TSH) and somatotropic hormone (STH) were unchanged. After pituitary stimulation, cortisol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release were decreased. Endurance training in EIT had no effect on peripheral hormone levels (cortisol, aldosterone, insulin, prolactin) except for a small decrease in free testosterone (Lehmann *et al*. 1993b).

Increases in endurance training intensity. In ITI, only a small, but significant, decrease in plasma catecholamines at a given exercise intensity was observed, which can be attributed to altered release and neuronal activity. The nocturnal excretion of catecholamines decreased significantly, but to a much lower extent than in ITV (Lehmann *et al*. 1991).

Mechanisms of endurance overtraining related to the endocrine system. During acute endurance exercise bouts, when muscle and liver glycogen are diminished, the normal hormonal reaction involves hypercortisolism and hypoinsulinism to maintain fuel homoestasis. With prolonged training, metabolic imbalance is prolonged and dysfunction of the HPA axis and hypoinsulinism may occur (Lehmann *et al*. 1999a). A similarity with the catabolic effects of calorie restriction is striking (Jenkins *et al*. 1993; Laughlin & Yen 1996; Argente *et al*. 1997). The hypothalamic down-regulation in overreaching and overtraining was described by Barron *et al*. (1985) and Lehmann *et al*. (1993b), who observed that insulin-dependent hypoglycemia or attenuated corticotropin-releasing hormone (CRH) corresponded with a lower ACTH and/or cortisol response in the state of overtraining.

The HPA axis is biphasically regulated. First, the acute stress and high metabolic load during an overreaching phase will lead to activation of the CRH and ACTH response and to a moderate adrenal desensitivation, meaning less cortisol response in relation to ACTH levels. Secondly, chronic stress and overtraining will lead to central and peripheral down-regulation (Lehmann *et al*. 1997b; Steinacker *et al*. 2004). Therefore, basal cortisol levels may be used for analysis of the effects of training (Steinacker *et al*. 2000). At an early stage during the 1996 training camp, when training load was highest, basal cortisol levels increased by 18% and decreased slightly afterwards (Steinacker *et al*. 2000). Increases in basal cortisol levels are indicative of a metabolic problem (e.g. glycogen deficiency) or of increased training-dependent stress (Lehmann *et al*. 1997b). A common metabolic cause can be seen in glycogen depletion with increased counterregulatory activity of hormones such as cortisol, growth hormone (GH) and catecholamines (Gray *et al*. 1993). Elevated basal cortisol levels are often seen as a normal stress response to high intensity training, whereas decreased basal cortisol levels and decreased pituitary–adrenocortical responsiveness are a late sign of overreaching or overtraining (Lehmann *et al*. 1993a, 1993b, 1997b). As discussed above, basal cortisol levels reflect an endpoint of the hypothalamic–pituitary–adrenocortical axis. Testing the axis by functional tests will give much more information, but they are time- and cost-consuming and are stressful for the athletes (Kuipers & Keizer 1988; Lehmann *et al*. 1998b; Steinacker *et al*. 2004).

Somatotropic hormonal reactions. The somatotrophic growth hormone–insulin-like growth factor I (GH–IGF-I) axis is also involved in this hormonal regulation. The pulsatile pattern of GH is stimulated and levels of GH rise by acute prolonged exercise (Kanaley *et al*. 1997). IGF-I levels increase, consecutively, after 1–2 weeks of training with moderate intensity and positive caloric balance (Snyder, D.K. *et al*. 1989; Engfred *et al*. 1994; Roelen *et al*. 1997; Roemmich & Sinning 1997) and decrease more profoundly when training is exhausting and catabolic

mechanisms are predominant (Koistinen *et al*. 1996). During prolonged exhausting training, the pulsatility of GH and IGF-I levels decrease simultaneously (Eliakim *et al*. 1998; Schmidt *et al*. 1995). There is evidence that depression of the GH–IGF-I axis is a common finding during overreaching. The effects are related to down-regulation of the HPA axis (Barron *et al*. 1985; Steinacker *et al*. 1998, 2004). Furthermore, down-regulation of the thyroid and gonadotropic axes will be observed (Lehmann *et al*. 1997b).

Leptin, an adipocyte-derived hormone, is involved the hypothalamic regulation of appetite, thermogenesis and metabolism (Friedman *et al*. 1997; Heiman *et al*. 1997). Leptin depresses the excitatory transmitter neuropeptide Y (NPY) expression in hypothalamic neurons (Carro *et al*. 1998). NPY neurons are activated by fasting and express a high degree of leptin receptors (Schwartz *et al*. 1996; Baskin *et al*. 1999). Increased NPY levels increase hypothalamic neuronal activity and therefore depress the HPA axes. Leptin is an example of a tissue hormone that has direct impact on the hypothalamic regulation and provides information about the metabolic state to other tissues like β cells or liver cells. Leptin is a potent stimulator of pulsatile GH secretion and the GH response to GH-releasing hormone (Tannenbaum *et al*. 1998). Leptin plasma levels are low and apparently decrease with training load (Leal-Cerro *et al*. 1998; Simsch *et al*. 2002). Whether leptin has the potential as a marker for overreaching or overtraining has to be clarified in prospective studies (Steinacker *et al*. 2004).

Muscle adaptations and heat shock proteins. Energy disturbances and glycogen depletion in skeletal muscle are common findings in early phases of overtraining, but it is now clear that restoring muscular glycogen will not decrease symptoms of overtraining (Lehmann *et al*. 1997b; Snyder, A.C. 1998). Muscular glycogen depletion is postulated as one of the metabolic causes that will initiate an overtraining syndrome in which peripheral thyroidal hormones and GH levels decrease as well as levels of IGF-I (Lehmann *et al*. 1997b; Steinacker *et al*. 1999).

All hormonal effects together will cause a shift in expression of myosin heavy chain expression in the direction of slow isoforms, and will lead to the expression of slow type fibers (Atalay *et al*. 1996; Pette & Staron 1997). Glycogen deficiency is associated with increased expression of local cytokines (interleukin-6) (Pedersen *et al*. 2001), decreased expression of glucose transporters (Richter *et al*. 2001), and increased cortisol and decreased insulin secretion and β-adrenergic stimulation which lead also to the loss of fast fibers (Pette & Staron 1997). The overtraining myopathy results from these metabolic and hormonal changes, and may be diagnosed in muscle biopsies by a shift to slow type myosin and a decreased number and fiber area of fast type fibers (Atalay *et al*. 1996; Steinacker *et al*. 2004).

Heat shock proteins are increased in muscle, possibly as hormonal molecular chaperones. A single bout of exercise leads to induction of heat shock protein 70 (HSP70) transcription but not of effective protein production; protein production rate is high after a week of training and peaks after approximately 2 weeks (Liu *et al*. 1999). High intensive training leads to an induction of HSP70 while endurance training with low intensity does not. It can be concluded that HSP70 response to training is dependent on exercise intensity rather than exercise volume (Liu *et al*. 2000). Whether accumulation of heat shock proteins is protective, or indicates a training response, or is related to trainability or stress stability has to be determined in further studies (Steinacker *et al*. 2004).

Intrinsic sympathetic activity and catecholamines during endurance overtraining. Increased intrinsic sympathetic activity may be found in the early phase of heavy training (according to the above mentioned classical model) which is an adaptive regulatory process (Fry, R.W. *et al*. 1991; Lehmann *et al*. 1997b, 1998a). However, if hypothalamic depression is more profound, intrinsic sympathetic activity decreases as indicated by decreased nocturnal catecholamine excretion and peripheral β-receptor down-regulation with compensatory increased plasma noradrenaline but ineffective catecholamine response (Lehmann *et al*. 1992b, 1998a; Wittert *et al*. 1996). The excretion of free catecholamines during overnight rest can be seen as the basal renal (urinary) catecholamine excretion reflecting the intrinsic activity or tone of the sympathetic nervous system, as activating

mechanisms are clearly reduced during night rest. Because noradrenaline concentrations in plasma and in cerebrospinal fluid are quite similar, circulating and excreted noradrenaline may also reflect the neuronal noradrenaline release in the brain. In overreaching and overtraining, resting and submaximal plasma noradrenaline concentrations may be increased and β-adrenoreceptor density is decreased (Jost *et al*. 1990; Lehmann *et al*. 1998a; Uusitalo *et al*. 1998). Down-regulation of the intrinsic sympathetic nervous system activity is a late finding in overtrained athletes and may be related to symptoms of fatigue (Lehmann *et al*. 1992b).

Neuromuscular excitability. There is also indication of desensitization of the motor end-plate (for an overview see Lehmann *et al*. 1998b). These findings are related to the subjective feeling of some overtrained athletes that their muscles may not respond as normally and of peripheral weakness. The hypothesis was tested that a deterioration in neuromuscular excitability (NME) also indicates an early stage in the overtraining process during high intensity training (Lehmann *et al*. 1997a). In the EIT trial, NME was slightly improved after 3 weeks of training, but deteriorated after 6 weeks, and was again normalized after 2 weeks of regeneration (Lehmann *et al*. 1995). The discrepancy between normalization of NME and still-deteriorated performance ability after 2 weeks of regeneration reflects additional significant, and probably central, mechanisms that explain persistent performance incompetence in overtraining. Deterioration in NME may indicate an early stage in the overtraining process during high-volume as well as high-intensity endurance overtraining, but normalization does not necessarily indicate sufficient regeneration (Lehmann *et al*. 1995, 1999b).

Overtraining in strength sports and activities

Resistance exercise and related sports. Many activities require considerable contributions from both anaerobic and aerobic energy systems (e.g. soccer, team handball, rugby, American football, military preparation). As such, these activities either use heavy resistance exercise for supplemental training or develop high muscular force and power during their events. Needless to say, these sports/activities are difficult to study due to their complex nature. Regardless, several attempts have been made to examine the manifestation of overtraining in these activites. It is likely that each of these studies actually observed states of overreaching rather that overtraining.

When supplemental heavy resistance exercise has been added to the sport-specific training program for American football (Murlasits *et al*. 1999) or team handball (Maso *et al*. 2004), decreases in resting levels of total testosterone and/or the T/C ratio have been reported. When interpreting these data, it must also be noted whether other aspects of the training program were concurrently altered. For example, Murlasits *et al*. (1999) observed that the addition of resistance exercise alone was not problemmatic, but when the volume of related conditioning drills was increased (i.e. sprints, agility, metabolic conditioning, etc.), attenuated resting testosterone levels resulted. These results should not be interpreted to mean resistance exercise should be avoided, but rather the cumulative training stresses of the entire program must be considered when determining the proper resistance exercise volume and intensity.

In the sport of rugby where high muscular forces are common, overtraining (as determined by questionnaire) was accompanied by decreased concentrations of resting total testosterone (Maso *et al*. 2004). With the limited data available about sports of this type, it appears that resting testosterone and the T/C ratio may be most sensitive to the training stresses. Indeed, during a collegiate soccer season, Kraemer *et al*. (2004) observed that those players beginning the season with the lowest T/C ratio also experienced the greatest performance decrements as the season progressed. In a non-sport setting, Chicharro *et al*. (1998) reported that the FT/C ratio identified Special Forces soldiers who appeared to respond poorly to the specialized training for these elite troops. As previously mentioned, it is likely that these situations represent overreaching and not true overtraining. It is readily apparent that much study needs to be done on these types

of activities, and that the T/C and FT/C ratios are sensitive enough to respond to the changing training stresses.

Increased resistance exercise volume. A more thorough understanding of resistance exercise overtraining requires an understanding of the variables that contribute to the resistance exercise training stress (Kraemer & Nindl 1998; Fry, A.C. 1999). For a single training session, these include the choice of exercise, order of exercise, exercise volume, exercise intensity and inter-set rest intervals. For the long-term resistance exercise program, this includes the variation inherent with a periodized training program. In order to more effectively determine the roles of each of these variables, it becomes necessary to attempt to control some or all of these variables for research purposes. Of course, it is not possible to completely eliminate the role of any of these training variables because of their interdependence.

The ability of chronic (≥ 2 yrs) training in elite competitive Olympic-style weightlifters to increase resting testosterone levels has been reported by Häkkinen *et al*. (1987). Although the magnitude of this change was small it, nevertheless, illustrates the plasticity of the endocrine system in highly resistance trained athletes. In a classic study from the same laboratory, changes in the training volume load (mass × repetitions; kilogram) for the Finnish national weightlifting squad were shown to be related to testosterone and cortisol levels and the T/C ratio (Häkkinen *et al*. 1987). As the volume load increased, resting concentrations of testosterone and the T/C ratio decreased, while resting cortisol increased. This pattern was reversed as the volume load was subsequently decreased. Although not an overtraining study, these findings demonstrate that changing training volume is reflected in the hormonal profile.

These findings were supported when high volume training of junior-age weightlifters resulted in an overreached state (Fry, A.C. *et al*. 1993). As was seen with the Finnish lifters, testosterone and the T/C ratio decreased, and cortisol increased when training volume was increased from 3–5 sessions per week to 2–4 sessions per day for a 1-week period. These decreases, however, were apparent for not only resting levels, but also for the exercise-induced responses. It appears that prior weightlifting training experience of ≥ 2 years can significantly influence the hormonal response to a single bout of exercise (Kraemer *et al*. 1992). Furthermore, it was subsequently demonstrated that weightlifters who had previous experience undergoing an overreaching phase of training were less impaired when exposed to a second overreaching phase 1 year later (Fry, A.C. *et al*. 1994b). This second overreaching phase exhibited an actual increase in both total testosterone and the T/C ratio at rest and in response to a standardized training session. The cortisol response did not change. Additionally, the most highly ranked lifters (but not more experienced) exhibited no relationship ($r = 0.00$) between relative (%) changes in performance and relative changes in the T/C ratio during the overreaching phase (Fry, A.C. *et al*. 2000). On the other hand, the relative change in T/C during this phase for lower ranked lifters was negatively related ($r = -0.70$) to relative changes in performance. Both groups of lifters appeared to benefit from the subsequent training taper phase, but the highly ranked lifters responded especially well from both performance and endocrine perspectives. As the T/C ratio increased during the taper, the increase in performance also increased ($r = 0.92$) for the highest ranked lifters, and less so for the lower ranked lifters ($r = 0.51$). Overall, the changes in the training volume as well as the actual weightlifting performances were strongly reflected by the testosterone and the T/C ratio.

Although study of the sport of weightlifting permits examination of a closely monitored training environment, many athletes incorporate more generic types of resistance exercise into their training. Several studies have examined the endocrine responses to increases in resistance exercise training volume, although this increase was accomplished differently for these studies. Raastad *et al*. (2001) increased the training volume load approximately fourfold (from < 20 000 $kg \cdot wk^{-1}$ to > 76 000 $kg \cdot wk^{-1}$) while training everyday for 2 weeks. Volek *et al*. (2004) and Ratamess *et al*. (2003) increased resistance training volume for 4 weeks by training all body parts five times per week. For their protocol, they

progressed from approximately 250 repetitions per day during week 1 to 75 repetitions per day for week 4. Concurrently, relative training intensity increased from 10–12-repetition maximum (RM) loads during week 1 to 3-RM loads for week 4. Strength perfomance was adversely affected in each of these studies, although an overreaching state appeared to result rather than an advanced overtraining syndrome.

In all three studies, resting concentrations of total testosterone decreased when volume load was increased (Raastad *et al*. 2001; Ratamess *et al*. 2003; Volek *et al*. 2004). Although a similar decrease was not apparent for direct measures of free testosterone, the ratio of testosterone to sex hormone binding protein (T/SHBG) did decrease, most likely due to a training-induced increase in the absolute concentrations of SHBG (Ratamess *et al*. 2003; Volek *et al*. 2004). As a result, not only did total testosterone decrease, but the bioavailable portion of testosterone also decreased. When several days of recovery were monitored, testosterone returned to baseline levels (Raastad *et al*. 2001). The decreases in testosterone occurred despite no alterations in LH. No changes or slight transient increases were observed for resting cortisol concentrations (Raastad *et al*. 2001; Ratamess *et al*. 2003; Volek *et al*. 2004). As with LH, no changes in ACTH were reported (Raastad *et al*. 2001), suggesting a decreased sensitivity of the Leydig cells in the testes and of the adrenal cortex to circulating levels of LH and ACTH, respectively. It is important to note that, to the authors' knowledge, no overtraining study has measured pulsatility characteristics of these trophic hormones, and thus these LH and ACTH data represent preliminary results. Only Raastad *et al*. (2001) examined measures related to the FT/C ratio, but reported that this was not altered.

In addition to the steroid hormones, increased resistance training volume has sometimes resulted in slight increases in resting levels of immunoreactive GH (Ratamess *et al*. 2003), although circulating levels of IGF-I have reportedly been unaffected (Raastad *et al*. 2001; Volek *et al*. 2004). Since IGF-I functions not only in an endocrine fashion, but also in paracrine and autocrine fashions, it is likely that these circulating values do not tell the entire story. Insulin concentrations have also been reported to decrease due to high volume resistance exercise overreaching (Ratamess *et al*. 2003; Volek *et al*. 2004). Although the insulin response appeared to be related to decreased glucose concentrations (Ratamess *et al*. 2003), the impact of this anabolic hormone on skeletal muscle and its function during overtraining remains to be determined. Only one resistance exercise study has reported augmented resting (nocturnal urinary) levels of epinephrine and norepinephrine in response to overreaching (Raastad *et al*. 2001). Such a response would be indicative of a sympathetic overtraining syndrome.

Increased resistance exercise intensity and power. In an attempt to determine the effects of resistance exercise relative intensity (% 1-RM) on the etiology of an overtraining syndrome, a series of studies were performed to isolate this training variable. When maximal loads were used daily (10 × 100% 1-RM) for 2 weeks on a squat simulating machine, 1-RM performance was depressed and required 2–8 weeks for recovery (Fry, A.C. *et al*. 1998). Contrary to what has been observed with high volume resistance exercise overtraining, total testosterone was unchanged at rest, and actually increased slightly in response to maximal exercise. This occurred simultaneously with a slight decrease in acute LH (preliminary data), suggesting that a mechanism other than the gonadotrophins were responsible for the testosterone response. Bioavailable testosterone was not altered as indicated by FT and %FT. No change was observed for resting levels of cortisol, but acute responses of cortisol actually decreased slightly. As with LH, no change was observed for single time point ACTH (preliminary data), again suggesting that the trophic hormone for cortisol was not responsible for the acute response. In actuality, the modest to non-existent hormonal responses to this high intensity protocol is not overly surprising, since it has been previously shown that there is little or no acute hormonal response to a single bout of high intensity, low volume resistance exercise (20 × 100% 1-RM) (Häkkinen & Pakarinen 1993).

Of particular interest for the high intensity resistance exercise overtraining protocol was the increased acute response of circulating concentrations of both

epinephrine and norepinephrine (Fry, A.C. *et al*. 1994a). When resting levels of catecholamines were monitored via nocturnal urine, only slight increases were observed (effect size, ES = 0.42), suggesting that the acute catecholamine responses are more sensitive to this high intensity overtraining protocol (Fry, A.C. *et al*. 2004b). As with Raastad *et al*.'s (2001) report for high volume resistance exercise, these results are symptomatic of the sympathetic overtraining syndrome. Attempts to non-invasively monitor sympathetic activity in this overtraining model via heart rate variability has not been successful to date (Weir *et al*. 2002). It was interesting to note, however, that although the relative changes (%) for the circulating catecholamines were strongly correlated to various measures of muscular force ($r = 0.79–0.96$) for the control subjects, none of these relationships were significant for the overtrained subjects. These results suggest that the high intensity resistance exercise overtraining protocol decreased sympathetic regulation of peripheral muscle activity. It should be noted that several of the pre-exercise hormonal responses (i.e. T, FT, T/C, FT/C) to this protocol were also correlated to muscular force measures ($r = 0.83–0.99$) for only the control subjects (Fry, A.C. *et al*. 1998). In the same study, significant correlations between the catecholamines and the hormonal responses were also observed (unpublished data), so it is speculated that, to some extent, the sympathetic system is driving both the acute hormonal responses as well as muscular performance. Whether the hormonal environment is influencing acute muscle performance is not clear.

In an attempt to understand some of the underlying physiological mechanisms for decreased performances with high intensity resistance exercise overtraining, skeletal muscle β_2-receptor density was examined (Fry, A.C. *et al*. 2004b). Not surprisingly, a 37% decrease in β_2-receptor density was observed after 2 weeks of the high intensity overtraining. When receptor densities were combined with resting catecholamine data, the ratio of epinephrine/β_2-receptor density (EPI/β_2) exhibited an extremely large increase (ES = 1.16). Although far from conclusive, these data are evidence of a decreased catecholamine sensitivity such as reported by Lehmann *et al*. (1998b) for endurance athletes.

Due to the apparent sensitivity of high velocity and power performances, recent advances in this line of study have examined resistance exercise overtraining using excessive high power resistance exercise (Fry, A.C. *et al*. 2004a). An overtrained state was induced by performing 10×5 speed squats at 70% 1-RM for 15 sessions over 7.5 days. As expected, no changes in resting levels of testosterone, cortisol or the T/C ratio were observed. Furthermore, nocturnal urine assessments exhibited no changes in the catecholamines. Although β_2-receptor density was not altered, the EPI/ β_2 increased dramatically. These results are similar to what was observed for the high intensity overtraining, and again indicate a decreased catecholamine sensitivity (Lehmann *et al*. 1998b).

Endocrine mechanisms of resistance exercise overtraining. When all the data available on resistance exercise overtraining are summarily examined, several physiological patterns emerge. First, during high intensity or high power overtraining, acute hormonal responses appear to change before resting levels do. Additionally, one of the 'classic' symptoms of overtraining (i.e. decreased testosterone) does not occur, rather there may actually be a slight increase. Peripheral maladaptation appears to occur via down-regulation of the β_2-receptors in skeletal muscle, resulting in a decreased catecholamine sensitivity. All of these symptoms are consistent with the sympathetic overtraining syndrome. Secondly, during high volume overtraining, the endocrine responses appear somewhat like they do with endurance overtraining. This is, testosterone is typically attenuated, while cortisol and the T/C ratio are either unaffected or attenuated. On the other hand, increases in sympathetic activity are beginning, at least at rest. While these are far from the classic parasympathetic overtraining syndrome, it is reflective of an overtrained state that is transitioning from a sympathetic type to one taking on a few of the characteristics of the parasympathetic type. To date, the data available on peripheral maladaptation of the β_2-receptors leaves much to question. Are these receptors disappearing or are they

just being turned off via phosphorylation or internalization? Are the β_2-receptor affinity or binding characteristics being altered? How do these peripheral maladaptations ultimately influence central regulation (i.e. central neurotransmitters, ANS, hypothalamic–pituitary axis) of the body? These and more questions remain to be studied.

Central regulation during overtraining

Is the brain involved? Over the last few years, there has been significant interest in determining specific peripheral markers for the metabolic, physiological and psychological responses to exercise that it is suggested are associated with overtraining. To date, relatively little attention has been placed on the role of the central nervous system in overtraining and fatigue during exercise and training (Meeusen 1999). We will focus on the involvement of the central nervous system and the role of neurotransmitters and neuromodulators in possible mechanisms that underly overtraining.

For several years it has been hypothesized that a hormonal mediated central dysregulation occurs during the pathogenesis of overtraining, and that measurements of blood hormones could help to detect the overtraining syndrome (Kuipers & Keizer 1988; Fry, R.W. *et al*. 1991; Urhausen *et al*. 1995, 1998; Fry & Kraemer 1997; Keizer 1998). The results of the research devoted to this subject is far from unanimous, mostly because of the differences in measuring methods, and/or detection limits of the analytical equipment used.

For a long time, the T/C ratio was considered a good indicator of the overtraining state. This ratio decreases in relation to the intensity and duration of training and, as previously mentioned, it seems likely that this ratio is an indicator of the actual physiological strain of training, rather than overtraining (Urhausen *et al*. 1995; Meeusen 1999). Most of the literature agrees that the many types of overtraining and overreaching must be viewed on a continuum, with a disturbance, an adaptation and, finally, a maladaptation of the HPA axis (Lehmann *et al*. 1993a, 1998b, 1999a, 1999b; Urhausen *et al*. 1995, 1998; Keizer 1998; Meeusen 1998, 1999). The HPA axis adaptation is often characterized by increased pituitary ACTH release. In the maladaptation stage, a decreased rise in pituitary hormones (ACTH, GH, LH, FSH) is reported (Barron *et al*. 1985; Lehmann *et al*. 1993b, 1998b, 1999a, 1999b; Urhausen *et al*. 1995, 1998; Wittert *et al*. 1996). But behind the seemingly uniform acute hormonal response to exercise, explaining the disturbance to the neuroendocrine system caused by overtraining is not that simple. The trigger that eventually leads to overtraining may be any of a number of mediators with separate regulatory mechanisms.

Which neurotransmitters? The observation that other stressors in addition to exercise (job, social, travel, inadequate nutrition, etc.) seem to predispose an athlete to overtraining (Foster & Lehmann 1997), links overtraining with the psychosocial signs of the maladaptive response to intense training. The work of Morgan and colleagues (Morgan *et al*. 1987, 1988; O'Connor *et al*. 1989, 1991; Raglin & Morgan 1994; O'Connor 1997) clearly indicates that psychological factors, especially mood state disturbances, are effective in predicting the onset of overtraining. The symptoms associated with overtraining, such as changes in emotional behavior, prolonged feelings of fatigue, sleep disturbances and hormonal dysfunctions are indicative of changes in the regulation and co-ordinative function of the hypothalamus (Meeusen 1999; Armstrong & Van Heest 2002). The hypothalamus is under the control of several 'higher' brain centers and several neurotransmitters (Meeusen & De Meirleir 1995). Amongst these transmitters, serotonin (5-HT) is known to play a major role in various neuroendocrine and behavioral functions, for example activation of the HPA axis, feeding and locomotion (Wilckens *et al*. 1992). The possibility that impaired neurotransmission at the various central aminergic synapses is associated with major disturbances of the central nervous system such as depression (and possibly overtraining), has been receiving more attention over the past several years. It has been suggested that exercise exerts its putative psychological effects via the same neurochemical substrate (the monoamines) as the antidepressant drugs known to increase the synaptic availability of transmitters (Meeusen 1999; Armstrong & Van Heest 2002; Uusitalo *et al*. 2004).

The target brain regions of interest seem to be the basal ganglia, the limbic system, the hippocampus and the hypothalamus, which have been found to play a role in motor behaviors and emotional and motivational states (Dunn & Dishman 1991). Likewise, study of the neurobiological aspects of exercise may add to a more complete understanding of the etiology and treatment of overtraining.

There is evidence that central serotonergic systems act upon the sympathetic nervous system and the HPA axis, and that reciprocally glucocorticoids and catecholamines (derived from sympathetic nerves and the adrenomedulla) affect central serotonergic systems (Chaouloff 1993). In pathological situations such as in major depression (Dishman 1997), post-traumatic stress disorders (PTSD) (Liberzon *et al*. 1999; Porter *et al*. 2004) and probably also in overtraining, the glucocorticoids and the brain monoaminergic systems apparently fail to restrain the HPA response to stress (Meeusen *et al*. 2004).

Which mechanisms? As it has been shown that exercise and training influence neurotransmitter release in various brain nuclei (Meeusen *et al*. 1994, 1995, 1996, 1997, 2001), possible dysregulation at this level could play a key role in the maladaptation to the 'stress' of exercise, training and overtraining. Meeusen *et al*. (1997) showed that endurance training decreases basal neurotransmitter outflow in the striatum of rats, while maintaining the necessary sensitivity for responses to acute exercise. These observations raise the possibility that there could exist an exercise-induced change in receptor sensitivity (Meeusen *et al*. 1997).

There is an extensive amount of anatomical, biochemical and physiological data lending support to an excitatory influence of central serotonergic systems upon the HPA axis (Chaouloff 1993; Porter *et al*. 2004). Norepinephrine is also linked to the regulation of the HPA axis, which is dysregulated not only in overtraining (Keizer 1998), but also in conditions such as in major depression and certain anxiety disorders (Chaouloff 1993; Dishman 1997). In these conditions, glucocorticoids and brain monoaminergic systems apparently fail to restrain the HPA response to stress. Activation of the brain noradrenergic and serotonergic systems during stress is believed to increase the secretion of ACTH by stimulating the secretion of CRH (Tuomisto & Mannisto 1985; Plotsky *et al*. 1989).

It has been shown previously that there exists a controlling or modulating role of the serotonin system on the function of nigro-striatal and ventral tegmental-limbic dopamine systems (Palfreyman *et al*. 1993). The fact that raphe cell bodies are densely innervated with noradrenergic cell terminals emphasize that the locus coeruleus norepinephrine and the dorsal raphe serotonergic system reciprocally innervate each other (Tuomisto & Mannisto 1985). We have to point out that interaction of serotonergic, dopaminergic, noradrenergic and other neurotransmitter and neuromodulator systems could (directly or indirectly) influence central and peripheral parameters.

Traditionally, the main criterion for a stress response is an increase in the secretion of stress hormones. On this basis, a decline in hormonal secretion when stress is repeated or prolonged is commonly interpreted as indicating stress adaptation (Stanford 1993). A large body of evidence indicates that stressful experiences also alter neurotransmitter metabolism and release in several brain areas (Kuipers & Keizer 1988; Abercrombie *et al*. 1989; Weizman *et al*. 1989; Keefe *et al*. 1990; Nisembaum *et al*. 1991; Imperato *et al*. 1992; Gresch *et al*. 1994; Jordan *et al*. 1994; Finlay *et al*. 1995; Kirby *et al*. 1997). Repeated exposure to stress may lead to a different responsiveness to subsequent stressful experiences depending on the stressor as well as on the stimuli paired with the stressor. This in turn may lead to either an unchanged, increased or decreased neurotransmitter and receptor function. Behavioral adaptation (release, receptor sensitivity, receptor binding, etc.) in higher brain centers will certainly influence hypothalamic output (Lachuer *et al*. 1994). It has been shown that immobilization stress not only increases hypothalamic monoamine release, but consequently CRH and ACTH secretion (Shintani *et al*. 1995). Chronic stress and the subsequent chronic peripheral glucocorticoid secretion could play an important role in the desensitization of higher brain center responses during acute stressors, since it has been shown that in acute (and also chronic) immobilization, the responsiveness of

hypothalamic CRH neurons rapidly falls (Cizza *et al*. 1993). These adaptation mechanisms could be the consequence of changes in neurotransmitter release, depletion of CRH and/or desensitization of hypothalamic hormonal release to afferent neurotransmitter input (Cizza *et al*. 1993). In overtraining it is assumed that a 'maladaptation' to chronic exercise (and other) stress occurs. There is evidence that apart from other brain areas, the hippocampus down-regulates the HPA axis in both rats and primates. Thorré *et al*. (1997) analysed the impact of streptozotocin (STZ) -elicited diabetes (a chronic stress for the body) on hippocampal extracellular 5-HT levels both under basal conditions and during restraint stress. Restraint stress is a procedure known to stimulate 5-HT synthesis/metabolism and release. The chronic stress decreased hippocampal 5-HT metabolism, but did not alter baseline 5-HT release (compared to controls), while restraint stress increased extracellular 5-HT levels in controls, but did not increase 5-HT in the diabetic animals (Thorré *et al*. 1997). This may indicate that in the chronically stressed animals the central response to an acute stressor (i.e. restraint) is impaired.

The examples cited previously illustrate that in several brain nuclei, chronic stress will create an adaptation mechanism (autoreceptor mediated, neurotransmitter interactions, or other mechanisms). When an animal is confronted with a novel stressful stimulus, sensitization will occur. However, in chronic, very intense stress situations (as in STZ diabetes where other peripheral hormonal mechanisms also play an important role), this sensitization of hippocampal 5-HT release does not occur. One might speculate that in overtraining (the step beyond coping with stress), a comparable mechanism occurs. It remains to be elucidated if these results from a 'non-exercise' chronic stress situation can be translated to another chronic stress such as overtraining (Meeusen 1999).

Assessment of overtraining. In overtrained athletes, a number of signs and symptoms have been associated with this imbalance between training and recovery. However, reliable diagnostic markers for distinguishing between well-trained, overreached and overtrained athletes are lacking. A hallmark feature of the overtraining syndrome is the inability to sustain intense exercise, and a decreased sports-specific performance capacity when the training load is maintained or even increased (Urhausen *et al*. 1995; Meeusen *et al*. 2004). Athletes suffering from overtraining are able to start a normal training sequence or a race at their normal training pace, but are not able to complete the training load they are given, or race as usual. Meeusen *et al*. (2004) recently published a test protocol with two consecutive maximal exercise tests separated by 4 h. With this protocol they found that in order to detect signs of overtraining and distinguish them from normal training responses or just overreaching, this method is a good indicator, not only of the recovery capacity of the athlete, but also of the ability to normally perform the second bout of exercise. The use of two bouts of maximal exercise to study neuroendocrine variations showed an adapted exercise-induced increase of ACTH, prolactin and GH to two successive exercise bouts. Particularly in overtrained athletes, the 'overshoot' of these hormones in the first exercise bout, followed by a complete suppression in the second exercise bout is interesting (Fig. 37.3). This could indicate a hypersensitivity of the pituitary followed by an exhaustion afterwards. Previous reports that used a single exercise protocol found similar effects (Meeusen *et al*. 2004). It appears that the use of two exercise bouts is more useful in detecting overreaching for preventing overtraining. Early detection of overreaching may be very important in the prevention of overtraining.

Central mechanism conclusions. Several neurotransmitters might play an important role in the onset of the central dysfunctions that occur in the maladaptation to chronic exercise stress or overtraining. The multifactorial aspect of central nervous functioning with different effects of increased neurotransmitter release in different brain regions, the complexity of several receptors and the mutual interaction of various neurotransmitters (having excitatory and inhibitory features) illustrates that this multifactorial disturbance in the onset of overtraining could give rise to more questions than answers. What remains to be seen is how individual elements of the several neurotransmitters and neuromodulators

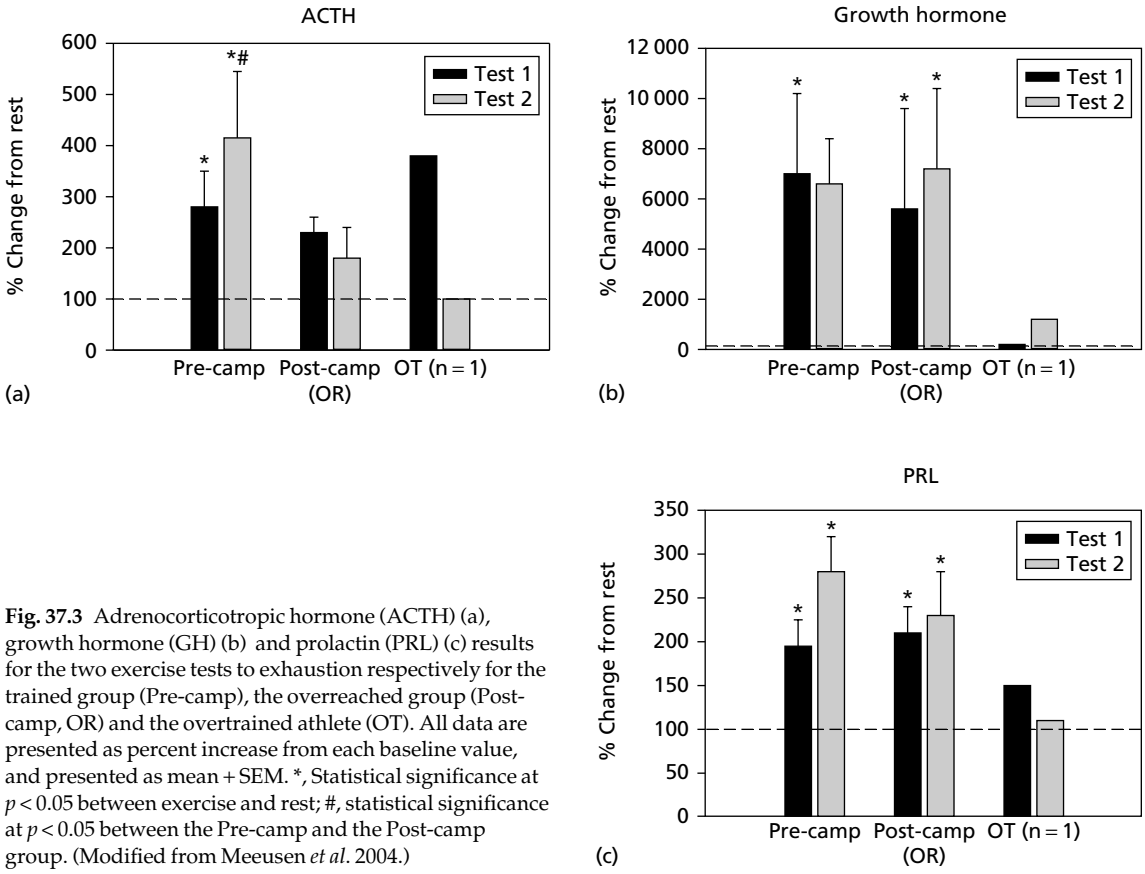

Fig. 37.3 Adrenocorticotropic hormone (ACTH) (a), growth hormone (GH) (b) and prolactin (PRL) (c) results for the two exercise tests to exhaustion respectively for the trained group (Pre-camp), the overreached group (Post-camp, OR) and the overtrained athlete (OT). All data are presented as percent increase from each baseline value, and presented as mean + SEM. *, Statistical significance at $p < 0.05$ between exercise and rest; #, statistical significance at $p < 0.05$ between the Pre-camp and the Post-camp group. (Modified from Meeusen *et al*. 2004.)

contribute to this scheme: how modulation of pre- and post-synaptic receptors, coupled with rapid or delayed changes in transmitter synthesis and release, accounts for the behavioral and performance impact of a stress called overtraining.

Finally, we need not look at the present research data as being totally contradictory or inconsistent, because it is clear that understanding brain function takes more than just one series of experiments. At this moment it seems that it will probably take more 'brains' than a human has available to understand only a part of brain functioning.

Summary

Needless to say, the neuroendocrine system in a healthy human body is an extremely complex scenario. When the additional complexities of overtraining are added to the story, it can become quite confusing. It is critical to remember that the actual definition of overtraining includes some type of long-term performance decrement. Even though some of the classical symptoms of an overtraining syndrome may be apparent, overtraining has not occurred until performance is adversely affected. Regardless, several aspects of overtraining and overreaching are becoming apparent. Much of the maladaptations associated with these performance decrements are due in part to regulation (or dysregulation) by the neuroendocrine system. How the various hormones and neurotransmitters respond is dependent on the type of exercise stress encountered. Although not all inclusive, Fig. 37.4 identifies some of the key endocrine and related variables that respond to the various types of training. What is readily apparent from this figure is that the

	Resistance exercise overtraining		Combined activities (team sports, military, occupational)	Endurance exercise overtraining	
	High intensity	High volume	High volume (usually)	High intensity	High volume
Testosterone	**NC**—rest ↑—acute LH–NC or ↓	↓ LH–NC	↓	**NC** LH–NC	↓ LH—↓
Cortisol	**NC**—rest ↓—acute ACTH–NC	**NC** or ↓ ACTH–NC	**NC**	**NC**—rest ↓—acute ACTH—↓ acute	↑ → ↓ ↑→↓ CRH & ACTH ↓sensitivity to ACTH
Tes/cort ratio	**NC**—rest ↓—acute	↓ or **NC**	↓	**NC**	↓
Catecholamines	**NC** or ↑—rest ↑—acute	↑ —rest		**NC** or ↓—rest **NC** ↓↑—acute	↓—rest ↑→↓—acute
β_2-Receptor density	**NC** or ↓ in skeletal muscle				↓ In lymphocytes
Catecholamine sensitivity	↓			↓	↓
Central nervous system					↑ 5-HT ↓ DA ↑ GABA ↓ NE ↓ GLU
Heat shock proteins (HSP70)			↑	↑	↑
Other		GH—↑ IGF-I— ↓ Insulin—↓	GH—NC or ↑ IGF-I—NC or ↓ Insulin—↑	GH—↓ acute Insulin—↓ acute	GH—↓ IGF-I— ↓ TSH—↓ T_3—↓ Leptin—↓ Insulin—↓ (rest)

Fig. 37.4 Overview of the endocrine and neuroendocrine responses and adaptations to various types of overtraining. ↑ = increase, ↓ = decrease; ACTH, adrenocorticotropic hormone; CRH, corticotrophin-releasing hormone; GABA, gamma-aminobutyric acid; GH, growth hormone; GLU, glucagon; IGF-I, insulin-like growth factor I; LH, luteinizing hormone; NC, no change; T_3, triiodothyronine; Tes/Cort, testosterone/cortisol; TSH, thyroid-stimulating hormone.

endocrine responses can be quite different depending on the physiological characteristics of the type of training. As a result, it is very important that those studying overtraining have an appreciation of the fundamental physiological and psychological differences inherent to each type of training and the sports that routinely utilize these training methods.

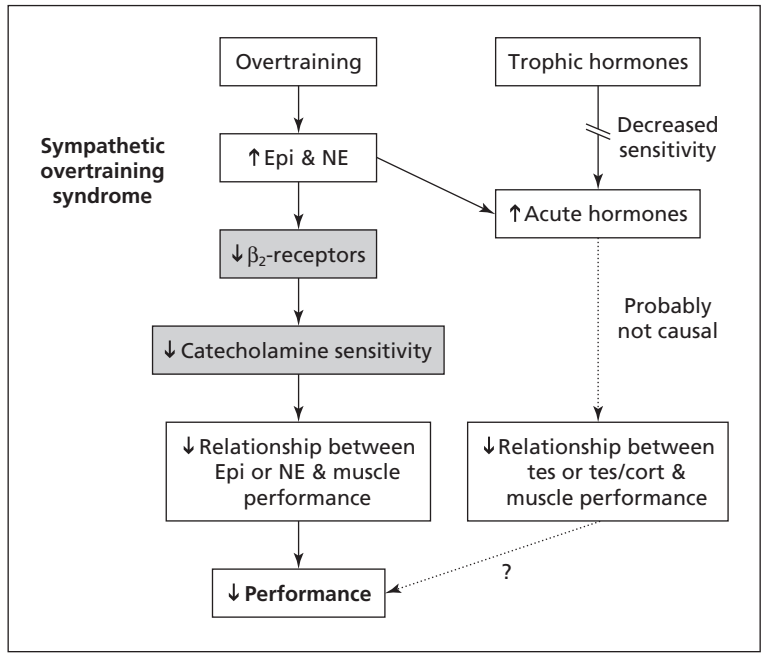

Fig. 37.5 Theoretical paradigm of the physiological progression of sympathetic (a) and parasympathetic (b) overtraining syndromes. The shaded areas indicate that the etiology of the sympathetic overtraining syndrome focuses on peripheral mechanisms, while that of the parasympathetic overtraining syndrome focuses on central mechanisms. CNS, central nervous system; Epi, epinephrine; NE, norepinephrine; tes, testosterone; tes/cort, testosterone/cortisol.

The role of the ANS in the onset of an overtraining syndrome is also becoming clearer. There appears to be a sequence in this response, with a sympathetic predominance occurring first, followed eventually by a parasympathetic predominance. Figure 37.5 illustrates theoretical progressions of each of these overtraining syndromes. As suggested in this chapter, the limiting factors for a sympathetic

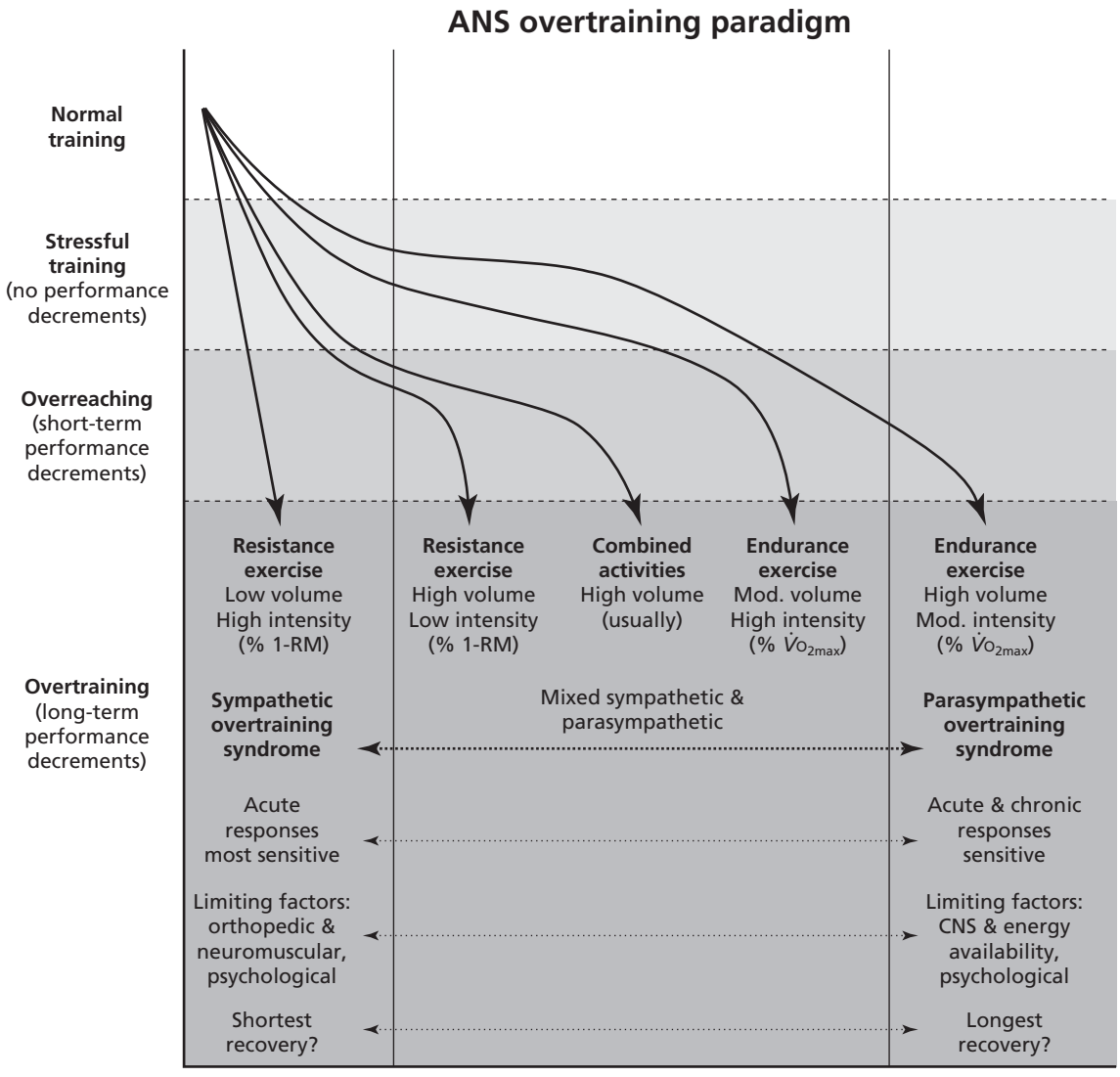

Fig. 37.6 Theoretical paradigm of the progression from normal training to overtraining for various types of sports and physical activity as it relates to the autonomic nervous system (ANS). Note that the sympathetic and parasympathetic overtraining syndromes represent the extremes of overtraining, and that many sports/activities fall in between these extremes. Also note that many symptoms of the overtraining syndromes may be present during lesser stages of overtraining, such as overreaching and phases of stressful training, that do not result in performance decrements.

overtraining syndrome appear to be mainly peripheral in nature, while those of the parasympathetic overtraining syndrome are central. Not all types of overtraining appear to make it all the way to a parasympathetic overtraining syndrome. It is likely that other limiting variables prohibit this occurring, such as orthopedic limitations with very intense resistance exercise or related activities. Figure 37.6 summarizes the likely progression of a variety of training stresses as they progress from normal training to stressful training to overreaching to overtraining. It should be apparent from Fig. 37.6 that

some of the symptoms sometimes associated with overtraining may appear before overtraining is an actual problem. This figure may also help illustrate why overtraining for so many sports is difficult to study, since so many sports fall in the middle of this continuum, and possess a combination of high-force and high-endurance characteristics.

Notes

J.M. Steinacker was responsible for the section on endurance sports, A.C. Fry was responsible for the section on strength sports and activities, and R. Meeusen was responsible for the section on central mechanisms.

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Chapter 38

Endocrinology of Sport Competition

JAY R. HOFFMAN

The primary role of hormones is to maintain internal equilibrium by interacting with various tissues and organs in the body to combat a variety of stresses. These stresses can either be physiological or psychological in nature, often stimulating a change in the secretory pattern of various hormones depending upon the specific stress encountered. Exercise is a potent stimulus to the endocrine system. The hormonal response to both acute exercise and prolonged training emphasizes the important role that the endocrine system has in responding to the metabolic demands of exercise and the recovery mechanisms involved in tissue repair and remodeling. Although a large body of literature exists concerning the hormonal response to various exercise stresses, relatively little information is available concerning specific hormonal responses to acute sport competition and during a season of sport competition. Despite the ability to simulate physiological stresses that may occur during competition in a laboratory setting, differences in stress levels between a laboratory measure of performance compared to actual competition are large, and may potentially have a much greater influence on the hormonal response. Therefore, the purpose of this chapter is to discuss the endocrine response to acute sport competition and during a season of athletic competition.

Precompetition hormonal response

Changes to circulating concentrations of hormones are generally seen in response to either a physiological or psychological stress. The mechanistic pathways controlling hormonal secretion patterns are similar for both types of stresses. The major difference is that the psychological stress is generally associated with the classic fight or flight response in which strong emotional components appear to have a key impact on the hypothalamic–pituitary–adrenal axis. Assuming that an athlete is in a state of homeostasis prior to competition, changes in hormonal concentrations are likely a function of precompetition anxiety. Anxiety is a common emotional expression of stress in sport and can influence athletic performance (Martens *et al.* 1990). Several studies have reported on the relationship between the hormonal response and anxiety (Filaire *et al.* 2001b; Salvador *et al.* 2003), and the hormonal response and behavior (Salvador *et al.* 1999). In addition, the athlete's mood has also been shown to play an important role in the endocrine response seen prior to exercise.

Competition is thought to cause both anxiety and arousal due to the uncertainty associated with the outcome and subsequent effects (Sapolsky 1994). As a result, this anxiety–arousal situation is perceived as a threat and causes an anticipatory rise in several different hormones. Elevations in cortisol, testosterone and catecholamines have been reported prior to the onset of the competitive activity. While cortisol elevations prior to exercise have been consistently reported in the literature, the response of testosterone has been less reliable. Studies that have examined the anticipatory catecholamine response to sporting events are less available, and are often inferred by research examining different modes of exercise.

Anticipatory elevations in cortisol have been seen in a number of studies examining primarily combative sports (e.g. wrestling and judo) (Elias 1981; Passelergue & Lac 1999; Salvador *et al.* 1999, 2003; Filaire *et al.* 2001b). Precompetition cortisol concentrations have been reported to be associated with winning (Elias 1981) and positively correlated to both self-confidence ($r = 0.64$) (Salvador *et al.* 2003) and anxiety (r ranging from 0.62 to 0.90) (Filaire *et al.* 2001b). An optimal level of anxiety is thought to be necessary for athletes to maximize performance (Gould & Krame 1992). The anticipatory rise in cortisol likely reflects a psychological mechanism used by athletes to increase precompetition arousal and as part of a coping mechanism to combat the stress of impending competition.

The role of an anticipatory rise in testosterone is not well understood. Several investigators have suggested that elevations in testosterone are related to aggressive behavior (Hannon *et al.* 1991; Bjorkqvist *et al.* 1994; Salvador *et al.* 1999). This would be beneficial for sports that have are physical in nature with a strong aggressive component. Salvador *et al.* (1999) reported a positive relationship between testosterone concentrations and the number of attacks during judo competition. In addition, precompetition testosterone concentrations have also been correlated to the mood state vigor (Tanaka *et al.* 1989) and negatively correlated ($r = -0.67$) to fatigue (Salvador *et al.* 2003). However, precompetition elevations in testosterone have not been consistently seen as several studies examining athletes performing contact sports have failed to support these findings (Gonzalez-Bono *et al.* 1999; Filaire *et al.* 2001b; Hoffman 2002; Salvador *et al.* 2003).

Studies examining the catecholamine response to competition are limited. In a study examining elite tennis players prior to Davis Cup competition, significantly higher (three to fourfold) epinephrine but not norepinephrine concentrations were seen prior to competition compared to practice conditions (Ferrauti *et al.* 2001). Kraemer *et al.* (1991) in a laboratory-controlled examination clearly showed that as subjects prepared to perform exercise at a maximal intensity an anticipatory elevation in epinephrine was seen compared to all other submaximal exercise intensities. This same pattern was not seen in norepinephrine, suggesting that the mechanism mediating the anticipatory catecholamine response is related to adrenal medulla activation where epinephrine is released in much greater quantities. Although a similar exercise protocol failed to elicit any anticipatory response from cortisol (Kraemer *et al.* 1989), the mechanistic pathway resulting in epinephrine release from the adrenal medulla may also be responsible for the anticipatory rise in cortisol that was previously discussed.

The potential benefit of an elevated sympathoadrenal response prior to a short duration, maximal intensity sporting event is likely related to an improved contractility of skeletal muscle. This may allow for greater power and force outputs during competition. In addition, the anticipatory catecholamine response also appears to be a phenomenon of training experience. Elevations in both epinephrine and norepinephrine concentrations have been reported prior to exercise in trained power lifters, but not in aged-matched untrained men (Kraemer *et al.* 1999). It is likely that the elevation in catecholamines is part of the psychological preparation (i.e. 'psyching-up') that experienced athletes use prior to competition.

Endocrine response to a sports competition

The endocrine response during competition likely plays an important role in substrate mobilization, cardiovascular regulation, blood flow and fluid balance adjustments, muscle (both cardiac and skeletal) contractility and augmentation of the secretion rates of other hormones. Due to the logistical problems associated with blood draws before, during and after competition, the volume of research that has examined the endocrine response to actual sport competition is limited. Most of our understanding is inferred by laboratory measures that attempt to simulate actual performance, but as discussed earlier the psychological component to actual competition may cause a different response pattern. In addition, differences in both the physiological and endocrine responses between trained competitive athletes and non-competitive individuals may

be large, limiting our complete understanding of the endocrine response occurring during actual competition.

Endocrine changes during endurance competitions

Endurance events such as a marathon are of sufficient duration to cause significant strain on metabolic systems. Often, both muscle and liver glycogen levels are exhausted, limiting the ability to provide fuel to exercising muscle. In addition, potential problems of exercising in the heat and dehydration may also cause additional strain on the athlete, causing significant changes to various hormones attempting to maintain thermoregulation and fluid balance.

Endurance athletes generally compete at an exercise intensity that does not exceed their anaerobic threshold. In well-conditioned endurance athletes this occurs between 80–90% of their maximal aerobic capacity (Hoffman 2002). During prolonged exercise at this intensity, such as in a marathon or triathlon, glycogen stores within the muscle are not sufficient to provide the needed fuel; thus, in addition to the body's reliance upon adipose tissue, the rate of hepatic glucose production will become markedly elevated (Wahren *et al.* 1971). Increased sympathetic nervous system activity (elevations in plasma catecholamine concentrations) and changes in glucagon and insulin concentrations are the primary factors regulating hepatic glucose production and lipid metabolism. In addition, growth hormone is also involved in lipolytic activity during prolonged endurance exercise.

Most of the knowledge attained of metabolic and endocrine responses during prolonged endurance exercise has been achieved under laboratory settings. In general, during prolonged exercise insulin concentrations will decline, while glucagon concentrations become elevated. Insulin depression appears to be a function of the duration of exercise. As exercise duration increases a greater decrease in insulin concentrations is seen (Galbo 1981). Although elevated glucagon levels will increase hepatic glucose production, the change in the molar ratio of glucagon to insulin has also been suggested to be as important in stimulating hepatic gluconeogenesis (Naveri *et al.* 1985).

Changes in catecholamine concentrations are also sensitive to blood glucose levels. Elevations in catecholamine concentrations have both a direct and indirect role in increasing substrate mobilization. During endurance exercise catecholamines also appear to respond in a differential manner. Increases in norepinephrine concentrations occur within 15 min of exercise without any change in epinephrine. Elevated norepinephrine concentrations are thought to stimulate hepatic gluconeogenesis by inhibiting insulin secretion (Glaster *et al.* 1981). As exercise duration continues a greater need for substrate mobilization is recognized by metaboreceptors stimulating an increase in epinephrine secretion. The elevation in epinephrine concentrations directly act to promote lipolysis (Smith 1980). In addition, both growth hormone and cortisol also have important roles in promoting lipolytic activity and increasing plasma free fatty acid concentrations during prolonged endurance exercise (Hoffman *et al*. 2002).

Endurance exercise appears to be a potent stimulus for both cortisol and growth hormone release. Both of these hormones appear to be positively related to the duration of exercise (Bunt *et al.* 1986). The influence of exercise intensity on the secretion patterns of these hormones though is less clear. It appears that exercise needs to be performed at a minimal intensity to elicit increases in the circulating concentrations of these hormones (Few 1974). However, most endurance events are performed at an intensity that appears to be sufficient to elicit elevation in both cortisol and growth hormone concentrations. Figure 38.1 provides a schematic figure outlining the proposed endocrine responses to an endurance event such as a marathon.

Only a limited number of studies have examined the endocrine response during endurance events such as marathons and triathlons. In these events a 170% elevation in catecholamine concentrations (Dearman & Francis 1983) and a 2–4.5-fold increase in cortisol concentrations (Dearman & Francis 1983; Hale *et al.* 1983; Urhaussen & Kindermann 1987; Ponjee *et al.* 1994) have been reported. Although the results of these studies clearly support the role of

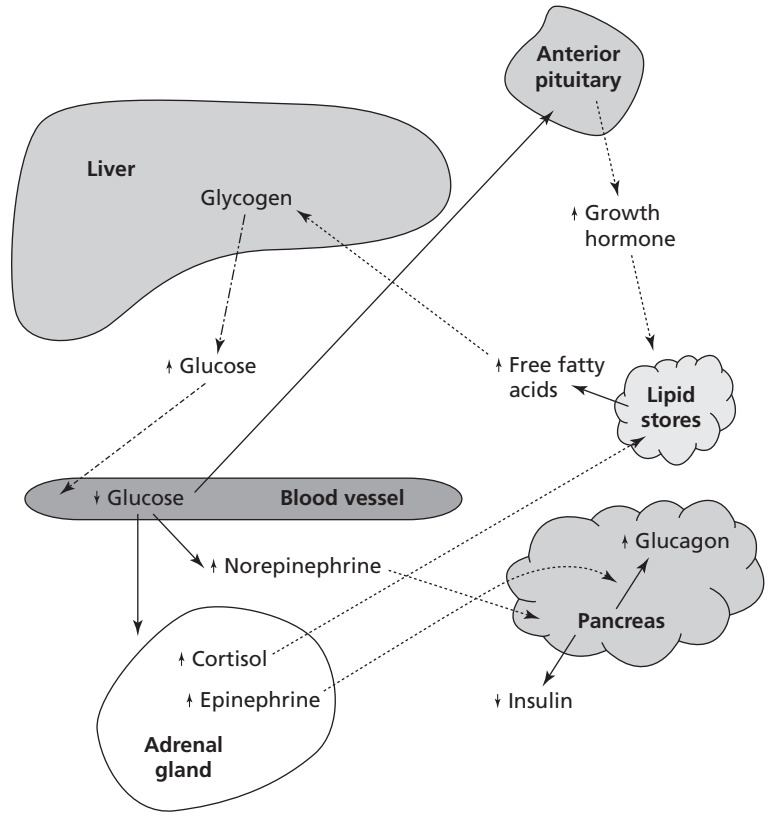

Fig. 38.1 Endocrine response to an endurance event.

these hormones in substrate mobilization during prolonged competitive endurance events, other studies have also shown that these athletic endeavors have a significant effect on the pituitary–testicular axis. During events that last less than 3 h no changes in circulating testosterone levels are seen; however, increases in sex hormone binding globulin (SHBG) were seen, resulting in a decrease in the testosterone/SHBG ratio and a decrease in the free, biologically active testosterone (Urhaussen & Kindermann 1987). As the endurance event exceeds 3–4 h, significant decreases in testosterone are seen (Tanaka *et al.* 1986; Lac & Berthon 2000), and may remain below baseline levels for up to 48 h post-competition (Tanaka *et al.* 1986).

The significance of this apparent anabolic deficit may be more important during the recovery period following competition than during the competition itself. Testosterone has been reported to influence the resynthesis of glycogen (Gillespie & Edgerton 1970) and creatine phosphate (Sutton *et al.* 1973). In addition, the elevated cortisol levels seen in conjunction with lower testosterone concentrations is indicative of greater catabolic activity and may limit the ability of skeletal muscle to repair damage associated with the endurance event. Interestingly, during ultraendurance events such as an 1100-km run testosterone levels will remain depressed for the entire 20-day event (Schurmeyer *et al.* 1984). This is in contrast to other stress hormones measured (i.e. thyroxine, cortisol and prolactin) that showed an ability to adapt to the prolonged exercise stress.

Endocrine changes during strength/power competitions

During competitions that emphasize strength and power (including sprint races) the demand on the metabolic energy system to fuel exercise is generally not a limiting factor. However, in competitions that

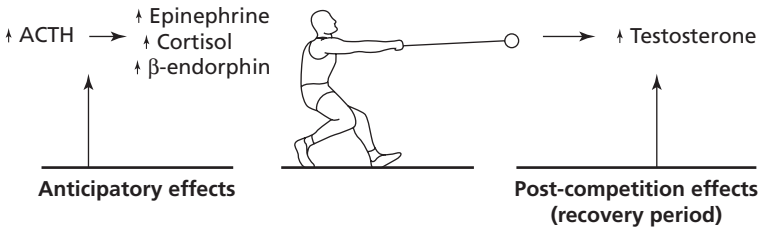

Fig. 38.2 Proposed endocrine responses to a high intensity, maximal effort. ACTH, adrenocorticotropic hormone.

rely on repetitive high intensity activity the hormonal responses and the stimuli that regulate their secretion may differ compared to responses observed during a single event (i.e. 100-m sprint). During such competitions the endocrine response may reflect both psychological preparation to the competition and the physiological demands that occur during the competition. Figure 38.2 provides a schematic example of potential endocrine responses to a high intensity competition involving a single maximal effort.

In athletes that participate in throwing events such as the discus or hammer throw, anticipatory elevations to pituitary and adrenal hormones (β-endorphin, adrenocorticotropic hormone [ACTH] and cortisol) have been reported, with no other hormonal changes seen post-competition (Petraglia *et al.* 1988). This is not surprising considering that the duration of the event is comprised of only a few seconds. During these maximal effort, short duration competitions the anticipatory hormonal response may be critical for optimal performance. As discussed earlier, increases in catecholamine concentrations are important in eliciting maximal strength performance, and changes in circulating epinephrine have been correlated to short-term alterations in strength (Fry *et al.* 1994). In addition, elevated sympathetic activity also appears to augment testosterone secretion (Jezova & Vigas 1981). This may have certain significance considering that recent research has shown that the testosterone/cortisol ratio (T/C ratio) is highly correlated ($r = 0.92$) to weightlifting performance in elite junior weightlifters (Fry *et al.* 2000). Although these investigators were careful not to imply a causal relationship based upon their correlational analyses, the importance of testosterone to strength performance has been suggested in several other studies examining elite weightlifters (Kraemer 1992; Fry *et al.* 1993; Kraemer *et al.* 1993). The role of testosterone in maximal strength expression may be related to its interaction with the nervous system and enhancement of the secretion rates of growth hormone and growth factors (Kraemer 1992). However, these latter interactions may have more importance during the preparation and recovery phases of competition, than having any acute effects during competition.

During 100- and 400-m sprint competition, elevations in both pituitary and adrenal hormones suggest that the primary stimulus stimulating secretion of these hormones is the psychological preparation preceding competition. During a 100-m sprint significant increases in β-endorphin, ACTH and cortisol concentrations have been reported prior to and following the race (Petraglia *et al.* 1988). While increases in ACTH and cortisol are part of the anticipatory response to the event, the increase in β-endorphin concentration could be associated with decreased pain sensitivity. This could explain how athletes despite suffering from muscle soreness or injury might still perform at optimal levels.

During a longer duration sprint (400-m sprint) significant elevations in luteinizing hormone (LH) and follicle-stimulating hormone (FSH), with significant decreases in total and free testosterone, have been observed (Slowinska-Lisowka & Majda 2002). Following 24 h of rest, testosterone concentrations returned to baseline levels. Considering the short duration of the event (45 s in these athletes) the depressed testosterone concentrations seen following the race may reflect interactions associated with the anticipatory hormonal response. As discussed earlier, both cortisol and testosterone concentrations may be elevated prior to a competitive event

involving maximal intensity exercise. However, the pre-event testosterone response has not been as consistent as that seen in other hormones. Since cortisol has been shown to display inhibitory actions on testosterone secretion (Cumming *et al.* 1983), it is possible that depressed testosterone concentrations observed immediately after the race reflect hormonal interactions occurring prior to the start of competition. Unfortunately, cortisol was not measured in this study making this only a speculative assumption. Regardless, the return of testosterone (total and free) to baseline levels was suggestive of a normal functioning recovery system.

Due to logistical difficulties there have been only a handful of investigations examining the hormonal response during prolonged high intensity sporting events such as wrestling tournaments or American football games. During a wrestling match in which wrestlers are engaged in combative competition for up to 7 min, under conditions that may include fluid and dietary restrictions, both psychological and physiological stresses are apparent. Passelergue and Lac (1999) observed an anticipatory rise in cortisol prior to each match during the 2-day study. Cortisol concentrations continued to elevate (2.5-fold from resting levels) throughout the competition, but fell rapidly to baseline levels (within 1.5 h) during the recovery period. Testosterone concentration remained at basal levels during the competition, but began to significantly elevate during the post-competitive period. During the recovery period subjective feelings of fatigue, and an inability to perform strenuous exercise was reported. However, during this time a significantly higher T/C ratio was observed, and remained elevated for 5 days following the tournament. This anabolic phase would likely enhance the recovery processes of the athletes allowing for a faster return to both training and competition.

Kraemer *et al*. (2001) examined wrestlers during 5 matches over a 2-day tournament. They reported that despite a 1-week weight loss period that resulted in a 6% loss of body mass by the athletes, no significant changes from baseline hormonal concentrations (epinephrine, norepinephrine, testosterone and cortisol) were observed prior to the first match. The investigators did not observe any anticipatory endocrine response prior to the first match. However, catecholamine levels were elevated at the end of each match and corresponded to an improved reaction time. The higher catecholamine response seen at the end of each match may reflect a greater arousal that contributed to the improved performance. Interestingly, norepinephrine responded in a more sensitive manner than epinephrine during the tournament. Norepinephrine was significantly elevated following each match, but epinephrine responses were not as consistent. The results were suggestive of a possible adrenal insufficiency that may have also contributed to a reduced force production observed during the study.

The testosterone and cortisol responses reported by Kraemer *et al.* (2001) contrasted from those observed by Passelergue and Lac (1999). However, it should be acknowledged that the endocrine analysis in the former study was from plasma samples, while in the latter study from saliva samples. Variability in hormonal measurements from different bio-compartments may have contributed to the differences in the endocrine response seen between these studies. Kraemer *et al*. (2001) observed a consistent reduction in testosterone concentrations during the 2-day tournament with significant elevations in cortisol seen only during matches 1, 2 and 5. Resting testosterone concentrations by the end of the tournament fell below normal, and was similar to levels seen in endurance runners performing high volume training (Wheeler *et al.* 1984) and in weightlifters participating in a week-long overtraining protocol (Fry *et al.* 1993). The low testosterone concentrations seen may have also contributed to the reduced strength capability of the subjects by delaying recovery between matches.

In high intensity, combative sports that are of longer duration (e.g. 60 min) such as American football the potential for greater performance decrements, tissue damage and stress may exist. Only one study has examined the endocrine and performance responses to participation in a competitive American football game (Hoffman *et al*. 2002). Cortisol concentrations were higher prior to the game than baseline levels measured the day before. However, this may have been a function of normal diurnal changes. Regardless, the 52% increase ($p >$

0.05) in cortisol concentrations in starters between baseline and pregame levels may also suggest a potential anticipatory response. During the game cortisol levels tended to return to baseline levels, but were still significantly higher than red-shirt freshman players who did not play and served as a control group. Since glucose levels were maintained during the game for both the players and the control group, the significant difference seen in cortisol likely represented a greater stress experienced by the players participating in the game. Considering the significant elevations seen in muscle damage markers seen as a result of game participation, the elevated cortisol concentrations appear to reflect the anti-inflammatory properties that cortisol possesses in response to tissue damage.

During the American football game no changes in testosterone concentrations were seen from baseline to the end of the game. However, testosterone concentrations were at the low end of normal. Considering that the game was the last game of the season, it is possible that low testosterone levels may inhibit recovery mechanisms during the recovery period. However, the players were not examined past the post-game period and any inference to recovery can only be speculative.

In high intensity sports that have limited physical contact (i.e. basketball) changes in hormonal concentrations would likely reflect physiological stresses arising from psychological preparation or metabolic demands, and a reduced risk of physiological stresses caused by tissue damage. In the only study known to have examined the hormonal response to a basketball game, saliva samples were collected from 16 members of two male professional European basketball teams (eight from each team) competing against one another 45 min before and 15 min after a game (Gonzalez-Bono *et al.* 1999). No change in testosterone was seen during the contest, while significant cortisol elevations were noted. The investigators in this study were primarily comparing the endocrine response to mood states in both winners and losers from the game. The increased cortisol concentrations seen at the end of the game does appear to be reflective of the metabolic stress that is associated with 40-min of high intensity activity.

In the majority of studies examining high intensity competitive sport activity the primary hormones examined have been cortisol and testosterone. The response of cortisol to a game has generally been seen to increase, while the response of testosterone has been less consistent. The physiological roles of cortisol (stimulation of gluconeogenesis, increase in blood glucose concentrations, facilitation of lipolysis and anti-inflammatory affects) do support the findings in these studies. On the other hand, the physiological roles of testosterone are primarily involved in mechanisms involved in recovery. Thus, making the measurement of testosterone during the recovery period has greater importance in the hours and days following the competitive performance. Unfortunately, often due to logistical problems the ability to obtain samples from competitive athletes for several days following a competition is quite difficult.

Endocrine changes during moderate intensity sporting events

During sport competitions of moderate intensity the metabolic demands are quite different than those seen during higher intensity sports. Although gluconeogenesis will still be of importance, a greater emphasis on lipid metabolism will likely be seen. In addition, the anticipatory rise in many hormones does not seem to be as prevalent in these sporting events compared to competitions involving high intensity, maximal efforts.

Soccer is an interesting sport considering that it is 90 min in duration and involves sprints and runs of various intensities and durations. In a limited number of studies, the pituitary–adrenal axis appears to be quite active during the course of a game. Significant elevations in ACTH, cortisol, growth hormone and prolactin have been reported (Lupo *et al.* 1985; Carli *et al.* 1986). Growth hormone and prolactin were seen to increase only at halftime, while ACTH and cortisol continued to rise throughout the game. Cortisol levels returned to baseline levels within 45 min following the game. These hormonal responses do appear to be reflective of the metabolic demands of soccer; high intensity sprints interspersed with periods of lower intensity activ-

ity. However, the varying intensities of activity during the course of a game do not appear to provide a sufficient stimulus to maintain growth hormone secretions. Previous research has shown that growth hormone responses are greater during continuous exercise in comparison to intermittent exercise, even when lactate levels are greater in the latter (Lugar *et al.* 1992). There apparently needs to be a minimum duration (10 min) of activity above the lactate threshold to stimulate consistent growth hormone release (Hoffman 2002).

Tennis is another sport that has an intermittent style of play involving periods of high intensity activity interspersed with numerous recovery periods. In contrast to soccer though, the high intensity activity seen in tennis is played over a much smaller area. As a result it appears that the metabolic demands of tennis players may be less than that seen in soccer players. In a study on a simulated singles match (85 min in duration) in NCAA Division I tennis players, the results observed appeared to support this (Bergeron *et al.* 1991). Blood glucose and plasma lactate concentrations were observed to remain at basal levels, while cortisol concentrations decreased and were significantly below baseline levels by the end of the match. At the end of the match testosterone concentrations were significantly elevated from baseline measures. The physiological role of testosterone during a tennis match however is not clear, but it likely has an important role during recovery.

In a separate study on tennis players, the importance of catecholamine concentrations was examined in a match during the Davis Cup competition (Ferrauti *et al.* 2001). A twofold increase was seen in both epinephrine and norepinephrine concentrations. Although data is limited, catecholamines may play an important role in fulfilling the metabolic demands of tennis.

The importance of catecholamines to performance may also be reflected by the ratio of norepinephrine to epinephrine (NE/EPI ratio). It has been suggested that the NE/EPI ratio provides important information concerning the athlete's ability to perform, especially in sports that require fine motor control for precise movements (Hoch *et al.* 1988; Ferrauti *et al.* 2001). Elevated epinephrine concentrations generally reflect the state of anxiety of the athlete. As previously discussed, there does appear to be an optimal level of anxiety for maximizing athletic performance, and several investigators have suggested that the NE/EPI ratio may provide an endocrine marker of optimal anxiety (Hoch *et al.* 1988; Ferrauti *et al.* 2001). With higher concentrations of epinephrine there exists a greater chance for hormone–receptor interaction in skeletal muscle potentially effecting contractile functioning of skeletal muscle. During a sport that requires maximal effort, augmentation of sympathetic activity appears to be beneficial. However, in a sport that requires fine motor control, elevated sympathetic activity may result in a loss of accuracy and movement economy resulting in performance impairment. The loss of co-ordination may further increase anxiety levels by the added stress of poor performance. When a physiological stress stimulates catecholamine release, the higher norepinephrine levels appear to compensate for the mental anxiety by reducing the NE/EPI ratio. The lower NE/EPI ratios appear to correspond to a calmer athlete, and the resulting catecholamine concentration reflects the physiological stress of exercise and not the mental anxiety produced by competition (Ferrauti *et al.* 2001).

Endocrine response to a competitive sport season

There have been a number of studies that have examined the endocrine response to a competitive sports season. The primary purpose motivating many of these studies was to provide a better understanding of hormonal changes to variations in training intensity and volume that occur during the course of a competitive season, and to provide insight to potential endocrine markers to overreaching or overtraining. Overtraining is a general term for describing an imbalance between training and recovery (Kuipers & Keizer 1988). Most studies have used testosterone and cortisol, hormonal indicators of anabolism and catabolism, respectively, as the primary endocrine measurement of training stress. In this section, the hormonal response to a season of competition will be discussed as it relates to endurance and strength/power sports.

Endocrine response to a season of competition in the endurance athlete

In most endurance sports, as the season progresses both the volume and intensity of training will elevate (Hoffman 2002). This training progression may be reflected by changes in circulating hormonal concentrations. In some athletes the changes are drastic and may reflect insufficient recovery and a possible overtraining syndrome, while other athletes performing the identical training program may have little to no changes in endocrine markers of recovery, signifying adequate recovery from training. During the course of a season the athlete will be competing in various competitions; however, the greatest emphasis is placed on the competitions that occur at the end of the season. Generally, the training program will be adjusted to help the athlete peak for these particular matches. This is referred to as a taper and involves a reduction in both training volume and intensity, with a heavier emphasis on volume reduction (Hoffman 2002).

Most studies examining endurance athletes have reported no changes in resting testosterone, growth hormone, norepinephrine, ACTH and cortisol during a season of competition (Vervoorn *et al.* 1991; Hooper *et al.* 1993; Lopez-Calbert *et al.* 1993; Flynn *et al.* 1994; Maresh *et al.* 1994; Mujicka *et al.* 1996; Bonifazi *et al.* 1998). In contrast, other studies examining endurance athletes reported significant decreases in testosterone concentrations following periods of high volume training (Flynn *et al.* 1994; Tyndall *et al.* 1996), while studies primarily on marathon runners have often reported chronically low testosterone concentrations (Barron *et al.* 1985; Houmard *et al.* 1990). Although the depressed testosterone concentrations in swimmers do appear to return to baseline levels during a taper, this does not appear to be the case for marathon runners (Houmard *et al.* 1990). It is likely that prolonged high volume training and longer training experience seen in these athletes contributes to a chronic reduction in testosterone. In collegiate cross-country runners the training volume progressed incrementally, and the athletes were relatively younger (range 18–22 years) than the marathon runners (range 22–36 years). It has been suggested that as endurance athletes continue to train testosterone concentrations are reduced as a result of hypothalamic dysfunction (Barron *et al.* 1985; MacConnie *et al.* 1986), or as a result of elevated cortisol levels that act to suppress testosterone secretions (Cumming *et al.* 1983). In these experienced endurance-trained athletes, relatively short periods of reduced training (3–4 weeks) may not be sufficient to restore normal testicular function.

The studies reporting on endocrine changes during a competitive season generally report on changes occurring to the entire team. However, individual athletes performing the same training program often respond differently. Hooper *et al.* (1993) examining 14 male and female swimmers reported no changes in any of the endocrine measures performed during the course of the competitive season. When subjects were examined on an individual basis three of the swimmers (all female) were identified as being stale. These athletes were also reported to have significantly higher norepinephrine concentrations during the taper period than the athletes that were not suffering from any overtraining syndrome. This highlights the importance of examining individual data. Further emphasizing this point, other investigators have also reported on a tendency ($p > 0.05$) for depressed testosterone and elevated cortisol levels (Lopez-Calbert *et al.* 1993). Though no significant changes were noted, five of seven athletes studied had reduced testosterone concentrations and a 29% reduction ($p > 0.05$) in the T/C ratio was seen.

A reduction in testosterone concentrations, elevation in cortisol concentrations and reduction in the T/C ratio are all potential indicators of a catabolic state and provides information concerning the recovery ability of the athlete, and the athlete's ability to synthesize protein and to maintain muscle mass. Changes in testosterone have been shown to significantly correlate ($r = 0.87$) to changes in lean body mass (Lopez-Calbert *et al.* 1993), and increases in the T/C ratio are also significantly correlated ($r = 0.86$) to improvements in swimming performance (Mujicka *et al.* 1996).

A competitive endurance sport season is quite

stressing, both on the physiological mechanisms to maintain homeostasis and on the psychological effects of training and competition. In an optimal situation the athlete is able to maintain normal physiological function reflected by little to no changes in the endocrine profile. Bonifazi *et al*. (2000) showed that following 18-weeks of training in elite swimmers, precompetition cortisol levels were reduced and corresponded to improvements in endurance swimming performance. In addition, 18-weeks of swim training has also been seen to result in elevated growth hormone concentrations (Bonifazi *et al*. 1998). This is a positive training adaptation considering that a greater growth hormone concentration may reflect an improved ability to maintain muscle mass, enhance fatty acid utilization and reduce utilization of glucose and amino acids (Rogol 1989). The psychological effect of a competitive sports season was also reflected by changes in epinephrine concentrations in competitive swimmers (Hooper *et al*. 1993). A significant depression in epinephrine concentrations was seen within 3 days of the conclusion of the competitive season. This likely reflects the reduced emotional stress and anxiety from the end of the competitive season.

Endocrine response to a competitive season in the strength/power athlete

The number of studies that have examined endocrine changes during a season in traditional strength/power sports (i.e. basketball, American football, sprinting, wrestling) are relatively few in comparison to endurance sports. Furthermore, no published studies are available that have examined seasonal hormonal changes in popular American team sports such as football or basketball. Similar to the research on endurance athletes, most studies examining the strength/power athlete have primarily measured testosterone and cortisol concentrations to provide information on the hormonal stress response and recovery capability in these athletes.

Soccer players, although not a typical strength/power athlete, have been reported to have a reduced T/C ratio during the course of a competitive season that did not correspond to any negative changes in soccer performance (Filaire *et al*. 2001a). Studies on swimmers involved in sprint events also reported no relationship between changes in cortisol, dehydroepiandrostenedione and performance (Chatard *et al*. 2002). In addition, unpublished data from this author's laboratory on American college football players have also shown that testosterone and cortisol levels are maintained during a season of competition. In contrast, Banfi *et al*. (1993) examining speed skaters have suggested that the free testosterone/cortisol ratio may be a useful indicator of temporary, incomplete recovery from training. This is supported by other investigators that have shown that the anabolic to catabolic hormonal ratio is a potential hormonal marker for incomplete recovery and overtraining syndrome (Adlercreutz *et al*. 1986). The lack of consistency seen in the literature suggests that hormonal changes during a season may not be a reliable indicator of performance changes, but that such markers may have more value when evaluating short duration training periods.

Wrestling has been shown to have a potent effect on endocrine function during a competitive season (Strauss *et al*. 1985; Roemmich & Sinning 1997). However, in contrast to other strength/power athletes, wrestlers also participate in periods of fasting and dehydration in order to make weight, which compounds the physiological stress of training. Decreases in body fat and body mass have been shown to be significantly correlated ($r = 0.72$) to reduced testosterone concentrations (Strauss *et al*. 1985). Roemmich and Sinning (1997) have also shown that dietary restriction and wrestling practice and competition produces a partial growth hormone resistance (elevated growth hormone concentration with a decrease in growth hormone binding protein) and a possible disruption of the pituitary–testicular axis (reflected by decreases in testosterone and free testosterone concentrations). Interestingly, low testosterone concentrations were not reported to be associated with any changes in LH concentrations suggesting a possible impairment of the hypothalamic–pituitary–gonadal axis. However, the reduced testosterone concentrations

in these athletes were still within normal physiological range.

A change in the circulating concentration of anabolic hormones is a positive adaptation for the strength/power athlete. The primary reason being the role that testosterone and growth hormone has in enhancing or facilitating the building of lean tissue (Hickson *et al.* 1994). Surprisingly, the ability of these athletes to alter endocrine function and the effects that hormonal changes have on strength and power performance is unclear. Experienced, competitive weightlifters have been shown to maintain testosterone concentrations during a year of training, while strength levels were improved. (Häkkinen *et al.* 1987). Only after 2 years of training did significant elevations in testosterone concentrations become apparent in these experienced weightlifters (Häkkinen *et al.* 1988). Alterations in the endocrine profile of these athletes appears to be a difficult to accomplish and may reflect an advanced adaptive strategy to increase force production (Kraemer 1992).

Conclusion

The endocrine response to acute sport competition was shown to reflect precompetition arousal and anxiety, the metabolic demands of the sport, the physical stress associated with competition and the recovery capability of the athlete. The endocrine response to competition appears to be dependent upon the nature of the sport. During competition requiring maximal effort over a short duration, the anticipatory hormonal response may have an important role in strength and power expression, while during endurance competition the hormonal response generally reflects the metabolic demands of prolonged activity. In the post-competition recovery phase, the hormonal profile may provide an indication for the athlete's ability to recover. Analyses of the endocrine changes during a season of competition have primarily examined the athlete's ability to adapt to various training stresses, and to serve as an endocrine marker of the overtraining syndrome.

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Index

Note: Page numbers in *italic* refer to figures and/or tables separate from the text