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Angiotensin

Vol. I

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Preface

The 1974 volume on angiotensin edited by Irvine H. Page and F. Merlin Bumpus expanded the *Handbook of Experimental Pharmacology* series. In the preface, the editors of the first edition commented on their subject matters as follows:

“...Initially, it seemed that the action of angiotensin was relatively simple but this proved grossly misleading... Even after two decades [since angiotensin was identified as the major effector peptide of the renin–angiotensin system (editors’ note)] the multiplicity of its actions appears not to have been fully discovered. To call attention to its many functions is one of the purposes of this book.”

Thirty years later, this statement still holds true. Nevertheless, this new edition of the volume on angiotensin attempts to provide an updated account of the knowledge and findings accumulated since the complexity of angiotensin was so accurately recognized.

Certainly, the editors of the first volume on angiotensin would have been gratified by the wealth of new data on their subject flooding the literature since 1974, adding to the complexity of actions of angiotensin peptides they had predicted. This, of course, does not make our present-day task of understanding the multiple facets of the renin–angiotensin system any easier. However, it justifies our current endeavor to take a new look at this venerable system that still offers so many hidden miracles to be unveiled and described and, after all, has proved in the last 30 years to be of utmost clinical importance.

It is indeed the advent of the inhibitors of the renin–angiotensin system, notably the angiotensin-converting enzyme inhibitors and the angiotensin AT₁ receptor antagonists, which has helped enormously in gaining deeper insights into the system, notwithstanding the fact that these compounds have become some of the most successful drugs ever developed, not only to control hypertension but also to protect target organs like the kidney, heart, blood vessels, and brain, and, most importantly, to reduce cardiovascular mortality.

Naturally, the focus of any published work on medical science changes over the years and between editions. In 1974, much emphasis was laid on the biochemistry of the renin–angiotensin system, since the major discoveries had been made in this area. The interaction of renin and converting enzyme with their substrates, newly developed assays to analyze the various components of the system, and angiotensin analogs and their structure–function relationships were in the center of interest.

In the years that followed, attention gradually shifted to other areas, such as the genetics of the renin–angiotensin system, angiotensin receptors, their regulation, signaling pathways and various functions, and the inhibitors of the renin–angiotensin system with their mechanisms of action and their clinical use.

Moreover, whereas 30 years ago angiotensin II was still predominantly seen as a regulator of blood pressure and body volume, this peptide and its active fragments together with another major effector molecule of the system, the adrenal steroid aldosterone, are now considered to play an important role in a variety of (patho-) physiological functions that may be as diversified as vascular growth and atheroma formation, renal protein handling and glomerulosclerosis, cardiac left ventricular hypertrophy, fibrosis and postinfarction remodeling, or central osmoregulation and neuroregeneration. And along with the immense progress in biological sciences that we have witnessed during the last decades, the renin–angiotensin–aldosterone system has been connected to a number of biological phenomena such as cellular differentiation, neuroplasticity, and apoptosis.

This shift in scientific interest and research activities is reflected in the present volume, although we strongly felt that the fundamental knowledge on the system accumulated and substantiated over the last 100 years should never be omitted but be present as an undercurrent to help our understanding and, even more importantly, to put our temporary knowledge of today into a historical perspective.

The editors of the 1974 volume finished their preface stating, “...Books today are expensive and time-consuming to read...”

Again, their statement holds true for the two volumes of this new edition. Today, electronic media provide us with virtually any information including, of course, what has been written on angiotensin to date, and a host of review articles has been published on almost every aspect of the renin–angiotensin–aldosterone system. However, as with Page and Bumpus in their day, we are convinced that even in our time, there is still a place for books of this kind which invite the scholar to in-depth reading of what acknowledged experts have compiled as the essentials in their field.

When we asked the authors if they were willing to contribute to this edition, we did so with a certain degree of apprehension for the above-mentioned reasons but were overwhelmed by their unanimous positive response to our request. We would like to thank all authors for their efforts to make this volume a solid source of comprehensive information. We would also like to express our gratitude to our secretaries, Miranda Schröder and Undine Schelle, as well as to Sibylle Melzer and Ellen Scheibe and also to Susanne Dathe, our partner at Springer-Verlag, for their continuous, invaluable support, which enabled us to achieve our task.

Berlin and Frankfurt, January 2004

Thomas Unger
Bernward A. Schölkens

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Part 1
General Aspects

General Physiology and Pathophysiology of the Renin–Angiotensin System

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Abstract The renin–angiotensin system (RAS), one of the oldest hormone systems, is a complex regulatory system with many identifiable actions. However, it may primarily be viewed as a powerful regulatory system for the conservation of salt and blood volume, and the preservation of an adequate blood pressure (BP). To circumvent the major threats of low blood volume and low BP, animals and our ancestors, with a diet relatively poor in sodium, needed powerful mechanisms for salt and water conservation, and their organisms relied heavily on the RAS. Many of the diverse actions of angiotensin II, the major end product of the RAS, can be viewed in a single conceptual framework, as serving to prevent life-threatening shrinkage of intravascular volume (rapid actions of angiotensin, in combination with the sympathetic nervous system), to help maintain volume homeostasis by minimizing the changes in arterial pressure and fluid volumes required to achieve sodium balance (prevention of salt-sensitivity), and to increase the efficiency of cardiovascular dynamics by promoting the growth of the heart and vessels, and sensitizing blood vessels to vasoconstrictor agents (slowest actions of angiotensin). Activation of the RAS is therefore a useful response in many demanding situations. However, an increased activity of the RAS, especially in combination with other cardiovascular risks factors, may lead to a cascade of deleterious effects. Many of these pathophysiological actions of angiotensin II may still be viewed as being homeostatic in principle, but harmful if carried to excess.

Keywords Renin release · Blood volume · Blood pressure · Sodium balance · Salt-sensitivity · Pressure natriuresis · Hypertension · Cardiovascular hypertrophy

Abbreviations

ACE	Angiotensin-converting enzyme
AGT	Angiotensinogen
Ang II	Angiotensin II
AT ₁ -R	AT ₁ -receptor
BP	Blood pressure
GFR	Glomerular filtration rate
NO	Nitric oxide
PNC	pressure-natriuresis curve
RAS	Renin–angiotensin system
TGF	Tubuloglomerular feedbacks

The renin–angiotensin system (RAS), one of the oldest hormone systems, is a strong control system for salt conservation, blood volume and blood pressure (BP) preservation. To circumvent the major threats of low blood volume and low BP, animals and our ancestors, with a diet relatively poor in sodium, needed powerful mechanisms for salt and water conservation, and their organisms relied heavily on the RAS. The purpose of this chapter is to give a general overview of the RAS, to stress its usefulness in daily homeostasis, but also to show how its effectiveness can be detrimental in certain circumstances.

1

The Major Players of the Renin–Angiotensin System

1.1

History of the Discovery of the RAS

The story of the discovery of the RAS began more than 100 years ago, on 8 November 1896, when the Finnish physiologist Robert A. Tigerstedt (1853–1923), who was at that time Professor of Physiology at the Karolinska Institute in Stockholm, and Per Gustav Bergman, a medical student, set up to do a crucial experiment. Inspired by the French physiologist Charles-Édouard Brown-Séquard, who started a trend for discovering “inner secretions” in organs by injecting extracts from donor organs into animals, Tigerstedt and Bergman injected cold extracts of donor rabbit kidneys into the jugular vein of recipient rabbits and showed that those extracts consistently increased BP. They concluded that the kidney contained a pressor substance they named renin. In further experiments, they showed that this pressor substance was located in the renal cortex and that the pressure response did not require an intact nervous system (Tigerstedt and Bergman 1898). Intriguingly, these observations were forgotten for many years.

The possibility that the kidney may release a pressor substance was revived by Harry Goldblatt, who could induce experimental hypertension in dogs by clipping one or both renal arteries (Goldblatt et al. 1934). These observations led to a renewed interest in a renal pressor substance, and in 1939 two independent laboratories, Page, Helmer and Kohlstaedt in Indianapolis, and Braun-Menendez, Fasciola and Leloir in Argentina, showed that renin was not itself a pressor substance, but an enzyme acting upon a protein to release a peptide vasoconstrictor that became known as angiotensin (Braun-Menendez and Page 1958).

1.2

The Renin–Angiotensin Cascade

Slowly over the years, the RAS was elucidated, as illustrated in Fig. 1. In response to certain stimuli, renin, a proteolytic enzyme produced by the kidney, is released into the circulation and acts on angiotensinogen (AGT), a circulating

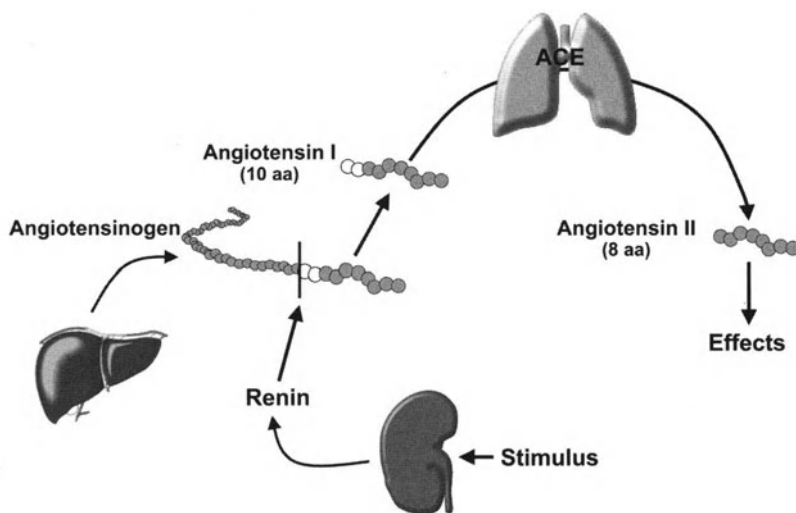


Fig. 1 Simple diagram of the RAS pathway

protein (α_2 -globulin) produced by the liver. Renin cleaves AGT to produce angiotensin I (Ang I) a small fragment of only 10 amino acids. Ang I has no biological action in itself, but is converted to angiotensin II (Ang II), an active octapeptide, by angiotensin-converting enzyme (ACE), an enzyme present on the cell surface of many cells and particularly on vascular endothelial cells. As all blood leaving the kidneys and liver eventually flows through the lung, the pulmonary vascular endothelium plays a major role in the rapid conversion of Ang I into Ang II. Finally, Ang II will bind to specific cell surface angiotensin-receptors to elicit multiple actions.

At this stage, for the sake of completeness, four important remarks should be made:

1. Ang I and Ang II can be generated by alternate enzymatic pathways (Fig. 2). Enzymes other than renin, such as tonin and cathepsin D, can promote the formation of Ang I. Similarly, enzymes other than ACE, such as trypsin, cathepsin G or heart chymase, can facilitate the conversion of Ang I into Ang II. However, the contribution of these alternative pathways in Ang II production in humans is still unclear.
2. ACE can act on substrates other than Ang I. Particularly, ACE can promote the degradation of bradykinin, substance P and other small peptides. Although the physiological role of this enzymatic conversion is unclear, pharmacological blockade of ACE with specific inhibitors leads to an accumulation of bradykinin and substance P, which may be responsible for some of the beneficial effects (antihypertensive), but also some of the adverse effects (angioedema, cough) of ACE inhibitors.

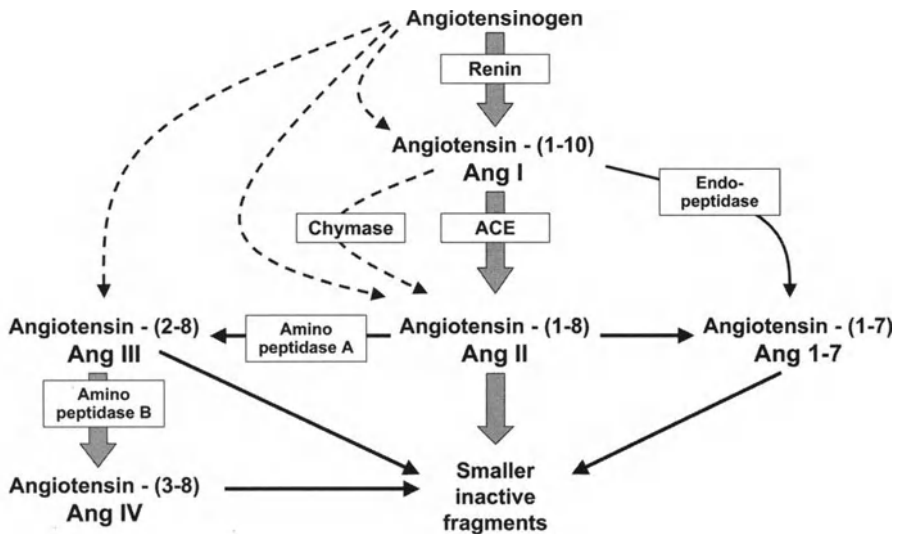


Fig. 2 Alternate pathways of angiotensin II formation and other angiotensin peptides

- There are several angiotensin peptides with biological effects (Fig. 2). Although Ang II [angiotensin-(1-8)] is the major end-product of the system, the action of other enzymes on Ang II may cleave a further one or two amino acids from the amino end, to yield Ang III [angiotensin-(2-8)] and Ang IV [angiotensin-(3-8)], respectively. Cleavage from the carboxyl end yields angiotensin-(1-7). Ang III and IV may play an important role in the brain, whereas angiotensin-(1-7) has vasodepressor properties and may contribute to the antihypertensive actions of ACE inhibitors; angiotensin-(1-7) may be formed directly from Ang I. These angiotensin peptides will not be discussed further in this Chapter, as they are the major focus of final two chapters of this volume.
- Components of the RAS reside within many tissues. Although most of the circulating renin comes from the kidney and most of the circulating AGT comes from the liver, components of the RAS (renin, AGT, ACE) may also be expressed locally within tissues. Circulating renin and AGT constitute the systemic RAS, and local components of the RAS constitute the tissue RAS. Local RAS have been described in many organs, such as the brain, heart, vascular wall, kidneys (interstitium), fat tissues, gut, pancreas, reproductive organs and the adrenals, and may play an important local role. For example: brain and intrarenal RAS are thought to contribute to salt balance and BP control; heart and vascular RAS are involved in cardiovascular pathology. The various tissue RAS will be discussed in detail in Part 4 of this book. In this chapter, we will focus on the physiological role of the systemic RAS.

1.3

Angiotensin II Acts on Specific Receptors

Two main cell surface receptors to Ang II have been identified: AT₁ and AT₂. In rodents, there are two isoforms of the AT₁ receptor, designated AT_{1a} and AT_{1b}. This distinction is, however, not relevant to humans, in which a single AT₁ receptor type is found. Other AT receptors have been described: AT₄R and AT₁₋₇R that mediate the effects of other angiotensin peptides and intracellular receptors.

Both the AT₁ and the AT₂ receptors have been cloned and belong to the superfamily of G protein-coupled receptors that contain seven transmembrane regions. They share about 34% homology and have distinct signal transduction pathways. The AT₁ receptor mediates all of the classical actions of Ang II (vasoconstriction, sodium retention, cell growth and proliferation), and can be selectively blocked with pharmacological agents known as sartans. AT₂ receptors are mainly expressed in fetal tissues and their number decreases in the postnatal period; however, their number increases again in tissue injury. AT₂ receptors promote vasodilatation, cell differentiation, inhibition of cell growth and apoptosis, and may play a counterbalancing role to the effects of Ang II on AT₁ receptors.

2

Regulation of Angiotensin II Formation

2.1

Synthesis of Circulating Renin

The human genome contains only one renin gene (Ren-1^c), whereas certain strains of mice, such as 129, have two distinct renin genes (Ren-1^d and Ren-2). Ren-1^c renin gene expression varies in different tissues, but the kidneys are the only organs that can contain substantial amounts of readily releasable active renin. Indeed, bilateral nephrectomy leads to practically undetectable levels of renin in the plasma. Renin is produced by the juxtaglomerular (JG) cells, specialized cells derived from vascular smooth muscle cells located at the end of the afferent arteriole. During sustained stimulation (such as with a low-salt diet), there is not only an increased expression in those cells, but also an expansion in expression to vascular cells situated upstream.

The initial step in renin synthesis is the formation of prorenin by renin messenger RNA, which is transported into the rough endoplasmic reticulum. The “pre” sequence is then cleaved, leaving prorenin, a likely inactive form of renin. Subsequently, prorenin is transported through the Golgi apparatus, glycosylated with mannose-6-phosphate residues, and deposited in granules where the “pro” sequence is cleaved to form renin the active 40,000-Da single-chain polypeptide enzyme. Renin can then be released lumenally into the circulation (or ablumenally into the renal interstitium) by exocytosis in a regulated response to specific mediators.

2.2

Regulation of Renin Release

The juxtaglomerular apparatus plays a central role in the regulation of renin release. It comprises the afferent arteriole, the glomerular mesangium and the macula densa cells of the distal tubule of the same nephron. Three classical stimuli, all elicited by a decrease in BP or blood volume, are known to increase renin synthesis and release:

1. Decreased stretch of the afferent arteriole. The smooth muscle cells of the afferent arteriole are very sensitive to stretch. An increase in intravascular pressure raises intracellular calcium, leading to both a contraction of vascular smooth muscle cells (myogenic vasoconstriction) and an inhibition of renin release. Conversely, a low intravascular pressure in the afferent arteriole stimulates renin release. This is a local effect, which does not require any neural input.
2. Decreased delivery of salt (sodium chloride) to the macula densa. Macula densa cells are modified tubular cells at the end of the loop of Henle that ensure a steady input of salt to the distal tubular cells by controlling both the tone of the afferent arteriole (tubuloglomerular feedback, TGF) and the release of renin. Such a mechanism helps maintain glomerular filtration rate (GFR) at a relatively constant level. Decreases in distal tubular salt delivery are sensed by macula densa cells (probably via the amount of salt which is transported through the luminal Na-K-2Cl cotransporter). This leads to a decreased release of some chemical mediators (ATP, adenosine, NO) by macula densa cells and to an increased release of other mediators (prostaglandin PGE₂). In turn, this chemical modulation of JG cells dilates the afferent arteriole and stimulates renin release.
3. Adrenergic stimulation. The JG cells are directly innervated by sympathetic nerve endings, which act on β_1 -adrenergic receptors expressed on cell surface. This results in an increased formation of cyclic adenosine monophosphate (cAMP) that stimulates renin release. Renal sympathetic nerves have a very potent effect on renin release, occurring at levels of sympathetic activity much lower than those required for acute sodium retention or renal vasoconstriction (DiBona and Kopp 1997).

All three stimuli operate simultaneously and are usually stimulated by the same conditions: a decrease in blood pressure (BP). When BP falls, there is decreased stretch of JG cells and salt delivery to the macula densa (due to a decrease in GFR and an increase in proximal tubular reabsorption). In addition, the low arterial pressure unloads carotid and aortic baroreceptors, leading to renal sympathetic nerve activation and thus to β_1 -receptor stimulation.

In addition to these three classical stimuli, some circulating hormones or substances can directly stimulate or inhibit renin release by the juxtaglomerular apparatus. For the purpose of this chapter, two substances that inhibit renin re-

lease are particularly worth mentioning: atrial natriuretic peptide (ANP), a hormone released by the atria in response to an increased blood volume, and Ang II. The inhibitory effect of ANP contributes to renin suppression at high salt intake. The inhibitory effect of Ang II constitutes a “short-loop” negative feedback that allows a rapid suppression of renin release, in contrast to a “long-loop” negative feedback (suppression of renin release by hypertension and hypervolemia), which would require many hours or days.

2.3

Modulation of Angiotensin II Production

Under normal circumstances, renin is the rate-limiting step in the formation of Ang II. However, modulation of Ang II formation by other components of the RAS may come into consideration in certain situations.

Modulation by Angiotensinogen. AGT is the only known precursor protein to the family of angiotensin peptides. Systemic AGT originates primarily from hepatocytes where it is constitutively secreted, and is present in the plasma in stable concentrations (half-life of 16 h, in contrast to 20 min for renin). AGT secretion can be modulated by various compounds, such as glucocorticoids, estrogens, thyroid hormones, insulin, selected cytokines, and Ang II itself (Brasier and Li 1996).

Because the normal concentration of AGT is near the K_m for its reaction with renin (Gould and Green 1971), one would expect any change in AGT levels to be accompanied by parallel changes in the formation and actions of Ang II. On the other hand, an AGT-mediated increase in Ang II levels (and action) should lead to a suppression of renin release via both the short and long negative feedback loops, and thereby a return to normal levels of plasma Ang II concentration. Yet there is indirect evidence that AGT may play a role in human hypertension (Jeunemaitre et al. 1992).

To understand this paradox, two explanations can be presented: (1) A hypertensive effect of AGT is expected in situations when renin release is poorly modulated, such as during renal damage or in 129 mice which have two distinct renin genes, one of which is submaxillary and is not subject to the usual negative feedback control (Wang et al. 2002). Indeed, 129 mice engineered to carry one to four copies of the AGT gene have AGT concentrations and BP levels that correlate to the number of AGT gene copies (Kim et al. 1995). (2) High levels of systemic AGT may also promote hypertension by increasing local formation of Ang II in various tissues, where it is not subjected to systemic feedback control.

Modulation by ACE. Various substances, such as NO and Ang II itself, have been shown to downregulate the activity of ACE in endothelium. However, the physiological role of these modulations remains unclear. Experimental data from animals and computer simulations have indicated that modest changes in ACE activity in either direction have little effect on the production of Ang II itself

(Smithies et al. 2000). As a matter of fact, mice engineered to carry one to three copies of the ACE gene do not show any variation in blood pressure (Krege et al. 1997).

On the other hand, ACE is present not only on vascular endothelial cells, but also on the cell membranes of many different cells. High levels of ACE in the microvilli of proximal tubular cells may produce high local levels of Ang II, promoting sodium reabsorption. The presence of ACE in inflammatory cells could also contribute to vascular disease (Dzau 2001). Furthermore, high levels of ACE may decrease bradykinin levels and thus contribute to the diabetic proteinuria observed in diabetic mice with genetically higher ACE levels (Huang et al. 2001), or in humans with the insertion/deletion ACE polymorphism.

In addition to the various factors that regulate or modulate Ang II formation, there are also a number of mechanisms by which Ang II action may be regulated: e.g. variations in AT receptor density, interactions between AT₁ and AT₂ receptors, or post-receptor modulation. Those complex interactions will be dealt in subsequent chapters of this book.

3

The RAS Is an Important Physiological Control System

The RAS is a complex regulatory system with many identifiable actions. However, it may primarily be viewed as a powerful regulatory system for salt conservation, blood volume and BP. A minimal intake of salt is required to compensate for obligatory salt losses by urine, sweat, faeces and epithelial desquamation. When animals are put on an extremely poor sodium diet, they exhibit hypovolaemia with possibly impaired exercise performance, thus becoming easier prey for predators. Salt depletion also endangers species survival due to poor reproductive functions such as decreased fertility, decreased number of pups in the litter and decreased pup size (McBurnie et al. 1999).

In the case of man, our ancestors routinely consumed a poor sodium diet (10–30 mmol/day) (Eaton and Konner 1985; MacGregor and de Wardener 1998). Stringent mechanisms for salt conservation were thus required to regulate the amount of fluid in our bodies. Without an efficient RAS, our ancestors would have never survived the additional stresses associated with starvation or haemorrhage, and would not have the required hemodynamic reserve for fight-or-flight reactions.

The RAS is a complex system, with more than 60 Ang II actions. In this chapter, we will show how many of these diverse actions of Ang II can be viewed in a single conceptual framework, as serving to prevent life-threatening shrinkage of intravascular volume (rapid actions of angiotensin), to help achieve sodium balance without large alterations in BP (slower actions of angiotensin) and to increase the efficiency of cardiovascular dynamics by promoting the growth of the heart and vessels, and sensitizing blood vessels to vasoconstrictor agents (slowest actions of angiotensin).

3.1

The Rapid Actions of Angiotensin Prevent Life-Threatening Hypovolaemia and Hypotension

Most of the rapid actions of Ang II can be viewed as a concerted response that supports the circulation when it is threatened by intravascular volume shrinkage and/or hypotension. Indeed, the main physiological stimuli for RAS activation are low salt intake, blood volume and BP. In turn, Ang II acts to help raise blood volume and BP via combined actions illustrated in Fig. 3: all are exerted via the AT₁ receptor.

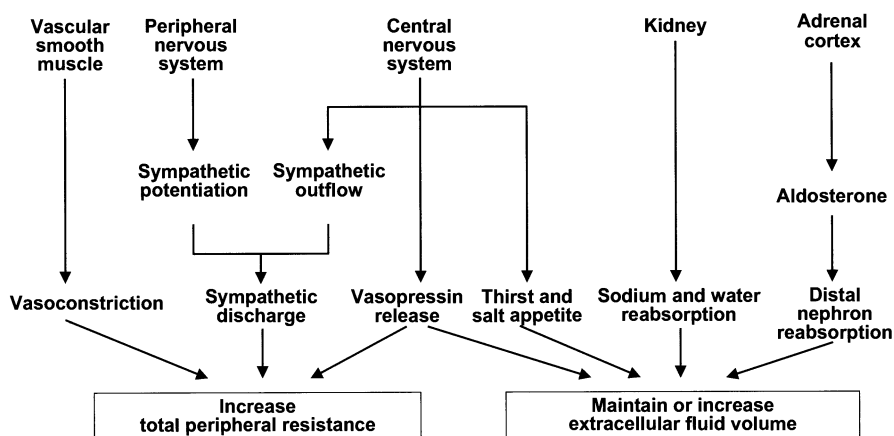


Fig. 3 Schematic diagram, showing the major effects of Ang II on total peripheral resistance and extracellular fluid volume preservation

3.1.1

Angiotensin Increases Total Peripheral Resistance

Ang II is a potent vasoconstrictor agent that elevates vascular tone by both direct and indirect mechanisms. Binding of Ang II to AT₁ receptors located on the surface of vascular smooth muscle cells leads to an immediate contraction. It is interesting to note that Ang II does not exert identical vasoconstrictor effects on all vessels. For example, renal post-glomerular (efferent) arterioles are exquisitely sensitive to Ang II, whereas pre-glomerular (afferent) arterioles show very little direct sensitivity to Ang II (Edwards 1983).

Ang II may also increase vascular tone by indirect mechanisms. Ang II increases sympathetic discharge via direct action at various brain structures that lack a blood–brain barrier, and can also potentiate the release of norepinephrine from adrenergic varicosities within peripheral tissues. This sympathetic effect is normally blunted or even suppressed *in vivo* by the vasoconstriction-induced rise in arterial pressure, which loads baroreceptors and results in a reflex de-

crease in sympathetic nerve activity (Lohmeier et al. 2000b). Situations associated with baroreflex impairment (such as heart failure or vasculopathies with aortic and carotid stiffness) may thus unmask the sympathoexcitatory actions of Ang II.

Another central action of Ang II is the stimulation of vasopressin release by the posterior pituitary gland. The quantitative contribution of this effect is not well established. However, very low levels of circulating vasopressin not only causes antidiuresis (via the V_2 -receptor), but can also increase total peripheral resistance (Montani et al. 1980) and favour a more efficient renal countercurrent system by vasoconstriction of renal vasa recta (Cowley 2000), thus facilitating renal retention of sodium and water.

3.1.2

Angiotensin Preserves Extracellular Fluid Volume

Ang II also acts to maintain or increase extracellular fluid volume (ECFV), both by promoting water and sodium intakes, and by decreasing water and sodium excretions. Intracerebral infusions of Ang II in experimental animals increase both thirst and salt appetite, leading to increased water drinking, and preferential drinking of a saline solution when the animal is offered both saline and water solutions. This effect is also seen in response to moderate elevation of circulating Ang II levels, due to its actions on various regions of the brain involved in thirst and salt appetite (Fitzsimons 1998).

Ang II acts on many other tissues with the same general goal of sodium and ECFV preservation. It enhances epithelial sodium transport in the gut. In the kidney, it promotes Na^+/H^+ exchange in the apical membrane of proximal tubular cells, augmenting sodium reabsorption. Renal vasoconstriction with predominantly post-glomerular constriction leads to a decrease in peritubular capillary hydrostatic pressure, and to an increase in filtration fraction that concentrates post-glomerular plasma protein concentration, further boosting sodium reabsorption (Hall 1986a). Constriction of efferent arterioles of juxtamedullary nephrons and/or a direct action on vasa recta lowers renal papillary blood flow, enhancing urine-concentrating capability. Finally, Ang II acts on the adrenal glands to promote secretion of aldosterone, a sodium-retaining hormone acting on the distal parts of the nephron.

3.1.3

Other Physiological Actions of Angiotensin Contribute to Corporal Integrity

Other actions of Ang II that may seem unusual or even harmful at first glance, do fit well in the general scheme of homeostatic functions. Some of these actions include:

- a. Preservation of glomerular filtration rate at low perfusion pressures. When arterial pressure increases suddenly, myogenic and TGF-induced vasocon-

strictions of the afferent arteriole protect the glomerulus, thereby preventing large increases in GFR. When arterial pressure decreases, these mechanisms are reversed; i.e. decreases afferent arteriolar tone. However, they are not very effective in maintaining GFR in situations of low perfusion pressures, and thus constriction of efferent arteriole by angiotensin then becomes crucial to preserve GFR. In situations when the RAS is activated (low salt intake, volume depletion), blockade of the RAS does indeed impair autoregulation of GFR at low perfusion pressures (Hall et al. 1977). This situation is well known to clinicians prescribing inhibitors of the RAS in hypertensive patients with renal artery stenosis (Hricik et al. 1983). Mechanisms for preservation of GFR during low salt intake and volume depletion are important as they allow the kidney to continue its filtrating and detoxifying functions.

- b. Haematopoiesis. ACE inhibitors often lead to a reduction in haematocrit, and RAS activation leads to erythropoiesis. Ang II stimulates the renal production of erythropoietin via an AT_1 receptor-dependent pathway (Gossmann et al. 2001) by decreasing oxygen concentration around peritubular fibroblasts. This action is due to the combined action of Ang II in decreasing renal blood flow and thus oxygen delivery, and in increasing tubular sodium reabsorption and thus oxygen consumption. Ang II may also act directly on AT_1 receptors present on erythroid precursor cells of the bone marrow (Rodgers et al. 2000). In situations of chronic volume depletion (e.g. during extreme salt deprivation or following haemorrhage), an increased haematocrit would compensate for the hypovolaemia-induced decrease in cardiac output.
- c. Procoagulatory effects. The mild stimulatory effects of Ang II on the coagulation cascade and on platelet activation (Larsson et al. 2000) can be viewed as a volume-conserving reaction during haemorrhage.
- d. Stimulation of liver glycogenolysis. Ang II infusion leads to an elevation in blood glucose levels (Machado et al. 1998) as a consequence of an AT_1 -receptor dependent action on hepatic glycogen phosphorylase (Keppens et al. 1993). Hyperglycaemia becomes useful in fight-or-flight situations, as this condition provides energetic fuel to the skeletal muscles.
- e. Inotropic actions. Ang II increases cardiac contractility (Mattiuzzi 1997). The exact mechanism of this action remains poorly understood, but it may be related both to a potentiation of norepinephrine release at adrenergic endings, and to a direct effect of Ang II on the myocardium. In any case, this represents a useful response during situations such as acute hypovolaemia (e.g. following haemorrhage).

3.1.4

Activation of the RAS Is a Useful Response in Many Demanding Situations

- a. An effective RAS attenuates orthostatic hypotension. Standing upright leads to an accumulation of blood in the legs, a decrease in venous return and thereby a decrease in cardiac output. In turn, arterial pressure decreases, which stimulates renin release in a matter of minutes, helping to restore BP.

Complementary systems (sympathetic nervous system and vasopressin) work concurrently to oppose acute drops in BP, and thus compensate for a poorly reactive RAS. Blockade of the RAS may lead to severe orthostatic hypotension if autonomic function or vasopressin release is impaired, or in situations of pre-existing hypovolaemia. Hypertensive patients with restricted sodium intake (10 mmol/day for 5 days) suffered from circulatory collapse during an orthostatic tilt, when renin release was prevented from rising by pretreatment with propranolol (Morganti et al. 1979).

- b. An effective RAS prevents hypotension during low salt intake or during dehydration. A poor sodium diet or a state of dehydration leads to a decrease in circulatory filling pressures and blood volume. However, activation of the RAS in these circumstances leads to a restoration of filling pressures and blood volume to near normal values, raising BP back to normal. The importance of RAS activation during low salt intake is illustrated by the fact that blockade of the RAS in dogs maintained on sodium intake of 5 mmol/day for 1 week lowered mean arterial pressure to about 68 mmHg (Hall et al. 1980)—a low value perhaps tolerated at rest, but probably not very well during exercise or orthostasis. Marked hypotension has also been described in patients treated with ACE inhibitors who experience gastrointestinal fluid loss or other types of volume depletion (McMurray and Matthews 1987).

3.2

The Slower Actions of Angiotensin Contribute to Sodium Balance

All the aforementioned renal actions of Ang II, not only help maintain ECFV and BP during prolonged periods of low salt intake, but play also an important role in defending ECFV and BP in the face of high salt intake. The beauty of the RAS is that it works in both directions. In one direction, the system is stimulated by low BP and blood volume induced by a low salt intake, and acts to oppose these perturbations by way of a classical negative feedback system. In the other direction, the system is suppressed in situations of increased BP and blood volume such as may be induced by high salt intake. One of the major roles of a normal RAS regulation is to prevent volume-dependent salt sensitivity. To illustrate this concept, a few words on the role of the kidney on long-term BP control are needed.

3.2.1

The Cardiovascular System Is More Than a Simple Closed Circuit

The cardiovascular system is often viewed as a simple closed circuit consisting of a pump (the heart) and a series of tubes with varying resistance (the vasculature). In this model, all that counts for BP control is the strength of the heart and the resistance of the peripheral vasculature. A more complete representation of circulatory function and BP control would be a cardiovascular system with an input from the outside (fluid and salt intakes) and an output to the out-

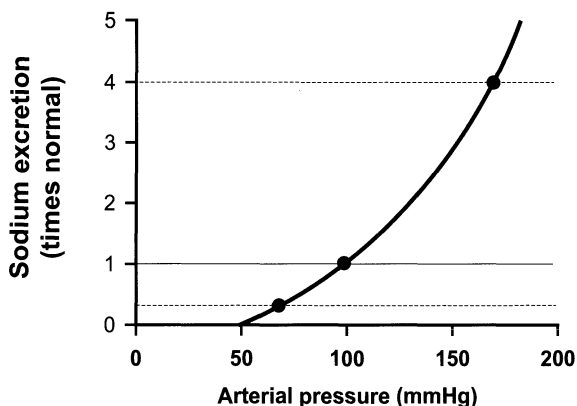


Fig. 4 The basic concept of pressure natriuresis to achieve sodium balance. Three levels of salt intake are depicted (normal, 0.2×normal and 4×normal). Equilibrium is reached at the intersection between the pressure natriuresis curve and the corresponding level of salt intake

side (urinary excretion). Any change in the input would alter blood volume and thereby BP, which in turn leads to changes in the output via pressure-natriuresis. In fact, changes in renal perfusion pressure, regardless of whether the kidney is studied *in vivo* or *in vitro*, lead to profound changes in sodium excretion, as illustrated by the solid curve in Fig. 4. The intrarenal mechanisms for this phenomenon have been detailed elsewhere (Granger et al. 2002).

3.2.2

Fluid Volume Equilibrium Is Reached When Salt Excretion Is Equal to Salt Intake

The pressure-natriuresis curve (PNC) in Fig. 4 is at the centre of blood volume and BP control. If the body gains too much fluid (e.g. acute volume load), BP increases. This leads to increased excretion of salt and water via the pressure-natriuresis mechanism, bringing blood volume and BP back towards normal. Conversely, if one loses fluids (e.g. haemorrhage), BP decreases and the kidneys retain salt and water, which helps return blood volume and BP to normal. Equilibrium is thus reached at the intersection point of the PNC with the corresponding salt intake level, as shown on Fig. 4.

Based on this concept, one can understand the general renal body fluid feedback mechanism (Hall et al. 1986b). Any imbalance between salt intake and output will lead to a cascade of events that oppose the initial disturbance; i.e. a classical negative feedback loop. For example, if salt intake is greater than output, ECFV increases, thereby increasing blood volume and thus the mean filling pressure of the circulation; this then increases cardiac output, and thus BP. In turn, the higher BP increases salt output, which opposes the effects of the initial increase in salt intake by way of the pressure-natriuresis mechanism. The system acts very slowly (hours or days), but it has an infinite gain and corrects completely any error in salt balance.

This simplified feedback loop may not explain the whole story. According to this analysis, a fourfold increase in salt intake would lead to volume retention until BP increases to well over 150 mmHg (Fig. 4). Sodium balance would be reached, but at the expense of profound volume retention and tremendous hypertension. Similarly, a poor sodium diet (for example, 1/5 of normal) would require a drop in BP by 30 or 40 mmHg to achieve a state of sodium balance. Yet, this sensitivity to salt does not fit with the small variations in BP that are normally observed when animals (Hall et al. 1980) and humans (Luft et al. 1979) are subjected to large variations of salt intake. Clearly, additional mechanisms must come into play.

3.2.3

The Pressure Natriuresis Curve Is Modulated by Changes in Salt Intake

The PNC depicted in Fig. 4 is not immovable. In fact, it becomes steeper and is shifted to the left during high salt intake. This allows the body to achieve sodium balance with minimal increases in blood pressure. Conversely, during low salt intake, the PNC becomes flatter and is shifted to the right. Joining the equilibrium points at the various salt intakes reveals a very steep “chronic” relationship with little changes in BP (Fig. 5). That is, the chronic relationship between salt intake and blood pressure has become relatively salt-*insensitive*.

Various neurohormonal mechanisms contribute to the adjustment of the PNC. RAS modulation, above all, plays a crucial role in the adaptation to changes in salt intake. RAS suppression at high salt intake facilitates sodium excretion, and RAS stimulation at low salt intake contributes to sodium conservation. The importance of this modulation is illustrated by the dramatic salt-induced changes in BP that occur when the RAS is blocked with an ACE inhibitor, or when circulating Ang II levels are fixed with an intravenous infusion of angio-

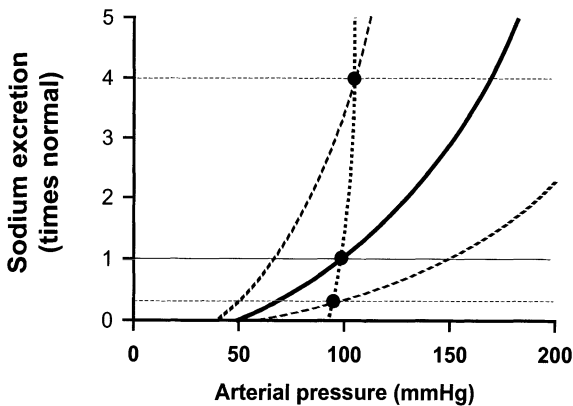


Fig. 5 The modulation of the pressure natriuresis curve during alterations in salt intake allows the body to achieve sodium balance with minimal changes in arterial pressure (*steep dotted line*)

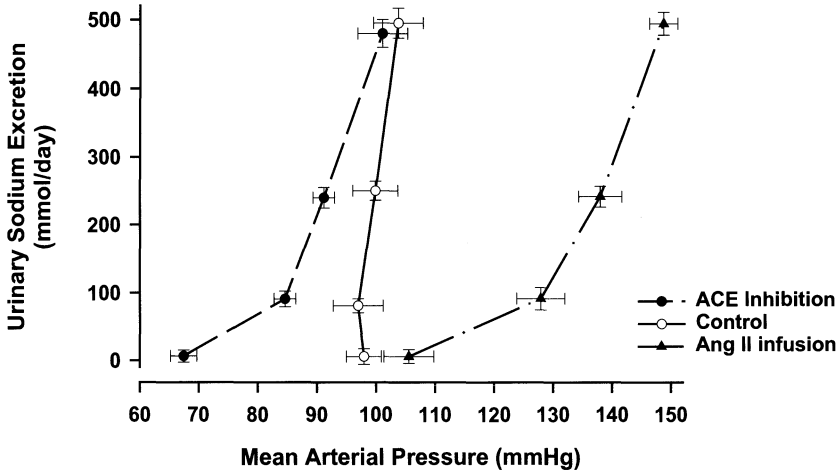


Fig. 6 Steady-state relationships between mean arterial pressure and urinary sodium excretion in dogs subjected to varying salt intakes from 5 mmol/day to 500 mmol/day, lasting 1 week at every level. The dogs were studied under three conditions: (1) with an intact RAS; (2) during chronic blockade of the RAS with captopril; (3) during fixed elevated circulating levels of Ang II via an intravenous infusion of Ang II at 5 ng/kg/min. (From Hall et al. 1980, by permission)

tensin (Hall et al. 1980) (Fig. 6). Lack of RAS activation during low salt intake leads to a dramatic decrease in mean arterial pressure to less than 70 mmHg.

But by which mechanisms do Ang II levels vary with changes in salt intake? The sequence of events is presented in Fig. 7. The initial increase in salt intake (salt with little water) leads to increased plasma osmolality, resulting in thirst

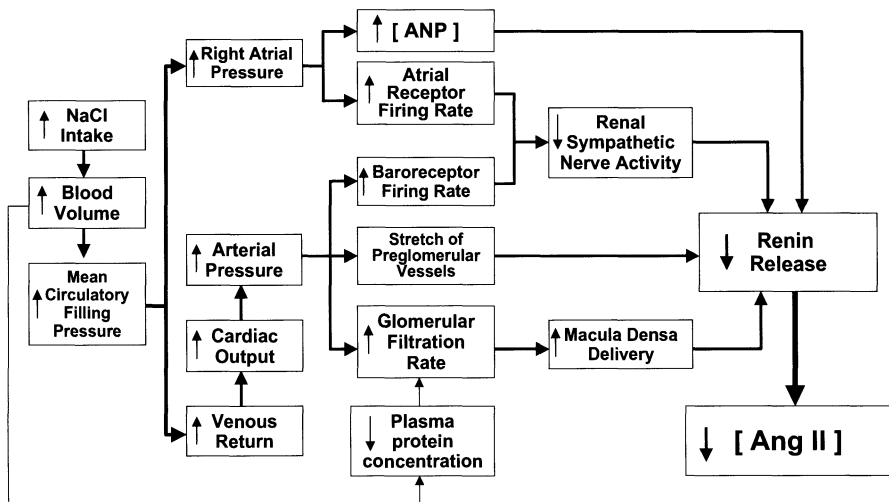


Fig. 7 Block diagram illustrating the mechanisms whereby an increased in salt intake leads to a decrease in Ang II formation

and drinking and thus an increase in extracellular fluid volume. This then increases blood volume and decreases, at least acutely, plasma protein concentration. Subsequently, a complex but logical sequence of events takes place. The decrease in plasma protein concentration favours an increased fluid filtration across the glomerular capillary membrane. The greater blood volume increases mean circulating filling pressure (greater content for the same container), resulting in both an increase in right atrial pressure and venous return.

The increased right atrial pressure stretches the right atrium, and loads low pressure receptors that reflexly decrease vasopressin secretion and renal nerve sympathetic activity. Atrial stretch leads also to a direct increased release of atrial natriuretic peptide (ANP), a hormone that has a direct inhibitory action on renin release (and aldosterone secretion). By its vasodilatory action on preglomerular vessels, ANP also promotes an increase in GFR.

The greater venous return increases cardiac output and thus arterial pressure, which in turn leads to three events: (1) loading of arterial carotid and aortic baroreceptors, and resulting in a decreased renal sympathetic nerve activity; (2) mechanical stretch of preglomerular vessels; (3) increase in delivery of fluid and salt to the macula densa, mediated by the small increase in GFR (favoured by physical forces and accentuated by the vasodilatory ability of ANP on the afferent arteriole). Altogether, these three events promote a decrease in renin release, and thus in Ang II levels.

A similar flowchart can be applied, but in the reverse direction, to explain the increased Ang II levels during low salt intake. Volume depletion leads to a decrease in filling pressures and arterial pressure. Unloading of atrial and arterial baroreceptors, decreased ANP concentration, decreased preglomerular stretch and decreased salt-delivery to the macula densa all contribute to the stimulation of renin release.

3.2.4

The Inability to Modulate the RAS Leads to Salt Sensitivity

As shown in Fig. 6, the ability to suppress renin release during high salt intake and to stimulate renin release during low salt intake is the cornerstone of having very little salt sensitivity. If the RAS is blocked with an ACE inhibitor or if Ang II levels are not allowed to fluctuate naturally in response to varying salt intakes, rapid, volume-dependent salt sensitivity ensues.

Thus one of the major roles of the RAS is to prevent a large drop in BP (and ECFV) during low salt intakes, and a large increase in BP (and ECFV) during high salt intakes. In other words, when the ability to suppress renin at high salt intakes is lost, volume-dependent salt sensitivity develops. This particularly may occur in the following two situations:

- a. Ageing. Circulating renin levels decrease steadily with age (Weidmann et al. 1975), possibly related to the observed decrease in glomerular number and size that occur with ageing (Nyengaard and Bendtsen 1992). The response

of renin in older individuals is also blunted when the RAS is either stimulated (volume contraction) or inhibited (volume expansion) (Luft et al. 1992). The lower basal levels of plasma renin activity and poor reactivity of the RAS may help explain the higher prevalence of salt sensitivity in older subjects.

- b. Low-renin essential hypertension. About one quarter of all essential hypertensive patients have low renin levels that are poorly stimulated by a low salt intake (Fisher et al. 2002). Because renin levels are low to start with, the incapacity to further suppress renin at high salt intake may explain the salt sensitivity frequently observed in low-renin essential hypertension.

On the other end of the PNC, the concept of RAS modulation is particularly useful to understand the increased effectiveness of ACE inhibitors or angiotensin-receptor blockers in lowering blood pressure, if treatment is combined with a reduction in salt intake or with the use of diuretics.

3.3

The Slowest Actions of Angiotensin Increase the Efficiency of the Cardiovascular System

3.3.1

Angiotensin Promotes Vascular Growth and Cardiac Hypertrophy

By way of its effect on the AT₁ receptor, Ang II is also a growth factor, acting on vascular smooth muscle cells and cardiac myocytes. The trophic response to Ang II leads to a slow structural remodelling that helps maintain a higher BP. With a greater vascular muscle mass that increases both the strength of vascular contraction and the sensitivity to chronic vasoconstrictors, the vascular system becomes more effective in maintaining a high vascular tone. With a larger myocardial mass that increases cardiac strength, the heart becomes more capable in maintaining a high blood pressure. In both cases, activation of the local tissue RAS may play an important role in this trophic response.

3.3.2

Angiotensin Stimulates Superoxide Anion Formation

The superoxide radical (O₂⁻) is produced endogenously during normal mitochondrial respiration and by various oxidases, especially NADH ((nicotinamide adenine dinucleotide, reduced) and NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidases. The superoxide anion may play a physiological role in various tissues as a signalling molecule and may contribute to the regulation of vascular tone (Touyz 2000), particularly in the renal microvasculature (Schnackenberg 2002). In normotensive anaesthetized rats, the administration of tempol, a mimetic of the enzyme superoxide dismutase that decreases superoxide levels, causes an increase in medullary blood flow, urine flow and sodium

excretion (Zou et al. 2001), but has no effect on basal afferent arterioles. This suggests that the superoxide anion may participate in maintaining the basal tone of the renal medullary microcirculation. In normal conditions, superoxide may also scavenge the NO formed in macula densa cells by neuronal NO synthase, thereby increasing the gain of the TGF (Ren et al. 2002). Both effects tend to increase the renal ability to retain salt.

Ang II can, via activation of the AT₁ receptor, stimulate NAD(P)H-oxidase and thereby the production of the superoxide anion. Some of the effects of Ang II on vasoconstriction and renal sodium retention may thus be mediated by oxygen radicals. For example, infusions of low doses of Ang II do not produce immediate hypertension, but a slow, progressive elevation of BP over hours or days. Furthermore, the administration of tempol attenuates or prevents Ang II-induced hypertension (Ortiz et al. 2001). The stimulation of oxygen radicals by Ang II may thus be viewed as a slow physiological response to enhance the long-term vasoconstrictor and sodium-retaining effects of Ang II.

4

Pathophysiology of the RAS

So far, we have reviewed the actions of the RAS from the general point of view of cardiovascular homeostasis. However, increased activity of the RAS, especially in combination with other cardiovascular risks factors, may lead to a cascade of deleterious effects such as hypertension, cardiovascular hypertrophy, oxidative stress with endothelial dysfunction, atherosclerosis and tissue inflammation. Many of these pathophysiological actions of Ang II will be reviewed in detail in other chapters of this book. At this stage, it is interesting to consider that many of these Ang II actions may still be viewed as being homeostatic in principle, but harmful if carried to excess.

4.1

The RAS May Contribute to the Higher Cardiovascular Risks of Males

Although being male is not exactly a pathological situation, men before the age of 50 show a higher prevalence of hypertension and a greater cardiovascular morbidity than premenopausal women. Blood pressure in a normotensive population is also higher in men than in women. Interestingly, men have higher plasma renin activities than women, and there is indirect evidence that increased levels of renin may contribute to increased cardiovascular risks. Indeed, hypertensive patients with high levels of plasma renin activity are at higher risk of developing stroke or myocardial infarction than those with low plasma renin activity (Brunner et al. 1972).

Plasma renin levels are higher in male spontaneous hypertensive rats (SHR) and their BP is 25–30 mmHg higher than in female SHR (Reckelhoff et al. 2000). On the other hand, castrated male SHR, female SHR and ovariectomized female SHR all show about the same level of BP. In contrast, female ovariectomized

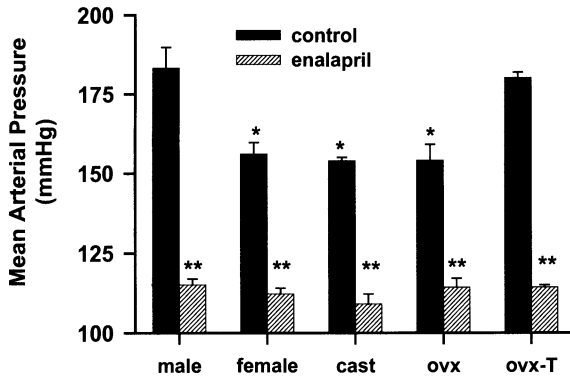


Fig. 8 Mean arterial pressure in five groups of SHR: male, female, castrated males (*cast*), ovariectomized females (*ovx*) and ovariectomized females receiving testosterone (*ovx-T*). Animals were studied with an intact RAS (*black bars*) or after chronic blockade of the RAS with enalapril (*shaded bars*). (From Reckelhoff et al. 2000, by permission)

SHR, given testosterone, show a degree of hypertension approaching the level observed in male SHR, pointing to the role of testosterone in the more severe hypertension of male SHR (Fig. 8). When the RAS is blocked with enalapril, all five groups of animals show remarkably similar BP levels, suggesting that the pressure difference between male and female SHR is due entirely to a more active RAS in male animals, and testosterone may contribute to the higher renin levels in males. This is consistent with the observation that normotensive castrated male Sprague Dawley rats, with undetectable serum testosterone levels, have low renin levels, and that implantation of testosterone pellets of increasing concentrations not only raise testosterone levels in blood, but also plasma renin activity with a significant linear correlation ($r=0.904$) between the two variables (Reckelhoff et al. 2001). Whether this animal observation can be extrapolated to humans remains to be further investigated.

4.2

The RAS Contributes to Many Forms of Hypertension

In humans, known causes of arterial hypertension account for less than 10% of all cases of hypertension (Kaplan 1998). Most often, a precise cause cannot be found and the hypertension is said to be “essential”. Since Ang II elevates BP, it is appealing to implicate an overreactivity of the RAS in the pathogenesis of certain forms of hypertension.

4.2.1

Role of the RAS in Renovascular Hypertension

Since the classical experiments of Goldblatt, many studies have appeared, inducing hypertension in animal models by clipping one or both renal arteries. How-

ever, the contribution of the RAS in renovascular hypertension depends on the type of stenosis.

- a. One kidney-one clip (1K1C) hypertension. Experimentally, a stenotic clip is placed on the renal artery of one kidney whereas the contralateral kidney is removed. The renal artery stenosis reduces renal perfusion pressure, which may explain many of the initial events, including sodium retention and stimulation of the RAS. However, as the animal retains volume over time and becomes hypertensive, the glomerular pressure tends to return towards normal and there is no longer a strong stimulus for renin release. At this stage, administration of an ACE inhibitor has little effect on BP. The hypertension is volume-dependent but no longer renin-dependent. The clinical equivalent of 1K1C is renal artery stenosis in a patient with a solitary kidney, or bilateral renal artery stenoses (2K2C) or stenosis of the aorta above the origin of the renal arteries. All three situations are characterized by a low renal perfusion pressure.
- b. Two kidney-one clip (2K1C) hypertension. The pathogenesis of hypertension in this model is more complex. The stenotic kidney is underperfused and thus secretes large amounts of renin. The resulting elevation in plasma Ang II acts on the intact contralateral kidney, both by a direct effect and via stimulation of aldosterone secretion to promote enhanced sodium reabsorption. Initially, both kidneys may retain salt, but the stenotic kidney with its lower distal renal artery pressure and its locally stimulated RAS retains much more salt than the contralateral kidney. As BP rises due to the volume expansion, systemic BP increases ultimately high enough to achieve sodium balance by the pressure natriuresis mechanism, sodium excretion being now slightly elevated in the intact kidney and slightly decreased in the stenotic kidney (Mizelle et al. 1993). Because there is a continuing stimulus for renin release from the stenotic kidney and possible accumulation of intrarenal Ang II in the nonstenotic kidney (Navar et al. 1998), this hypertension is highly angiotensin-dependent and responds well to blockers of the RAS.

4.2.2

Role of the RAS in Essential Hypertension

Low renin levels would be expected in essential hypertension because of the higher renal perfusion pressure. However, the observation that most hypertensive patients have either normal or high renin levels has led to the view that renin may play a critical role in the pathogenesis of many forms of essential hypertension (Laragh 1992). Several factors have been presented to explain these “inappropriate” high levels of renin, such as a state of high sympathetic drive found in many hypertensive patients (Julius 1988), nephron heterogeneity with a subpopulation of ischaemic nephrons responsible for increased tonic renin release (Sealey et al. 1988) or a defective feedback regulation with nonmodulation

of the RAS (Williams et al. 1992). As expected, patients with normal or high renin levels respond better to β -adrenergic blockers and ACE inhibitors, whereas low-renin hypertensive patients respond better to diuretic treatment.

4.3

Angiotensin-Induced Cardiac and Vascular Hypertrophies Are Risks Factors

As mentioned above, the growth-promoting actions of Ang II may be viewed as an appropriate response in conditions in which increased heart strength and prolonged vasoconstriction are required. A certain degree of vascular hypertrophy is also useful in volume-loading hypertension, as it minimizes the amount of volume retention needed within the vascular system to maintain an elevated blood pressure. Indeed, the various forms of volume-dependent hypertension (mineralocorticoid-induced hypertension, high salt intake with a reduced renal mass) are characterized experimentally by an initial increase in cardiac output followed by a secondary autoregulatory vasoconstriction that returns blood volume and cardiac output towards normal (Guyton 1980). Were it not for autoregulation and vascular hypertrophy, reestablishment of sodium balance would be accompanied by much larger changes in fluid volumes.

Although cardiac and vascular hypertrophies may be considered adaptive from the point of view of enhancing short-term survival, they are clearly detrimental when allowed to continue to progress over prolonged periods of time. The increased stiffness of the hypertrophied heart impairs ventricular relaxation and filling. In fact, left ventricular hypertrophy is considered an independent risk factor for cardiovascular events. Vascular hypertrophy makes arteries stiffer, leading to increased pulse pressure and increased pulse wave velocity. An increased pulse pressure for a given mean arterial pressure is in itself a cardiovascular risk factor: the higher systolic pressure constitutes an elevated left ventricular afterload while the lower diastolic pressure reduces the driving pressure for coronary blood flow. Increased pulse wave velocity results in a rapid return of reflection waves to the heart, increasing systolic pressure and decreasing diastolic pressure further. In this context, one can understand the beneficial health effects of blockers of the RAS in reversing cardiac and vascular hypertrophy.

4.4

Activation of the RAS Worsens Congestive Heart Failure

An activation of the RAS may be a natural response to the initial insult of heart failure. Various models of experimental heart failure (rapid ventricular pacing, pulmonary artery occlusion) in which the same animal could be studied before and after induction of heart failure, are characterized by a decrease in arterial pressure (Mizelle et al. 1989; Lohmeier et al. 1995, 2000a), which is expected to stimulate the RAS. Although there is only a modest activation of the RAS in the early compensated phase of heart failure (Lohmeier 2002), even small increments in Ang II concentration may favour fluid retention. The resulting increase

in fluid volumes could be beneficial, allowing cardiac output and arterial pressure to return towards normal.

However, excessive activation of the RAS in heart failure is clearly harmful. When exogenous Ang II was administered for 4 days to dogs in compensated heart failure, decompensation occurred with profound sodium retention and marked increases in plasma norepinephrine (Lohmeier et al. 2000a). Cardiopulmonary baroreflex suppression of sympathetic nerve activity, which is impaired in heart failure, could play a critical role in the transition from compensated to decompensated heart failure. Impaired sympathoinhibition would unmask the sympathoexcitatory actions of Ang II, resulting in a positive feedback. Ang II would increase sympathetic nerve activity, which stimulates renin secretion further. In turn, higher plasma levels of Ang II would further stimulate sympathetic activity. As a result, there would be a progressive fluid retention and progressive cardiac dysfunction. Consistent with this hypothesis is the observation that ACE inhibitors have been shown to delay the progression of heart failure and to improve symptoms and prolong survival in patients with ventricular dysfunction.

4.5

Systemic and Local Angiotensin May Initiate and Amplify Vascular Disease

Ang II has direct effect on endothelial and vascular smooth muscles cells, and may play a key role in initiating and amplifying vascular disease. In the normal state, there is a homeostatic balance between locally produced NO and oxygen radicals, such as the superoxide anion and hydrogen peroxide. Under these conditions, NO can exert all of its protective functions as vasodilator, inhibitor of platelet aggregation, inhibitor of vascular smooth muscle growth and migration, and inhibitor of the expression of proinflammatory molecules.

As aforementioned, Ang II by its action on AT₁ receptors can stimulate NAD(P)H oxidases, leading to the production of the oxygen radical superoxide. Quenching of NO by the superoxide anion not only reduces the bioavailability of NO (and thereby of all its protective functions), but also forms peroxynitrite (ONOO⁻), a powerful oxidant. The combined action of excess Ang II and oxidative stress may unleash a cascade of harmful effects, such as increased vasoconstriction, increased expression of chemoattractant proteins and leukocyte adhesion molecules, stimulation of thrombosis and vascular remodelling. The local inflammatory response promotes an accumulation of various inflammatory cells that can release enzymes that generate Ang II. For example, macrophages are rich in ACE, neutrophils in cathepsin G, mast cells in chymase. The increased local production of Ang II may further promote oxidative stress, leading to a vicious cycle of inflammation and subsequent increase in tissue Ang II (Dzau 2001).

If endothelial function is preserved, this positive feedback can easily be dampened by the actions of NO and antioxidants. However, in the presence of cardiovascular risk factors, the homeostatic balance between pro-oxidants and antioxidants is perturbed. Dyslipidaemia, diabetes and cigarette smoking can all

initiate endothelial dysfunction and promote oxidative stress. Excessive activity of the RAS potentiates the vicious cycle described above, inducing vascular remodelling, promoting atherosclerosis and upsetting the balance between the fibrinolytic and coagulation systems. The observations may explain why ACE inhibitors and angiotensin-receptor blockers have beneficial effects on cardiovascular events far beyond blood pressure reduction.

5 Conclusions

Without efficient mechanisms for conserving salt, our ancestors living on a diet relatively poor in sodium would have never survived, as they would not be able to respond to even moderate haemorrhage, and not have the required haemodynamic reserve for fight-or-flight reactions. Ang II, the major end-product of the RAS, has multiple actions that work in a concerted manner to maintain cardiovascular integrity and efficiency. Most of the rapid actions of Ang II can be viewed together, in combination with the sympathetic nervous system, to support the circulation when it is threatened by acute disturbances such as hypovolaemia and hypotension. Slower actions of Ang II help maintain volume homeostasis by minimizing the changes in arterial pressure and ECFV required to achieve sodium balance (prevention of salt sensitivity). Some of the very slow actions of Ang II, such as cardiac and vascular hypertrophy and oxidative stress, although potentially harmful, may be viewed as adaptive responses to improve the efficiency of fluid volume and BP preservation. However, such responses are clearly detrimental if carried to excess.

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Phylogeny and Ontogeny of the Renin–Angiotensin System

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Abstract Renin substrate in plasma, biological renin activity, and/or granulated cells in the kidney evolved at an early stage of vertebrate phylogeny. Angiotensin (Ang) I and II molecules have been biochemically identified in representative species of all vertebrate classes, and Ang II structure is well preserved throughout the phylogenetical scale. Ang receptors have also been identified pharmacologically and, in limited species, molecularly characterized. The renin–angiotensin system (RAS) is important in maintaining blood pressure/blood volume homeostasis and ion-fluid balance. Recently, the regulatory role

of Ang in cell growth and vascularization, possibly via paracrine action, has been an intensive focus of research. The RAS has been detected during early fetal development, and genetic or pharmacological blockade of Ang signaling results in striking developmental abnormalities of the kidney. This chapter will provide: (1) a brief overview of current knowledge of RAS phylogeny and ontogeny; (2) a comparison of these two systems in terms of structural, biochemical, and functional properties of each component of the RAS; and (3) perspectives for future study. Discussions are focused on: (1) the most fundamental functions of the RAS that have been conserved throughout phylogenetical advancement as well as developmental maturation; (2) the physiological significance of the shifting of expression of renin-producing cells in the renal arterial trees with vertebrate advancement or ontogenic maturation; and (3) the roles of environments, or microenvironments, in modulating the expression of component members of the RAS during phylogenetic and ontogenic processes. Although ontogeny does not directly recapitulate phylogeny, comparison of these two time-dependent processes should provide new insight into the molecular and functional evolution and significance of the RAS.

Keywords Renin–angiotensin system · Native angiotensin · Renal growth and development · Blood pressure regulation · Juxtaglomerular apparatus · Angiotensin-converting enzyme · Tissue renin–angiotensin · Evolution and development · Renin-secretory cells · Angiotensin receptor subtypes

Abbreviations

ACE	Angiotensin-converting enzyme
AGT	Angiotensinogen
AL	Ascending limb
Ang	Angiotensin
AQP	Aquaporin
BP	Blood pressure
BW	Body weight
cAT ₁	Chicken angiotensin type-1 receptor
CD	Collecting duct
D7–D15	Newborn days 7–15
DCS	Distal convoluted tubule
DHES/s	Dehydroepiandrosterone-sulfate
E18	Embryonic day 18
EDRF	Endothelium-dependent relaxation factor
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
JG	Juxtaglomerular
JGG	Juxtaglomerular granules
NS	Neck segment
PRA	Plasma renin activity
RAS	Renin–angiotensin system

SHR	Spontaneously hypertensive rats
tAT ₁	Turkey angiotensin type 1 receptor
UT	Urea transporters
VEGF	Vascular endothelium growth factor
VSM	Vascular smooth muscle
WKY	Wistar-Kyoto
ZCatD	Zebrafish cDNA

1

Introduction

The renin–angiotensin system (RAS) appears to have evolved during an early stage of vertebrate evolution. Although the molecular identification of each component of the RAS has not been completed in nonmammalian species, biochemical and pharmacological evidence suggests that the RAS and angiotensin (Ang) receptors are present in all vertebrate classes (Fig. 1). Increasing evidence also indicates that the RAS plays an important role during fetal life in promoting growth and development and that lack or impairment of a component of the RAS induces pathological structure and function at birth and/or in adult life. This chapter will provide a brief overview of current knowledge of RAS phylogeny and ontogeny and perspectives on their integration. Following Haeckel’s hypothesis, a number of investigators have tried, but failed, to elaborate more complete taxonomies of the relationship between ontogeny and phylogeny. Although it is not my intent to discuss whether ontogeny recapitulates phylogeny, both are time-dependent processes and show certain similarities. Details of mo-

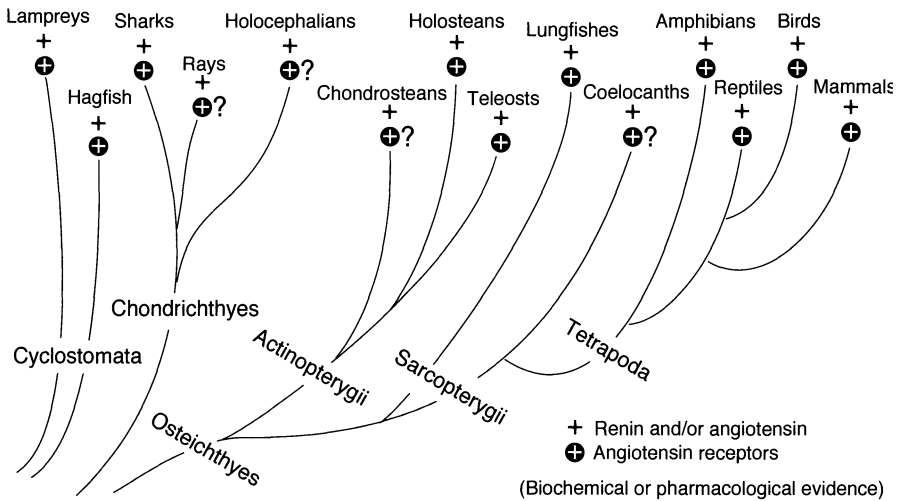


Fig. 1 Diagrammatic presentation of vertebrate phylogenetic tree and presence of biochemical activities of renin and/or angiotensin (+) and molecular, biochemical, or pharmacological evidence for the presence of angiotensin receptors (+ in closed circle). A question mark (?) indicates that the presence of an angiotensin receptor is presumed but not confirmed. (Reproduced from Nishimura 2001)

lecular, biochemical, or physiological properties of each component of the RAS and mechanisms behind them are beyond the scope of this chapter. It is not possible to include all organs and tissues that express the RAS; this review is focused on the kidney and cardiovascular system and their functions.

2 Phylogeny of the Renin–Angiotensin System

2.1 Morphology, Biochemistry, and Molecular Biology

2.1.1 Juxtaglomerular Apparatus in Primitive Vertebrates

The juxtaglomerular (JG) apparatus of the mammalian kidney is composed of JG cells in the afferent arteriole with sympathetic innervation, efferent arterioles, the convoluted early distal tubule (macula densa), and the extracellular

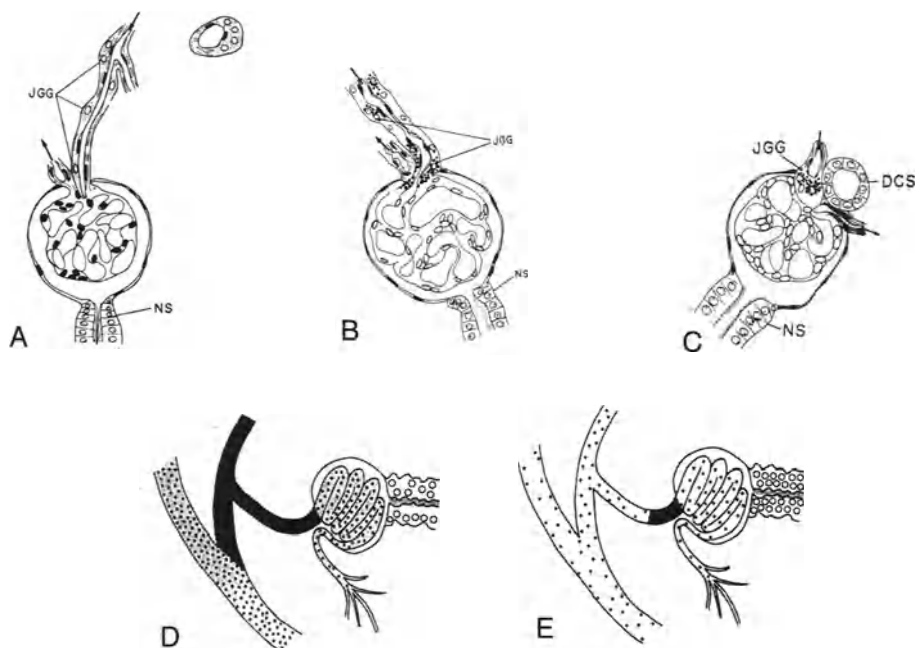


Fig. 2A–E Diagrams of nephron and juxtaglomerular (JG) apparatus showing distribution of JG granules (JGG) in teleost fish (A), amphibian (B), and bird (C) examined histochemically and by electronmicrography. (Reproduced with permission from Sokabe et al. 1969). DCS, distal convoluted tubule; NS, neck segment. Diagrams D (newborn rat) and E (adult rat) indicate the expression of angiotensinogen (open circles), renin (black area), angiotensin-converting enzyme (gray area) and AT₁ receptor (black dots) (Reproduced with permission from Harris and Gomez 1997). Both in primitive vertebrates, such as teleosts, and in newborn rat, expressions of renin (JG granules, renin mRNA and protein) are seen at upstream of renal arteries

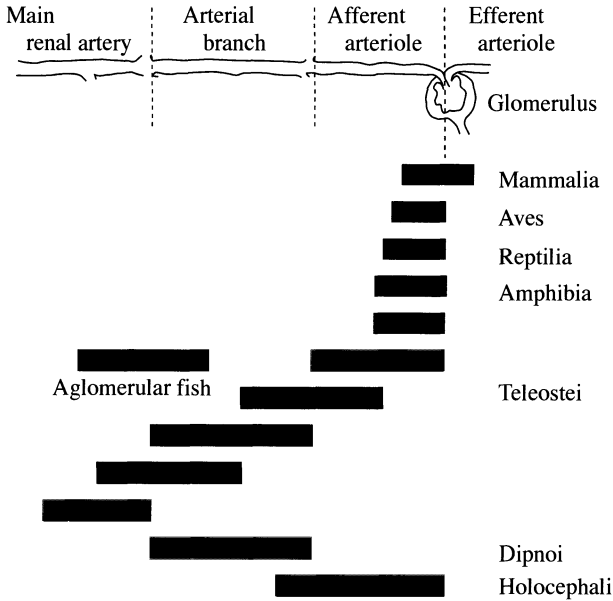


Fig. 3 Distribution of granulated cells along renal arteries and arterioles in various vertebrate animals. Granulated cells are more localized at the juxtaglomerular area with phylogenetic advancement of vertebrates. (Reproduced with permission from Sokabe and Ogawa 1974)

mesangium (Barajas and Latta 1967). The JG apparatus in nonmammalian vertebrates is incomplete (for review, Sokabe and Ogawa 1974; Nishimura 1980). Primitive bony fish, such as holosteans (for example, longnose gar), teleosts, lungfish, amphibians, and reptiles have granulated epithelioid cells resembling mammalian JG cells (Fig. 2). Granulated cells are widely seen in the media of large and small renal arteries and arterioles (Sokabe et al. 1969; Nishimura et al. 1973) (Fig. 3). Kidneys from agglomerular teleosts, in which glomeruli and a part of the proximal tubules have degenerated, also have renin secretory granulated cells. Granulated cells either have not been found (cyclostomes) or are present but negative for Bowie's staining (elasmobranchs) (Lacy and Reale 1990) or the appearance and distribution pattern of the granules differ (holocephalians, such as ratfish) in the more primitive vertebrates (Nishimura et al. 1973; Nishimura 1980).

In the majority of the nonmammalian species examined, either the distal tubules do not attach to the vascular pole of their parent glomeruli (hence, they lack a macula densa) or they return to their own glomeruli and attach to the afferent arterioles as seen in elasmobranchs, holocephalians, and in some amphibians, but no cells resembling mammalian macula densa cells are present (Sokabe et al. 1969; Nishimura et al. 1973, 1980). Avian kidneys possess macula densa cells that have some characteristics of a mammalian macula densa (Sokabe et al. 1969). Therefore, the JG apparatus in birds represents a transitional JG apparatus between the primitive and mammalian types. It therefore appears that the vascular component of the JG apparatus evolved phylogeneti-

cally earlier than the macula densa, the tubular component. This is important when we consider the function of the RAS in nonmammalian vertebrates.

2.1.2

Angiotensinogen and Renin

Angiotensinogen (AGT) levels, determined as maximally yielded Ang I from homologous plasma, appear to be higher in taxonomically more advanced animals than in lower vertebrates (Nishimura et al. 1973). The molecular structure of zebrafish AGT has been identified (Vallon et al. 2000). Renal renin activities (determined as Ang I-forming activities by incubating kidney extracts with homologous plasma under adequate inhibition of angiotensinases and by testing the vasopressor activity of the product in anesthetized rats) have been found in representative species of all vertebrates (Nishimura et al. 1973; Sokabe and Ogawa 1974; Nishimura 1980; Henderson et al. 1993; Hazon et al. 1999) (Fig. 1). This suggests that the RAS evolved at an early stage of phylogeny and is widely distributed among vertebrates. The renal renin activity agrees in general with the occurrence of granulated cells except in cartilaginous fish and primitive bony fishes, which show some discrepancy, suggesting that the properties of renin granules may differ in these fish. To date, no renin has been molecularly identified in nonmammalian vertebrates. Zebrafish cDNA (zCatD) that has high homology to human cathepsin D was cloned from a zebrafish kidney cDNA library using human renin cDNA as a probe (Chen et al. 2000); zCatD generates Ang I from both porcine AGT and zebrafish peptide substrate at an acidic pH (Chen et al. 2000; Vallon et al. 2000). Since renin, pepsin, and cathepsin D appear to have been derived by gene duplication from an ancestral gene encoding for an aspartyl protease, it is possible that aspartyl protease acts as an Ang-forming enzyme in primitive vertebrates. Renin from teleost fish, however, has a neutral optimum pH and substrate specificity and clearly differs from cathepsin (Nishimura et al. 1977). The cleavage of homologous AGT by zCatD should be performed. Zebrafish renin, distinct from cathepsins, has been identified recently (K. Gross, personal communication), but the details have not yet been reported. Plasma renin activity (PRA) has also been measured in several species (for review see Nishimura 1987; Henderson et al. 1993).

2.1.3

Angiotensin

Native Ang ligands appear to be stable molecules throughout the phylogenetic scale, with variation occurring in the amino acid at positions 1 (Asn, Asp, or additional side chain of tri-peptide), 3 (Val, Ile, or Pro), 5 (Ile or Val), and 9 (His, Ser, Tyr, Gly, Thr, etc.) of Ang I (Table 1) (for review, Kobayashi and Takei 1996; Nishimura 2001). Dogfish Ang I (Pro³) (Takei et al. 1993a) and *Crinia* skin Ang II (Ile³) (Erspamer et al. 1979) show unique amino acid variations in position 3. Structure–function relationship studies indicate that changes in the amino acid

Table 1 Native angiotensins of various vertebrate species (reproduced from Nishimura 2001)

Cyclostomes		
Sea lamprey, <i>Petromyzon marinus</i>	[Asn ¹ ,Val ⁵ ,Thr ⁹]Ang I	Takei et al. 2003
Lamprey, <i>Lampetra fluviatilis</i>	[Asn ¹ ,Val ⁵ ,Thr ⁹]Ang I	Takei 1999
Elasmobranchs		
Dogfish, <i>Triakis scyllia</i>	[Asn ¹ ,Pro ³ ,Ile ⁵ ,Glu ⁹]Ang I	Takei et al. 1993a
Bony fish		
Holoosteans		
Bowfin, <i>Amia calva</i>	[Asp ¹ ,Val ⁵ ,Asn ⁹]Ang I	Takei et al. 1998
Teleosts		
Japanese goosefish, <i>Lophius litulon</i>	[Asn ¹ ,Val ⁵ ,His ⁹]Ang I	Hayashi et al. 1978
Chum salmon, <i>Oncorhynchus keta</i>	[Asn ¹ ,Val ⁵ ,Asn ⁹]Ang I	Takemoto et al. 1983
Japanese eel, <i>Anguilla japonica</i>	[Asp ¹ ,Val ⁵ ,Gly ⁹]Ang I	Hasegawa et al. 1983a
American eel, <i>Anguilla rostrata</i>	[Asn ¹ ,Val ⁵ ,Gly ⁹]Ang I	Khosla et al. 1985
Trout, <i>Oncorhynchus mykiss</i>	[Asn ¹ , or Asp ¹ ,Val ⁵]Ang II	Conlon et al. 1996
Flounder, <i>Platichthys flesus</i>	[Asn ¹ ,Ile ⁵ ,Thr ⁹]Ang I	Balment et al. 2003
Lungfish		
Australian lungfish, <i>Neoceratodus forsteri</i>	[Asn ¹ ,Val ⁵ ,Thr ⁹]Ang I	Joss et al. 1999
Amphibians		
Axolotl, <i>Ambystoma mexicanum</i>	[Asp ¹ ,Val ⁵ ,His ⁹]Ang I	Takei et al. 2003
Three-toed amphiuma, <i>Amphiuma tridactylum</i>	[Asp ¹ ,Val ⁵]Ang II	Takei et al. 2003
Bullfrog, <i>Rana catesbeiana</i>	[Asn ¹ ,Val ⁵ ,Asn ⁹]Ang I	Hasegawa et al. 1983b
Australian frog, <i>Crinia georgiana</i> (skin)	Ala-Pro-Gly-[Asp ¹ ,Ile ³ ,Val ⁵]Ang II	Erspamer et al. 1979
Reptiles		
Snake, <i>Elaphe climacophora</i>	[Asp ¹ ,Val ⁵ ,Tyr ⁹]Ang I	Nakayama et al. 1977
Turtle, <i>Pseudemys scripta</i>	[Asp ¹ ,Val ⁵ ,His ⁹]Ang I	Hasegawa et al. 1984a
Alligator, <i>Alligator mississippiensis</i>	[Asp ¹ ,Val ⁵ ,Ala ⁹]Ang I	Takei et al. 1993b
Birds		
Chicken, <i>Gallus gallus</i>	[Asp ¹ ,Val ⁵ ,Ser ⁹]Ang I	Nakayama et al. 1973
Emu, <i>Dromiceus novaehollandiae</i>	[Asp ¹ ,Val ⁵ ,Asn ⁹]Ang I	Takei et al. 2003
Quail, <i>Coturnix coturnix</i>	[Asp ¹ ,Val ⁵ ,Ser ⁹]Ang I	Takei and Hasegawa 1990
Mammals		
Human, horse, dog, rat	[Asp ¹ ,Ile ⁵ ,His ⁹]Ang I	Khosla et al. 1974, 1983
Cow	[Asp ¹ ,Val ⁵ ,His ⁹]Ang I	

at position 1 (Asn or Asp) or position 5 (Ile or Val) cause relatively minor effects with respect to function and binding (Khosla et al. 1974). Removal of the first amino acid, or the change from Asp¹ to Asn¹, reduces vasopressor activity in the rat, whereas native Ang II appears to exert more potent vasopressor/catecholamine-releasing actions in fishes (Nishimura 1985a; Takei et al. 1993a).

Position 9 of the Ang I is variable and is likely to show different specificity to Ang-converting enzyme. Furthermore, antibodies raised against specific Ang I molecules often fail to bind to other Ang I ligands that have a different amino acid in position 9 (Nishimura et al. 1977, 1981a). All mammalian species studied to date have histidine at the 9th position, but variation in the 9th amino acid

may exist in mammals (Best et al. 1974). Change in the first amino acid from Asp to Asn or in the 5th from Val to Ile requires a single nucleotide change. Conversion of the amino acid at the 9th position from alanine (alligator Ang I) (Takei et al. 1993b) to serine (avian Ang I) (Nakayama et al. 1973) or from tyrosine (snake Ang I) (Nakayama et al. 1977) to histidine (turtle and mammalian Ang I) requires a one-point mutation of the triplet sequence of the DNA code (Takei et al. 1993b), whereas a two-nucleotide difference exists between alanine and tyrosine (or histidine) (Takei et al. 1993b) and between serine (avian Ang I) and histidine. This structural difference in Ang I agrees with the notion that the evolutionary line leading to the mammals departed from the reptilian-avian line at a very early stage of tetrapod evolution and that the crocodylians are phylogenetically closer to avians. The differences at position 3 (Pro, Ile, Val) may induce conformational changes and thus significantly modulate ligand binding or signal transduction. Indeed, [Pro³]Ang II shows much lower vasopressor action in the rat compared to other Ang II (Takei et al. 1993a). Furthermore, Ang receptors possess dynamic structures that may undergo conformational changes by shifting between active and inactive forms in response to ligand binding (Pérodin et al. 1996).

2.1.4

Angiotensin Receptor Subtypes and Signaling

AT₁-Homolog Receptors and Signaling. The recent development of novel non-peptide Ang receptor antagonists has led to the discovery of Ang receptor subtype 1 (AT₁) and subtype 2 (AT₂) (Murphy et al. 1991; Sasaki et al. 1991; Kambayashi et al. 1993; Mukoyama et al. 1993). The biochemical properties and signal transduction pathways (Griendling et al. 1997) of AT₁ and AT₂ will be discussed in other sections of this book. Ang receptors sharing part of the AT₁ receptor protein/nucleotide sequences (referred to as AT₁-homolog receptors) have been identified in several nonmammalian species (Table 2) (Nishimura 2001). Cobb and Brown (1993), Brown and coworkers (1997), and Parkyn and coworkers (1997) have molecularly identified Ang receptors in the rainbow trout. A partial clone indicates relatively low homology (47%) to the mammalian AT₁ receptor. Teleost Ang receptors show considerable affinity to losartan (trout; Brown et al. 1997) or [Sar¹, Ile⁸]Ang II (toadfish; Qin et al. 1999) and stimulate cytosolic Ca²⁺ (Cobb et al. 1999; Qin et al. 1999). Tran Van Chuoi and coworkers (1999) have cloned a cDNA encoding the eel Ang receptor from the intestinal brush border membrane that has 50% homology to the rat AT_{1A} receptor and 35% to rat AT₂ receptor subtypes (Fig. 4; H. Ji and K. Sandberg, personal communication).

The AT₁-homolog receptor has been identified in amphibians (Ji et al. 1993; Bergsma et al. 1993) (Table 2). Myocardial AT receptors from *Xenopus laevis* consisting of 362 or 363 amino acid residues show ~60% amino acid identity and 65% nucleotide homology with the coding region of the mammalian AT₁ receptor. AT₁-homolog *Xenopus* receptors are coupled to GTP binding protein

Table 2 AT₁ homologous receptors

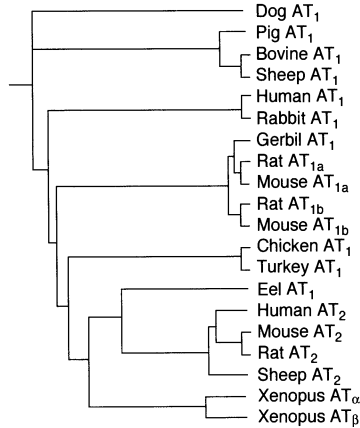
	AT ₁ homology (%)	Amino acid	Binding affinity (nM)			Structure	Signaling	Function/Action	Reference
			Ang II K _d or IC ₅₀	[Sar ¹ , Ile ⁸] Ang II	Losar- tan PD CGP				
Teleost fish									
Rainbow trout Glomerulus			0.38	19.3	—		[Ca ²⁺] _i ↑	Control of GFR	Cobb and Brown 1993; Brown et al. 1997; Cobb et al. 1999 Parkyn et al. 1997
Liver	47 (partial clone)								
Toadfish VSM			1.0	15.8	2500		[Ca ²⁺] _i ↑	Vasopressor	Qin et al. 1999
Eel Intestine, BBM	50		3.4						Marsigliante et al. 1994; Tran van Chuoi et al. 1999
Amphibians									
<i>Xenopus laevis</i> Myocardium	63	362	1.0	0.40	—	540	[Ca ²⁺] _i ↑	7 transmem- brane domain, G protein coupled	Bergsma et al. 1993; Aiyar et al. 1994
	60–65	363	2.0	0.91	—	1200	[Ca ²⁺] _i ↑	7 transmem- brane domain, G protein coupled	Sandberg et al. 1991; Ji et al. 1993

Table 2 (continued)

	AT ₁ homology (%)	Amino acid	Binding affinity (nM)	Structure	Signaling	Function/Action	Reference
Avians							
Turkey Adrenal	75	359	Ang II K _d or IC ₅₀	29*	[Ca ²⁺] _i ↑ IP ₃ ↑	Mineralo-corticoid secretion?	Murphy et al. 1993
Chicken Adrenal	75	359	Losar-tan Ang II	3400*	IP ₃ ↑	Mineralo-corticoid secretion?	Kempf et al. 1996
Endothelium	Ex-pressed		~10		EDRF/ cGMP↑	Vaso-relaxation	Yamaguchi and Nishimura 1988; Nishimura et al. 1994; Nishimura et al. 2003

BBM, brush border membrane; EDRF, endothelium-dependent relaxing factor; GFR, glomerular filtration rate; VSM, vascular smooth muscle; †, transfected cells; -, no selective binding.

A. AT₁, AT₂, and AT₁-homologue Subtypes



B. Undefined Subtypes

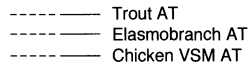


Fig. 4 **A** Phylogenetic dendrogram of cloned mammalian and nonmammalian angiotensin II receptors. The length of the horizontal connecting bars is inversely proportional to the pairwise similarity scores calculated from the alignment of sequences with use of the Clustal algorithm of PC/GENE. Courtesy of Ji and Sandberg, modified from Sandberg (1994). **B** Unidentified AT subtypes in nonmammalian vertebrates. *AT*, angiotensin receptor; *VSM*, vascular smooth muscle. (Reproduced from Nishimura 2001)

and stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, leading to the formation of inositol triphosphate (IP₃) and to cytosolic Ca²⁺ release. The *Xenopus* AT receptors show high affinity to Ang II and [Sar¹, Ile⁸]Ang II and low affinity to CGP-42112A (Ciba-Geigy), but show no binding to AT₁-selective (losartan) or AT₂-selective PD (Parke-Davis) compounds.

Furthermore, AT₁-homolog receptors (359 amino acid residues) have been cloned in adrenal glands of two avian species, turkeys (tAT₁) (Murphy et al. 1993) and chickens (cAT₁) (Kempf et al. 1996). cAT₁ and tAT₁ are nearly identical, show 75% amino acid identity with the mammalian AT₁ receptor, and involve the inositol phosphate-IP₃ pathway. cAT₁ and tAT₁ transfected to Cos cells exhibit high affinity to Ang II and considerable affinity to [Ile⁸]Ang II analogs and CGP (in tAT₁), whereas both show no specific binding to AT₁- or AT₂-selective nonpeptide antagonists. cAT₁ mRNA is expressed in the endothelia (Kempf et al. 1996) of arteries and arterioles in the kidney, to a lesser extent in the epithelial cells of small arteries of the adrenals, and abundantly in the glomeruli and subcapsular region of the adrenals of chick embryos (Nishimura et al. 2003).

Unidentified Angiotensin Receptors and Signaling. Pharmacological and molecular studies suggest the presence of another unidentified receptor(s) in vertebrates (Smith 1996) (Table 3). The dogfish, an elasmobranch, has a CGP-sensitive receptor in the rectal gland and an interrenal receptor that has considerable

Table 3 Unidentified angiotensin receptors

	Binding affinity (nM)				Signaling	Function/ Action	Reference
	Ang II K_d or IC_{50}	[Sar ¹ , Ile ⁸] Ang II	Losar- tan	PD CGP			
Elasmobranchs							
Japanese dogfish							
Rectal gland	0.41–0.75		~100				Tierney et al. 1997
Interrenal	0.25		~10		~50	[Ca ²⁺] _i ↓	1 α -hydroxycorticosterone secretion? Armour et al. 1993; Hazon et al. 1997
Avians							
Chicken							
VSM	0.15	1710	–	41000		No [Ca ²⁺] _i ↑ ?	Takei et al. 1988; Stallone et al. 1989; Nishimura et al. 1994
Liver	30	55000	–	49000			Walker et al. 1993
Liver	8.2		–	–		Glycogenolysis?	Bouley et al. 1997

VSM, vascular smooth muscle.

affinity to both losartan and CGP (Tierney et al. 1997; Hazon et al. 1997). Chicken vascular smooth muscle (VSM) has a high-affinity Ang receptor (Takei et al. 1988; Stallone et al. 1989) that shows low binding to 8th amino acid-replaced peptide antagonists (Stallone et al. 1989) and no binding to nonpeptide AT₁ or AT₂ antagonists (Nishimura et al. 1994). The chicken VSM Ang receptor does not mediate cytosolic Ca²⁺ signaling (Walker et al. 1993; H. Nishimura, unpublished). When the endothelium is removed, fowl Ang II ([Asp¹, Val⁵]Ang II) causes neither relaxation nor contraction (Hasegawa et al. 1993) of fowl abdominal aortae. Fowl Ang II does not stimulate thymidine incorporation into cultured adult fowl VSM cells (Shimada et al. 1998). Furthermore, cAT₁ mRNA is not expressed in VSM layers of aortae or in small arteries and arterioles of various organs from adult fowl (Kempf et al. 1996; Nishimura et al. 2003), indicating that the Ang receptor in fowl VSM is distinct from the cAT₁ cloned from the fowl adrenal gland. A similar receptor that has no selectivity to nonpeptide antagonists is also present in the liver (Walker et al. 1993; Bouley et al. 1997).

2.2

Function and Regulation

2.2.1

Control of Renin Release

Effects of Blood Volume and Blood Pressure. Since the kidneys from primitive vertebrates lack a macula densa, the possible baroreceptor function in JG cells may be important in the regulation of renin release in these animals. Cumulative hemorrhage or a single massive one increased PRA more than 15 times in the conscious glomerular toadfish (*Opsanus tau*; Nishimura et al. 1979), and also increased plasma Ang II levels in conscious Japanese eels (*Anguilla japonica*; Kobayashi et al. 1980). Hemorrhage increased PRA in the turtle (*Pseudemys scripta*; Stephens and Creekmore 1984), pigeon (Chan and Holmes 1971), and chicken (Nishimura and Bailey 1982). Furthermore, intraarterial injection of papaverine (10 mg/kg) in the toadfish increased PRA (5- to 20-fold) with a concomitant decrease in blood pressure (BP). Likewise, reduction in renal perfusion pressure caused renin release in the rainbow trout (Bailey and Randall 1981) and in chickens (Wideman et al. 1993). It appears, therefore, that primitive vertebrates, in spite of the absence or incomplete form of a macula densa, have a functional renal baroreceptor mechanism for controlling renin release. In contrast, isosmotic volume expansion in the Australian lungfish (*Neoceretodus forsteri*) decreased PRA and increased the mean dorsal aortic pressure by 2 mmHg (Blair-West et al. 1977).

Furthermore, injection or infusion of Ang-converting enzyme (ACE) inhibitor decreases resting BP levels and increases PRA in conscious bony fish (Nishimura et al. 1978; Nishimura and Bailey 1982), suggesting that the RAS may have a role as a physiological regulator of BP. Since kidneys of lower vertebrates are not, or are only poorly, autoregulated, the changes in aortic pressure readily influence glomerular filtration rate (GFR) and renal blood flow, which appear to be a mechanism for controlling fluid and electrolyte excretion.

Involvement of Neural Mechanisms and Macula Densa. A question rises as to whether the renal nerves are also involved in stimulation of renin release induced by hemorrhage or hypotension. It appears that the large arteries of teleosts are supplied by both “constrictor adrenergic” and “constrictor cholinergic” nerves (Kirby and Burnstock 1969). Cholinergic vasoconstrictor nerves comprise both sympathetic and parasympathetic fibers. Adrenergic receptors and adrenergic innervation in the blood vessels are present, though less marked, in teleost fish (Nakamura et al. 1992); and adrenergic innervation increases, whereas cholinergic innervation diminishes, as phylogeny advances (Burnstock 1969).

In mammals, β -adrenoceptors are localized on the JG cells; and intrarenal infusion of isoproterenol or norepinephrine, or electrical stimulation of renal nerves causes renin release (Davis and Freeman 1976). In glomerular teleost fish, isoproterenol evoked an increase in PRA with a concomitant reduction in BP, both of which are inhibitable by propranolol (Nakamura et al. 1992). Neither

propranolol alone nor depletion of catecholamines by repeated treatment with 6-hydroxydopamine, however, altered the basal level of PRA, suggesting that isoproterenol-induced renin release is likely due to activation of baroreceptors via decreased renal arterial perfusion pressure. Indeed, there is no specific relationship between renin-containing granulated cells and adrenergic nerve endings in renal arterioles (Nakamura et al. 1992).

The role of the macula densa in the control of renin release and the existence of a tubuloglomerular feedback mechanism have not been much explored in nonmammalian vertebrates. Although a close topographical relationship between the distal tubule and the glomerular vascular pole exists in elasmobranch kidneys, communication and signaling between these structures has not been examined. We observed that slow infusion of hypertonic saline into the renal portal system of fowl in which shunt pathways bypassing the kidney were occluded caused diuresis and natriuresis on the infused side without altering GFR, whereas infusion of the same dose into the systemic circulation showed no effect (Bailey and Nishimura 1984). PRA was decreased, suggesting that, in fowl, some functional link may exist between NaCl concentration in tubular fluid and renin secretion via a macula densa mechanism. In mammalian kidneys, a reduced chloride transport across macula densa cells or an application of bumetanide to the tubular lumen stimulates renin secretion from JG cells (Lorenz et al. 1991), and this Cl-dependent renin release is inhibited by blockade of cyclooxygenase 2 (Traynor et al. 1999).

Cellular Mechanism of Control of Renin Release. In aglomerular toadfish, potassium-induced depolarization increases cytosolic Ca^{2+} in VSM (Qin et al. 1999) and decreases renin release from renal slices superfused in vitro (Nishimura and Madey 1989). This depolarization-induced reduction of renin release was inhibited by blockade of Ca^{2+} influx by nifedipine and restored by the Ca^{2+} channel agonist Bay K8644 or by phorbol ester (Nishimura and Madey 1989). Likewise, removal of extracellular Ca^{2+} stimulates renin release. Since renin-secreting cells are modified VSM cells, mechanical stretch induced by increased renal perfusion pressure is likely to cause membrane depolarization and activation of a voltage-gated calcium channel. It appears, therefore, that cytosolic Ca^{2+} levels may be a cellular regulator for control of renin release. In contrast, isoproterenol, dibutyryl, chlorophenylthio-cAMP, 8-bromo-cGMP, and acetylcholine each failed to stimulate renin release or to restore potassium-induced renin suppression. This supports the concept that β -adrenoceptor-mediated control of renin release has not yet evolved in teleost fish. Similarly, prostaglandin E_2 , phenylephrine, Ang II, and atrial natriuretic peptide showed no effect on renin levels (H. Nishimura, unpublished).

In mammalian JG cells, intracellular regulation of renin secretion appears to occur at three levels, including vesicle volume, the state of contraction of the actin-myosin complex, and the docking process of the vesicles with the cell membrane. The swelling of renin storage granules evoked by the proton pump-induced influx of KCl may be an important cellular process for the exocytosis of

renin secretory granules (Kurtz and Wagner 1999). While an increase in cytosolic Ca^{2+} via a release from the internal store or via stimulation of Ca^{2+} influx stabilizes exocytosis, cAMP protein kinase stimulates this process by weakening the actinomycin shield (Kurtz and Wagner 1999).

2.2.2

Biological Action of Angiotensin

Cardiovascular Effects and Release of Catecholamines. Ang increases BP in selected species of all vertebrate classes and also stimulates cardiac contractility in some species (Nishimura 1980, 1987). In nonmammalian vertebrates, Ang II causes catecholamine release (Nishimura et al. 1981b; Carroll and Opdyke 1982; Bernier et al. 1999b). $[\text{Asn}^1, \text{Val}^5]\text{Ang II}$ (Opdyke and Holcombe 1976; Carroll and Opdyke 1982) and dogfish Ang II ($[\text{Asn}^1, \text{Pro}^3, \text{Ile}^5]\text{Ang II}$) (Bernier et al. 1999a) dose-relatedly increase plasma catecholamines and arterial pressure of the dogfish (*Squalus acanthias*). Treatment with α -adrenoceptor antagonists, phentolamine (Opdyke and Holcombe 1976) and yohimbine (Bernier et al. 1999a), completely abolished Ang-induced vasopressor action but not the catecholamine-releasing effect (Bernier et al. 1999a), suggesting that the vasopressor effect of Ang II is indirectly mediated by catecholamine release via Ang receptors at the adrenergic nerve endings and in the adrenal medulla. Ang releases catecholamines from chromaffin tissues in rainbow trout via a specific Ang II binding site (Bernier and Perry 1997). In teleost fish (Carroll 1981), amphibians (Corwin et al. 1984), and reptiles (Stephens 1984), Ang II contracts isolated arterial preparations. These findings suggest that the receptors mediating catecholamine release may have evolved phylogenetically earlier than the vascular Ang receptors that mediate the direct vasocontractile action of Ang II.

In domestic fowl, Ang II-induced vasopressor action is solely ascribable to catecholamine release from the adrenal medulla and adrenergic nerve endings (Nishimura et al. 1982; Nakamura et al. 1982). Furthermore, Ang II specifically binds to the aortic endothelium and causes endothelium-dependent relaxation (Yamaguchi and Nishimura 1988; Stallone et al. 1990; Nishimura et al. 1994), accompanied by a sharp rise in aortic cGMP levels. Hemoglobin completely inhibits and N_w -nitro-L-arginine methyl ester (L-NAME) partially inhibits Ang II-induced vasorelaxation in fowl aortic rings, suggesting that part of the signal pathway of the endothelial Ang receptor may be via nitric oxide and cGMP (Hasegawa et al. 1993). Ang II-induced depressor and/or vasorelaxing effects of Ang II are inhibitable by 8th amino acid-replaced Ang antagonists but not by inhibitors for cyclooxygenase or monooxygenase, β -adrenoceptor antagonists or cholinergic blockers, or by inhibitors for serotonin, vasotocin, histamine, or phospholipases (Nakamura et al. 1982; Yamaguchi and Nishimura 1988). Ang II-induced relaxation appears partly ascribable to a K^+ -channel and endothelium-dependent hyperpolarization factor (Nishimura et al. 2003; H. Nishimura, unpublished).

Ang II induces neither inotropic nor chronotropic action in perfused rainbow trout heart preparations (Olson et al. 1994). In intact rainbow trout, Ang II increases the heart rate, and the increase can be attenuated by [Sar¹, Ala⁸]Ang II (Fuentes and Eddy 1998). In fresh water-adapted conscious eels, Ang II increases the heart rate and cardiac contractility (Oudit and Butler 1995).

Dipsogenic Action. Intravenous or intracranial application of Ang II stimulates water intake in various classes of vertebrates (extensively reviewed in Nishimura 1987; Kobayashi and Takei 1996; Takei 2000), suggesting that, as in mammals, Ang receptors in the brain mediate dipsogenic action. Furthermore, stimulation of the endogenous RAS by hemorrhage or papaverine-induced hypotension enhances water intake in elasmobranchs (Anderson et al. 2001), freshwater-adapted flounder (Balment and Carrick 1985), and freshwater- and seawater-adapted eels (Tierney et al. 1995) with a concomitant increase in plasma Ang II (Tierney et al. 1995). Captopril decreases both the plasma Ang II level and drinking rate in seawater-adapted eels (Tierney et al. 1995), suggesting that endogenous RAS plays a role in maintaining fluid intake in fish. It remains to be determined, however, whether systemic or brain Ang physiologically maintains extracellular and intracellular fluid volume homeostasis via its dipsogenic effect. The role of the subfornical organ in Ang II-induced drinking has been reported in Japanese quail (Takei 1977). Increases in water intake and plasma Ang II levels following hemorrhage have been reported in the conscious Japanese eels adapted to fresh water (Kobayashi et al. 1980). The evolution of brain Ang receptors responsible for drink stimulation, and their precise localization and properties, however, remain to be elucidated in nonmammalian vertebrates.

Stimulation of Adrenal Steroids. In mammals, Ang stimulates aldosterone production in early and late stages of steroidogenic pathways (Fraser and Lantos 1978; Fraser et al. 1979), respectively, by stimulating conversion of cholesterol to pregnenolone and by stimulating 18-hydroxydehydrogenase activity. It has been reported that Ang II stimulates 1- α hydroxycorticosterone and cortisol that act as mineralocorticoids in, respectively, elasmobranchs and teleost fish (for review, Nishimura 1987; Henderson et al. 1993; Hazon et al. 1999). In ducks, [Asp¹, Val⁵]Ang II (native fowl Ang II) stimulates production of aldosterone and 18-hydroxycorticosterone and corticosterone from adrenal cell suspensions (Vinson et al. 1979). Although the available information is limited, a linkage between endogenous RAS activity and adrenal steroids apparently evolved early and may be conserved. For example, in flounder, injection of the vasodilator papaverine evoked hypotension and an increase in plasma cortisol, whereas both the elevation of cortisol and BP restoration after papaverine-induced hypotension were prevented by captopril (Perrott and Balment 1990). Likewise, the transfer of freshwater-adapted eels to seawater increased the plasma levels of both renin and cortisol, which presumably play a role in the stimulation of drinking and water absorption (Henderson et al. 1976). Although aldosterone has been identified in bony fish, amphibians, reptiles, and birds, the role of Ang

II in the control of aldosterone is not as clear as in mammals; rather, ACTH appears a more potent stimulator for both glucocorticoids and mineralocorticoids (Klingbeil 1985; Holmes et al. 1991; for review, Nishimura 1987). Furthermore, in nonmammalian animals, the role of aldosterone or other adrenal steroids in the control of renal Na^+ and K^+ transport has not been established, whereas mineralocorticoid functions of adrenocortical hormones have been clearly demonstrated in extrarenal transport epithelia. Thus, the manifestation of Ang function in hydromineral balance would vary depending on species and target organs. In fowl, the mRNA encoding AT_1 -homolog receptor cDNA has been extensively expressed in fowl adrenocortical tissues (Kempf et al. 1996), particularly the subcapsular region of embryonic adrenals (Nishimura et al. 2003). The role of Ang in steroidogenesis has not been investigated in embryonic adrenals.

Renal Action and Role in Osmoregulation. The RAS could influence renal function (1) through its effect on the systemic renal perfusion pressure and flow, (2) by renal action of systemically delivered renin and Ang, and (3) by local action of intrarenally formed renin and Ang (for review, Nishimura 1985b, 1987; Brown et al. 1993; Russell et al. 2001). Furthermore, the renal actions of Ang include its effects on the renal vasculature and renal tubules. In general, high pressor doses of Ang II produce glomerular diuresis via an increase in GFR in bony fish and amphibians (pressure diuresis); whereas Ang II reduces GFR, urine flow, and renal blood flow by constriction of preglomerular arterioles in rainbow trout (glomerular antidiuresis) (Brown et al. 1980). Inhibition of ACE activity by captopril causes profound diuresis in the in situ perfused trout kidney and a reduction of vascular resistance of the trunk (Brown et al. 2000). Thus the effect of Ang on renal hemodynamics and function differs depending on the location of Ang receptors. High cAT_1 mRNA levels are detected by RT-PCR and in situ hybridization in glomeruli of mesonephric kidneys of chick embryos and, to a lesser degree, in metanephric kidneys (Nishimura et al. 2003; H. Nishimura, unpublished). Strong expression in glomerular tufts, presumably mesangial cells, during development may indicate that the cAT_1 receptor plays a role in the growth/maturation of renal glomeruli.

Direct action of Ang II in renal tubule epithelial cells remains unclear. Ang altered neither urine flow nor Na excretion in aglomerular teleosts (Zucker and Nishimura 1981) in which urine is formed via excretion and reabsorption across the renal tubules. In contrast, native Ang II infused into the renal portal system of fowl, after the shunt between the systemic and renal portal circulation was closed, produced marked natriuresis without significantly altering GFR (Nishimura 1987), suggesting that Ang directly influences Na transport in renal tubules. In addition to the effect on the kidney, Ang II influences NaCl and water transport across epithelial tissues such as the urinary bladder, gastrointestinal tract, and nasal salt glands in some species (for review, Nishimura 1987; Kobayashi and Takei 1996). In general, however, the Na-retaining effect of Ang II, either via stimulation of mineralocorticoid secretion or renal tubule transport, is not established in nonmammalian species; rather, it varies depending on the

osmoregulatory function of the target organs. In euryhaline teleosts, Ang II receptor concentration and Na^+/K^+ ATPase activity of gill chloride cells increase during adaptation to seawater (Marsigliante et al. 1997), reflecting an enhanced dipsogenic effect of Ang II and possibly NaCl extrusion from the gill in seawater fish.

3

Ontogeny of the Renin–Angiotensin System

Embryonic and fetal development is a result of integrated programs of growth and differentiation leading to organogenesis. A number of reports, primarily on rodent or ovine models, as well as on human subjects, suggest that the RAS plays a critical role in fetal growth and development and serves as a modulator of the ontogenic process. Defect or deletion of a component of the RAS results in abnormal development in architecture and function. All components of the RAS are detected during the fetal period; and gene expressions of AGT, renin, ACE, and Ang receptors are developmentally regulated in a tissue- and time-specific manner. Furthermore, impairment of the RAS during the fetal or perinatal period appears to induce organ dysfunction in maturity via so-called perinatal programming, or the fetal origin, of adult disease. Recently emerging gene-targeting techniques provide useful tools for elucidating the roles of specific genes and their physiological manifestation.

3.1

Biochemistry and Molecular Biology

3.1.1

Renin Substrate and Renin

All components of the RAS are present and functioning early in fetal life (Gomez et al. 1991; Tufro-McReddie and Gomez 1993). In the mesonephric kidney of the human fetus, AGT mRNA determined by *in situ* hybridization is first detected at the proximal portion of the renal tubules (Corvol et al. 1998). AGT mRNA is also present in the proximal tubules, mesangial cells of the glomerulus, and renal arterial system of the rat kidney at embryonic day 18 (E18); it is most intensely expressed during the neonatal period and is reduced to less than half in mature adults (higher in females than in males) (Darby and Sernia 1995) (Table 4). AGT mRNA from the fetal rat liver (total RNA or poly A+ enriched RNA) is very low and increases by 20- to 50-fold after birth; it gradually reaches the adult level by three postnatal months (Gomez et al. 1988b; Tufro-McReddie and Gomez 1993). In contrast, AGT mRNA is found abundantly in the brain, brown fat, and kidneys, suggesting that the liver may not be the primary source of AGT in fetal life (Gomez et al. 1988b; Harris and Gomez 1997). Differences in AGT mRNA were more clearly seen in spontaneously hypertensive rats (SHR) than in control Wistar-Kyoto (WKY) rats (Gomez et al. 1988b).

Table 4 Time-dependent expression of the renin–angiotensin system in the developing rat kidney

Component (mRNA or protein)	Embryonic day				
	E11~15	E17~18	E20~21	Newborn/ perinatal	Adult
Angiotensinogen	±	+	++	+++++ ^a	++
Renin	±	+	+++++++~ ^b	+++++++~ ^c	+~++
Angiotensin-converting enzymes ^d		±	++	+++	+++++
AT ₁	±	++	+++	+++++	++++
AT ₂	++	++++	+++++	+++++	±
AT ₁ /AT ₂		AT ₁ <<<AT ₂	AT ₁ <<AT ₂	AT ₁ ≥AT ₂	AT ₁ >>AT ₂
Angiotensin 11				+++++	+

Data (relative levels within each component) are summarized from Darby and Sernia 1995; Gomez et al. 1988a,b, 1989, 1991; Harris and Gomez 1997; Norwood et al. 1997; Shanmugam et al. 1994, 1995; Tufro-McReddie and Gomez 1993; Tufro-McReddie et al. 1993, 1994; Wallace et al. 1978, 1979; Yosipiv and El-Dahr 1996.

^a Fifty-fold increase in liver.

^b Twenty-fold of adult level.

^c Ten-fold of adult level.

^d Higher ACE activity in fetal lung.

In human mesonephros at 5–6 weeks, detectable renin is expressed in the glomerular capillaries and in the walls of renal arteries from arterioles up to aortae (Corvol et al. 1998). Renin mRNA and protein (histochemical manifestation of JG cells) increase with gestation, whereas expression in the JG apparatus does not increase when normal fetal growth and the growth of the renal cortex are restricted (Kingdom et al. 1999). In metanephric kidneys, the distribution of renin immunoreactive cells (Celio et al. 1985) and renin mRNA is confined to the JG area and arterioles adjacent to the vascular pole (Corvol et al. 1998) and, to a lesser extent, proximal tubules (Celio et al. 1985).

During ontogeny of metanephric rat kidneys, renin mRNA levels and distribution change as maturation progresses. Renin protein and mRNA signals are detectable at E17 in the renal artery, and localization of renin synthesis (mRNA) and storage (protein) shift from arcuate and interlobular arteries to the classical JG apparatus vascular pole and afferent arteries (Gomez et al. 1989) towards term. Renin mRNA and renin protein levels (renin-containing JG cells) are higher in newborns and decrease progressively as animals mature (Gomez et al. 1988b, 1991) (Table 4). Although the reasons for the high and widely distributed renin expression in fetal kidney are unclear, it may be the result of developmentally regulated transacting factors that interact with the 5' flanking region of the renin gene (Harris and Gomez 1997). Likewise, the transgene of human renin promoter with a *LacZ* reporter gene to mouse embryo at E15 mimics endoge-

nous renin expression and is detected in the interlobular arteries of the kidney, as well as in large arteries outside the kidney (Fuchs et al. 2002).

Pan and coworkers have identified the renin promoter element as a *Hox* protein binding site and verified that *Hox* genes and their cofactors determine the sites of renin expression (Pan et al. 2001). Since *Hox* genes are members of a homeobox family of transcription factors and control cell morphogenesis and differentiation, strong renin expression may coincide with growing structures during development (Pan et al. 2001). Interestingly, in adult rats, chronic inhibition of ACE enhances intrarenal renin synthesis and renin mRNA upstream from the glomeruli in cells that do not express renin in their basal state (Gomez et al. 1988a; Jones et al. 2000). The distribution of renin-expressing cells in ACE-treated adult kidneys resembles that in fetal kidneys, suggesting that the adult vasculature is capable of recruiting JG cells and reproducing the fetal pattern of renin and renin gene expression.

3.1.2

Angiotensin and Angiotensin-Converting Enzymes

Immunoreactive ACE activity is detected in apical and basolateral membranes of differentiating human proximal nephrons and in glomerular endothelial cells of developing capillaries (Mounier et al. 1987). During renal development and postnatally, ACE protein is consistently found on the brush border of proximal tubule cells (Mounier et al. 1987; Corvol et al. 1998). In fetal rat lungs, ACE activity is first detected at E18 and increases two- to fourfold by the first day of birth (Wallace et al. 1978, 1979; Tufro-McReddie and Gomez 1993). ACE activity further increases during postnatal maturation and is stabilized in adult life (Table 4).

Reflecting high levels of AGT and renin levels, intrarenal Ang I and Ang II levels are three- to sixfold higher in newborn than in adult rat kidneys (Yosipov and El-Dahr 1996) (Table 4). It is not known, however, whether any Ang other than [Asp¹, Ile⁵]Ang II or [Asp¹, Val⁵]Ang II is expressed during mammalian fetal development, as in the case of neurohypophysial hormones. It will be interesting to examine whether nonmammalian Ang I and Ang II (Table 1) are expressed during mammalian ontogeny. ACE activity is detected in fetal lambs and increases with gestation parallel to the increases in plasma cortisol levels and BP; levels are high in newborns and decrease with time (Forhead et al. 1998).

3.1.3

Angiotensin Receptors

In human mesonephric and metanephric kidneys, AT₁ mRNA is detected in the glomeruli, perhaps in mesangial cells, beginning at stage 12, while AT₂ mRNA is also expressed in undifferentiated mesenchymal tissues at stage 11 (Corvol et al.

1998). AT_2 mRNA is highest at 8–9 weeks of gestation and declines after 20 weeks of gestation, although it is still detectable at birth.

In rat kidneys (Table 4), AT_1 mRNA is minimum at E14, highly expressed at E20, and persists, although becoming lower with maturation, into adulthood (Norwood et al. 1997). The AT_1 receptor is found in the undifferentiated mesenchymal tissues at E14, whereas it is localized in nephron segments (Norwood et al. 1997) at E17. During maturation, localization of AT_1 mRNA in the rat kidney shifts from a diffuse distribution in the nephrogenic cortex to more selective areas such as the glomeruli, adjacent arterioles (media/adventitia; weak signal), and vascular bundles (Tufro-McReddie et al. 1993). Also, the evolution and distribution of two AT_1 receptor subtypes, AT_{1A} and AT_{1B} , slightly differ: from E15–E19 gestation, both subtype mRNAs are present in the kidney and adrenal glands; whereas only AT_{1A} mRNA is expressed in the liver, lung, heart, aorta (media and adventitia), and undifferentiated mesenchymal tissues (Shanmugam et al. 1994). AT_{1A} mRNA is found in mesenchymal cells associated with differentiating glomeruli that later mature to mesangial cells, interstitial tissues, maturing tubules, vasa recta, and JG apparatus in the postnatal kidney; whereas AT_{1B} expression is limited to preglomerular structures and mature glomeruli (Shanmugam et al. 1994).

In contrast, AT_2 expression in the rat cardiopulmonary system is easily detectable from E14–15 through postnatal days 7–15 (D7–D15) and rapidly declines (Shanmugam et al. 1995, 1996). AT_2 mRNA is abundantly found in undifferentiated nephrogenic tissues, but not in glomeruli (Shanmugam et al. 1995). During the fetal period, AT_2 represents the majority of Ang receptor subtypes; whereas AT_1 is the dominant functioning receptor after birth, although AT_2 expression remains in some organs/tissues. Rat kidneys continue to mature after birth; and AT_1 mRNA, but not AT_2 , is expressed in immature glomeruli of neonatal day 2 (D2) kidneys (Aguilera et al. 1994; Shanmugam et al. 1995) and in the medulla at D7. The dissociation constants of AT_1 and AT_2 are similar to those of adult receptors (Norwood et al. 1997). The time-dependent expression of AT_1 and AT_2 and selective localization suggest that these receptors may have different roles in nephrogenesis. In situ radioligand binding autoradiography indicates that, at E14, specific binding to AT_1 and AT_2 is, respectively, 24% and 76% (Norwood et al. 1997); but the ratio of AT_1 and AT_2 receptors (as much as 1:10) during fetal life differs, depending on the time and method of measurements.

Adrenal glands contain cells from two origins: (1) undifferentiated mesenchymal tissue (cortex, 3 zones) and (2) a cluster of neural crest cells by migration (medulla). In human adrenal glands, the AT_2 subtype represents the dominant specific Ang II binding site in the fetal zone at gestation week 14, whereas the AT_1 is expressed only at the periphery of the gland (Breault et al. 1996). No detectable AT_2 mRNA (in situ hybridization) is present, however, in the zona glomerulosa (Schütz et al. 1996). At E17–21 of rat fetal life, AT_1 mRNA is detected in the zona glomerulosa, medulla (less extensively), and capsule regions (Shanmugam et al. 1994). After birth, AT_1 expression remains high in the

glomerulosa. In rat adrenals, strong AT₂ mRNA signals are also detected in the zona glomerulosa, whereas the zona fasciculata and zona reticularis do not express AT₂ mRNA (Shanmugam et al. 1995). The adrenal medulla contains clusters of cells with positive signals. After birth until D15, the zona glomerulosa continues to express AT₂ mRNA (Shanmugam et al. 1995).

Likewise, AT₁ is expressed predominantly in the zona glomerulosa of fetal lambs and to a lesser extent in the zona fasciculata (Coulter et al. 2000), or equally in both zones but not in the medulla (Wintour et al. 1999). AT₁ mRNA can be detected as early as 60 days of gestation and remains high throughout adult life, whereas AT₂ mRNA dramatically decreases at term and after birth (Wintour et al. 1999). AT₁ mRNA has also been detected in the forebrain of the E19 rat, which contains an area important for fluid homeostasis; whereas AT₁ mRNA is nearly absent in medullary nuclei until after birth (Nuyt et al. 2001). AT₂ mRNA expression is found only in medullary zone in 8-day-old mice (Hubert et al. 1999).

In normal adults, the AT₂ receptor is expressed in only limited tissues, including adrenal glands, ovary, and brain (Pucell et al. 1991; for review see de Gasparo et al. 1994; Horiuchi et al. 1999). The AT₂ receptor is also expressed during pathophysiological conditions such as skin wound healing and vascular remodeling. The signal pathways of AT₁ and AT₂ receptor subtypes have been studied in adult tissues (discussed in detail in other chapters of this book), but information on fetal tissues/organs is scant.

3.2

Function and Regulation

3.2.1

Angiotensin Action

The RAS appears to play important roles in cardiovascular and fluid–mineral homeostasis during both fetal and mature mammalian life. The role of the RAS in regulation of BP and fluid–mineral balance evolved early, whereas its role in the control of renal hemodynamic forces may have developed at a later stage of fetal life (Harris and Gomez 1997). Increasing evidence suggests that Ang regulates fetal growth and development by initiating a growth-related signal pathway involving proto-oncogenes and various growth factors and cytokines (Harris and Gomez 1997). The effect of the RAS on body growth and organogenesis has been studied by (1) blockade of the RAS using pharmacological tools, (2) use of gene-targeting approaches, and (3) examining the effects of an impaired RAS during fetal life that result in abnormal manifestation at adulthood (McMillen et al. 2001; Battista et al. 2002). It has been shown that deletion or blockade of any component of the RAS results in striking maldevelopment of kidney architecture and function, affecting both the nephrons and the vascular system (Gomez 1998).

Role of Angiotensin in Body Growth and Renal Development. As in cultured cells or tissues from mature animals, Ang II (10^{-11} M) stimulates both somatic and yolk sac/allantoic development and growth of rat embryo cultured in vitro, and this effect is inhibited by PD123319 (Tebbs et al. 1999). The time and localization of expressions suggest that AT₂ is important for early stages of organogenesis, stimulating differentiation and apoptosis; whereas AT₁ plays a role in renal vascularization and trophic actions, including cell hypertrophy, synthesis of cell matrix, and development of glomeruli and mesangial cells (for review, Harris and Gomez 1997; Ardaillou 1999). The signal pathway involving fetal growth via AT₂ is not well understood. In adult tissues, AT₂ receptor over-expression induced by transfecting a vector into balloon injury-induced carotid neointima causes a reduction in neointima formation; whereas in cultured cells it reduces mitogen-activated protein (MAP) kinase and Ang II-induced (losartan-inhibitable) cell proliferation, suggesting that AT₂ mediates antiproliferative action that counteracts the growth-promoting action of the AT₁ receptor (Nakajima et al. 1995). It thus appears that both AT₁ and AT₂ are needed for normal cell differentiation and organogenesis.

Homozygous AGT gene null-mutant mice, five postnatal weeks old, exhibit structural defects of the kidney, including hypoplastic papilla and prominent medial hypertrophy of interlobular arteries and afferent arterioles (Okubo et al. 1998). AGT-null mice have labile GFR after different amounts of Na intake, while urine osmolality does not increase in response to the vasopressin agonist 1-deamino-D-Arg-8 vasopressin (DDAVP) (Okubo et al. 1998). Animals are hypotensive and exhibit lesions in the cortex resembling nephrosclerosis in which platelet-derived growth factor (PDGF)-B and transforming growth factor (TGF)- β -1 mRNA were upregulated (Niimura et al. 1995).

Pharmacological blockade of Ang I to Ang II conversion by ACE inhibitors induces fetopathy in humans (Barr 1994; Sedman et al. 1995). Maternal intake of ACE inhibitor during the second and third trimester of pregnancy often evokes profound fetal growth restriction, hypotension, renal tubule dysplasia, hypocalvaria, and death (Sedman et al. 1995). Likewise, chronic ACE inhibition in neonatal rats arrests renal growth and nephrovascular maturation (Tufro-McReddie et al. 1995) and induces maldeveloped renal structure similar to that evoked by AGT gene deletion, such as dilated Bowman's capsule space and irregular size/shape of glomeruli (Barr 1994). Similarly, treatment of newborn SHR and WKY rats with captopril or enalapril induces renal dysfunction and structure, including (1) reduced urine osmolality, (2) increased urine production and increased water intake, and (3) renal papilla atrophy and tubulo-interstitial inflammation (Friberg et al. 1994). Mice lacking ACE (mice in which modified ACE allele was used for targeted homologous recombination in embryonic stem cells) exhibit renal papilla atrophy, vascular hyperplasia, and defects in urine-concentrating ability (Esther et al. 1996). Thus, the various investigative approaches indicate that a lack of functioning ACE activities exerts a significant influence on the development of renal architectural and functional defects—ones similar to those observed in the AGT gene deletion model (AGT knockout mice).

Blockade of Ang receptors, either pharmacologically, using AT₁- or AT₂-selective antagonist, or with gene-targeting approaches, also causes maldevelopment of the kidney (Barr 1994; Sedman et al. 1995; Woods and Rasch 1998). Treatment of weaning rats with losartan for 3 weeks reduces the DNA content of the kidney, whereas renin-secreting cells are seen more widely on arteries and arterioles (Tufro-McReddie et al. 1994). Deletion of both AT_{1A} and AT_{1B} genes evokes severe abnormal phenotypes resembling those of homozygous AGT null mutant mice (Tsuchida et al. 1998; Okubo et al. 1998), including low ex utero survival rate, growth retardation, hypotension, and abnormal kidney development (impaired glomerular maturity, plastic papilla, hypertrophy of renal arteries). In contrast, targeted deletion of the AT_{1B} receptor gene did not evoke any distinctive abnormal morphological phenotype (Chen et al. 1997). These findings indicate that AT₁ receptor is responsible for somatic renal growth. AT_{1A} receptors, which are predominantly expressed in mouse kidneys, may compensate for the loss of the functioning AT_{1B} subtype (Chen et al. 1997).

AT₂ gene-null mutant mice also display structural defects of the kidney and urinary tract (Nishimura et al. 1999). Of particular interest is the delayed apoptosis of undifferentiated mesenchyme. Since timely programmed apoptosis of mesenchymal tissues that surround the differentiating Wolffian duct and ureters is required for the normal development of the kidney and urinary tract, the lack of the AT₂ receptor that plays a role in differentiation and time-dependent apoptosis may lead to structural defects of the kidney and urinary tracts. In summary, currently available studies indicate that during fetal and neonatal development, AT₁ mediates proliferation, whereas AT₂ mediates differentiation and apoptosis, both of which are essential for normal development/maturation of kidney structures and functions.

Role in Perinatal Programming of Growth. Increasing evidence suggests that adult diseases originate in abnormal events during the fetal period. It has been postulated that maternal dietary protein restriction suppresses the fetal RAS, leading to impaired fetal growth and renal development (Woods 2000; Langley-Evans 2001). This in turn results in permanently altered renal structure and function in adult life, such as a reduction in the number and size of glomeruli, followed by a reduction in GFR and impaired tubule function, which may further alter the RAS function (Woods 2000). Furthermore, although phenotypes at birth may be normal, abnormal fetal experience determines in adulthood whether an individual is resistant or prone to stress and a variety of health problems, including hypertension, cardiovascular diseases, and renal dysfunction (Ingelfinger and Woods 2002). The RAS, in interaction with various other hormonal systems, appears to play an important role in this perinatal programming, while suppression of Ang action during the perinatal period may trigger abnormal renal and cardiovascular function in adulthood. For example, newborn rat pups treated with losartan for 12 days of the postnatal period demonstrated retarded growth at weaning day (D22) compared to controls. Both groups had similar body weight (BW) and kidney-BW ratios at 22 weeks (adult);

but, in the losartan-treated rats, BP was higher while GFR, effective renal plasma flow, urine-concentrating ability, and the number of glomeruli were significantly lower than in the untreated group (Woods and Rasch 1998). Similarly, intrauterine growth restriction induced by treating the mother rats with a low-Na diet for the last 7 days before term produced pups with retarded growth and with an age-dependent increase in BP (Battista et al. 2002). At 12 weeks of age, renin and aldosterone levels were elevated. Since kidney weight is consistently lower during development/maturation of pups of Na-restricted mothers (although the size of glomeruli and the number of glomeruli/area are similar to those in control rats), the high renin and BP may be a direct consequence of reduction of renal mass or impaired vascular development. Furthermore, captopril reduced BP in adult sheep exposed to growth restriction during the fetal period, but not in control groups (McMillen et al. 2001), suggesting that the RAS plays a greater role in the control of BP in growth-restricted animals. The cellular and molecular mechanisms for this fetal adaptation to insufficient substrate supply remain to be determined.

Role of Angiotensin in Cardiovascular Function. The possible role of the RAS in controlling cardiovascular–renal function during development has been investigated using molecular and pharmacological approaches. The transduction of the mouse Ren-2 gene to the rat increased plasma prorenin and increased BP, whereas renal renin and Ang I levels were low (Mullins et al. 1990). Captopril treatment of fetal lambs at 120–130 days' gestation markedly increased PRA (Gomez and Robillard 1984); and hemorrhage increased PRA in both control and captopril-treated fetal lambs (Gomez and Robillard 1984), suggesting that feedback control between BP/blood volume (BV) and renin activity exists in the fetus. Interestingly, renal blood flow decreased while renal vascular resistance increased in response to hemorrhage in captopril-treated fetuses (Gomez and Robillard 1984), indicating that the RAS is not the essential factor for regulating renal hemodynamic forces in the fetus. Hence, it appears that the RAS plays an important role in control of BP/BV during fetal life, whereas its effect on renal hemodynamic function may have evolved at a later stage of development (Robillard and Nakamura 1988).

Role of Angiotensin in Water and Ion Balance. As in adults, Ang II increased aldosterone levels in fetal lambs in the third trimester (Robillard et al. 1982; Harris and Gomez 1997). Exogenous administration of aldosterone to fetal lambs of the same gestational period decreased PRA, suggesting that, as above, negative feedback control of renin via volume/Na regulation exists in the fetus. Human adrenal glands in midgestation express abundant AT₂ in the fetal zone that secretes dehydroepiandrosterone-sulfate (DHES/s) (Breault et al. 1998). DHES/s is, however, primarily regulated by adrenocorticotrophic hormone (ACTH), and Ang II exerts no effect on basal steroid secretion. When human fetal adrenal cells were cultured in serum-free medium, Ang II potentiated the ACTH-induced secretion of cortisol, aldosterone, and DHES secretion (Breault

et al. 1998). Central administration of L-NAME into fetal lambs near term reduced Ang II-induced swallowing, suggesting that Ang II and nitric oxide interact in facilitating swallowing (Ei-Haddad et al. 2000).

3.2.2

Regulation of the Renin–Angiotensin System During Development

As discussed above, all components of the RAS exist in the fetus and are developmentally regulated. The factors controlling the time-dependent expression of the RAS, however, are not clear. In rats, renin levels are higher in the fetus than at other times of life, possibly because of increased renin synthesis/secretion and decreased metabolism (Harris and Gomez 1997). In contrast, the sensitivity of the Ang receptor is lower in newborns than in adults. Although the mechanism is unclear, this relative insensitivity may compensate for high Ang II levels (Yosipiv and El-Dahr 1996) in the newborn. The Ang receptor K_d appears similar in fetuses and adults (Norwood et al. 1997).

It has been reported that several factors shown to regulate renin gene expressions and/or renin release in mature animals also control them during fetal life. First, stimulation of the renal nerve in fetal sheep evoked renin release via the β -adrenergic system and increased renal vascular resistance with a concomitant reduction in renal blood flow (Robillard et al. 1987; Robillard and Nakamura 1988). The magnitude of these changes, however, was smaller than in adult kidneys. Bilateral renal denervation in fetal sheep (125 days gestation) decreased the basal level of active PRA and reduced isoproterenol-induced increases in prorenin and active renin levels in vivo, as well as renin mRNA in renal cortical cells (Draper et al. 2000). Second, PRA is higher in fetal lambs (mid- to end gestation) than in mothers and, as discussed above, is increased by hemorrhage (Smith et al. 1974). Ang II infusion (1 $\mu\text{g/h}$ for 3 days) into the ovine fetus at midgestation increased BP and urine flow, whereas PRA and the levels of AT_1 , AT_2 , and renin mRNA decreased, suggesting that Ang II exerts a negative feedback control on the RAS (Mullins et al. 1990; Moritz et al. 2000). Likewise, Ang II reduced the number of renin-secreting cells equally in both newborn and adult rats.

4

Integration of Phylogeny and Ontogeny

4.1

Phylogeny and Ontogeny

The doctrine of Ernst Heinrich Haeckel that ontogeny recapitulates phylogeny and his interpretation of the gill slits of human embryos as a re-expression of ancestral adult fish features are the subject of arguments among evolutionary biologists (Gould 1977). Attempts to elucidate an intimate relationship between ontogeny and phylogeny have not had much success. In this chapter, I have de-

scribed the RAS during phylogeny and ontogeny in a parallel fashion so that readers may find similarities and differences between these two categories. Although it is not my intent to discuss whether some characteristics of the RAS in the mammalian fetus/embryo may be a repeat of the adult stage of ancestral vertebrates or whether they merely represent a common feature of the early ontogeny of all vertebrate animals, comparison of phylogeny and ontogeny may provide new insight into the interpretation of existing findings and provide a perspective for future investigations. Furthermore, molecular and physiological changes observed during phylogenetic or ontogenic processes may be related to, and thus have an impact on disease-/tissue-specific gene expressions and phenotypic modulations during pathogenesis (Kintscher and Unger 2003).

4.2

Biochemistry of the Renin–Angiotensin System

At present, molecular information on renin substrate and renin in primitive vertebrates is scarce. Since Ang I and Ang II show variation in the amino acid in positions 1, 3, 5, and 9, it is expected that multiple AGT genes exist among vertebrates. Although mammalian fetuses express native AGT mRNA and/or proteins, this does not exclude the possibility that heterologous AGT genes are expressed during the early ontogeny process. As mentioned in Sect. 2.1.2, the renin-like enzyme identified in zebrafish has high homology to cathepsin D. It is possible that aspartyl proteases other than renin act as Ang I-forming enzymes in primitive vertebrates. Likewise, the RAS in some mammalian tissues differs from the RAS in the kidney and systemic circulation, bypassing the traditional cascade to form Ang (Danser 1996).

In primitive vertebrates renin-secreting cells are diffusely distributed along small arteries and arterioles in the kidney (Figs. 2 and 3). With the phylogenetic advancement of vertebrates, renin-secreting cells are localized in the JG area. A similar time-dependent change in renin expression has been recognized during ontogeny of mammalian kidneys. In the vascular system of fetal kidneys, renin-secreting cells (Gomez et al. 1989), renin transcripts (Jones et al. 1990, 2000), and renin/AGT gene expression (Gomez et al. 1988b; Fig. 2) are widely distributed. As the renal arterial tree develops, renin mRNA-containing cells are progressively localized to more distal blood vessels and finally to JG apparatus (Jones et al. 1990). A reported construct with a renin 5'-flanking sequence fused to green fluorescent protein (GFP) cDNA accurately recapitulates endogenous renin expression profiles (Jones et al. 2000). This renin/GFP transgene expression was also detected in developing VSM cells of renal arteries in mouse embryos, and the expression was localized at the JG area in adults (Jones et al. 2000). Furthermore, a diffuse distribution of renin mRNA and protein in adult rats (Gomez et al. 1988a) and the renin/GFP transgene expression in adult mice (Jones et al. 2000) similar to the fetal type have been reproduced during ACE inhibition, suggesting that adult renal arteries have the ability to express renin or recruit renin-secreting cells in response to physiological stimulation. As shown in Table 1,

native Ang in various steps of mutation has been identified in nonmammalian vertebrates. It remains to be determined whether some of these nonmammalian Ang are recapitulated during mammalian fetal life.

Likewise, Ang receptors with considerable homology to the AT₁ subtype have been identified in nonmammalian vertebrates (Nishimura 2001; Table 2; Fig. 4). The degree of homology increases with vertebrate advancement, suggesting that all AT₁-homolog receptors may have evolved from the same progenitor receptor. AT₁ mRNA and protein have been detected around E14 of embryonic life (Table 4) by using AT₁ probes constructed on the basis of AT₁ structures identified in adults. It has not been investigated whether the AT₁-homolog receptors in nonmammalian vertebrates may be expressed during the early embryonic stages of mammals.

4.3

Function and Regulation

The comparison of phylogeny and ontogeny in terms of function and regulation of the RAS has several interesting aspects. First, in both the mammalian fetus and nonmammalian vertebrates, the vasopressor/vasoconstricting action of Ang evolved early. Inhibition of ACE activity decreases the basal level of BP in conscious teleost fish (Nishimura and Bailey 1982) and in human and other mammalian fetuses (Sedman et al. 1995), but usually not in mature mammals. Furthermore, circulating Ang or renin levels increase in response to the reduction in BP/BV in both primitive vertebrates and mammalian fetuses. It thus appears that the role of the RAS as a physiological regulator of BP/BV and the feedback control of renin secretion via the baroreceptor function of JG cells may be a fundamental mechanism of the RAS. In contrast, evidence suggests that, in both fetal life and primitive vertebrates, the tubular action of Ang in the control of ion/water transport and the involvement of the adrenergic nervous system in the control of renin secretion may be later additions during the ontogenic (Harris and Gomez 1997) or phylogenetic (Nakamura et al. 1992) processes. This concept is supported by the facts that (1) a close spatial relationship between renin-containing cells and nerve fibers has been detected at E17 in rat fetal kidneys and (2) the density of nerve fibers around renin-secreting cells increases as renin is localized in the JG area (Pupilli et al. 1991). In teleost fish (Nishimura and Madey 1989), cytosolic Ca²⁺ may be a fundamental cellular mechanism controlling renin secretion. Also of interest is the question of whether fetal kidneys contain a Ca²⁺-dependent mechanism similar to the one seen in adult renin-secreting cells (Fray et al. 1987). Stimulation of adrenocorticosteroid hormones by Ang II has been reported in primitive vertebrates (see Sect. 2.2.2.3). ACTH appears to be a more potent regulator for mineralocorticoid, at least in some species. It will be interesting to determine the relative potencies of ACTH and Ang II in the regulation of mineralocorticoid synthesis/secretion during the ontogeny of mammalian adrenal glands.

As discussed above, a major physiological role of Ang in developing mammals appears to be stimulation of growth and differentiation via AT₁ and AT₂ receptors (Tufro-McReddie and Gomez 1993; Hilgers et al. 1997). In nonmammalian vertebrates, the growth-promoting action of Ang has been studied in only a few species. Le Noble and coworkers (1993, 1997) have demonstrated that Ang II causes pronounced angiogenesis of pre- and postcapillary vessels in the chorioallantoic membrane of chick embryos, suggesting that the Ang receptor plays a role in vascular growth and organization during avian development; the response is inhibitable by CGP42112A, but not losartan or PD123319 (Le Noble et al. 1993). Vascular endothelium growth factor (VEGF) appears to play an important role in the development of the vascular system in the avian embryo (Eichmann et al. 1998), but the effect of Ang II on VEGF gene expression or on VEGF receptors has not been determined. Ang II stimulates thymidine incorporation into cultured VSM cells from chicks; but this effect decreases with maturation/age, and no growth-promoting action of Ang is seen in adult birds (Shimada et al. 1998). The paracrine action of Ang as a growth-stimulating factor in nonmammalian species remains to be examined.

4.4 Perspectives

During development, the expression level of the RAS shows time-dependent changes (Table 4). In rat embryos, AGT is first detected at E11 of gestation. AGT mRNA or protein shows 2- to 3-fold (kidney) and 50-fold (liver) increases within 24 h of birth (Darby and Sernia 1995; Gomez et al. 1988b), remaining elevated for 3 weeks, and then it reaches a plateau at the adult level (Tufro-McReddie and Gomez 1993). Renin is first detected in rats at E15–E17 in the aorta and its major branches, extends to the renal vasculature by the end of gestation, and is localized at the vascular pole of the JG area and afferent arterioles after birth (Gomez et al. 1989). Ang receptors appear at E11 of gestation and are broadly expressed in developing tissues; their abundance increases as gestation progresses, with maximum binding near term (Tufro-McReddie and Gomez 1993). During fetal life, AT₂ receptor expression is about tenfold higher than that of AT₁; but it rapidly decreases at birth. ACE activity also shows time-dependent changes (Table 4). ACE activity shows the same substrate affinity at all ages examined, while the V_{max} of the enzyme is two- to threefold lower in fetal than adult kidneys and lungs, reflecting low ACE activity (Wallace et al. 1978, 1979).

It is interesting to note that the evolution of AGT and Ang receptors in rat kidneys occurs a few embryonic days earlier than that of renin. Does this indicate that an aspartyl protease other than renin acts as renin-like activity at early gestation? Does this agree with the fact that, in teleost fish, cathepsin D appears to exert renin-like activity? The physiological significance of the progressive elevation of the RAS near term and during the neonatal period is not completely understood. The role of Ang II in differentiation and apoptosis via AT₂ receptors and its growth-promoting effect via AT₁ receptors may be more crucial for de-

velopment and maturation near term and continue to play a maturation role during the neonatal period. Deletion or impairment of any component of the RAS results in serious abnormality in organogenesis (see Sect. 3.2.1.1). It has been suggested that the RAS is partly responsible for tonic vasoconstriction of renal vessels and mesangium, resulting in limited glomerular blood flow and GFR in developing animals (Robillard and Nakamura 1988). cAT₁ mRNA is also abundantly expressed (PCR and *in situ* hybridization) at E19 (term, 21 days), primarily on mesangial cells of glomeruli (Nishimura et al. 2003); and its expression decreases in chicks and pullets. Furthermore, cAT₁ mRNA has been detected in the adventitia and outer layer of the media of the abdominal aorta at E19 and in 2- to 3-week-old chicks, but not in adult birds. The time and site of cAT₁ mRNA expression suggest that Ang plays a role in embryonic growth and maturation.

An interesting example of the similarities of ontogeny and phylogeny can be found during the development/maturation of renal tubule transport in the rat and chick kidneys. Liu and coworkers (Liu et al. 2001) compared medullary nephrons from the kidneys of (1) quail (looped nephron; for review, Nishimura and Fan 2002), (2) neonatal rats (long-looped nephron), and (3) mature adult rats (long-looped nephron) and found the following structural and functional similarities between the first two. First, both quail and neonatal rat kidneys lack the thin ascending limb, and the entire thick limb shows characteristics of diluting segments. During maturation of rat kidneys, apoptosis occurs in the lower segment of the ascending limb (AL), where the Na⁺-K⁺-2Cl⁻ cotransporter is replaced by a thin AL-specific Cl channel. Second, the descending limb of both kidneys has low water permeability and no aquaporin (AQP)1 water channel expression. Third, AQP2 is present in the collecting duct (CD) of neonatal rats and quail, but the AQP2 responses (increases in water permeability, AQP2 protein level, etc.) to vasopressin/AVT are only modest. Fourth, the thin AL and CD of neonatal rats (and presumably quail kidney) lack urea permeability and urea transporters (UT), whereas UTA₁ mRNA is expressed in mature rat kidneys. Urea recirculation utilizing exchange mechanisms between the vasa recta and tubule segments contributes to the development of an osmotic gradient in mammalian kidneys, whereas uric acid is a primary nitrogen metabolite in birds that does not contribute to enhancement of the medullary osmotic gradient. Similarly, an effective mechanism for maintaining high papillary osmolality utilizing urea recirculation has not yet developed in neonatal rats. It will be interesting to determine whether Ang is involved in these growth/differentiation processes of the renal medulla.

It is difficult to assess whether these time-dependent changes in the RAS in developing mammals are a recapitulation of the changes seen during phylogeny because the time scales involving these two biological processes are entirely different. Moreover, simple comparisons of the structural, functional, or molecular properties of mammalian fetuses and existing nonmammalian vertebrates have little meaning because most of the currently available species represent the end-products of various taxonomical branches. Hence, the collection of information

from existing nonmammalian vertebrates does not necessarily reflect the true evolutionary process of modulation of the RAS. It is therefore important to select species taxonomically close to the origin of evolution and the trunk of phylogenetic advancement.

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Angiotensin as a Cytokine Implicated in Accelerated Cellular Turnover

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Abstract Several models of essential hypertension have revealed abnormalities in pathways regulating cell proliferation and programmed cell death (apoptosis). The increased proliferative phenotype found as early as birth in hypertensives is accompanied by age-dependent alterations in apoptosis, contributing to neonatal hyperplasia of the heart, aorta, and kidneys. During the course of life, accelerated cell turnover occurs and is modifiable by antihypertensive therapy, notably by inhibitors of the renin-angiotensin system. We consider the hypothesis that hypertension may be a case of accelerated aging. Part of this process may involve the defective regulation of cell proliferation in cardiovascular target organs via a putative specific senescence pathway. Candidates include abnormalities in cell cycle control genes, the renin-angiotensin pathway, and regulation of the telomerase pathway. Abnormal activity of angiotensin II-regulated Na⁺ transporters and augmented production of endogenous ouabain-like substances have been detected in experimental models of primary hypertension. Recent data show that both ouabain and intracellular Na⁺ are involved in the regulation of gene expression and apoptosis. The relevance of neonatal and early life development as a predictor of cardiovascular disease outcomes later in life is an in-

triguing issue that remains to be better defined. In this regard, understanding the complex genetic and epigenetic influences contributing to aging and age-related diseases will be a major goal. Because phenotype development can be analyzed longitudinally during the course of life in recombinant inbred rat strains, these models will allow a systematic approach to the molecular analysis of senescence pathway regulation, their determinants early in life, and their control by hereditary and epigenetic factors, including pharmacotherapy.

Keywords Hypertension · Apoptosis · Proliferation · Cellular senescence · Renin-angiotensin system · Telomeres · Recombinant inbred strains · Genetics · Epigenetics · Hypertrophy · Ion transport · Mortalin

Abbreviations

AII	Angiotensin II
ACE	Angiotensin-converting enzyme
BP	Blood pressure
cGMP	Cyclic guanosine monophosphate
chr	Chromosome
ERG	Early response genes
ESPI	Endogenous Na ⁺ ,K ⁺ pump inhibitors
HSP	Heat stress protein(s)
MAPK	Mitogen-activated protein kinase
MOT-2	Mortalin
NHE1	Na ⁺ -H ⁺ exchanger
NKCC1	Na ⁺ ,K ⁺ ,Cl ⁻ cotransport
NO	Nitric oxide
PI3 K	Polyphosphoinositide-3-kinase
PKC	Protein kinase C
PLC- γ	Phospholipase C
QTL	Quantitative trait locus
RAS	Renin-angiotensin system
REC	Renal epithelial cells
RIS	Recombinant inbred strains
SHR	Spontaneously hypertensive rats
SMC	Smooth muscle cells
VSMC	Vascular smooth muscle cell(s)
VSMC-E1A	E1A-adenoviral protein

1 Introduction

Angiotensin II (AII) was initially considered a main blood pressure (BP) regulatory substance due to its vasoconstricting effects. Relatively recently, its growth-promoting effects have been recognized, mainly as being mediated by AT_1 receptors, but it was only when AT_2 receptor-mediated inhibition of cell proliferation (and apoptosis) became known (Stoll et al. 1995; Stoll and Unger 2001) that the extent of AII's impact on the balance of proliferation/apoptosis was realized. This has major implications for cardiovascular disorders of generalized proliferation imbalance, such as hypertension (Hamet 1995), or localized abnormalities, such as occurring in atherosclerosis (Choy et al. 2001).

In this chapter, we will review the evidence of accelerated cellular turnover associated with defective proliferation and apoptosis in target organs of hypertension with specific attention to the putative role of AII in this process and in genetic determinants of early development, ion transport, and the impact of remodeling via the implication of apoptosis.

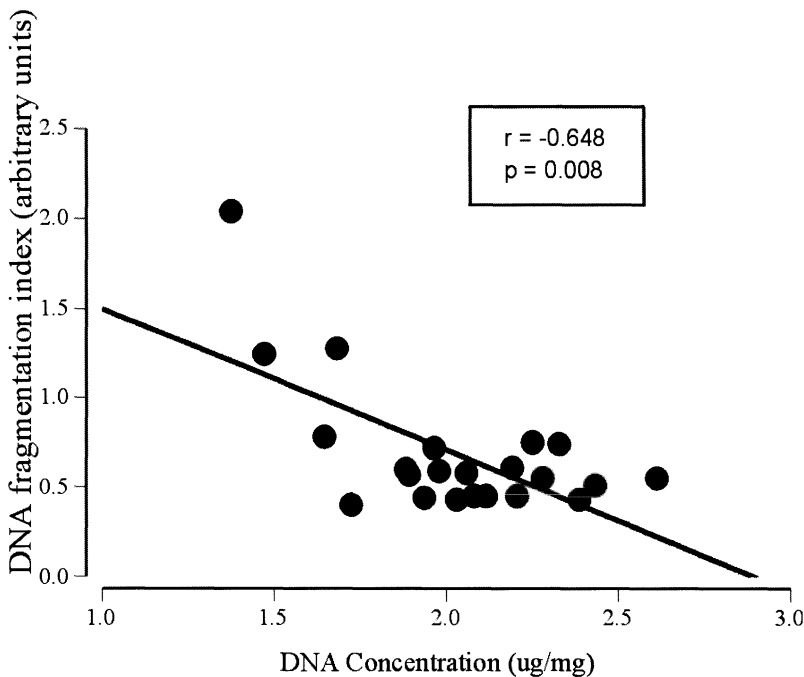


Fig. 1 Correlation between neonatal cardiac DNA fragmentation indicative of apoptosis and DNA concentration ($\mu\text{g}/\text{mg}$) in 20 recombinant inbred strains and 2 progenitor strains. There was negative correlation between newborn cardiac DNA concentration and apoptosis in the heart ($r=-0.64$, $p<0.001$). Data shown are mean values of 5 rats per strain. (Reproduced from Hamet et al. (2001) with permission from *Hypertension*)

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Abnormal Proliferation/Apoptosis in Hypertensive Neonates

In hypertension, the heart shows both increased cell size (mainly cardiomyocytes) and cell number (mainly fibroblasts) (Anversa et al. 1997; Walsh and Dorn 1998), while the aorta presents augmented smooth muscle cell (SMC) DNA due to SMC polyploidy and hyperplasia. Several groups, including our own, have reported hyperplasia in the “prehypertensive period” in the heart (Tanase et al. 1982; Walter and Hamet 1986) and kidney of neonates from diverse strains with genetic hypertension (Pang et al. 1986). An altered balance between cell replication and apoptosis is present in the newborn heart and kidney of spontaneously hypertensive rats (SHR), leading to greater relative organ size and even DNA content, while apoptosis is significantly suppressed (Moreau et al. 1997). DNA accumulation is actually significantly negatively correlated with DNA fragmentation (Fig. 1). In this neonatal period, the ratio of DNA synthesis over apoptosis nevertheless favors proliferation in the heart, kidney, and aorta (Hamet et al. 1996a, 2001; Orlov et al. 2002).

Table 1 summarizes the major findings by our group and several others, supporting the concept of proliferation/apoptotic disorders and accelerated senescence in hypertension

Table 1 Major phenotypes supporting evidence of proliferative/apoptotic disorder and accelerated senescence in hypertension

Phenotype	Year	Reference(s)
Cardiac and renal hyperplasia in neonates from SHR	1982, 1986	Tanase et al. 1982; Pang et al. 1986
Increased thymidine incorporation into DNA of SHR neonates (heart, kidney, aorta)	1986	Walter and Hamet 1986
Persistence of increased VSMC proliferation in culture	1989, 1992	See Table 2
Shortening of the cell cycle (G ₁ -S, G ₂ -M)	1992	See Table 2
Apoptosis in target organs of hypertension	1995	Hamet et al. 1995
Time window of apoptosis	1996	Hamet et al. 1996a
Apoptosis in regression of hyperplasia/hypertrophy	1997–2000	deBlois et al. 1997; Diez et al. 1997; Tea et al. 1999; Intengan and Schiffrin 2000
Suppression of apoptosis in neonatal SHR	1997	Moreau et al. 1997
QTL of elevated heart/ and kidney/body weight ratios, proliferation and apoptosis in hypertension	1995–2001	Hamet et al. 1998
First proposition of accelerated aging with increased cell turnover	1997	Hamet 1997
Altered in vivo vascular cell turnover in SHR	2001	Thorin-Trescases et al. 2001
Inverse relationship between telomere length and pulse pressure	2000	Jeanclous et al. 2000
Excess growth and apoptosis as accelerated aging with increased telomere restriction fragments	2001	Hamet et al. 2001
Telomerase activation in VSMC as a cause of proliferation in hypertension	2001	Cao et al. 2002

Table 2 Markers of proliferative activity in cultured cells from hypertensive rats

Rat strains/cell type	Parameter measured	Data	Reference(s)
SHR vs WKY, VSMC	Rate of cell growth, DNA and protein synthesis	Increased in SHR	Yamori et al. 1981; Clegg and Sambhi 1989; Hadrava et al. 1989; Paquet et al. 1989; Scott-Burden et al. 1989; Bukoski 1990; Hamada et al. 1990; Hadrava et al. 1992; Saltis and Bobik 1992; LaPointe et al. 1995; Nakayama et al. 1999
GH vs WKY, VSMC	DNA synthesis	Increased in GH	Harris et al. 1990
SHR vs WKY, VSMC	Cell cycle analysis	Shortening of the G ₀ /G ₁ phase in SHR	Uehara et al. 1991; Hadrava et al. 1992
SHR vs WKY, VSMC	Cell cycle analysis	Increased in SHR, accumulation of cells in the S phase	Hamada et al. 1990
Rats with DOCA-salt hypertension, VSMC	DNA synthesis, cell cycle analysis	Not altered	Hamada et al. 1990
SHR vs BN.lx, VSMC	DNA synthesis	Increased in SHR	Champagne et al. 1999
SHR-SP vs WKY, VSMC	Rate of cell growth, DNA synthesis	Increased in SHR-SP	Devlin et al. 1995, 2000
SHR vs WKY, skin fibroblasts	Rate of cell growth, DNA synthesis	Increased in SHR	Guicheney et al. 1991
SHR vs WKY, adventitial fibroblasts	Rate of cell growth, DNA synthesis	Increased in SHR	Zhu et al. 1991
SHR vs WKY, adventitial fibroblasts	Cell cycle analysis	Faster exit from G ₀ in SHR	Venance et al. 1993
SHR-SP vs WKY, endothelial cells	Rate of cell growth, DNA synthesis	Increased in SHR-SP	Ito et al. 1995
SHR-SP vs WKY, astrocytes	Rate of cell growth, DNA synthesis	Increased in SHR-SP	Yamagata et al. 1995

GH, New Zealand strain of spontaneously hypertensive rats; SHR-SP, stroke-prone SHR. For other abbreviations see text.

cence in hypertension, from enlarged organ size in genetically hypertensive neonates to the recent description of heightened telomerase activity in vascular SMC (VSMC). The phenotype increase of proliferation persists in culture, as initially observed by Yamori and colleagues (1981). The markers of heightened proliferative activity reported in cultured cells from hypertensive rats are summarized in Table 2. Finally, quantitative trait locus (QTL) mapping of elevated heart and kidney weight/body weight ratio, DNA accumulation and apoptosis is summarized in Table 3.

QTLs of enlarged organ size map to the same chromosome (chr)—for example, relative heart weight on rat chr 2—in different genetic models of hypertension, but some studies point to different loci. The availability of “permanent ho-

Table 3 QTLs of elevated heart- and kidney-body weight ratio, proliferation, and apoptosis in hypertension

Chromosome (QTL)	Phenotype	Crosses	Significance	Reference
1(a)	HW	SS/JR×LEW	4.0*	Gu et al. 1996
1(b)	HW	SS/JR×LEW	2.8*	Gu et al. 1996
1(c)	AKW	SHR×BN.lx	(-)-1.2*	Hamet et al. 1998
2	HW	SS/JR×MNS	3.0*	Deng et al. 1997
	HW	SS/JR×WKY	4.0*	Deng et al. 1997
	HW	GH×BN	1.3*	Harris et al. 1995
3	NKW	SHR×BN.lx	(-)-2.0*	Hamet et al. 1998
	AKW	SHR×BN.lx	(-)-2.0*	Hamet et al. 1998
5(a)	HW	SS/JR×LEW	4.0*	Gu et al. 1996
5(b)	AKW	SHR×BN.lx	2.0*	Hamet et al. 1998
8	HW	SHR×BN.lx	2.0*	Kren et al. 1997
10	HW	GH×BN	1.3*	Harris et al. 1995
12	HW	SHR×BN.lx	2.0*	Hamet et al. 1996b
13	HW	GH×BN	3.0*	Harris et al. 1995
17(a)	HW	SHR×BN.lx	3.4	Pravenec et al. 1995
17(b)	HW	SS/JR×LEW	3.7	Gu et al. 1996
18(a)	AHN	SHR×BN.lx	(-)-1.3*	Hamet et al. 2001
18(a)	PHN	SHR×BN.lx	(-)-1.3*	Hamet et al. 2001
18(b)	AHN	SHR×BN.lx	1.3*	Hamet et al. 2001
18(c)	AHN	SHR×BN.lx	(-)-2.0*	Hamet et al. 2001
18(d)	AHN	SHR×BN.lx	(-)-1.3*	Hamet et al. 2001
X	HW	GH×BN	2.0*	Harris et al. 1995

Note: statistical significance is presented as the value of a logarithm of odds ratio (LOD score) or as a log of the statistical values (asterisks).

AHN, apoptosis in the heart of newborn rats (a relative amount of fragmented DNA was used to estimate this parameter); AKW, kidney-body weight ratio of adult rats; HW, heart-body weight ratio; LEW, Lewis normotensive rats; NKW, kidney-body weight ratio of newborn rats; PHN, proliferative activity in the heart of newborn rats (DNA content per wet tissue weight was used to estimate this parameter); SS/JR, salt-sensitive strain of Dahl rats; -, negative impact of the SHR allele.

mozygous replicas” of the F₂ generation, obtained by 25-generations of inbreeding after intercrossing between BN.lx and SHR (Pravenec et al. 1989), has allowed us to relate neonatal to adult phenotypes in recombinant inbred strains (RIS); while neonatal kidney weight at least partially determines the phenotype in adulthood, the latter is negatively influenced by BP increases with age (Hamet et al. 1998) (Fig.2).

The renin-angiotensin system (RAS) is known to be excessively stimulated in the neonatal period. It is therefore relevant if one of the significant QTLs of apoptosis inhibition (phenotype of excess shorter fragments over long ones) (Moreau et al. 1997) includes the D17Mit2 locus ($p=0.004$) on rat chr 17, which contains the AT_{1A} receptor gene as the main positional candidate (Moreau P, Sun Y, Kren V, and Hamet P, unpublished data).

To obtain continuous “recordings” of cellular death, we recently developed a novel approach of in utero prelabeling with prolonged follow-up of DNA-specific activity (Hamet et al. 2001; Thorin-Trescases et al. 2001). Despite its limita-

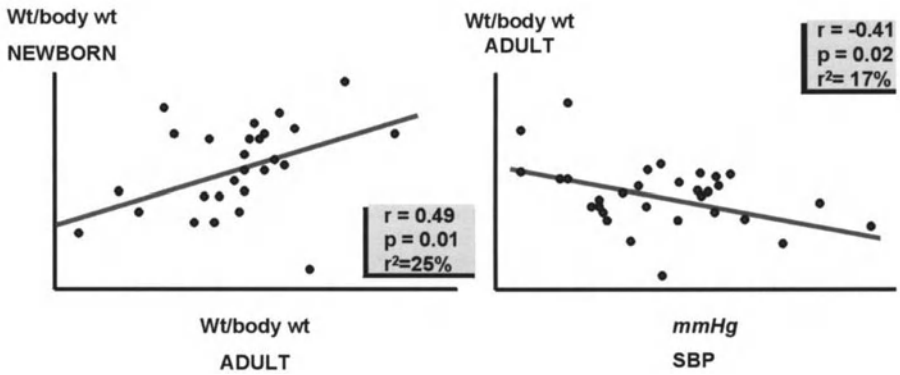


Fig. 2 Correlation between relative kidney weight in newborn and adult male rats of the RIS panel (*left side*). Correlation between adult relative kidney weights and systolic blood pressure in the panel of RIS (*right side*). (Reproduced from Hamet et al. (1998) with permission from *Kidney International*)

tions, this technique showed that increased aorta, kidney, and heart size in the neonatal stage of hypertension reverses by the age of 5-8 weeks in rats, followed by the appearance of secondary hypertrophy (heart, aorta) or atrophy (kidney) with time (Hamet et al. 2001; Thorin-Trescases et al. 2001). Cellular half-life was shorter in the SHR aorta: $t_{1/2}$ was 20 weeks in normotensive Wistar-Kyoto rats, but only 7 weeks in SHR (these values in the kidney were 14 and 8 weeks, respectively). Our most recent studies have demonstrated the presence of increased telomere restriction fragments, particularly of smaller size (Hamet et al. 2001), in the neonatal kidney. Clearly, telomerase activity should also be monitored (Aisner et al. 2002). Taken together, these investigations led us to propose that heightened cell turnover in SHR is related to initial apoptosis inhibition in neonates, resulting in heart, kidney, and aortic hyperplasia (Hamet et al. 2001), shortening of the cell cycle (Uehara et al. 1991; Hadrava et al. 1992), and increased apoptosis later in life (Hamet et al. 1995). While the AT_1 receptor is an attractive target to modulate proliferation/apoptosis imbalance, its direct involvement remains to be tested.

3 Role of Cell Cycle Genes and Chaperones

The above events could result from abnormalities within cell cycle control genes, including p53, MDM 2, p21^{cip1} and p16^{INK4a} as well as genes controlling their proteolytic degradation (Okorokov et al. 1997; Kubbutat and Vousden 1997; Ariyoshi et al. 1998; Pochampally et al. 1999; Nakayama et al. 2001; Heessen et al. 2002) and chaperoning at G₁-S and G₂-M checkpoints. The interaction of chaperones (represented mainly by heat stress proteins, HSP) with the p53 molecule is now better understood (King et al. 2001). In this context, it is relevant to point out that the targeted disruption of mouse AT_2 receptor gene impacts on

stress-induced hyperthermia (Watanabe et al. 1999). We have previously demonstrated that HSP27 polymorphism is in linkage with left ventricular hypertrophy and that HSP70 expression is higher in SHR VSMC (Tremblay et al. 1992; Hamet et al. 1996b). Prior HSP expression, on the other hand, has been shown to partially protect against hyperthermic cell cycle arrest (Champagne et al. 1999).

Human aging, according to the gene profiling studies of Ly et al. (2000), is mainly characterized by a cumulative slowdown in G₂-M. It is therefore relevant that a newly identified hypertension-related calcium-regulated gene with heightened expression in SHR kidneys (Solban et al. 2000) induced G₂-M arrest of renal epithelial cells (REC) by increasing p21^{cip1/WAF-1} and decreasing p27^{kip1} (Devlin et al. 2003). AII is involved in cardiomyocyte hypertrophy and cell death via its impact on extracellular signal-regulated kinase 1/2 (MEK-ERK1/2 pathway and JAK-STAT signaling) (Booz et al. 2002; Bueno and Molkenstein 2002). Part of the pathway downstream of AT₁ and AT₂ receptors is distinct but a part is shared, mainly by activation of nuclear factor- κ B transcription factors (Ruiz-Ortega et al. 2000, 2001). In addition, the AT₂ receptor involves activation of protein phosphatases, the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) system and phospholipase A₂ (Nouet and Nahmias 2000), and its antiproliferative effect appears to include downregulation of SM-20 (Wolf et al. 2002), a mitochondrial protein implicated in apoptosis and expressed in vascular smooth muscle (Wax et al. 1996). It has been reported that apoptosis is heterogeneously distributed in VSMC, depending in part on the cellular phenotype (Bascands et al. 2001). AT₂ receptor expression is not obligatorily related to apoptosis, such as, for instance, apoptosis induced during ischemia/reperfusion-dependent cardioprotection (Moudgil et al. 2002). AII induces p53 expression (Bonnet et al. 2001); via the same downstream pathway implicating p21^{cip1/WAF-1}, p27^{kip1} and p53, it also evokes proliferation via superoxide production (Mueller et al. 2002).

Our studies using proteomics have identified another HSP, mortalin (MOT-2 or HSPA9), as a major mediator of the [Na⁺]_i-induced delay in VSMC apoptosis (for more details, see below). MOT-2 was initially cloned as a highly homologous member of the HSP70 family, not inducible by heat stress, but interacting with other chaperones (Domanico et al. 1993). Injection of anti-MOT-2 antibodies led to transient division of senescent cells (Wadhwa et al. 1993a), whereas transfection with MOT-2 cDNA resulted in induction of senescence in NIH 3T3 fibroblasts (Wadhwa et al. 1993b). It has now been demonstrated to inactivate p53 and extend fibroblast lifespan (Kaul et al. 2000). This action is exercised in concert with HSP90 under control of the co-chaperones Bag-1, Hop and HSP40 (King et al. 2001). The regulation of p53 degradation (by ubiquitination) is the consequence of a complex interplay of these HSPs with wild and mutated p53 (King et al. 2001). Actually, HSP90 inhibits the MDM 2 stabilization of p53 (Peng et al. 2001).

4 Genetics of Growth-Related Phenotypes

MOT-2 is localized on rat chr 18 in a region syntenic to human 5q31-3. This region is highly relevant to hypertension in both rats and humans (Jacob et al. 1991; Krushkal et al. 1998). It has been known since publication of the first total genome scan in SHR (Jacob et al. 1991), and confirmed by many others (Cowley et al. 2000; Garrett et al. 1998), that the locus on chr 18 bears significant components of salt sensitivity (Fig. 3). This is of interest as $[Na^+]_i$ elevation induces MOT-2 expression (Taurin et al. 2002a).

In the largest set of genotypic/phenotypic profiling studies published to date by the Medical College of Wisconsin and accessible on <http://brc.mcw.edu/phy-prof> (Stoll et al. 2001), a significant cluster of loci was identified on rat chr 18. BP, renal and anthropometric phenotypes correlated only on chr 18, suggesting the presence of a “gene cassette” (Fig. 3).

To avoid an untargeted and tedious search for candidate genes determining neonatal growth-related phenotypes, we performed total genome scan in RIS as a unique tool to investigate neonatal and adult phenotype/genotype concordance (Hamet et al. 1998). This approach has demonstrated a strain distribution pattern of RIS for the DNA fragmentation index and DNA concentration in the kidney, allowing their chromosomal mapping (Hamet et al. 2001). The most significant QTLs were found on chrs 1 and 18. Of interest here is the locus on chr 18 at Mit4R525 which is the locus of MOT-2 (Fig. 3). Direct testing of this candidate locus/gene and its putative interaction with components of the RAS is under way.

5 Replicative Senescence Versus Accelerated Aging

Aging-related phenotypes, including disease states, are both genetically and epigenetically defined. The aging process is biologically complex, and the distinction between replicative senescence, old age, and accelerated aging (such as progeria) must be made (Slagboom et al. 2000). Thus, for instance, fibroblasts from the elderly share more molecular determinants with progeria than with replicative senescence. Some markers, such as p53 and p21^{WAF-1}, are augmented in all three conditions, while cdc42 and CAMK2D increase only in progeric and elderly fibroblasts (Park et al. 2001). Clustering demonstrated hierarchical similarities between progeria and old age but not with replicative senescence (Eisen et al. 1998). A detailed analysis of middle age, old age, and progeria was provided by Ly et al. (2000) who related aging with mitotic misregulation, particularly affecting G₂-M transition; of 6,000 genes analyzed, 61 (1%) were modified, 25% of them in cell cycle progression, and 31% in extracellular matrix remodeling. Progeria, while extremely rare, is characterized by the presence of severe hypertension (Ogihara et al. 1986) and VSMC depletion (Stehbens et al. 2001) leading to cardiovascular death (Rosman et al. 2001). Its mechanism is unknown, but

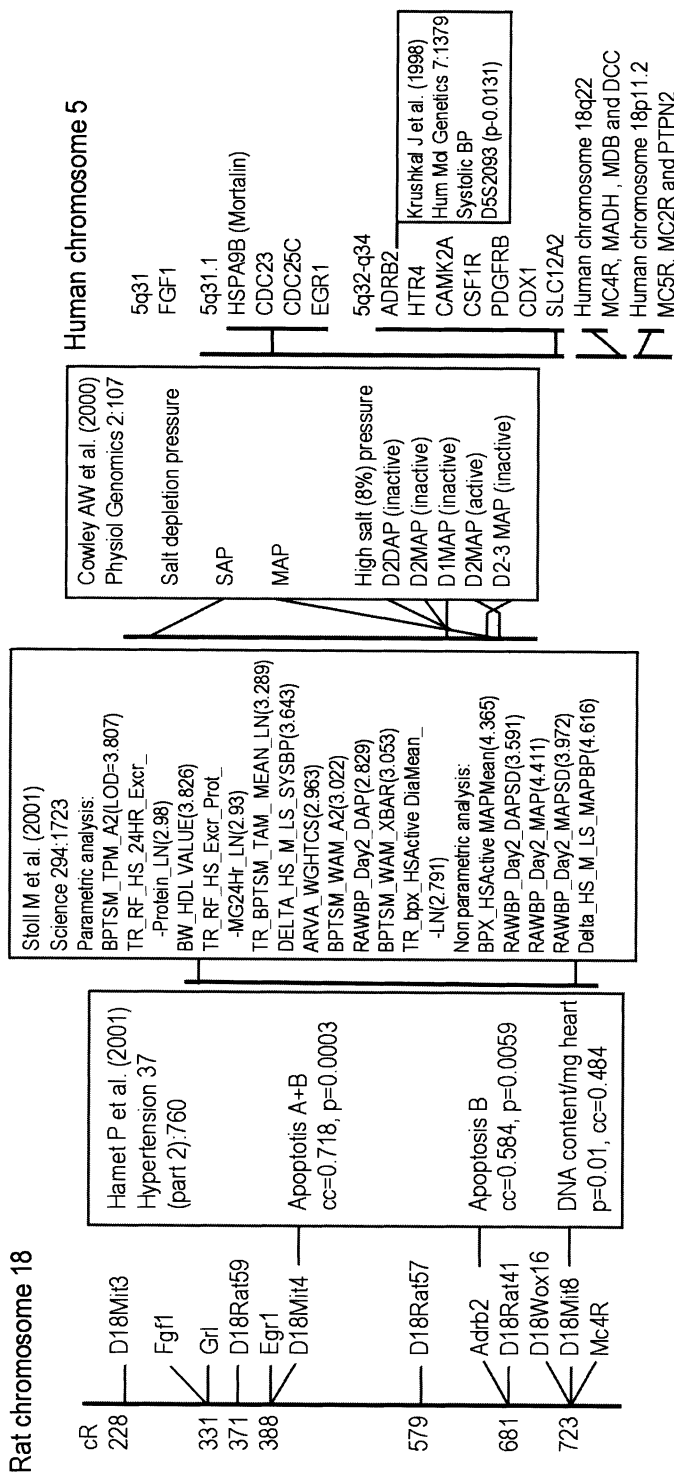


Fig. 3 Quantitative trait loci (QTL) clusters mapped on the long arm of rat chr. 18 and its syntenic region on human chr 5q31-q32

sporadic dominant mutation is suspected (Sarkar and Shinton 2001). Telomerase activity is putatively involved, but normal telomere length has been observed in neonatal progeroid (Wiedemann-Rautenstrauch) syndrome (Korniszewski et al. 2001).

6 Biomarkers of Senescence

Senescence-associated β -galactosidase is widely used as a distinct biomarker of senescence as it is not expressed in pre-senescent cells and is absent in “immortal cells” (Dimri et al. 1995). Increased p53-induced gene 3 expression, in response to environmental stimuli such as reactive oxygen species, is long-lasting (Flatt et al. 2000). In human atherosclerotic plaque, Rb and p53 have been shown to act cooperatively in the control of proliferation, cell senescence, and apoptosis, leading to a high level of proliferation and suppression of apoptosis (Bennett et al. 1998). Disappearance of angiotensin-converting enzyme (ACE) activity was identified as a phenotype associated with several karyotypic changes induced by the aging of human endothelial cells in culture (Johnson et al. 1992). More recently, in a seminal study, Tyner and colleagues (2002) have demonstrated that p53 mutant mice display aging-associated phenotypes, including cutaneous atrophy, osteoporosis, impaired stress tolerance, and reduced longevity. Noticeably, several of these phenotypes are also linked with cardiovascular diseases, such as diminished stress tolerance (Dumas et al. 2000a,b) and even osteoporosis, SHR being used as a “model of osteoporosis” (Tsuruoka et al. 2001).

7 Role of Ion Transporters and Intracellular Monovalent Cations

Cell physiology studies from several laboratories demonstrated a potent role of AII in monovalent ion handling. Thus, in VSMC, AII activates ubiquitous isoforms of $\text{Na}^+\text{-H}^+$ exchanger [NHE1 (Hatori et al. 1987; Berk et al. 1990)], $\text{Na}^+, \text{K}^+, \text{Cl}^-$ cotransport [NKCC1 (Smith and Smith 1987; Orlov et al. 1992; Peiro et al. 1997; Akar et al. 1999)] and the Na^+, K^+ pump (Orlov et al. 1992). Activation of NHE1 and the Na^+, K^+ pump by AII was also revealed in secretory epithelial cells (Hou and Delamere 2002). In REC, AII activates the NHE3 isoform of the $\text{Na}^+\text{-H}^+$ exchanger (Tsuganezawa et al. 1998) and $\text{Na}^+, \text{HCO}_3^-$ cotransport (Horita et al. 2002; Robey et al. 2002) and inhibits the $\text{Na}^+, \text{glucose}$ cotransporter SGLT1 (Kawano et al. 2002). Activation of $\text{Na}^+, \text{HCO}_3^-$ cotransport was also observed in AII-treated cardiomyocytes (Baetz et al. 2002). Recently, it was shown that similarly to blood cells (Avdonin et al. 1990), non-selective intracellular Ca^{2+} store-operated cation channels contribute to $[\text{Na}^+]_i$ elevation in AII-treated arterial myocytes (Arnon et al. 2000). In neuronal cells, AII activates delayed rectifier K^+ channels, thus leading to membrane hyperpolarization (Kang et al. 1994; Matsukawa and Ichikawa 1997).

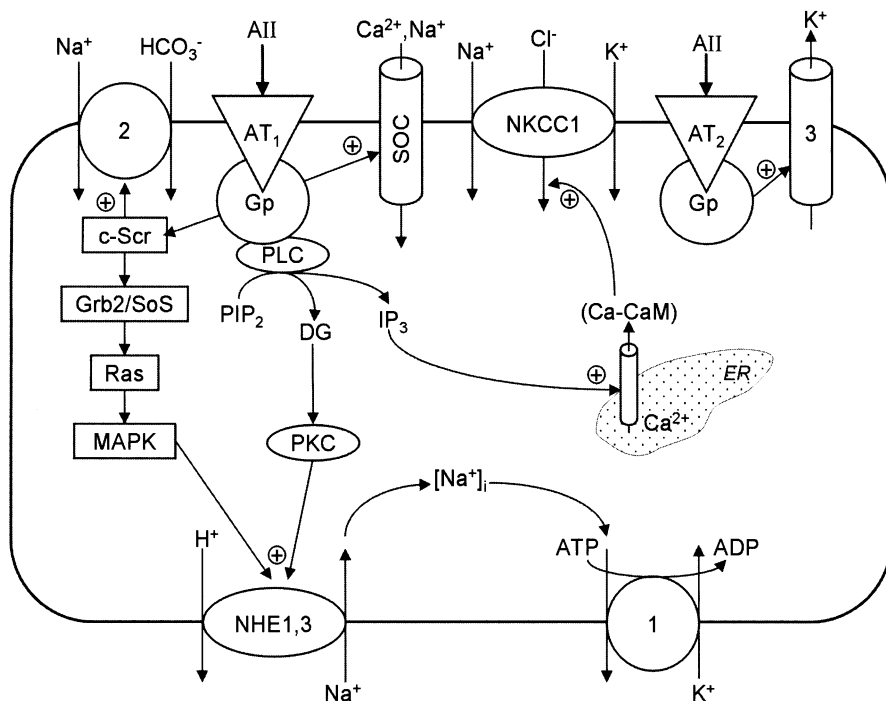


Fig. 4 Mechanism of the regulation by AII of intracellular monovalent ion handling. *G_p*, GTP-binding proteins; 1, Na⁺,K⁺-ATPase; 2, Na⁺,HCO₃⁻ cotransporter; 3, delayed rectifier K⁺ channels. For more details, see text

Our current knowledge on the regulation of intracellular monovalent ion handling by AII is schematically presented in Fig. 4. In VSMC and the secretory epithelium, NHE1 activation is mediated by AT₁ receptors (Ye et al. 1996; Hou and Delamere 2002), protein kinase C (PKC) (Hatori et al. 1987; Grinstein et al. 1989), and ERK1/2 mitogen-activated protein kinase (MAPK) (Kusuhara et al. 1998). In VSMC, transient elevation of [Ca²⁺]_i contributes to enhanced NKCC1 (Smith and Smith 1987; Orlov et al. 1992) whereas in REC, NHE3, and Na⁺,HCO₃⁻ cotransport is activated by a c-Src-dependent pathway (Tsuganezawa et al. 1998; Robey et al. 2002). In the majority of cells studied so far, activation of the Na⁺,K⁺ pump is a secondary event caused by the NHE1-mediated rise of [Na⁺]_i (Orlov et al. 1992; Hou and Delamere 2002; Rangel et al. 2002). Indeed, in VSMC and REC, AII led up to threefold elevation of [Na⁺]_i that was completely abolished by potent NHE1 blockers (Ye et al. 1996; Touyz and Schiffrin 1999; Hou and Delamere 2002). In contrast to the above-listed ion carriers, AII activates K⁺ channels via an unknown signaling cascade triggered by AT₂ receptors (Kang et al. 1994; Matsukawa and Ichikawa 1997).

It is well-documented that AII signaling efficiency is increased in SHR compared to normotensive rats. Importantly, these data were obtained by analysis of the [Ca²⁺]_i response, inositol-4,5-triphosphate production, and MAPK activity

(Touyz et al. 1994; Lucchesi et al. 1996; Phan et al. 1997) as well as $[Na^+]_i$ and pH_i modulation triggered by AII in cultured VSMC (Touyz and Schiffrin 1997, 1999) and REC (Thomas et al. 1990; Cheng et al. 1998) from SHR. It should be stressed that apart from increased sensitivity to AII, augmented increment of $[Na^+]_i$ might be caused by enhanced baseline activity of NHE1 and other ion carriers documented in several cell types from SHR, the Milan hypertensive strain, and patients with essential hypertension (Roskopf et al. 1993; Siffert and Dusing 1995; Orlov et al. 1999a). In addition, intracellular monovalent cation content is subjected to regulation by endogenous Na^+, K^+ pump inhibitors (ESPI) identified as immunoreactive ouabain-like (Ludens et al. 1991; Tymiak et al. 1993) and marinobufagenin-like (Bagrov et al. 1995; Hilton et al. 1996; Sich et al. 1996) steroids.

Several research teams have proposed that abnormal intracellular monovalent ion handling is involved in long-term maintenance of elevated BP via enhanced contractility of VSMC and exaggerated salt and water reabsorption in REC (Blaustein 1984; Postnov and Orlov 1985; Blaustein 1996; Cheng et al. 1998; Hall et al. 1999; Orlov et al. 1999a; Ortiz and Garvin 2001). The latter hypothesis was partially proved by identification of genes whose mutation leads to the development of monogenic (Mendelian) hypertension forms (Lifton et al. 2001). More recently, several research teams have reported modulatory effects of monovalent ion transport inhibitors on cell proliferation and death. Thus, it has been shown that ouabain at low concentrations increases the proliferation of cultured SMC (Golomb et al. 1994; Aydemir-Koksoy et al. 2001; Chueh et al. 2001). In essential hypertension, the level of ESPI correlated positively with left ventricular mass (Manunta et al. 1999). These results suggest that AII-induced remodeling in hypertension can be caused by the altered activity of ion transporters.

Based on data showing that tissue hypertrophy/hyperplasia in hypertension is limited to a few organs, such as the heart, blood vessels, and kidneys, we proposed that abnormalities of cell cycle progression and apoptosis, i.e., the two major counterparts of organ remodeling seen in hypertension, are evoked by mutations within genes encoding their cell type-specific regulators rather than ubiquitous intermediates, such as cyclins, cyclin-dependent kinase, p53, proteins of the Bcl-2 family, etc. (Orlov et al. 1999b, 2002). We focused our initial interest on the study of such regulators in VSMC apoptosis.

Wild-type VSMC are resistant to apoptosis, so that even after 24 h of serum deprivation, the amount of morphologically defined apoptotic cells does not exceed 2%-4% (Bennett et al. 1994, 1995). Therefore, to elaborate the mechanism of VSMC apoptosis, we adopted the chromatin cleavage assay which allows us to measure intracellular chromatin fragments in amounts less than 1% of total DNA content (Hamet et al. 1995; Orlov et al. 1996) and we used VSMC transfected with the functional c-myc analog, E1A-adenoviral protein (VSMC-E1A), which are highly sensitive to apoptotic stimuli (Bennett et al. 1995; Champagne et al. 1999). In these cells, apoptosis can be prevented by growth factors, such as insulin-like growth factor, platelet-derived growth factor, and epidermal growth factor (Orlov et al. 1999b), whose receptors are coupled to phospholipase C

(PLC- γ), polyphosphoinositide-3-kinase (PI3 K) and MAPK. Our team (Orlov et al. 1999b) and Bai with co-workers (1999) reported that PI3 K inhibition with wortmannin potentiated apoptosis in serum-supplied VSMC. We failed to observe the involvement of any other intermediates of intracellular signaling, such as PLC, $[Ca^{2+}]_i$, and MAPK, in the protection of serum-deprived VSMC against apoptosis (Orlov et al. 1999b).

In addition to the above-listed intermediates of intracellular signaling, activation of AT_2 receptors in REC leads to NO production and activation of cGMP signaling. Recently, Dulin with co-workers reported that vasoconstrictors, including AII, transiently activated PKA (Dulin et al. 2001; Davis et al. 2003). To study the role of this enzyme, we treated VSMC with activators of cyclic adenosine monophosphate signaling and observed that these compounds blocked the development of apoptosis during the initial 10-12 h of serum-deprivation at a step upstream of caspase-3 (Orlov et al. 1999c). In contrast, we did not detect any effect of activators of soluble (NO donors) and membrane-bound atrial natriuretic peptide guanylate cyclase on apoptosis in VSMC-E1A (Orlov et al. 1999c).

It was shown that inhibitors of NHE1 abolished the proliferative action of AII (Sachinidis et al. 1996) and AII-induced hypertrophy of cultured VSMC (Peiro et al. 1997). The role of intracellular monovalent cations in organ remodeling is also supported by data demonstrating the suppression of myocardial hypertrophy with NHE1 inhibitors (Cingolani 1999; Morris 2002) and parallel normalization of cardiac hypertrophy and NHE activity in cardiomyocytes from SHR subjected to antihypertensive therapy (Alvarez et al. 2002). It should be underlined, however, that long-term application of NHE blockers leads to numerous side effects, such as inhibition of PKA, PKG, and PKC (Orlov et al. 1989). Considering this, we used ouabain, i.e., a potent and selective Na^+,K^+ -ATPase inhibitor, to study the role of intracellular monovalent ions in apoptosis.

We observed that preincubation of VSMC from the rat aorta with ouabain and in K^+ -free medium blocks the development of the apoptotic machinery at a step upstream of caspase-3 (Orlov et al. 1999d). Importantly, Na^+,K^+ pump inhibition protects VSMC against distinct apoptotic stimuli, such as serum-deprivation, inhibitors of serine-threonine protein kinases (staurosporine), phosphoprotein phosphatases (okadaic acid) (Orlov et al. 1999d) and DNA damage caused by its extensive $[^3H]$ labeling (Orlov et al. 2003). Soon after our publication, it was reported that ouabain prevents apoptosis in rat cerebellar granule cells (Isaev et al. 2000) and REC (Zhou et al. 2001). In contrast to these cell types, Na^+/K^+ pump inhibition potentiated the development of apoptosis in lymphocytes (Olej et al. 1998), Jurkat cells (Orlov et al. 1999d; Bortner et al. 2001), and several tumor cell lines (McConkey et al. 2000; Penning et al. 2000; Yeh et al. 2001). In rat VSMC (Orlov et al. 2001), human lymphocytes (Falciola et al. 1994), and HEK-293 cells (unpublished data), long-term inhibition with ouabain did not exhibit any cytotoxic action, whereas in REC (Sato and Ozawa 1977; Ledbetter et al. 1986; Bolivar et al. 1987), ischemic neuronal cells (Urenjak and Obrenovitch 1996), and the mesothelioma cell line (Marklund et al. 1999), it

elicited massive cell death. The limited number of markers employed in these studies complicates a definitive conclusion on the relative contribution of necrosis and apoptosis to overall cell death. However, the prevalence of necrosis can be proposed based on the lack of protective effect of bcl-2 overexpression (Gilbert and Knox 1997) and insensitivity to caspase inhibitors (Pchejetski et al. 2003).

Two approaches have been employed to identify genes whose expression is triggered by elevated $[Na^+]_i/[K^+]_i$ ratio. The candidate gene approach suggests the expression of early response genes (ERG), i.e., transcriptional factors leading to massive RNA synthesis seen in ouabain-treated cells (Piechaczyk and Blanchard 1994; Tamai et al. 1997; Ahmad et al. 1998; Sylvester et al. 1998; Rivard and Andres 2000; Orlov et al. 2001). Indeed, it was shown that inhibition of the VSMC Na^+,K^+ pump with ouabain or in K^+ -depleted medium leads to rapid and massive expression of c-Fos followed by the appearance of c-Jun protein (Taurin et al. 2002b). It might be suggested that modulation of $[Ca^{2+}]_i$ rather than monovalent cations is an upstream signal of ERG expression. However, we demonstrated that c-Fos expression in ouabain-treated cells was insensitive to the presence of extracellular Ca^{2+} and Ca^{2+} chelators, and the kinetics of c-Fos mRNA expression correlates with $[Na^+]_i$ elevation rather than with $[K^+]_i$ loss (Taurin et al. 2002b). Using luciferase *trans*- and *cis*- reporter assays, it was shown that the activity of Elk-1, serum response element, cAMP-response element binding protein, and activator protein-1 transcription factors interacting with major transcriptional control elements of the c-fos promoter was potentiated by serum but was insensitive to the presence of ouabain (Taurin et al. 2002b). These data suggest the presence of a novel $[Na^+]_i$ -mediated, $[Ca^{2+}]_i$ -independent mechanism of excitation-transcription coupling (Taurin et al. 2002b).

To search for $[Na^+]_i$ -sensitive genes involved in the inhibition of apoptosis, the proteomes of control and ouabain-treated VSMC have been compared. This study led to the identification of more than 20 soluble proteins whose expression was increased by more than tenfold after a 3-h incubation with ouabain, including MOT-2 (Taurin et al. 2002a). We observed that similarly to ouabain, transient transfection with MOT-2 causes transient inhibition of apoptosis in VSMC, as discussed above.

The data presented in this section show that exposure of VSMC and REC to AII leads to transient $[Na^+]_i$ elevation. Both the increased activity of AII-sensitive ion carriers, such as NHE1 and NKCC1, and enhanced ESPI production contribute to the augmented AII-induced $[Na^+]_i$ response seen in hypertension. In VSMC, $[Na^+]_i$ elevation triggered by ouabain evokes the expression of MOT-2 and probably of other genes involved in the suppression of apoptosis (Fig. 5). In contrast to VSMC, ouabain leads to massive necrotic type of REC death. However, using Madin-Darby canine kidney cells, it was shown that this effect is mediated by conformational transition of Na^+,K^+ -ATPase rather than by ion fluxes provided by this enzyme (Pchejetski et al. 2003). Which protein(s) interact(s) in REC with the Na^+,K^+ -ATPase α -subunit in the presence of ouabain to induce the necrotic type of cell death? Which intermediate(s) of this signaling cascade are

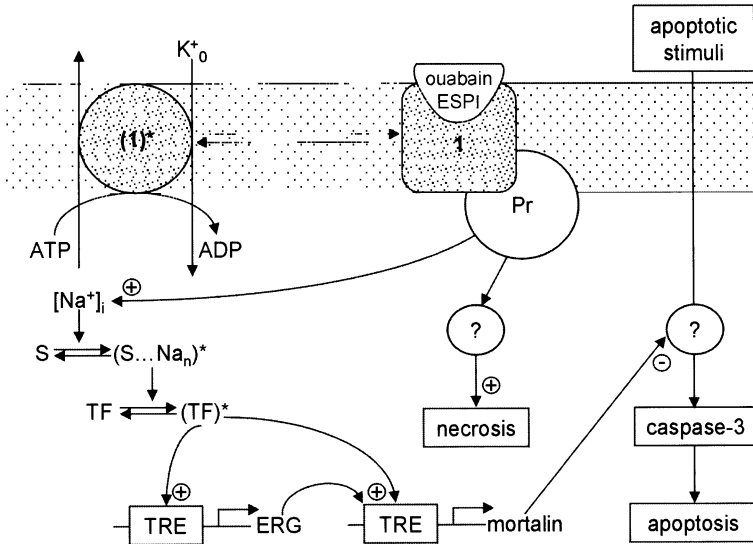


Fig. 5 Mechanism of the involvement of Na^+, K^+ -ATPase and intracellular Na^+ in the apoptotic and necrotic modes of cell death. 1, Na^+, K^+ -ATPase; Pr, unknown protein interacting with inactivated Na^+, K^+ -ATPase and triggering the death signal; S, $[\text{Na}^+]_i$ sensor; TF, transcription factor; TRE, transcription response element; (*), activated state of the molecules; ?, unknown intermediates. For more details, see text

missing/suppressed in VSMC and other cells resistant to the toxic action of ouabain? Does the enhanced production of ESPI seen in hypertension (Dmitrieva and Doris 2002) contribute to the premature death of REC and protection of VSMC against apoptosis? These questions should be addressed in forthcoming studies.

8 Antihypertensive Therapy, Cell Growth, and Apoptosis

As mentioned above, features of cell senescence include increased oxidative stress and a greater incidence of nuclear polyploidy. Both of these features are stimulated in the cardiovascular system not only with aging but also with hypertension. Moreover, AII stimulation of AT_1 receptors results in the potent activation and gene expression of oxidative stress-generating enzymes, such as nicotinamide adenine dinucleotide (phosphate), reduced $[\text{NAD(P)H}]$ oxidase (Touyz et al. 2002). There is a growing body of pharmacological evidence suggesting that cardiovascular cell fate is modulated by antihypertensive therapies. The increased turnover of SMC in hypertension is prevented by these agents, including enalapril (Thorin-Trescases et al. 2001) (Table 4). This effect in SHR is achieved mainly because of decreased cell growth with no change in cumulative cell death rates. ACE inhibitors reduce cell turnover in the neonatal rat heart (Choi et al. 2002). Long-term treatment with the AT_1 receptor antagonist losartan in SHR

Table 4 Effect of chronic antihypertensive treatment on aortic weight, relative aortic weight corrected for body weight (BW), and aortic DNA content. Treatment with hydralazine (40 mg/kg/day), nifedipine (30 mg/kg/day), or enalapril (30 mg/kg/day) was initiated in 5-week-old SHR and maintained for 15 weeks. Data represent values for the different parameters in 20-week-old SHR compared with age-matched WKY. Data are mean±SEM

Rat (n)	Aorta weight (mg)	Aorta weight/BW (mg/g)	DNA content (μg)
WKY (9)	29.6±2.5	0.12±0.01	36.3±2.0
SHR (6)	42.0±3.5 ^a	0.17±0.01 ^a	51.9±6.2 ^a
Hydralazine (8)	37.3±3.6	0.14±0.01	31.9±3.7 ^b
	<i>p</i> =0.289	<i>p</i> =0.111	<i>p</i> =0.001
Nifedipine (7)	31.9±2.2 ^b	0.12±0.09 ^b	33.6±1.9 ^b
	<i>p</i> =0.032	<i>p</i> =0.015	<i>p</i> =0.001
Enalapril (8)	31.8±2.7 ^b	0.13±0.02 ^b	31.5±1.5 ^b
	<i>p</i> =0.026	<i>p</i> =0.034	<i>p</i> =0.001

SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

^a *p*< 0.05 SHR vs WKY.

^b *p*< 0.05 treated SHR vs untreated SHR.

Reproduced from Thorin-Trescases et al. (2001) with permission from *Journal of Cardiovascular Pharmacology*.

alters the phenotype and suppresses the proliferative behavior of SMC cultured from the aorta (Bravo et al. 2001). In the heart, cardiomyocyte polyploidy is reduced by RAS inhibitors (Panizo-Santos et al. 1995). We recently obtained evidence that cardiomyocyte polyploid nuclei may be removed by apoptosis during treatment with RAS inhibitors (Der Sarkissian et al. 2003).

Our studies have shown that, irrespective of the class of antihypertensive drug administered to SHR, those that are able to induce the regression of cardiac or aortic hypertrophy also evoke a transient increase of apoptosis in these organs, in large part via pressure-independent mechanisms (deBlois et al. 1997; Tea et al. 1999). Drugs effective in these regards include antagonists of AT₁ receptors, such as valsartan and losartan, and ACE inhibitors, such as enalapril. The induction of cardiovascular apoptosis in SHR is transient and significant only within the first weeks after the initiation of drug treatment, even when it is maintained for several weeks. Moreover, the induction of SMC apoptosis is temporally dissociated from inhibition of cell growth since the time window of apoptosis occurs before the sustained inhibition of SMC DNA synthesis and organ mass reduction. Polyploidy is a common feature of senescent cells (Ly et al. 2000). AII-dependent hypertension is associated with increased SMC polyploidy (Owens 1989, 1995). The age-dependent accumulation of cells arrested in G₂ (i.e., polyploid cells) occurs typically faster in hypertensive individuals (Barrett et al. 1983; Lee et al. 1992; Panizo-Santos et al. 1995), a feature that is reversed (Dominiczak et al. 1996) by the same classes of antihypertensive drugs that induce apoptosis and regression of cardiovascular hyperplasia (deBlois et al. 1997; Tea et al. 1999). Administration of quinapril decreases the percentage of tetraploid cardiomyocytes in SHR (Pochampally et al. 1999), an effect that reflects either nuclear division or

polyploid cell deletion, suggesting that the accumulation of aberrant cells with aging and hypertension may be reversed by programmed cell death during drug therapy. While the AT₁ receptor has been implicated in the inhibition of SMC apoptosis (Pollman et al. 1996), recent evidence indicates that a subpopulation of SMC responds to AT₁ receptor stimulation with sustained transmembrane calcium influx and apoptosis induction rather than survival or growth (Bascands et al. 2001). This is reminiscent of the recent report that AII infusion for 7 days, a condition known to be associated with SMC growth (deBlois et al. 1996), also activates the Bax/caspase-3 pathway (Diep et al. 1999). Together, these observations provide further evidence that under certain conditions AII may be stimulating SMC turnover in vivo. SMC apoptosis induction, growth inhibition, and vascular regression evoked by AT₁ antagonists in the SHR aorta are completely dependent on AT₂ receptors for AII (Tea et al. 2000).

In the heart, AT₁ antagonists and ACE inhibitors increase apoptosis selectively in fibroblasts, an effect independent of AT₂ receptors and consistent with a possible role of AT₁ receptors in cell survival (Der Sarkissian et al. 2003). Cardiac apoptosis induction resulted in caspase-3 activation and selective deletion of cardiac fibroblasts without affecting cardiomyocyte number or capillary density (Der Sarkissian et al. 2003). We propose that the stimulation of apoptosis in fibroblasts is an important determinant of cardiac fibrosis suppression. In contrast, long-term prophylactic antihypertensive treatment (initiated before the onset of hypertension) inhibits vascular hyperplasia in adult rats without affecting the rate of cell death (Thorin-Trescases et al. 2001).

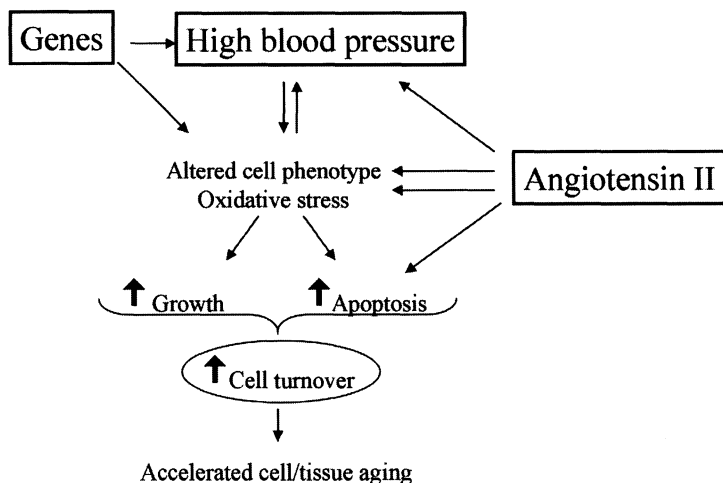


Fig. 6 Angiotensin II and senescence: a hypothesis. Key features of senescent cells are enhanced in hypertensive individuals, including cardiovascular cell polyploidy and oxidative stress. These features are in part genetically determined but can be modulated by pharmacotherapy. Recently, angiotensin II has emerged as a potent pro-oxidant factor and a key regulator of cell phenotype. Depending on cardiovascular cell phenotype, angiotensin II can stimulate growth (via AT₁ receptors) or apoptosis (via AT₁ and AT₂ receptors). Enhanced cell growth and apoptosis in tissues will result in enhanced cellular turnover and accelerated aging causing target organ damage

In conclusion, overall, complex epigenetic and genetic factors contribute to aging and age-related diseases, including hypertension. Part of this process involves the defective regulation of cell proliferation and death in cardiovascular target organs via a putative specific senescence pathway which may involve the RAS (Fig. 6).

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Regulation of Angiogenesis by Angiotensin II

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Abstract The renin/angiotensin system can exert a double-edged role in angiogenesis signaling. The pro-angiogenic activity of angiotensin II is predominantly mediated by stimulation of the angiotensin II receptor 1 (AT1R), whereas the angiotensin receptor 2 (AT2R) inhibits angiogenesis. The pro-angiogenic activity of angiotensin II is believed to be predominantly mediated by stimulation of VEGF expression. Moreover, a transactivation of the growth promoting epidermal growth factor receptor (EGFR) and the enhanced expression of pro-angiogenic transcription factors such as Cyr61 and Krüppel-like factor may contribute to the pro-angiogenic effects of angiotensin II. In contrast, stimulation of the AT2R inhibits angiogenesis by preventing endothelial cell proliferation and inducing apoptosis of endothelial cells, which counteracts angiogenesis. The apoptosis induction is mediated by an activation of phosphatases and subsequent down-regulation of the anti-apoptotic protein Bcl-2.

Keywords Angiogenesis · Apoptosis · Cyr 61 · Double-edged role · Transactivation · VEGF

1

Introduction

Adequate oxygen delivery is essential to maintain normal tissue function. Understanding of the signaling processes that regulate the formation of new blood vessels has become a major challenging objective of the last decade. Postnatal neovascularization is essential after tissue ischemia in order to adapt blood supply. Moreover, neovascularization plays an important role in wound healing. However, in a number of pathophysiological situations, neovascularization may accelerate disease progression (Folkman 1995). Thus, neovascularization of tumor tissue allows the tumor to grow beyond a diameter of 2 mm (Folkman 1995). Neovascularization has also been implicated in atherosclerotic plaque growth, although the contribution of neovascularization for plaque rupture and the initiation of myocardial infarction is controversial (Isner 2001). Taken together, strategies to either improve neovascularization in order to improve oxygen supply, e.g., after myocardial infarction, or concepts to inhibit neovascularization in order to limit tumor growth are important therapeutic options (Isner and Asahara 1999).

During the last few years, the understanding of the processes leading to neovascularization has dramatically changed. In the past, the vascularization of ischemic tissue was believed to be caused by the migration and proliferation of mature endothelial cells—a process termed “angiogenesis” (Isner and Asahara 1999; Carmeliet 2000). Meanwhile, increasing evidence suggests that circulating progenitor cells home to sites of ischemia and contribute to the formation of new blood vessels. In analogy to the embryonic development of blood vessels out of primitive endothelial progenitors (angioblasts) and the subsequent organization into a primary capillary plexus, this process is called “vasculogenesis” (Isner and Asahara 1999; Carmeliet 2000). Since these novel insights into the processes underlying post-natal vessel growth have become evident during the past 2 years, most of the cited studies do not discriminate between angiogenesis and vasculogenesis. Therefore, the use of the term angiogenesis in this review article does not necessarily exclude effects on vasculogenesis.

2

Role of the Renin–Angiotensin System in Angiogenesis

Pharmacological approaches revealed the existence of two subtypes of angiotensin receptors, namely angiotensin II types 1 and 2 receptors (AT1R and AT2R) (Unger et al. 1996). In mice, the AT1R is further subdivided into AT1aR and AT1bR (Martin et al. 1995). The AT1R is ubiquitously and abundantly distributed (Murphy et al. 1991; Sasaki et al. 1991). Most of the well-known functions in the cardiovascular system are mediated through the AT1R and especially through the AT1aR in rodents (Martin et al. 1995). In contrast, little is known regarding the physiological roles of the AT2R and its signal-transduction pathways. Several lines of evidence, however, suggest that the AT2R might mediate

opposite effects to those related to the AT1R activation. It is very well established that angiotensin II-induced smooth muscle cell proliferation is mediated by the AT1R (Rao et al. 1994; Berk 1999). In contrast, studies on the AT2R demonstrate that stimulation of the AT2R induce apoptosis in vascular smooth muscle cells *in vitro* and in spontaneously hypertensive rats *in vivo* (Rao et al. 1994; Berk 1999), suggesting the opposing effects of the two receptors.

Another interesting field where angiotensin II has now been implicated is angiogenesis, the development of new vessels from preexisting vessels. The first reports on the effects of the renin-angiotensin system on angiogenesis were obtained from studies using angiotensin-converting enzyme (ACE) inhibitors regardless of the AT receptor subtypes. Volpert et al. showed that captopril suppresses tumor angiogenesis and growth (Volpert et al. 1996). In contrast, Fabre et al. reported that quinapril increases angiogenesis in a rabbit model of hindlimb ischemia (Fabre et al. 1999). Taken together these studies give no precise picture of the renin-angiotensin system in angiogenesis. Different reasons can be found to explain such differences. One rationale to explain the discrepancy of the two mentioned studies might be the use of different ACE inhibitors. Thus, quinapril was shown to more potently inhibit tissue ACE activity in comparison to captopril, and moreover, captopril contains a free SH-group which may inhibit Zn²⁺-dependent metalloproteinase activity that is required to respond to an angiogenic stimulus (Volpert et al. 1996; Fabre et al. 1999).

Generally, ACE inhibition not only prevents angiotensin II synthesis, but also increases bradykinin production, which leads to stimulation of the endothelial nitric oxide synthase. Activation of the nitric oxide synthase has been reported to promote angiogenesis (Murohara et al. 1998). Therefore, the promoting effects of ACE inhibition could be rather a side effect than a direct function of angiotensin II blockade. Moreover, angiotensin II is not only produced by the angiotensin-converting enzyme *in vivo*, but also by other enzymes, such as chymase (Muramatsu et al. 2000).

Therefore, recent studies used the advantage of knockout mice either from the AT1aR or from the AT2R to understand the direct role of angiotensin II and the role of the two receptor subtypes in angiogenic processes. Sasaki and coworkers examined the role of the AT1R receptor in a mouse model of ischemia-induced angiogenesis (Sasaki et al. 2002). Well-developed collateral vessels were observed in wild-type mice in response to hindlimb ischemia, whereas ischemia-induced angiogenesis was significantly reduced in AT1aR knockout mice (Sasaki et al. 2002). Moreover, infiltration of mononuclear cells was suppressed in the ischemic tissues of AT1aR knockout mice. Interestingly, the phenotype of reduced angiogenesis observed in AT1aR knockout mice could be rescued by muscular transplantation of mononuclear cells from wild-type mice (Sasaki et al. 2002). These data suggest that AT1aR knockout mice show an impairment of circulating progenitor cells or monocytic cells, which are known to contribute to vasculogenesis and arteriogenesis, respectively.

In contrast, AT2R knockout mice showed the opposite results as obtained in the AT1aR knockout mice. Angiogenesis, again analyzed in the model of hin-

dlimb ischemia, was increased in AT2R knockout mice compared to their wild-type littermates (Silvestre et al. 2002). Of note is that Silvestre et al. did not detect a further enhancement of the angiogenic score in AT2R knockout mice by angiotensin II infusion (Silvestre et al. 2002). Likewise, no improvement of ischemia-induced angiogenesis was achieved in wild-type mice by infusion of the well-established AT2R blocker, PD123319 (Tamarat et al. 2002). These findings are in contrast to pharmacological blockade of the AT2R in the rat subcutaneous sponge granuloma, where angiotensin II-induced angiogenesis was reduced by PD123319 (Walsh et al. 1997). But nevertheless, all these studies further contribute to the concept that the AT1R and the AT2R exert opposite effects on angiogenesis.

Therefore, the following sections will separately discuss the mechanisms underlying the pro-angiogenic effects of the AT1R and the inhibitory effect of AT2R.

3 Pro-angiogenic Effects of Angiotensin II

3.1 Stimulation of VEGF, bFGF, and Nitric Oxide Synthase by Angiotensin II

Over the past few years several angiogenic factors have been identified. Among them, vascular endothelial growth factor (VEGF), is one of the most important players in angiogenesis. This was most impressively demonstrated by the findings that targeted deletion of VEGF or the VEGF-receptors 1 and 2 showed lacks or abnormalities in vessel formation (Carmeliet et al. 1996). Recent studies suggest that the pro-angiogenic effects of angiotensin II depend on the stimulation of VEGF expression *in vitro* and *in vivo* (Tamarat et al. 2002). In cultivated bovine aortic endothelial cells, angiotensin II induced expression of the VEGF receptor and thereby enhanced VEGF-induced proliferation and tube formation of the cells. These effects of angiotensin II were blocked by the AT1R antagonist, losartan, demonstrating that these processes are regulated by the AT1R in endothelial cells (Otani et al. 1998). Moreover, several studies were undertaken to show the effect of ACE and/or AT1R blockade on VEGF expression. Microvascular angiogenesis in skeletal muscle induced by electrical stimulation and short-term exercise was inhibited by ACE inhibitors and AT1R blockade (Amaral et al. 2001). Another scenario of more pathological angiogenesis, namely tumor growth, resulted in differential effects of ACE inhibitors and AT1R blockade. Yoshiji et al. showed that the ACE inhibitor, perindopril, but not AT1R blockade significantly inhibited the progression of tumor growth with a concomitant suppression of VEGF expression (Yoshiji et al. 2001). Overall, these studies demonstrate that angiotensin II stimulates VEGF production; however, the up-stream signaling cascade involved is not clearly defined but may include the transactivation of growth factor receptors and stimulation of several transcription factors (see Sects. 6 and 7).

Another intermediate which could link the renin-angiotensin system and VEGF is thrombin. Thrombin is a tightly regulated serine proteinase that plays a central role in hemostasis. Besides the well-known functions of thrombin to convert fibrinogen into fibrin and to stimulate platelet aggregation and secretion, thrombin is a key factor in tissue repair and wound healing. Thrombin has been shown to increase VEGF expression via the thrombin receptor PAR-1 (Williams et al. 1995; Richard et al. 2000) and has been implicated as another angiogenic factor. Browder et al. suggested that angiotensin II-induced VEGF expression led to generation of the tissue factor which will increase intracellular thrombin production and thereby may act as a feedback loop to further enhance VEGF production by angiotensin II through thrombin (Browder et al. 2000).

Besides the stimulation of the expression of VEGF and its receptors, angiotensin II also increased the expression of the endothelial nitric oxide synthase (eNOS) protein content within the ischemic leg in the hindlimb ischemia model (Silvestre et al. 2002) and thereby exerts proangiogenic effects. Indeed, angiotensin II administration did not improve the angiographic score and blood perfusion within the ischemic leg of eNOS knockout mice (Tamarat et al. 2002), suggesting that angiotensin II-mediated VEGF release requires the nitric oxide system for promoting angiogenesis signaling. This is in accordance with various studies that demonstrate that endothelial-derived NO is necessary for VEGF-induced angiogenesis and endothelial function (Murohara et al. 1998; Dimmeler et al. 2000).

Basic fibroblast growth factor and one of its receptors, named endoglin, represent another target of angiotensin II action (Lastres et al. 1996). Recently, it has been demonstrated that angiotensin II stimulates the expression of endoglin (Li et al. 2001). Since endoglin knockout mice showed defects in angiogenesis (Li et al. 1999) and inhibition of endoglin expression in endothelial cells attenuates angiogenesis (Li et al. 2000), one may speculate about a potential contribution of endoglin up-regulation in the pro-angiogenic effects of angiotensin II. The mechanism, however, by which angiotensin II increases the expression of endoglin requires further investigation.

3.2

Transactivation of Growth Factor Receptors by Angiotensin II

It is well established that G protein-coupled receptors stimulate growth factor receptor transactivation, like EGFR and platelet-derived growth factor receptor (PDGFR) (Prenzel et al. 1999; Heeneman et al. 2000). Therefore, transactivation of growth factor receptors is one important signaling mechanism for angiotensin II-induced angiogenesis. Growth factor receptors belong to the family of receptor tyrosine kinases, which are tyrosine phosphorylated upon stimulation. Angiotensin II stimulated classical signaling pathways via transactivation of growth receptors (Fig. 1). Upon stimulation with angiotensin II, pro heparin-binding EGF is cleaved by metalloproteinases and HB-EGF is released, which results in activation of the EGFR (Prenzel et al. 1999). The EGFR is rapidly au-

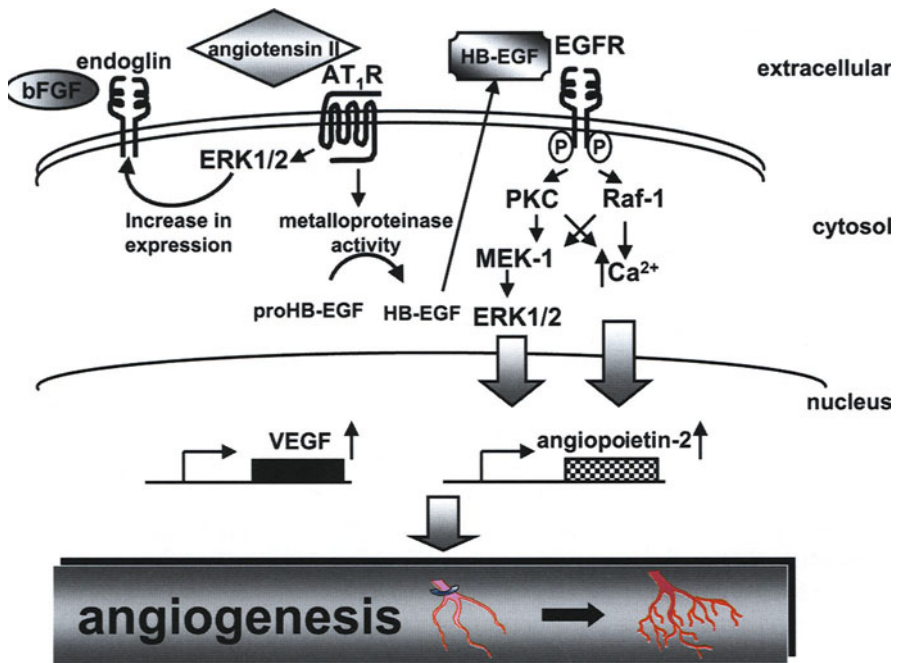


Fig. 1 Transactivation of growth factor receptors by angiotensin II leading to angiogenesis. Model illustrating the different signaling pathways involved. *bFGF*, basic fibroblast growth factor; *EGFR*, epidermal growth factor receptor; *ERK1/2*, extracellular signal-regulated kinase

tophosphorylated leading to protein kinase C activation, release of intracellular Ca^{2+} , and concomitant extracellular signal-regulated kinase (ERK)1/2 activation after 30 min. Activation of these kinases and the intracellular Ca^{2+} release results then in transcriptional activation of angiogenic factors like VEGF and angiopoietin-2. This finally leads to induction of angiogenic processes (Fujiyama et al. 2001). The AT1R as a G protein-coupled receptor requires heterotrimeric, small G proteins for activation. Heterotrimeric G proteins consist of an α , β , and γ subunit and are defined by the identity of their α subunit. Sixteen α subunit genes have been cloned, and on the basis of sequence similarities they are divided into four groups: $G\alpha_{1/o}$, $G\alpha_q$, $G\alpha_s$, $G\alpha_{12/13}$ (Simon et al. 1991). The angiotensin II/AT1R-activated increase in intracellular Ca^{2+} was selectively inhibited by injection of antisense oligonucleotides directed against mRNAs coding for the $G\alpha_{13}$, $G\beta_1$ and $G\gamma_3$ subunits (Macrez-Lepretre et al. 1997), suggesting an important role for these subunits in angiotensin II-induced angiogenesis. This has been most notably underscored by the disruption of the gene encoding for $G\alpha_{13}$. $G\alpha_{13}$ knockout mice indicate a recessive lethal phenotype. Homozygous embryos could only be recovered until day 9.5, showing an impaired ability of endothelial cells to develop into an organized vascular system and demonstrating an important role for $G\alpha_{13}$ in developmental angiogenesis (Offermanns et al.

1997). However, a direct involvement of $G\alpha_{13}$ in transactivation of growth receptors by the AT1R has not been described yet and requires further investigation.

3.3

Regulation of Pro-angiogenic Transcription Factors by Angiotensin II

Recent findings demonstrate that angiotensin II induces expression of the Krüppel-like zinc-finger transcription factor Klf5 (Shindo et al. 2002) (Fig. 2). Initially described as a molecular marker of phenotypically modulated smooth muscle cells, Klf5 also exerts diverse functions during cell differentiation and embryonic development (Aikawa et al. 1995; Bieker 2001). Importantly, heterozygous Klf5 mice showed impaired angiogenic activity in the hindlimb ischemia model, and moreover, angiogenic responses to implanted tumors were markedly attenuated (Shindo et al. 2002). Therefore, one may speculate that Klf5 might be a new candidate to mediate the pro-angiogenic effects of angiotensin II.

Recently, another transcription factor termed Cyr61 has been considered as a downstream target of angiotensin II (Hilfiker et al. 2002) (Fig. 2). Cyr61 is a heparin-binding, secreted cysteine-rich protein that integrates into the extracellular matrix and binds directly to integrins (Perbal 2001). Cyr61 gene transfer potently stimulated limb revascularization, thereby promoting even greater improvement than achieved with VEGF (Fataccioli et al. 2002). Hilfiker et al. have demonstrated that Cyr61 is induced by angiotensin II in vascular cells and tissue and that the expression of Cyr61 colocalized with angiotensin II in small vessels of human arteriosclerotic lesions (Hilfiker et al. 2002).

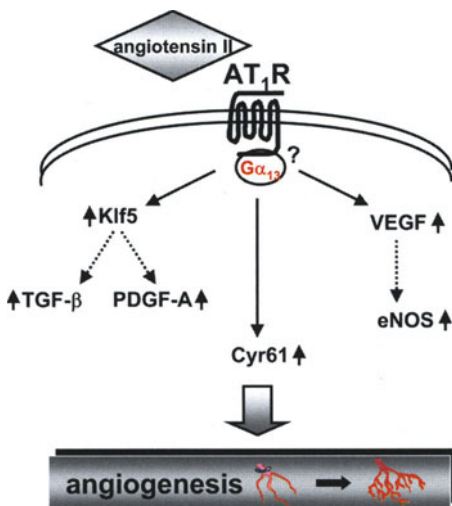


Fig. 2 Activation of transcription factors leading to angiogenesis. *eNOS*, endothelial NO-synthase; *Klf5*, Krüppel-like factor 5; *PDGF-A*, platelet-derived growth factor-A; *TGF-β*, transforming growth factor-β

Although the causal involvement of Klf5 and Cyr61 in angiotensin II-induced angiogenesis has to be proved, these factors might play a role in the complex mechanisms regulating angiogenesis signaling in response to angiotensin II.

4

Antiangiogenic Effects of Angiotensin II Via the AT2R: Important Role for Apoptosis

Little information is available regarding the physiological roles of the AT2R and its signaling pathways. In cultured cells the AT2R seems to be difficult to study, since its expression is unstable. However, initial studies in 1995 demonstrated that stimulation of the AT2R in endothelial cells inhibits proliferation (Stoll et al. 1995). Moreover, angiotensin II or pharmacological stimulation of the AT2 receptor were shown to induce apoptosis of cultivated endothelial cells (Dimmeler et al. 1997). In addition, similar pro-apoptotic effects are described in AT2R-expressing cells (Yamada et al. 1996). The inhibition of endothelial cell proliferation and the induction of endothelial cell death obviously can counteract angiogenesis (Dimmeler and Zeiher 2000). Indeed, recent *in vivo* studies support an anti-angiogenic activity mediated by the AT2R (Silvestre et al. 2002). AT2R knockout mice or pharmacological AT2R blockade by PD123319 revealed a pronounced angiogenic response (Walsh et al. 1997; Silvestre et al. 2002). In addition, endothelial cell apoptosis was reduced in mice lacking the AT2R (Silvestre et al. 2002). The pro-apoptotic activity of AT2R stimulation was mainly attributed to the activation of mitogen-activated protein (MAP) kinase phosphatases (MKP), which subsequently reduce the activity of the survival promoting ERK1/2 kinase and diminish Bcl-2 expression (Horiuchi et al. 1997). In endothelial cells mRNA expression of MKP-3 is increased by angiotensin II stimulation (Rössig et al. 2002), whereas other studies reported that angiotensin II induced the expression of another isoform, MKP-1, in smooth muscle cells (Horiuchi et al. 1997). In both cell types the expression of the anti-apoptotic protein Bcl-2 was reduced by AT2R stimulation. This has further been confirmed in AT2R knockout mice, which revealed significantly higher levels of Bcl-2 as compared to wild-type mice (Silvestre et al. 2002).

Beside the induction of apoptosis, AT2R stimulation also prevents angiotensin II-induced up-regulation of VEGF and eNOS. Thus, administration of PD123319 in mice inhibited the increase in VEGF protein expression and prevented transactivation of EGFR by angiotensin II (Fujiyama et al. 2001). The signaling seems to involve activation of protein tyrosine phosphatases, since tyrosine phosphorylation of the EGFR was inhibited by PD123319 (Fujiyama et al. 2001). Moreover, the increase in VEGF and eNOS expression induced by angiotensin II was abolished in AT2R knockout mice (Silvestre et al. 2002).

5

Conclusions

The renin/angiotensin system can exert a double-edged role in angiogenesis signaling. The pro-angiogenic activity is predominantly mediated by stimulation of the AT1R. In contrast, stimulation of the AT2R induces apoptosis of endothelial cells and prevents angiogenesis. The balance of receptor expression may thus determine the angiogenic response to angiotensin II.

Acknowledgements. We apologize for the failure to cite many of the important and relevant papers in this field due to space limitations.

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Immunohistochemistry of Angiotensin

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Abstract Immunocytochemical approaches have achieved an outstanding level for characterizing angiotensinergic systems in the brain and periphery. The development of affinity-purified antibodies for angiotensin II has resulted in distinct loss of background activity and produced discrete staining of neurons and small angiotensinergic fibres. The improvement of immunocytochemical techniques has enhanced the ability to develop maps of angiotensinergic pathways. Furthermore, the production of specific monoclonal antibodies to angiotensin receptor subtypes, such as AT₁ and AT₂, has led to the characterization and localization of angiotensin hormonal and neurotransmitter systems in brain and peripheral tissues. Such affinity-purified antibodies have revealed that angiotensin and vasopressin are colocalized in hypothalamo-neurohypophysial systems, implying that both peptides are released together in the median eminence and the neurohypophysis. Recent studies have shown that in spite of using the same antigen molecule for immunization as well as for affinity purification, different staining patterns are obtained, suggesting that the different carrier proteins forced the production of different antibodies to different epitopes. Furthermore, the finding supports the hypothesis that angiotensin II is not the

only effector peptide in the renin–angiotensin system but a member of a family of biologically active angiotensinergic peptides. The presented studies support once more that immunocytochemical approaches remain important tools for characterizing angiotensinergic systems in the central nervous and peripheral tissues.

Keywords Renin–angiotensin system · Immunocytochemistry · Affinity purification · Anti-idiotypic antibody · Monoclonal antibody · Colocalization · Angiotensinogen · Angiotensin receptor subtypes · AT₁ receptor · AT₂ receptor

1 Introduction

The brain renin–angiotensin system (RAS), which plays an important role in controlling cardiovascular function and maintenance of body fluid homeostasis, has been the subject of many reviews (Bunnemann et al. 1993; Bottari et al. 1993; Wright and Harding 1994; Mosimann et al. 1996; De Gasparo et al. 2000). The action of angiotensin II is mediated by the activation of its surface receptors, which are located in the central nervous system and in peripheral tissues.

In the rat brain, two angiotensin II receptor subtypes, AT₁ and AT₂ have been established using autoradiography and selective ligands (Tsutsumi and Saavedra 1991; Rowe et al. 1992; Song et al. 1992). Most of the known central actions of angiotensin II have been shown to be mediated through the AT₁ receptor. This receptor is coupled to G proteins and stimulates classical second messenger systems such as inhibition of adenylcyclase or activation of phospholipase C (Timmermans et al. 1992). Genetically different AT₁ receptor isoforms, AT_{1A} and AT_{1B} have been localized by *in situ* hybridization using specific riboprobes (Jöhren et al. 1995). AT₁ receptor expression has been demonstrated in human breast tissues, suggesting that the RAS may be involved in normal and tumour tissue function (Inwang et al. 1997). Recent results demonstrated the presence of AT₁ receptor in malignant glial cells and a favourable therapeutic response in glioma cells by a selective blockade of this receptor subtype (Rivera et al. 2001). Furthermore, blockade of the AT_{1A} receptor becomes an effective novel strategy for tumour chemoprevention, since such blockade reduced tumour growth, angiogenesis and metastasis (Fujita et al. 2002).

In contrast, the function of the AT₂ receptor is far from being understood. AT₂ is found to exert growth inhibitory and proapoptotic effects (Ishida and Fukamizu 1999). This receptor seems to exert opposite effects in terms of cell growth and cardiovascular haemodynamics (Xoriuchi et al. 1999). An upregulation of AT₂ expression under certain pathological conditions exists, such as vascular injury, myocardial infarction and heart failure (Nakamura et al. 1999; Malendowicz et al. 2000). Of high interest are results which suggest that the AT₂ receptor promotes vascular differentiation, contributes to vasculogenesis (Yamada et al. 1999) and cell migration (Cote et al. 1999).

Most published results about the RAS are obtained from radioreceptor assays of tissue homogenates or from autoradiography with specific antagonists in tissue sections. However, such methods are not suitable for studying exact cellular location of binding. In this respect, immunocytochemical approaches have achieved an outstanding level from both a methodological and scientific point of view. Especially, the specific affinity purification of polyclonal antibodies and the development of monoclonal antibodies offer a powerful tool for localization as well as colocalization studies at the cellular level. In addition, these antibodies can be used for Western blot analysis from tissue under distinct conditions. The combined immunocytochemical and biochemical approaches are certainly suitable for understanding principles of different angiotensin functions. Furthermore, with the aid of immunocytochemical techniques, possible interrelationship of angiotensin with other peptides may be clarified. For such purposes, the production and use of different monoclonal antibodies will be a prerequisite for the characterization and localization of the hormonal and neurotransmitter systems in the brain and in peripheral tissues.

2

The Specificity of Immunocytochemical Techniques

2.1

Affinity Purification of Angiotensin Antiserum

The sensitivity, specificity and also simplicity of immunocytochemical techniques have made them useful tools in the field of neurobiology over the past few years. The brain RAS involves selected populations of neurons within the brain. It is primarily the domain of immunocytochemical studies that determine the exact location of selected populations of neurons and their projection fields necessary for a complete understanding of the distribution of centrally produced angiotensin II (Imboden et al. 1987a,b). Implicit in the use of immunocytochemistry for such purposes is the belief that the staining produced is due to authentic angiotensins. The validity of this assumption deserves special scrutiny since only a small number of angiotensin antisera proved suitable for angiotensin immunocytochemistry. The use of crude angiotensin antiserum resulted in the staining of large varicosities and cell bodies. Fibres, however, were all but invisible owing to extensive background staining. Biochemical and histochemical analysis of crude angiotensin antiserum indicated that the background staining seen with the crude antiserum was neither angiotensin II nor several angiotensin-derived fragments (Imboden et al. 1987a).

The enhancement in immunocytochemical resolution was drastically improved when Imboden and co-workers (1987a,b) prepared affinity-purified antisera. Antibodies were purified by chromatographing crude antiserum (rabbit DE) with an angiotensin II affinity column prepared with CH-sepharose 4B (Pharmacia). Such affinity purification yielded an antibody preparation that produced staining which confirms the presence of angiotensin II. This staining

sufficiently improved the level of resolution to visualize extensive fine fibres as well as the more precise location of angiotensin immunoreactive neurons. Furthermore, the purified antibodies yielded only discrete staining without background.

A major advantage of the purified antibodies over the crude antiserum is the ability of the former to stain clearly fine fibres and their accompanying varicosities. These studies have been done without colchicine pretreatment of the animals. Such improved capability to visualize fibres allows one to directly map angiotensinergic pathways. The potential of this mapping capability is exemplified in Fig. 1 in which a part of the paraventricular-hypophysial pathway is shown (Imboden et al. 1987b).

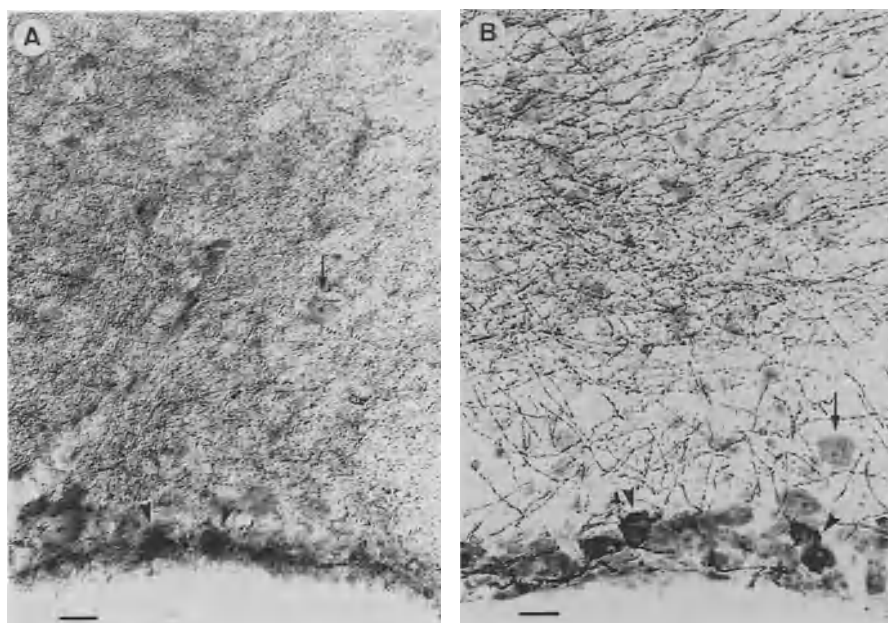


Fig. 1A, B Comparison of angiotensin II-like immunoreactive product in fibre tracts of the dorsal and ventral supraoptic commissure and in cells of the ventral base of the hypothalamus. Bars=20 μ m. **A** Staining with the crude antiserum. Only a few cells at the hypothalamic border show faint and diffuse reaction (*arrowheads*). No clear fibre pathways were observed in the commissure. **B** Staining with the affinity-purified antibody. Angiotensin II-like immunoreactive fibre pathways are clearly visible. Immunoreactive cells (*arrowheads*) can be distinguished from toluidine blue-counterstained, nonreactive cells (*arrows*). (Reproduction by permission from Imboden et al. 1987b, by courtesy of Marcel Dekker, Inc., NY)

2.2

Anti-idiotypic Angiotensin Antibody

Another approach to the crucial angiotensin receptors has been the use of anti-idiotypic antibodies, which is based on the network theory of the immune system (Jerne 1974). One anti-idiotypic antibody reacting with angiotensin II receptors (Couraud 1987), whose capacity served as a reliable tool for mapping immunohistochemical angiotensin II receptor distribution, has been reported by Pfister et al. (1993). Specificity of these antibodies toward angiotensin II receptor has been demonstrated with a number of biochemical experiments. The conclusion of this immunohistochemical study shows it to be a reliable tool for mapping angiotensin II receptor distribution. The established experimental approaches to angiotensin II receptors are thus supplemented with the possibility of immunocytochemical investigations. Using anti-idiotypic antibodies, punctate immunoreactive granules in or on neurons of different hypothalamic nuclei were demonstrated (Pfister et al. 1993).

2.3

The Use of Different Anti-peptide Antibodies

As mentioned above, angiotensin II mediates its biological actions via two subtypes of receptors, termed AT₁ and AT₂. In rat and mouse, two further subtypes of AT₁, namely AT_{1A} and AT_{1B} exist (Iwai and Inagami 1992). Therefore, the primary goal for immunohistochemists is to raise monoclonal anti-peptide antibodies specific to the different angiotensin receptor subtypes and to angiotensin II itself and using them to determine the intraorganic and/or the intracellular localization. Until now, only scarce immunohistochemical investigations have been performed in which polyclonal anti-idiotypic antibodies were used; these studies were unable to distinguish between the different receptor subtypes (Pfister et al. 1993). Comparable results have been published concerning the use of an antibody to a portion of the third cytoplasmic loop of the AT₁ receptor (Phillips et al. 1993). Further polyclonal antibodies (Zelezna et al. 1992; Rakugi et al. 1997; Benarroch and Schmeichel 1998; Giles et al. 1999) and one monoclonal antibody (Barker et al. 1993; Harrison-Bernard et al. 1997) to the AT₁ receptor exist. Regarding the AT₂ receptor, only two polyclonal and no monoclonal antibody have been published (Yiu et al. 1997; Nora et al. 1998). So far, only the monoclonal antibody against the AT₁ receptor has been proved useful for immunohistochemical investigations (Harrison-Bernard et al. 1997). In a recent study, Frei et al. (2001) reported the production and immunocytochemical characterization of three novel murine monoclonal antibodies directed against angiotensin II receptor subtypes AT₁ and AT₂, and against angiotensin II. Immunocytochemical characterization were carried out in the rat adrenal, in which AT₁-reactive cells were found in the zona glomerulosa and the medulla, whereas the AT₂ antibody bound specifically to medullar cells. Cells in the medulla and the zona glomerulosa reacted both with the angiotensin II monoclonal antibody.

In Western blots, both AT₁ and AT₂ antibodies detected a major 73-kDa protein band from adrenal homogenates but did not recognize a protein of 41 or 44 kDa as reported for COS-7 cells transfected with AT_{1A} receptor cDNA (Barker et al. 1993; Rakugi et al. 1997). These observations suggest that the previously identified 75-kDa protein (Sen and Rajasekaran 1991) obtained from cross-linking experiments of [¹²⁵I]-labelled angiotensin II was the biological relevant glycosylated form of the AT₁ receptor. This finding is of importance, since the new monoclonal antibodies specifically recognize angiotensin II and AT₁ and AT₂ receptors, representing powerful tools for further subcellular and effector pathway investigations (Frei et al. 2001).

2.4 Intracellular Staining of Angiotensin Receptors

Despite the knowledge of the existence of angiotensin II itself as well as its receptors in specialized brain areas, the coexistence of components of angiotensin and angiotensin receptors in individual cells have been reported only recently. For such investigations, the lack of pretreatment of the animals with colchicine and the purification of the specific antibodies were important steps. With this procedure it was possible to stain not only neurons in hypothalamic regions but also to visualize angiotensinergic fibres in the hypothalamic paraventricular-hypophysial pathway. Spengler et al. (1996) used 5- μ m thick sections, allowing a chance to get at least three adjacent sections out of a single neuron. With these serial sections they were able to carry out immunoreaction studies in one single cell using an anti-idiotypic angiotensin antibody, an affinity-purified angiotensin II antibody ("BODE") and an antibody against the third intracellular loop of the AT₁ receptor ("ELAINE"). As an example, Fig. 2 illustrates the coexistence of angiotensin and its receptor subtype AT₁ in individual neurons.

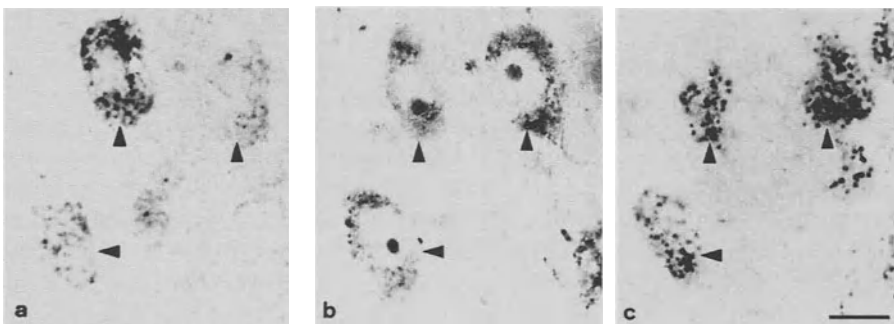


Fig. 2a–c Coexistence of angiotensin and angiotensin receptors in neurons of the supraoptic nucleus. **a** Immunoreactive staining with the anti-idiotypic antibody for angiotensin II receptors. **b** Immunoreactive staining with the affinity-purified antibody to angiotensin II. **c** Immunoreactive staining with the antibody to the angiotensin AT₁ receptor oligo-sequence. Scale bar=10 μ m. (Reproduction by permission from Spengler et al. 1996, by courtesy of Birkhäuser Verlag Basel/Schweiz)

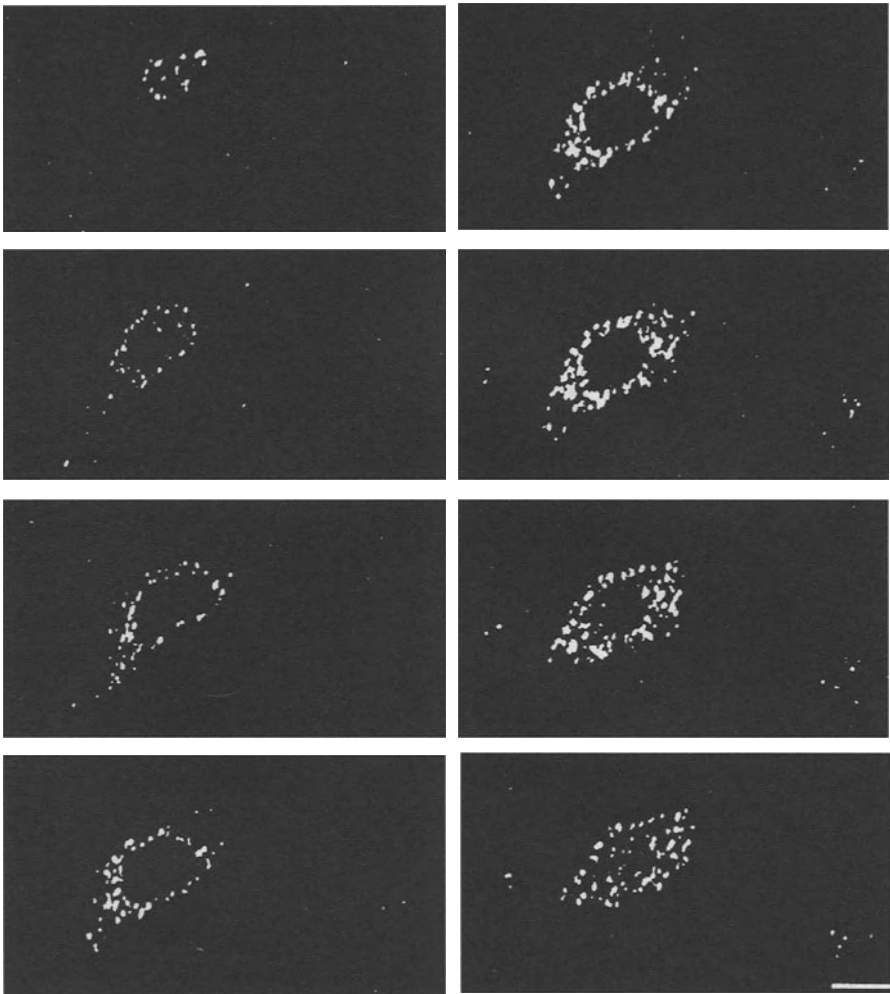


Fig. 3 Confocal laser-scan microscopic analysis of a neuron in the paraventricular nucleus (PVN). The sections show cytoplasmic localization of angiotensin II-receptor immunoreactivity. The series (*from left to right, top to bottom*) show optical sections in the z-direction of a neuron that was incubated with an anti-AT₁ receptor antibody. Scale bar=10 μ m. (Reproduction by permission from Pfister et al. 1997, by courtesy of Elsevier Science)

Although several individual studies using immunocytochemical (Phillips et al. 1993) or autoradiographic (Tsutsumi and Saavedra 1991) techniques reported the existence of angiotensin or angiotensin receptors, this was the first report of coexistence of the peptide and its receptors within the same neuron. The fact that angiotensin-containing neurons possess receptor sites as well, raises the possibility of an angiotensinergic input on the same neuron. The advantage of using thin sections allowed testing the different antibodies in individual slices of the same neuron, thereby preventing any possible interaction with each other.

To summarize, the chosen approach offers a good tool for any further studies on colocalization of the components of hypothalamic peptidergic systems.

The immunocytochemical reaction product from intracellular staining of angiotensin receptors showed characteristic focal appearance. Using anti-idiotypic antibodies, such focal immunoreactive granules were described in or on neurons of the supraoptic nucleus and of the paraventricular nucleus in the hypothalamus of rats (Pfister et al. 1993). Comparable results have been published concerning the use of an antibody to a portion of the third cytoplasmic loop of the AT₁ receptor (Phillips et al. 1993). To clarify whether the mentioned immunoreactivity indeed resulted from intracellular staining, Pfister et al. (1997) tried three different approaches: (1) use of the fluorescein isothiocyanate (FITC)-conjugated anti-idiotypic antibody with confocal laser-scan microscopic analysis; (2) use of the anti-AT₁ receptor antibody ("ELAINE") in combination with a FITC-conjugated second antibody and subsequent confocal laser-scan microscopic analysis; (3) use of the anti-idiotypic antibody with the PAP/DAB-method and subsequent light-microscopic analysis of semi-thin sections. The use of the different approaches has shown punctate immunoreactive material homogeneously distributed in the cytoplasm shown in Fig. 3.

In contrast, the nuclei of marked neurons were devoid of staining. With the pre-embedding semi-thin sections, it was possible to localize punctate staining at the border of the cell membrane. The intracellular distribution pattern of angiotensin II receptor immunoreactivity could indicate that the epitope being recognized by the anti-receptor antibodies already appears during receptor biosynthesis and/or during degradation.

3 Combination of Immunocytochemical Methods with Other Techniques

The current knowledge on brain RAS has been derived mostly from a synthesis of many studies with different methods of investigations. The disadvantage of all these studies, however, is the lack of information to the same neuron. Such information can only be gained with a combination of different methods focused on a single structure and performed in one experimental approach. The advantage is to develop a relation between the results obtained. For instance with a combination of electrophysiology, morphology and immunocytochemistry, a neuron could be investigated with regard to its sensitivity to specific neurotransmitters, the content of different peptides, enzymes, receptors, and its morphology.

Egli et al. (2000) used different methods to characterize single neurons in the hypothalamic paraventricular area of the brain. Starting with intracellular recordings and a pharmacological identification, not only neurotransmitters but also involved receptors were defined with the application of receptor agonists and antagonists. Finally, the injection of Lucifer yellow into the electrophysiologically investigated cell using confocal laser-scan microscopically generated optical sections allowed a 3D-model to be created. The use of different antibod-

ies on 1- μ m semi-thin sections from the investigated cell showed a possible presence of diverse substances. The electrophysiological method was used to find angiotensin II-sensitive neurons in this brain area. An application of the angiotensin II-specific AT₁ receptor antagonist Losartan (Dup 753) during the angiotensin II application confirmed its evoked reaction, followed by a dye injection into the cell, defining its morphology. Finally, an immunocytochemical method was used to look for vasopressin and components of the angiotensin system inside or outside the neuron (Egli et al. 2000). Such techniques offer new insight into the function of neurotransmitter systems in general, and the results enables us to gain improved information on interactions of the angiotensinergic system within the RAS or other systems in specialized areas of the brain or in peripheral tissues.

4

Localization of the Components of the Renin–Angiotensin System

4.1

Angiotensinogen in Brain

Each of the components of the brain RAS including angiotensinogen (Lewicki et al. 1978; Campbell et al. 1984), renin (Ganten and Speck 1978; Ganten et al. 1984), converting enzyme (Ganten et al. 1984) and angiotensin II itself (Hermann et al. 1984) have been identified in the brain. Although the presence of all of the components in the brain seems indisputable, the exact cellular locations of several key components, such as angiotensinogen (Deschepper et al. 1986; Sernia 1995) have been disputed. While angiotensin II appears to be exclusively located in neurons (Lind et al. 1985; Imboden et al. 1987a), angiotensinogen has been reported to reside only in astrocytes and ependymal cells. In the hypoglossal nucleus of the rat, angiotensinogen was found in astrocytes, with small amounts in neuronal dendrites (Tham et al. 2001). The cellular localization of angiotensinogen mRNAs in the subfornical organ is predominantly expressed in astroglial cells (Lippoldt et al. 1993).

Imboden et al. (1987c) confirmed, with affinity-purified polyclonal antibodies against the tetradecapeptide, that a large portion of the total angiotensinogen immunoreactivity was present in astrocytes. However, it appeared that only a subpopulation of astrocytes contained angiotensinogen, having the highest concentration in the arcuate nucleus. Of potential importance was the tendency of angiotensinogen-containing astrocytes to contact brain microvessels. Therefore, astrocytic angiotensinogen could represent the precursor pool for a second brain-angiotensin system that is exclusively associated with blood vessels and involved with the regulation of cerebral blood flow. In contrast to earlier reports, Imboden et al. (1987c) could show that clear angiotensinogen staining of neurons was evident in paraventricular, supraoptic and accessory magnocellular hypothalamic nuclei. Interestingly, these same nuclei always contained for angiotensin II, suggesting a unique relationship between angiotensinogen-containing

astrocytes and angiotensin-producing neurons. Such a system would require direct contact between these elements so that angiotensinogen could be taken up by the neurons. The final location of angiotensinogen is the choroid plexus, indicating that angiotensinogen is being actively secreted into the cerebrospinal fluid (Imboden et al. 1987c).

The localization of angiotensinogen in three cell types of the brain, using immunohistochemical methods, are consistent with multiple functions for brain angiotensinogen as a precursor for neuronal angiotensin II and as a potential source for angiotensin II that is locally produced in the brain (Schelling et al. 1983). Regional distribution of angiotensinogen in fetal and neonatal rat brain showed that most of the immunopositive cells in the hypothalamus and brainstem were astrocytes, while those in the cortex were almost exclusively neurons (Mungall et al. 1995).

4.2 Angiotensin Receptors

Although angiotensin II receptors were among the first peptide receptors to be characterized, the discovery of receptor subtypes and the elucidation of the signal transduction mechanisms linking receptors to effectors have revealed new possibilities concerning the role of an angiotensin system in the brain (Bottari et al. 1993). Most of the known central actions of angiotensin II, such as dipsogenic response, increase in blood pressure and vasopressin release, have been shown to be mediated through the AT₁ receptor. In the past, many immunohistochemical studies have focused on the distribution pattern of this receptor subtype. The precise localization and the physiological role of angiotensins in circumscribed areas of the brain especially in the third ventricle area (subfornical organ, organum vasculosum laminae terminalis, anterior third ventricle area), the hypothalamic areas and the hypothalamo-neurohypophysial axis have shed light on the understanding of the role of the brain RAS (Imboden and Felix 1991; Paxton et al. 1993; Phillips et al. 1993; Mosimann et al. 1996; Benarroch and Schmeichel 1998). Besides the third ventricular and hypothalamic area, direct evidence exists of neuronal localization of the AT₁ receptor in distinct areas of the brain stem (Yang et al. 1997; Benarroch and Schmeichel 1998) and the limbic system (von Bohlen et al. 1998).

As already mentioned, the function of the AT₂ receptor is far from being understood. High AT₂ receptor mRNA expression was localized at the cellular level in the lateral septum, in several thalamic nuclei, in the subthalamic nucleus, in the locus coeruleus and in the inferior olive. In all regions examined, AT₂ receptor immunoreactivity was associated with the cytoplasm and cell membrane and was not localized within the nucleus (Reagan et al. 1994). Schelman et al. (1997) reported an AT₂ receptor-mediated inhibition of NMDA (*N*-methyl-D-aspartate) receptor signalling in neuronal cells. The results suggest that angiotensin II inhibits NMDA-mediated nitric oxide and cyclic guanosine monophosphate (cGMP) production through a mechanism involving AT₂ receptor subtype.

In the adrenal gland both angiotensin receptor subtypes have been characterized. Immunocytochemical studies revealed AT₁ reactive cells in the zona glomerulosa and the medulla, whereas the AT₂ antibody bound specifically to medullar cells (Bird et al. 1996; Frei et al. 2001). Since the AT₂ is expressed in the adrenal medulla, it is tempting to speculate whether this receptor subtype might play a role in adrenal medullar apoptosis (Shenoy et al. 1999).

5

Colocalization of Central Angiotensinergic and Vasopressinergic Systems

Since the discovery of angiotensin receptor subtypes and the development of specific angiotensin receptor antagonists, new aspects concerning the functional role of these receptors in the central nervous system have been elucidated. There is a close interrelationship between angiotensin receptors and other neurotransmitter systems. Using microdialysis, Stadler et al. (1992) showed that stimulation of periventricular AT₁ receptors leads to a release of noradrenaline in hypothalamic nuclei. Noradrenaline, on the other hand, acts on postsynaptic adrenoceptors to release vasopressin from the neurohypophysis.

The existence of the two peptides angiotensin and vasopressin in individual magnocellular cell groups of the hypothalamus has been demonstrated by using immunocytochemical methods (Imboden and Felix 1991). These neurosecretory magnocellular groups consist of the paraventricular nucleus and the supraoptic nucleus, as well as different accessory cell groups (Riva et al. 1999). From a functional point of view, these peptide systems are closely related and have dramatic vasoconstrictive effects when given peripherally and both exhibit pressor activity when applied centrally (Matsuguchi et al. 1982; Phillips 1987; Langhans 1988; Janiak et al. 1989). Their axons project into the pituitary, hindbrain structure, and other regions of the central nervous system (Healy and Printz 1984; Lind et al. 1985; Imboden et al. 1989). An immunocytochemical comparison of the angiotensin and vasopressin hypothalamo-neurohypophysial systems in rats by Imboden and Felix (1991) confirmed the colocalization of the two peptides in neurons of the paraventricular nucleus and the supraoptic nucleus (Kilcoyne et al. 1980; Hoffman et al. 1982; Silverman and Zimmerman 1983; Lind et al. 1984).

Furthermore, with affinity-purified antibodies it has been shown that the angiotensin and vasopressin hypothalamo-neurohypophysial systems are located in the same fibre system (Imboden and Felix 1991) as shown in Fig. 4.

Arching fibre tracts were formed mainly by projections emanating from cell bodies in the paraventricular nucleus and supraoptic nucleus, extending as far as the median eminence and the neurohypophysis, where major terminal fields exist. The implication of this finding is that angiotensin and vasopressin may be released together from the median eminence and the neurohypophysis. An important question remains to be answered as to how the release of such colocalized peptides is controlled. Functional studies have indicated that the AT₂ receptor subtype may participate in the regulation of vasopressin release by angioten-

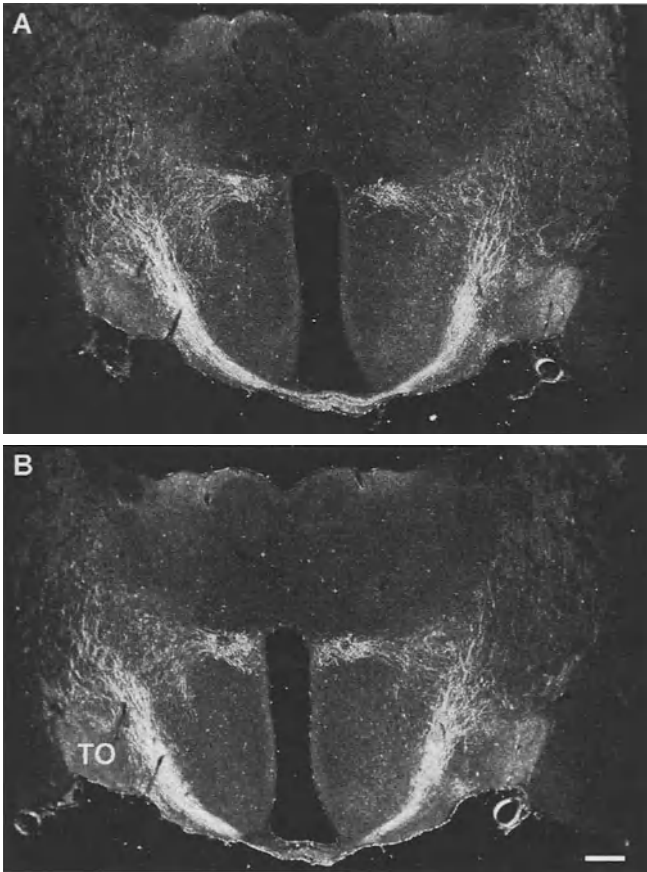


Fig. 4A, B The angiotensinergic (**A**) and vasopressinergic (**B**) fibre systems in coronal adjacent sections in the rat hypothalamus. *TO*, tractus opticus; *III*, third ventricle. Scale bar=500 μ m

sin II. Furthermore, subtype AT_2 receptor-directed antiserum has been immunohistochemically detected AT_2 receptors in identified vasopressinergic neurons within the paraventricular and the supraoptic nuclei (Shelat et al. 1998).

6

A New Angiotensinergic System in the Central Nervous System

Through immunocytochemical techniques, the localization of angiotensin II-like immunoreactivity has mainly been demonstrated in the hypothalamo-neurohypophysial pathway, neurosecretory paraventricular nucleus, supraoptic nucleus, suprachiasmatic nucleus, as well as in the median eminence (Brownfield et al. 1982; Lind et al. 1985). Similar results were obtained by the use of a polyclonal, affinity-purified, monospecific antibody to angiotensin II called "BODE", produced by Imboden et al. (1987a,b). Recently, a new polyclonal rab-

bit antibody to angiotensin II, called "BODE 1" was developed by the same group of researchers (Burkhalter et al. 2001). Nitrocellulose tests revealed for both antibodies a high degree of crossreactivity with different angiotensin peptides. They did not, however, interact with other neuropeptides like vasopressin, indicating that both antibodies are uniquely specific for angiotensins. Interestingly, the use of the two antibodies revealed dissimilar distribution of angiotensin II immunoreactivity within the rat central nervous system. The angiotensin II-like material detected using BODE was concentrated in the neurosecretory hypothalamic nuclei, in the inner layer of the median eminence and in the posterior lobe of the pituitary. In contrast, the BODE 1 antibody did not stain the hypothalamo-neurohypophysial angiotensinergic system; the staining pattern was much more broadly distributed throughout the central nervous system. This distribution of angiotensin-like material is new and indicates the existence of a new angiotensinergic system. BODE 1 is the first antibody that can be used to verify the locations of endogenous angiotensin and their receptor sites in the central nervous system (Burkhalter et al. 2001).

The results, however, do not reveal which exact types of angiotensins are detected. It is interesting to note that in spite of using the same antigen molecule for immunization as well as the same antigen molecule for affinity purification, these two polyclonal antibodies showed a dissimilar staining pattern. The findings suggest that the different carrier proteins forced the production of different antibodies to different epitopes. Therefore, it has to be taken into account, that, depending on how the different antibodies are able to bind to the exposed epitopes in the paraformaldehyde-fixed sections, different staining pattern can be obtained.

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Concluding Remarks

Early immunohistochemical studies that described the detection of angiotensin II-immunoreactive material were forced to rely on a small number of antisera because most angiotensin antibodies had proved unsuitable for immunohistochemistry. Although useful tools, these antisera have suffered from high background staining. With the development of affinity-purified antibodies, the resolution has been greatly enhanced. The purification results in a distinct loss of background activity and produces a discrete staining of neurons and even small angiotensin fibres in animals not treated with colchicine. Such low background and discrete staining obtained through the improvement of immunocytochemical techniques has enhanced our ability to develop accurate maps of angiotensinergic pathways.

Another important aspect of the immunocytochemical approach is the production of different specific monoclonal antibodies to angiotensin AT₁ and AT₂ receptor subtypes and to angiotensin II as well as to components of the RAS. The development and use of such angiotensin antibodies will be a prerequisite for the characterization and localization of angiotensin hormonal and/or neuro-

transmitter systems in the brain and in peripheral tissues. It has to be taken into account that in spite of using the same antigen molecule for immunization as well as the same antigen molecule for affinity purification, different staining patterns can be obtained. The finding that in the central nervous system at least two different angiotensin systems exist supports the hypothesis that angiotensin II is not the only effector peptide in the RAS, but a member of a family of biologically active angiotensin peptides. Nevertheless, immunohistochemical techniques remain important methodological tools for characterizing and localizing angiotensinergic systems in the central nervous system and in peripheral tissues.

Whereas most of the immunohistochemical studies concerning the RAS have dealt in the past with the central nervous system and the adrenal, there is an increasing need for immunocytochemical approaches in normal and pathological peripheral tissues, e.g. cancer. With few exceptions, such studies are missing. It will be of importance that clinical trials using angiotensin receptor antagonists for treatment, e.g. glioblastoma multiforme and other malignant tumours, take place. Therefore, the immunocytochemical approach will become an important tool to place such trials on a firm scientific basis.

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Renin–Angiotensin System: Plasma Versus Tissues

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Abstract The classical view of the renin–angiotensin system (RAS) as an endocrine system, designed to deliver its active end-product, angiotensin II, to tissues via circulating blood, has been challenged in the past two decades by studies demonstrating high tissue levels of angiotensin II, as well as release of angiotensin I from tissue sites. The high tissue levels of angiotensin II could not be explained on the basis of diffusion and/or receptor-mediated uptake of circulating angiotensin II, thereby supporting the concept that tissue angiotensin II is largely, if not completely, synthesized locally. Remarkably, in many organs (in particular the heart and vessel wall) this local synthesis depends on the uptake of circulating (i.e. kidney-derived) renin and/or prorenin, either via diffusion into the interstitial space or through binding to (pro)renin receptors. Renin and/or prorenin binding to (pro)renin receptors located on the cell membrane, combined with the fact that angiotensin-converting enzyme (ACE) also is a membrane-associated enzyme, favours angiotensin generation on the cell surface. Although renin has also been demonstrated intracellularly, intracellular angiotensin generation is unlikely to occur, due to the lack of angiotensinogen internalization. Tissue angiotensin generation is further supported by clinical studies demonstrating that RAS blockade, with either ACE inhibitors or angiotensin receptor antagonists, results in beneficial cardiac, vascular and renal effects that are independent, at least in part, of the blood pressure-lowering effects

of these drugs. Taken together therefore, angiotensin II is generated at tissue sites, where it acts as a paracrine and/or autocrine hormone, and RAS blockers exert their effects mainly through interference with tissue angiotensin II.

Keywords Angiotensin · Renin · Prorenin · AT₁ receptor · AT₂ receptor · Mannose 6-phosphate/insulin-like growth factor II receptor · Chymase · Intracellular · ACE inhibitor

1

Introduction

Classically, the renin–angiotensin system (RAS) has been viewed as an endocrine system, designed to deliver its effector peptide, angiotensin (Ang) II, via the circulation to target organs, where it binds to Ang II type 1 (AT₁) and type 2 (AT₂) receptors in order to induce effects, the most important of which are vasoconstriction, sodium and water retention, and growth stimulation. According to this concept, liver-derived angiotensinogen is cleaved in the circulating blood by kidney-derived renin, to form Ang I. Ang I is subsequently converted to Ang II by angiotensin-converting enzyme (ACE) located on the luminal side of the vascular endothelium. ACE also occurs as a soluble form in blood plasma, but the contribution of soluble ACE to Ang II generation is negligible (Ng and Vane 1968; Admiraal et al. 1993).

Following the introduction of the ACE inhibitors in the early 1980s, it soon became clear that these drugs exerted effects in the face of unaltered or even increased levels of Ang II in circulating blood (Nussberger et al. 1985, 1986; Mooser et al. 1990; van den Meiracker et al. 1992). Moreover, beneficial effects were observed that occurred independently of their blood pressure-lowering effects (Linz et al. 1992; Yusuf et al. 2000). Although the former may have been due, at least in part, to inaccurate measurements of Ang II (Nussberger et al. 1985, 1986), these observations have led to the concept that angiotensins are also synthesized outside the circulation, i.e. at tissue sites (Dzau 1988). Recent studies with other RAS blockers (renin inhibitors and AT₁ receptor antagonists) further support this view (Fischli et al. 1994; Linz et al. 2000).

Remarkably, despite an overwhelming number of studies on “tissue Ang II generation” in the past 20 years, the precise mechanism underlying this phenomenon is still not completely understood. Originally, it was thought that all components required to synthesize Ang II locally, i.e. renin, angiotensinogen and ACE, were also synthesized in tissues, thus allowing organs like the heart, vessel wall, brain and adrenal to synthesize Ang II independently of angiotensin generation in the circulation. Protein and mRNA measurements have been performed to support the existence of local RASs in organs. Protein measurements, however, cannot be taken as evidence that the protein is synthesized locally. Moreover, such measurements are often hampered by a lack of specificity. For example, the presence of “renin” activity in tissue extracts may be caused by enzymes other than renin that are capable of generating Ang I (Katz et al. 2001).

Furthermore, detection of mRNA, particularly when the concentrations are low, does not necessarily mean that the corresponding protein is synthesized locally in physiologically relevant amounts (von Lutterotti et al. 1994). These uncertainties, as well as observations that renin could not be detected in cardiac and vascular tissue following bilateral nephrectomy (Loudon et al. 1983; Danser et al. 1994), have led to the idea that tissue Ang II generation might depend on one or more RAS components taken up from the circulation. Consequently, the term local (or tissue) RAS has been replaced by “local Ang II-generating system”.

2

Release of Angiotensins from Tissue Sites into the Circulation?

Local production of angiotensins has been studied extensively, both through in vitro and in vivo experiments. In studies of isolated perfused rat Langendorff hearts, cardiac production of Ang I and II could only be demonstrated after the addition of renin to the perfusion buffer (Lindpaintner et al. 1990; de Lannoy et al. 1997, 1998). This suggests that angiotensinogen and ACE, but not renin, are present in the isolated rat heart. Results from isolated perfused rat hindquarter experiments parallel these findings (Fig. 1), in that the spontaneous release of angiotensins from these preparations decreased to low or undetectable levels when the animals had been nephrectomized prior to hindquarter perfusion (Hilgers et al. 1993; Kato et al. 1993).

Attempts have been made to study tissue angiotensin production in the intact animal by measuring arteriovenous differences of Ang I and II across the various vascular beds. However, such differences are small (Admiraal et al. 1990, 1993; Danser et al. 1992a; Neri Serneri et al. 1996), and it should be taken into account that a large proportion of circulatory Ang I and II is metabolized during

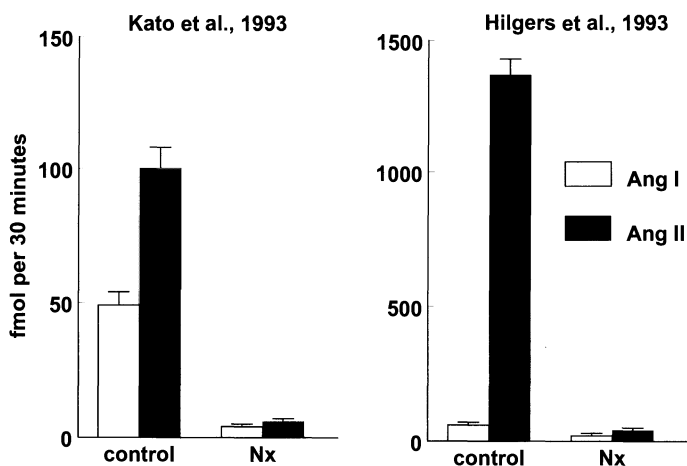


Fig. 1 Angiotensin (*Ang*) release from isolated perfused rat hindlimbs before and after bilateral nephrectomy (*Nx*). (Hilgers et al. 1993; Kato et al. 1993)

a single passage through an organ. In early studies in sheep (Fei et al. 1981), Ang II clearance across the pulmonary and combined systemic vascular beds was calculated from arteriovenous differences measured across these beds during infusion of high doses of Ang II into the pulmonary artery. These studies already indicated that Ang II is rapidly degraded and that plasma renin activity (PRA) is probably insufficient to generate the endogenous levels of circulating Ang II.

More recently, the regional clearance and metabolism of Ang I and II was quantified, both in humans and pigs, by constant infusions of radiolabelled ^{125}I -Ang I or II (Admiraal et al. 1990, 1993; Danser et al. 1992b, 1998; Neri Serneri et al. 1996). Blood was sampled from various arterial and venous sites for measurements of radiolabelled Ang I and II. Additional measurements of the levels of endogenous Ang I and II, and of PRA at physiological pH made it possible to estimate how much of venous Ang I could be attributed to arterial delivery, vs de novo synthesis, and what proportion of de novo synthesized Ang I depends on the action of circulating renin on circulating angiotensinogen (PRA). It was also possible to calculate how much of venous Ang II originated from arterial delivery, and how much from the conversion of arterially delivered Ang I.

It was found that in virtually all vascular beds studied (heart, lung, kidney, head, arm, leg, liver), a major proportion of venous Ang I originated from de novo production and that PRA contributed little to this de novo production

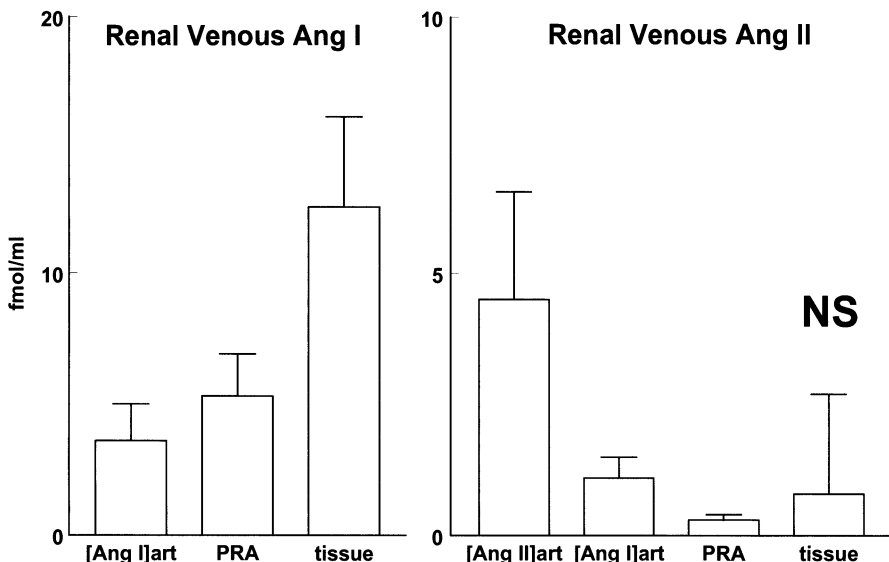


Fig. 2 Origin of renal venous angiotensin (Ang I and II) in humans (Danser et al. 1998). *[Ang I]art* and *[Ang II]art* represent arterial Ang I and Ang II, respectively. *PRA*, plasma renin activity. The majority of renal venous Ang I originates from tissue sites, whereas the release of Ang II from tissue sites is not significant (*NS*) from zero. The latter implies that de novo synthesized renal venous Ang II is derived only from arterial Ang I and Ang I generated by PRA during passage of the renal vascular bed

(Fig. 2). One can therefore conclude that Ang I is produced at tissue sites and that part of it is released into the circulation. While most of venous Ang I appears not to be generated by the action of circulating renin on circulating angiotensinogen, the level of venous Ang I produced at (and released from) tissue sites correlated strongly with the level of PRA (Danser et al. 1992b). This, together with findings in pigs and rats that tissue angiotensin levels are greatly reduced after nephrectomy (Campbell et al. 1993; Danser et al. 1994; Nussberger 2000), strongly suggests that it is kidney-derived renin that is a major determinant of tissue Ang I production.

For Ang II, we were unable to demonstrate release from tissue sites (Admiraal et al. 1993; Danser et al. 1998). Most, if not all, venous Ang II appeared to originate from delivered by the artery and from Ang II generated by conversion of arterially delivered Ang I. Thus, Ang I produced at tissue sites and released into the circulation may have escaped conversion to Ang II. It is possible that Ang I produced in the tissue enters the blood at a level distal to the site where arterially delivered Ang I is converted to Ang II by the vascular endothelium, so that this conversion site is bypassed. For instance, Ang I formed at tissue sites may enter the circulation at the level of the capillaries or venules, whereas Ang I to II conversion may occur only at the level of the arterioles. Alternatively, Ang II is produced in the tissue and remains there.

3 Origin of Tissue Angiotensin I and II

The studies described above are limited to measurements of angiotensins in the circulation. Although they support the concept of Ang I release from tissue sites into the circulation, further proof for Ang I (and II) generation at tissue sites can only be obtained by measuring tissue levels of Ang I and II. Such measurements are technically difficult because of *ex vivo* metabolism and generation of angiotensins, and because the antibodies used to quantify Ang I and II usually cross-react with other angiotensin metabolites and non-related substances. Thus, great care must be taken when measuring the “true” *in vivo* levels of Ang I and II. Furthermore, a correction should be made for (active) uptake of angiotensins from circulating blood, for instance through AT₁ receptor binding, as net tissue angiotensin levels are the sum of uptake from blood and local production. Uptake from the circulation can be quantified by measuring steady-state tissue and plasma levels of ¹²⁵I-labelled Ang I and II during ¹²⁵I-Ang I and II infusion.

The results obtained from pigs (van Kats et al. 1997, 1998, 2001) showed that ¹²⁵I-Ang II, but not ¹²⁵I-Ang I, accumulated in tissues (Fig. 3). The absence of significant tissue ¹²⁵I-Ang I accumulation already indicated that the presence of Ang I in tissues cannot be attributed to uptake from blood plasma. Steady-state levels of ¹²⁵I-Ang II at tissue sites were up to 20 times higher than those in blood plasma. This accumulation was largely prevented by pretreatment with an AT₁ receptor antagonist (Fig. 3), suggesting that it is mediated via AT₁ receptor-de-

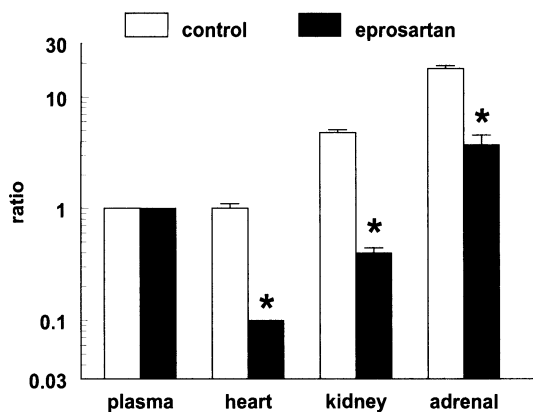


Fig. 3 ^{125}I -Angiotensin II levels (expressed as a ratio versus the steady-state plasma ^{125}I -angiotensin II level) during systemic infusion of ^{125}I -angiotensin II in pigs, both under control conditions and following AT_1 receptor blockade with eprosartan (van Kats et al. 1997, 2000, 2001). Eprosartan reduces the tissue/plasma concentrations of ^{125}I -angiotensin II by more than 80% without affecting plasma ^{125}I -angiotensin II. *, $p < 0.05$ vs control pigs

pendent endocytosis. A role for AT_2 receptors in this process is unlikely because these receptors do not internalize Ang II (Matsubara 1998). Similar conclusions were drawn from Ang II infusion studies in rats (Zou et al. 1996a,b). Comparison of the ^{125}I -labelled and endogenous angiotensin levels (Fig. 4) revealed that, despite the significant uptake of ^{125}I -Ang II in various tissues, in all organs the majority (>90%) of tissue Ang II is not derived from circulation, i.e. is locally synthesized from locally generated Ang I.

Thus, both Ang I and II are generated at tissue sites, and of these locally generated angiotensins, only Ang I is released into the circulation, and Ang II remains in the tissue. The reason for this discrepancy may be that Ang II,

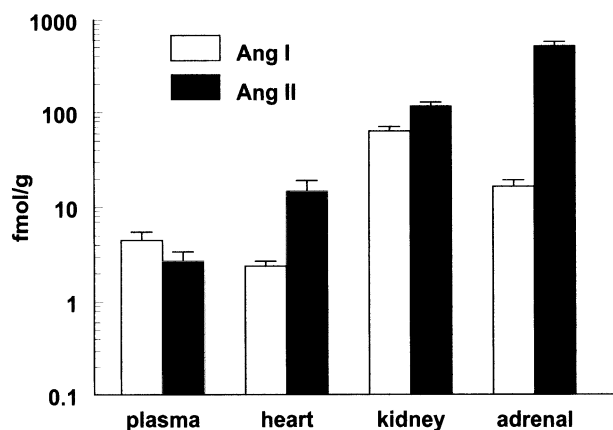


Fig. 4 Plasma and tissue levels of angiotensin I and II in normal pigs. (van Kats et al. 1997, 2000, 2001)

like ^{125}I -Ang II, rapidly binds to AT_1 receptors following its local generation, thereby preventing it from “leaking” into the circulation. Two lines of evidence support this view. First, the tissue Ang II content (adrenal>kidney>heart; Fig. 4), like the tissue ^{125}I -Ang II content (Fig. 3), parallels AT_1 receptor density in these organs (Whitebread et al. 1989; Zhuo et al. 1994; Regitz-Zagrosek et al. 1995). In this regard, it is important to note that the tissue Ang II levels, even in the adrenal (where the highest tissue Ang II levels are found), are too low to occupy all receptors, and thus that a large reserve of unoccupied AT_1 receptors exists. Second, the subcellular distribution of Ang II mimics that of ^{125}I -Ang II (van Kats et al. 2001), and as discussed above, the latter peptide accumulates in tissues via AT_1 receptor-mediated endocytosis only.

The half-life of AT_1 receptor-bound Ang II is roughly 15 min; i.e., 20–30 times longer than the half-life of Ang II in extracellular fluid (Schuijt et al. 1999; van Kats et al. 2001). A large number of free AT_1 receptors, combined with a significant increase of Ang II half-life following its binding to AT_1 receptors, would allow tissue Ang II to reach (much) higher levels than blood plasma Ang II, even when the level of Ang II generation is similar in both compartments.

4

Origin of Renin in Tissues: (Pro)Renin Receptor(s)?

Renin mRNA has been demonstrated in many tissues (Ekker et al. 1989; Iwai and Inagami 1992), in confirmation with the aforementioned studies providing direct evidence for tissue angiotensin generation. However, few attempts have been made to either combine renin mRNA measurement with tissue renin protein measurements (using renin-specific antibodies and inhibitors), to demonstrate renin release from tissues *in vivo* and/or from cultured cells, or to measure tissue renin following a bilateral nephrectomy. Tissues that convincingly show local renin synthesis are the ovary, testis, adrenal and eye (Itskovitz et al. 1987, 1992; Sealey et al. 1988; Danser et al. 1989; Peters et al. 1996; Wagner et al. 1996). Remarkably, in most of these tissues, prorenin (the inactive precursor of renin) rather than renin was detected. In addition, recent studies have reported the existence of a renin transcript that lacks the coding sequence for the prosegment; i.e. the transcript cannot be targeted to secretory pathways and thus remains intracellular (Clausmeyer et al. 1999, 2000; Lee-Kirsch et al. 1999). The functions of prorenin, without evidence for its local conversion to renin, and intracellular renin [which appears to be located in mitochondria (Clausmeyer et al. 1999)] remain unclear.

Renin and angiotensin disappear from cardiac and vascular tissues following bilateral nephrectomy, under both normal and pathological conditions (Hilgers et al. 1993; Danser et al. 1994; Katz et al. 1997). Moreover, 48 h after nephrectomy the angiotensin levels in muscle, liver, lung, and adrenal (but not in the brain) also decrease by 60%–95%, suggesting that they too, depend on renal renin (Campbell et al. 1993; Nussberger 2000). The fact that these levels, particularly in the adrenal, did not decrease to zero within 48 h, may either suggest that

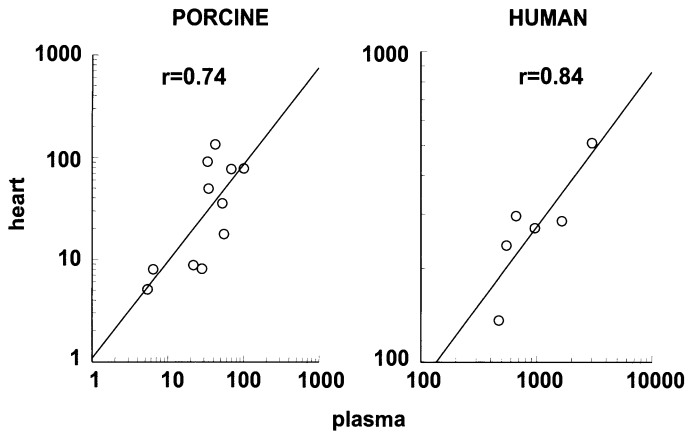


Fig. 5 Relationship between the plasma and cardiac tissue levels of renin in normal pigs (*left panel*) and in humans with dilated cardiomyopathy (*right panel*). (Danser et al. 1994, 1997)

renal renin, once present in tissues, has a long half-life, or that a percentage of angiotensin in these organs has indeed been synthesized by locally generated renin.

The large contribution of renal renin to angiotensin production in many tissues, in particular the heart and vascular wall, implies that tissues are capable of taking up circulating (i.e. kidney-derived) renin and/or prorenin, and possibly of locally converting prorenin to renin. Levels of renin in the heart, under both normal and pathological circumstances (Danser et al. 1994, 1997; Heller et al. 1998), correlate directly with plasma levels of renin (Fig. 5). "Uptake" might mean diffusion into interstitial space and/or binding to (pro)renin receptors. Evidence for renin diffusion/binding was obtained 20 years ago, when it was observed that vascular renin disappeared more slowly than circulating renin following a bilateral nephrectomy (Loudon et al. 1983). Subsequent studies confirmed that tissue renin is partly membrane-associated and that renin-binding proteins/receptors exist (Campbell and Valentijn 1994; Danser et al. 1994). Three such proteins have now been identified.

The first is an intracellular renin-binding protein (RnBP), that reduces the Ang I-generating activity of renin by more than 80% (Takahashi et al. 1992). This RnBP was found to be identical to the enzyme *N*-acyl-D-glucosamine 2-epimerase (Maru et al. 1996). It does not co-localize with renin, and mice lacking the RnBP display normal blood pressure and renin activity (Leckie et al. 2000; Schmitz et al. 2000). Thus, it is most likely unrelated to renin. The second is the mannose 6-phosphate insulin-like growth factor II (M6P/IGFII) receptor. This receptor not only binds and internalizes phosphomannosylated (i.e. M6P-containing) proteins like renin and prorenin with high affinity (van Kesteren et al. 1997; Admiraal et al. 1999; Saris et al. 2001; van den Eijnden et al. 2001), but it also activates prohormones (including native human prorenin) intracellularly (Helseth and Veis 1984). Its expression is developmentally regulated (Kornfeld

1992). The third is a high-affinity (pro)renin receptor that was originally discovered in human mesangial cells (Nguyen et al. 1996). It probably also occurs in membrane preparations obtained from rat tissues (Sealey et al. 1996). The gene encoding this binding site has recently been cloned (Nguyen et al. 2002). It encodes for a 350-amino acid protein showing no homology with known proteins. The binding site is predominantly present in vascular smooth muscle cells and glomerular mesangial cells. Interestingly, when bound to this receptor, renin displays increased catalytic activity as compared to soluble renin. Finally, there is evidence for a fourth receptor/binding site in adult rat cardiomyocytes that results in internalization of non-glycosylated prorenin (Peters et al. 2002). No attempts have been made so far to identify this protein.

The functional importance of these (pro)renin receptors should now be addressed, and the percentage of renin and prorenin containing the M6P signal should be determined under normal and pathological conditions. Studies in transgenic animals with liver-specific overexpression of renin and/or prorenin have already confirmed the uptake of circulating (pro)renin in cardiac tissue, as well as the subsequent generation of Ang I in the heart (Prescott et al. 2000, 2002). Unravelling the precise mechanism(s) underlying this phenomenon might result in the development of drugs that prevent generation of tissue Ang II by blocking (pro)renin uptake/binding.

5

The Site of Tissue Angiotensin Generation: Intra- or Extracellular?

Despite numerous reports suggesting that Ang II is an intracrine hormone, i.e. a hormone that is synthesized and acts intracellularly, convincing evidence for this concept is currently lacking. Despite the effects exerted by intracellularly applied Ang II (de Mello 1994; Filipeanu et al. 2001), and the reported presence of Ang II in and its release from cells (Dostal et al. 1992; Sadoshima et al. 1993; Leri et al. 1998), cellular Ang II synthesis remains unproven. Firstly Ang II accumulates in cells via AT₁ receptor-mediated internalization (van Kats et al. 1997), and at least part of its effects depend on such internalization (Griendling et al. 1987), and secondly, it cannot be excluded that cellularly released Ang II (e.g. after stretch) had been previously internalized (van Kesteren et al. 1999).

Intracellular angiotensin generation requires the presence of renin and angiotensinogen in the same subcellular compartment. As mentioned above, there is evidence for renin occurring intracellularly. An alternative renin transcript gives rise to a renin variant that lacks the coding sequence for the prosegment (and thus cannot be secreted); and M6P/IGFII receptor-mediated internalization of renin and prorenin, followed by intracellular activation of prorenin, also results in renin accumulation in cells (Admiraal et al. 1999; Clausmeyer et al. 1999; Saris et al. 2001). Yet, the addition of angiotensinogen to the medium of cultured, renin-containing cardiomyocytes and endothelial cells did not result in intracellular Ang II generation (van den Eijnden et al. 2001; Saris et al. 2002). The main reason for this appeared to be a complete lack of angiotensinogen in-

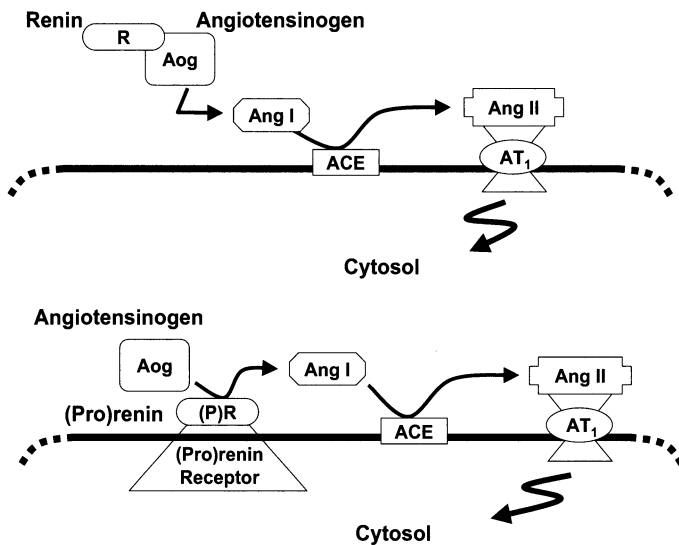


Fig. 6 Model of cell surface angiotensin (*Ang*) II generation and subsequent AT₁ receptor activation. *Ang* I synthesis from angiotensinogen (*Aog*) by renin (*R*) may either occur in the interstitium (*top panel*) or on the cell surface (*bottom panel*). The latter would require the existence of a (pro)renin [(*P*)*R*] binding protein/receptor. Prorenin, when bound to this receptor, may display enzymatic activity. (Nguyen et al. 2002)

ternalization (van den Eijnden et al. 2001; Saris et al. 2002). In view of this finding, and also taking into consideration that angiotensinogen-synthesizing cells normally release angiotensinogen into the extracellular fluid (without storing it intracellularly, due to the presence of a signal peptide that leads to secretion) (Klett et al. 1993), the question arises whether intracellular angiotensin generation is possible at all. In fact, a recent study in rat hepatoma cells demonstrated that such intracellular angiotensin generation could only occur following transfection of the cells with a mutated angiotensinogen cDNA that produces a non-secreted form of angiotensinogen (Cook et al. 2001). Taken together, intracellular *Ang* II generation is highly questionable under physiological conditions.

This conclusion leaves the possibility that tissue angiotensin generation occurs extracellularly, either in the interstitial fluid or on the cell surface (Fig. 6). Circulating renin diffuses into the interstitial fluid compartment, reaching steady-state levels that are equal to those in plasma (de Lannoy et al. 1997). The efficient cleavage of angiotensinogen by membrane-bound renin particularly supports cell surface angiotensin generation (Nguyen et al. 2002). In tissues where local angiotensin generation depends entirely on the sequestration of circulating renin, such efficient cleavage could result in interstitial angiotensin levels that are somewhat higher than the plasma angiotensin levels (de Lannoy et al. 1998; Hilgers et al. 1998; Schuijt et al. 2002). Larger differences, corresponding with several orders of magnitude (Siragy et al. 1995; Dell'Italia et al. 1997; Nishiyama

et al. 2002), are unlikely to occur following sequestration of circulating renin only, and could therefore be suggestive for an important contribution of locally synthesized renin.

Extracellular angiotensin generation implies that Ang II is an autocrine and/or paracrine hormone. It does not argue against Ang II exerting intracellular effects following internalization of the AT₁ receptor-Ang II complex. If not leading to intracellular angiotensin generation, M6P/IGFII receptor-mediated (pro)renin internalization may represent (pro)renin clearance, thereby indirectly determining extracellular Ang I-generating activity. The function of mitochondrial renin remains unknown.

6 Plasma and Tissue Angiotensin Levels During Renin–Angiotensin System Blockade: How Do ACE Inhibitors and AT₁ Receptor Antagonists Work?

RAS blockade with ACE inhibitors or AT₁ receptor antagonists immediately results in renin release from the kidney, because it no longer allows Ang II to suppress renin release via AT₁ receptor activation. As a consequence, Ang I generation will increase (Mooser et al. 1990). This will lead to a rapid rise in the levels of Ang II during AT₁ receptor blockade (Fig. 7), and depending on the degree of ACE inhibition, a rise in Ang II levels during treatment with an ACE inhibitor (Campbell et al. 1993, 1995; van Kats et al. 2000). Other explanations for the rise in plasma and tissue Ang II during prolonged ACE inhibition (Fig. 7) are ACE upregulation and/or a role for alternative converting enzymes such as chymase (Urata et al. 1990; Balcells et al. 1997). ACE upregulation occurs both as a consequence of chronic ACE inhibitor therapy and during the progression of cardiovascular diseases (Farquharson and Struthers 2002). In support of the former,

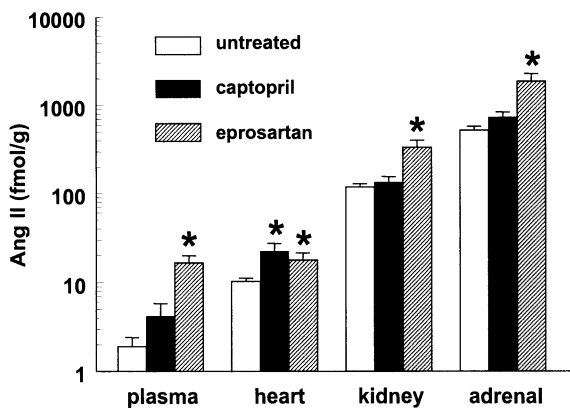


Fig. 7 Plasma and tissue levels of angiotensin II in untreated pigs and in pigs that were treated for 3 weeks with either the ACE inhibitor captopril or the AT₁ receptor antagonist eprosartan. (van Kats et al. 1997, 2000, 2001). *, $p < 0.05$ vs untreated pigs

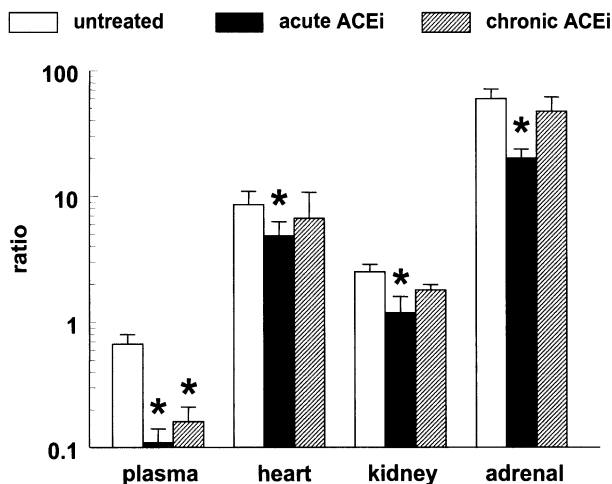


Fig. 8 Angiotensin II/angiotensin I ratios in untreated pigs and in pigs that were treated for either 3 days (acute ACEi) or 3 weeks (chronic ACEi) with the ACE inhibitor captopril. (van Kats et al. 1998, 2000, 2001). The ratio is taken as a measure for ACE activity. Note that the 45%–85% decrease in ACE activity following acute ACE inhibition disappears at tissue sites, but not in the circulation, upon prolonged treatment. *, $p < 0.05$ vs untreated pigs

the tissue Ang II/I ratio in captopril-treated pigs is acutely decreased, but increases to normal values following prolonged ACE inhibitor treatment (Fig. 8) (van Kats et al. 1998, 2000, 2001). This phenomenon may be counteracted by increasing ACE inhibitor dosage (Jorde et al. 2000). As far as chymase is concerned, it is important to realize that chymase is largely located intracellularly (Urata et al. 1993). Studies demonstrating its significance, invariably measured Ang II generation in homogenized tissues, and evidence for chymase-mediated conversion of endogenous Ang I in humans is currently not available. In fact, when infusing large amounts of Ang I into the forearm of healthy volunteers (raising plasma Ang I by nearly four orders of magnitude), we were unable to demonstrate significant Ang II generation or Ang II-mediated vasoconstriction in the presence of the ACE inhibitor enalaprilat (Saris et al. 2000).

Furthermore, the phenotypes of ACE knockout mice and angiotensinogen knockout mice are similar (Tanimoto et al. 1994; Krege et al. 1995). This suggests that, also in mice, ACE is the main contributor to Ang II generation.

How do RAS blockers exert their effects if they result in increased or unaltered, rather than decreased plasma and tissue Ang II levels? One explanation might be that these increased Ang II levels result predominantly in AT_2 receptor activation. AT_2 receptors counteract the effects mediated by AT_1 receptors [they may in fact function as natural antagonists of AT_1 receptors (AbdAlla et al. 2001)], and their number increases under pathological conditions (Matsubara 1998; Siragy and Carey 1999; Schuijt et al. 2001) (Fig. 9). During AT_1 receptor blockade, it is obvious that increased Ang II levels result in AT_2 receptor stimulation. During ACE inhibition, increased Ang II levels will result in stimulation

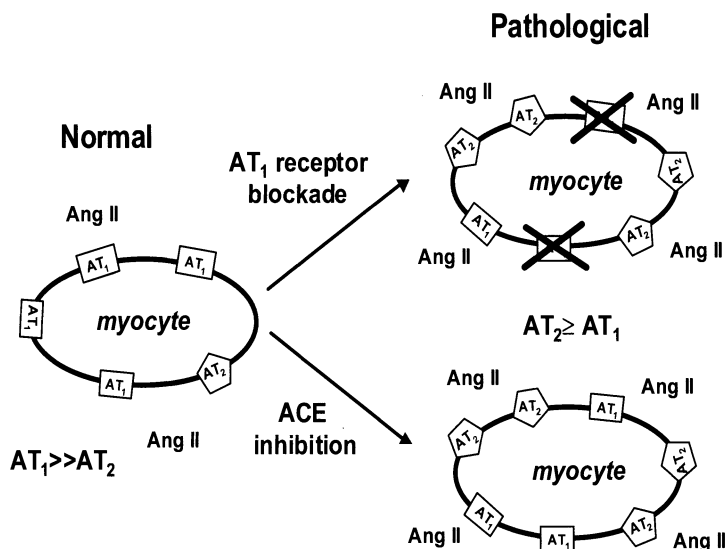


Fig. 9 Model explaining the cardiovascular effectiveness of ACE inhibitors and AT₁ receptor antagonists despite their ability to increase the levels of angiotensin (Ang) II at tissue sites. The rise in AT₂ receptor density under pathological conditions results in a predominance of AT₂ receptor-mediated effects, even when AT₁ receptors are not completely blocked

of both AT₁ and AT₂ receptors. However, the increase in AT₂ receptor density alters the net effect of Ang II. This concept is supported by studies showing that AT₂ receptor antagonism prevents the beneficial effects of AT₁ receptor blockade (Liu et al. 1997; Xu et al. 2002).

7

Conclusions

In conclusion, there is convincing evidence to support the production of both Ang I and Ang II at tissue sites. Intriguingly, in many tissues this production depends on circulating (i.e. kidney-derived) renin and/or prorenin. Circulating renin and prorenin diffuse into the interstitial space and bind to specific (pro)renin receptors, leading to generation of extracellular Ang I, either in the interstitium or on the cell surface. Although renin and prorenin are also internalized by various cells, the lack of intracellular angiotensinogen accumulation argues against significant intracellular angiotensin generation. Following its generation, tissue Ang II rapidly binds to AT₁ receptors and thereby increases its half-life from less than 30 s in the extracellular space to more than 10–15 min. This phenomenon may explain why tissue Ang II levels, particularly in organs with high AT₁ receptor densities (e.g. the adrenal), are several orders of magnitude higher than the levels of Ang II in blood, even when the level of Ang II generation is similar in both compartments. In addition, a large number of “free” AT₁ receptors, combined with low (i.e. comparable to blood plasma)

interstitial Ang II levels, offers an explanation for the high sensitivity of organs like the kidney and adrenal, to modest changes in arterial Ang II. Finally, because of the AT₁—AT₂ receptor shift that occurs under pathological conditions, the increase in tissue Ang II generation that is sometimes observed during treatment with RAS blockers (including ACE inhibitors) may result in net AT₂ receptor stimulation, thereby counteracting AT₁ receptor-mediated vasoconstriction and growth stimulation.

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Angiotensin–Endothelin Interactions

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Abstract Angiotensin II (Ang II) has been shown in several experimental conditions to stimulate the production of endothelin (ET), an endothelium-derived constricting factor. It is therefore not surprising that some of the physiological or pathological effects of angiotensin II are in fact mediated by endothelin through interaction with its specific receptors. For instance, ET receptor antagonists block part of the pressor effect of Ang II, especially when the treatment is initiated early. Accordingly, Ang II-induced vasoconstriction appears to be dependent on ET release, especially in smaller arteries. Moreover, blockade of ET receptors has a profound effect on Ang II-induced hypertrophic remodeling of small arteries. However, the contribution of ET appears to be limited to the initial phase of hypertrophy, as ET receptor antagonists do not regress already established vascular hypertrophy, but completely prevent it. Interestingly, ET also

appears to mediate part of Ang II-induced vascular superoxide production *in vivo*. Thus, blocking ET receptors may provide similar cardiovascular benefits as inhibiting the renin–angiotensin–aldosterone system, and the combination of inhibitors of both systems therefore appears counter-intuitive. However, experimental data strongly support added benefits of a combined treatment. Therefore, the interaction between the renin–angiotensin system and ET may become clinically significant if clinical trials confirm the already available experimental data.

Keywords Endothelin · Vascular remodeling · Hypertension · Oxidative stress

1 Introduction

The biology of the endothelin (ET) system has been reviewed in details elsewhere (Schiffrin and Touyz 1998; Miyauchi and Masaki 1999), but highlights will be introduced to provide an appropriate background to discuss the interactions between this system and the renin–angiotensin–aldosterone system (RAAS). In addition, the effect of ET and its antagonists (ETRA for ET receptor antagonists) will be discussed in pathological conditions relevant to the RAAS. Thus, this chapter will not discuss all the effects of ET and ETRA, but only those involved in interactions with local or circulating elements of the RAAS.

1.1 Biology of Endothelin

Endothelin-1 (ET-1), a 21 amino acid peptide, is the most studied member of a family of three peptides that includes ET-2 and ET-3 (Yanagisawa et al. 1988); all three peptides are encoded by different genes (Inoue et al. 1989a). It is mainly, but not exclusively, produced by endothelial cells (Miyauchi and Masaki 1999). Indeed, other cell types, including vascular smooth muscle cells (VSMC) and cardiomyocytes have also been shown to produce the peptide (Hahn et al. 1990; Miyauchi and Masaki 1999). ET-1 is generally considered a local factor that has both autocrine and paracrine functions. Most of its release, in fact, occurs abnormally rather than in the circulation (Wagner et al. 1992).

The promoter region of the gene contains consensus sequences for several transcription factors, such as nuclear factor-1 and activator protein (AP)-1 (Inoue et al. 1989a,b). Induction of prepro-ET mRNA transcription results in a rapid onset, and appears to be the rate-limiting step (Yanagisawa et al. 1988). ET-1 is generated from its precursor prepro-ET, in two steps: the first intermediate generated is a 38 amino acid peptide called Big-ET (Kido et al. 1997). The second cleavage is made by an ET converting enzyme (ECE), and seems to occur in vesicles that may act as temporary storage (Harrison et al. 1995; Russell et al. 1998). Mechanical factors such as shear stress and pulsatile stretch are known to modulate ET transcription. Several endogenous substances stimulate ET pro-

duction: thrombin, transforming growth factor β , tumor necrosis factor α , immunoglobulin-1, insulin, angiotensin (Ang) II, vasopressin, bradykinin and norepinephrine (Miyachi and Masaki 1999). Interestingly, the signaling pathways required to produce ET are similar to those used by ET (and other vasoactive substances such as Ang II) to elicit its cellular responses (Schiffrin and Touyz 1998; Miyachi and Masaki 1999). Indeed, increased intracellular calcium concentration, and phospholipase C (PLC), protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) activation are linked to ET production and to its biological actions. In contrast, nitric oxide, atrial natriuretic peptide, prostacyclin, and heparin are known inhibitors of ET production (Schiffrin and Touyz 1998; Miyachi and Masaki 1999).

Two ET receptors have been cloned in most mammalian species. Both belong to the superfamily of G protein-coupled receptors with seven-transmembrane domains. Both ET_A and ET_B receptors mediate contraction of VSMC, although the former seems the more important, efficaciously dominant (Seo et al. 1994; Moreau et al. 1997a). ET_B receptors on endothelial cells are also responsible for the release of endothelium-derived relaxing factors, such as nitric oxide and prostacyclin, which leads to vasodilatation (Moreau et al. 1997a). However, when injected systemically, ET elicits a short interval of hypotension followed by a long-lasting increase in blood pressure (Yanagisawa et al. 1988), suggesting that the vasoconstriction it induces is far more important than vasodilatation. In addition to its pressor effects, ET regulates cellular growth (hypertrophy) and proliferation, and could also influence cellularity by inhibiting apoptosis (Sharifi and Schiffrin 1997; Shichiri et al. 1997; Dao et al. 2001). In that respect, it is not clear if ET is a trophic factor or a mitogen *in vivo*, as *in vitro* data conflict (Hirata et al. 1989; Grainger et al. 1994; Miyachi and Masaki 1999). Interestingly, a similar debate on Ang II has been going on for years. ET is also involved in wound healing, a process that may be of importance in cardiovascular remodeling (Guidry 1992). The physiological effects of ET will be discussed in more details in relation with the RAAS in the next sections.

1.2

RAAS Modulation of ET Production

Cultured endothelial cells stimulated with Ang II show an increased expression of prepro-ET mRNA, (Emori et al. 1991). This was later confirmed by *in vivo* administration of Ang II (Dohi et al. 1992). In addition, we have observed that *in vivo* (aorta, kidney, and mesenteric resistance arteries) ET content is increased when rats are chronically treated with Ang II (Barton et al. 1997; Moreau et al. 1997b). Furthermore, Larivière et al. have shown that tissue ET levels correlate better with local Ang II concentrations than with circulating Ang II levels (Larivière et al. 1998). Interestingly, cultured VSMC, fibroblasts, and cardiomyocytes also express ET-1 when stimulated with Ang II, extending the interaction to non-endothelial cells (Hahn et al. 1990; Resink et al. 1990; Ito et al. 1993; Sung et al. 1994). Moreover, in transgenic mice expressing both human re-

nin and angiotensinogen genes, cardiac concentrations of ET are elevated (Maki et al. 1998). ET may amplify its own production by an autocrine mechanism operating through ET_A receptors, possibly by increasing ECE activity (Alberts et al. 1994; Fujisaki et al. 1995; Barton et al. 1997).

The opposing paradigm (i.e., inhibition of the RAAS) is associated with reductions of vascular and renal ET levels, demonstrating that the interaction is physiologically relevant and operates endogenously (Larivière et al. 1998; Dumont et al. 2001). Furthermore, ACE inhibitors have been found to reduce plasma ET-1 levels in patients with hypertension (Horky et al. 1993), congestive heart failure (Galatius-Jensen et al. 1996), and myocardial infarction (Dipasquale et al. 1997). Thus, *in vitro* and *in vivo* data suggest that the RAAS can stimulate endogenous ET production.

The question that arises from this positive interaction is whether endogenous ET mediates some of the effects attributed to Ang II. The best approach for determining the contribution of endogenous substances on physiological functions is to use their receptor antagonists. Fortunately, and because of the potential role of ET in several cardiovascular pathologies, its receptor antagonists (ETRA) were developed rather quickly after the discovery of the peptide in 1988. Orally active, non-peptidic ETRA are now available and one of them, bosentan, has recently been approved for treatment of pulmonary hypertension. Since Ang II stimulates ET production, it was logical to evaluate the contribution of ET in Ang II-induced cardiovascular effects.

1.3

ET Modulation of the RAAS

Contrary to the mechanisms discussed in the previous section, several lines of evidence also support the concept of ET-induced modulation of RAAS activity. Indeed, numerous studies have reported an inhibition of renin release from juxtaglomerular cells by ET, suggesting that ET can decrease the activity of circulating RAAS (see Rossi et al. 1999 for a review). Several mechanisms, including ET_B-mediated release of NO or prostacyclin, may contribute to this inhibition. However, the physiological relevance of this modulation remains unclear. It must be emphasized that ET should not be considered as a circulating hormone, but rather as a local regulator. As such, its effects on the RAAS activity should be considered only when local synthesis of the peptide is evident. Thus, *in vitro* results should be interpreted with caution. Accordingly, under control conditions or after renal clipping, the administration of an ETRA did not modulate renin expression or plasma renin activity (PRA) in rats (Schricker et al. 1995). In dogs, however, ETRA treatment increased PRA twofold and enhanced the sensitivity of pressure-dependent renin release at lower perfusion pressures (<80 mmHg), suggesting a physiological role for that interaction (Berthold et al. 1999).

In contrast, ET has been reported to stimulate the production of aldosterone equipotently to Ang II in the adrenal cortex (Rossi et al. 1997, 1999). According-

ly, zona glomerulosa cells express both subtypes of ET receptors (Imai et al. 1992). It also appears that, in the rat, ETB receptors are involved in the production of aldosterone (Belloni et al. 1996), while both subtypes are involved in man (Rossi et al. 1997). Thus, the ET system could amplify the release of aldosterone, which is now becoming an important facilitator of end-organ damage in cardiovascular diseases.

2 Hemodynamic Effects

2.1 Angiotensin II-Induced Hypertension

Elevation of arterial pressure is a global hemodynamic index that reflects more than just the balance between vasoconstriction and vasodilatation; it is also a clinically meaningful parameter that is partially under the control of the RAAS. Several mechanisms are thought to contribute to this hemodynamic effect of Ang II and aldosterone; notably, vascular constriction and regulation of renal sodium. Inhibition of ET receptors was reported to blunt Ang II-evoked elevations in blood pressure, in normotensive and hypertensive rats (Balakrishnan et al. 1996). Most studies on chronic administration of Ang II reported some hypotensive effects with ETRA treatment (Moreau et al. 1997b; Rajagopalan et al. 1997; Herizi et al. 1998; see Dao and Moreau 2001 for a review). As the blood pressure reduction was only partial (approximately 50%), it is likely that some hypertensive actions of Ang II are not ET-mediated. Interestingly, the hypotensive effect of ETRA is mainly observed when administered during the development of hypertension (Fig. 1). When treatment is undertaken after establishment of hypertension (10–14 days of Ang II administration), ETRA often fails to lower arterial pressure (Dao et al. 2001; Ficai et al. 2001). In the 2 K/1C model, where the early phase of hypertension development depends on the RAAS, only one in four chronic studies reported a partial reduction of hypertension (Li et al. 1996; Ehmke et al. 1999; Hocher et al. 1999; Bianciotti and de Bold 2001). In the positive study, the ETRA administration occurred early, i.e., concurrently with the clipping of the renal artery. In a 2-day study on the same model, a blunting of pressure elevation was observed following ETRA treatment started at the time of the clipping (Schricker et al. 1995).

In the transgenic (mREN2)²⁷ model, which was obtained by inserting the mouse Ren-2 gene into the genome of normotensive Sprague-Dawley rats, acute ETRA treatments were effective in lowering arterial pressure (Gardiner et al. 2000). However, when started late (4 weeks of age), the chronic ETRA treatment did not induce hypotension in this model (Rossi et al. 2000). In another model in which both human renin and angiotensinogen genes were inserted, rats treated from week 4–7 with an ETRA had a reduction of 40% of their systolic pressure (Muller et al. 2000), while treatment from weeks 6–10 was ineffective

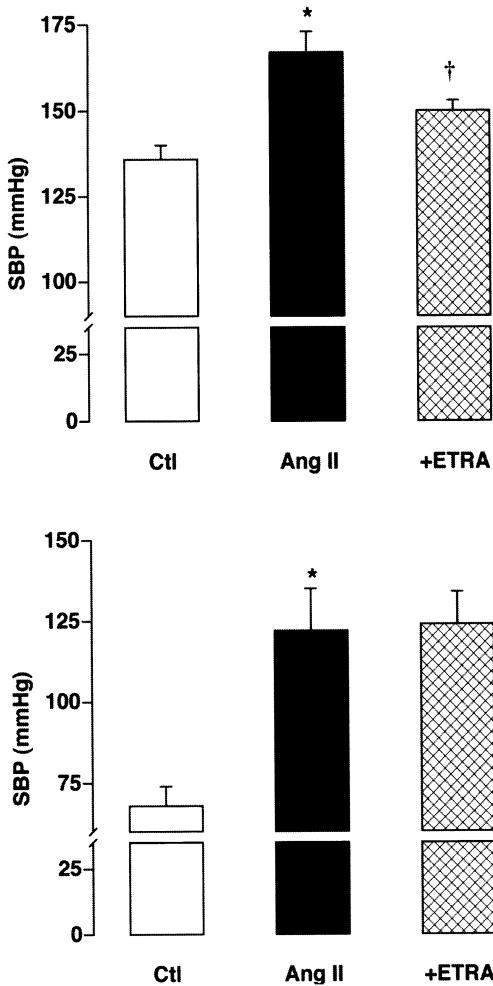


Fig. 1 Upper panel: systolic blood pressure (SBP) measured by tail cuff plethysmography in conscious rats treated for 2 weeks with angiotensin II alone (*Ang II*, 200 ng/kg/min) or in combination with an endothelin receptor antagonist (*+ETRA*, darusentan 30 mg/kg/day). (Adapted from Moreau et al. 1997b). The lower panel represents the same hemodynamic parameter measure in anesthetized rats treated for 4 weeks with *Ang II* alone or with an ETRA only for the last 2 weeks (weeks 3 and 4). (Adapted from Dao et al. 2001). *, $p < 0.05$ as compared to control values; †, $p < 0.05$ as compared to *Ang II* values

(Bohlender et al. 2000). This, again, suggests that early rather than late treatments with ETRA are more effective to lower arterial pressure.

Very recent observations also suggest an involvement of ET in the pressor effect of aldosterone (Park and Schiffrin 2001a). Indeed, simultaneous administration of an ETRA completely prevented the pressure elevation, suggesting a greater involvement of the peptide in aldosterone-induced hypertension than in the *Ang II*-induced form of the disease.

2.2

Vascular Reactivity

One mechanism whereby ET can participate in the hypertensive effect of Ang II is direct vasoconstriction. ET released from the endothelium was found to mediate part of the vasoconstriction induced by Ang II (Chen et al. 1995). In a very elegant study, Chen et al. showed that although the involvement of ET in Ang II-induced vasoconstriction is absent in the aorta, its contribution progressively increases as arteries decrease in size (Chen et al. 1995). This is of importance, considering that the control of arterial pressure lies downstream in the vascular tree. Moreover, in the human skin microcirculation, a selective ET_A-receptor antagonist blunted the Ang II vasoconstriction, suggesting that this interaction is also relevant in man (Wenzel et al. 2001).

Very low concentrations of ET, although not inducing vasoconstriction, have been shown to amplify the vascular response to other vasoconstrictors (Yang et al. 1990). Such an effect is physiologically important because in pathological conditions the peptide is present in low basal concentrations and its fluctuations are limited. Dohi et al. have reported that the ET produced by Ang II is sufficient to amplify norepinephrine (NE)-evoked contractions (Dohi et al. 1992).

Exogenous Ang II-induced hypertension is associated with blunted acetylcholine-induced relaxation (d'Uscio et al. 1997), and as ACE inhibitors generally improve endothelium-dependent vasorelaxation (Kahonen et al. 1995), endogenous Ang II was also postulated to influence this vasodilatory homeostatic mechanism. Ang II-induced alteration of endothelium-dependent response was normalized by an ETRA selective for ET_A receptors (d'Uscio et al. 1997). The unopposed endothelial ET_B-receptor stimulation, and subsequent release of NO and prostacyclin, represents a probable mechanism for improvement of endothelium-dependent relaxations, considering the enhanced local ET levels produced by Ang II. Blocking ET receptors during Ang II administration could therefore lower arterial pressure by reducing ET-mediated vasoconstriction, preventing its amplification of other vasoconstrictors, and restoring endothelial vasodilatory functions.

3

Cardiovascular Remodeling

Both Ang II and ET are trophic factors capable of influencing cardiovascular structure. Cardiovascular remodeling is believed to be initially beneficial, but has deleterious consequences for system integrity under chronic conditions. Since these changes are structural, they are not as easily reversible as vasoreactivity changes, and their prevention or regression still presents therapeutic challenge.

3.1 Small Arteries

Changes in the structure of small arteries induced by hemodynamic changes or vascular remodeling, can also contribute to the maintenance of hypertension (Heagerty et al. 1993; Mulvany et al. 1996). Ang II stimulates hypertrophic remodeling of resistance arteries (Griffin et al. 1991; Simon and Altman 1992; Moreau et al. 1997b; Simon et al. 1998), defined as increased media thickness, media/lumen ratio, and cross-sectional area (Heagerty et al. 1993). Since Ang II stimulates local ET production, we postulate the involvement of endogenous ET

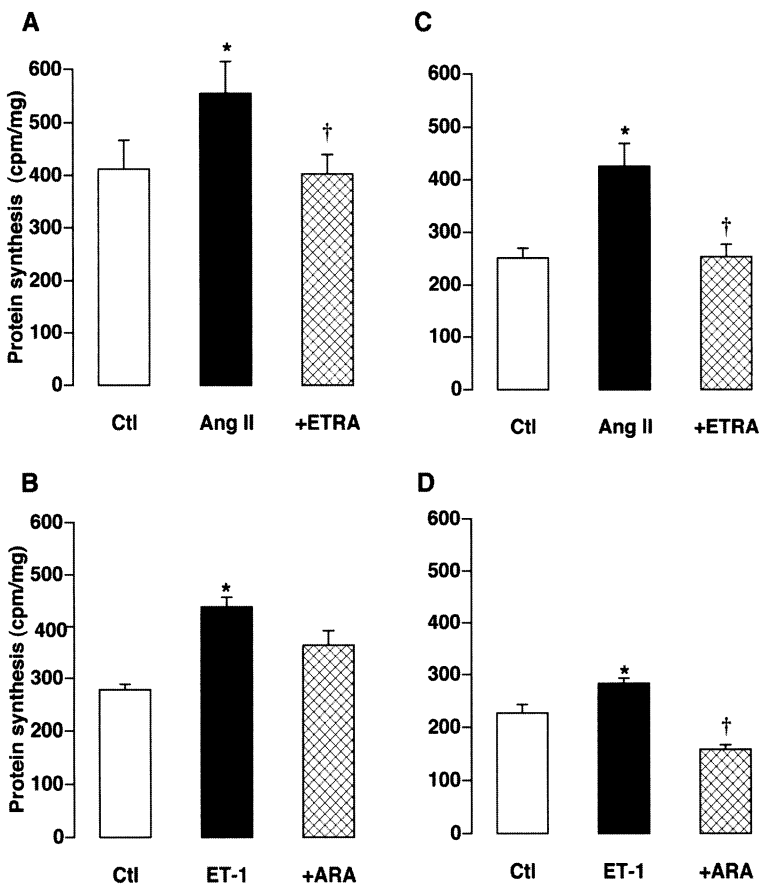


Fig. 2A–D In vivo protein synthesis measurements in small mesenteric arteries (A) and in the aorta (C) expressed as cpm of incorporated [3 H]-leucine in vivo in control rats (Ctl) and rats treated for 24 h with exogenous angiotensin II alone (Ang II, 400 ng/kg/min) or in combination with an endothelin receptor antagonist (+ETRA, darusentan of 30 mg/kg/day). The same measurements in rats treated with exogenous ET-1 alone (5 pg/kg/min) or combined with an angiotensin receptor antagonist (irbesartan 10 mg/kg/day) in mesenteric arteries (B) and in the aorta (D). (See Martens et al. 2002 for methodological details.) *, $p < 0.05$ as compared to control values; †, $p < 0.05$ as compared to Ang II or ET-1 values

in Ang II-induced vascular hypertrophy. The administration of an ETRA, which blocks ET receptors specifically, totally prevented Ang II-induced hypertrophic remodeling of small arteries (Moreau et al. 1997b). Similar results were obtained, with an ET_A-selective ETRA for intramyocardial arteries, in the 2 K/1C model (Hocher et al. 1999). The most likely explanation for this phenomenon is that Ang II stimulates local production of ET that, in turn, mediates vascular hypertrophy. Blockade of ET_A-receptors on VSMC then inhibits the trophic response. In a model developed in our laboratory to acutely measure *in vivo* vascular protein synthesis, a good predictor of hypertrophy, (Martens et al. 2002; Voisin et al. 2002), concomitant administration of an ETRA prevented Ang II-induced protein synthesis in small mesenteric arteries during the first 24 h of administration (Fig. 2A). In contrast, an AT₁ receptor antagonist failed to influence exogenous ET-induced protein synthesis, suggesting that the interaction is not reciprocal (Fig. 2B). Treatment with an ETRA also prevented vascular remodeling of small arteries in a model of aldosterone-induced hypertension (Park and Schiffrin 2001a).

Our group recently found that when hypertrophy of small arteries is allowed to proceed, the inhibition of ET receptors did not regress the process (Dao et al. 2001). Thus, it can be concluded that ET is transiently involved in development of Ang II-induced hypertrophy, but not in the maintenance phase. Similar conclusions can also be drawn from norepinephrine- and pulse pressure-induced hypertrophy (Dao et al. 2001, 2002). This concept may help explain some discrepancy in the literature: where early treatments with ETRA were more effective in limiting hypertrophic remodeling than those started after remodeling has occurred. Similarly, the hypotensive effects of ETRA in Ang II-dependent models of hypertension also appear to be optimal when treatments are started early (see above).

3.2

Large Arteries

One of the most striking observations concerning the interaction between the two peptides is that even in cell culture conditions, an ETRA can prevent Ang II-stimulated cell growth (Ito et al. 1993; Fujisaki et al. 1995). Thus when Ang II is used to stimulate hypertrophy in cultured VSMC, some signaling events are involved in the production of ET, while others mediate the trophic actions of ET. Consequently, using the more direct effector (ET in this example) as the stimulus would be more appropriate when studying mechanisms related to vascular hypertrophy. If this paradigm were transposed *in vivo*, VSMC should also use ET as an autocrine growth regulator. ETAs have been shown to prevent the effect of Ang II-induced large artery remodeling (Herizi et al. 1998; Ficai et al. 2001). In the aorta, Ang II-induced *in vivo* protein synthesis was blunted by an ETRA (Fig. 2C). As in small arteries, the effect of the ETRA was restricted to early treatments, since giving the treatment after the establishment of hypertension with Ang II did not regress carotid artery hypertrophy (Ficai et al. 2001).

In contrast to the situation in small arteries, blocking AT₁ receptors also reduced ET-1-induced aortic protein synthesis (Fig. 2D). Thus, the trophic interaction between the two peptides may be mutual. However, to the best of our knowledge these preliminary observations, with ET as the agonist, were never tested *in vivo*. Exogenous ET amplifies neointimal formation after balloon injury in the rat (Barolet et al. 2001). Although treatment with an AT₁ receptor antagonist (ARA) could prevent or regress neointimal hyperplasia (Lemay et al. 2000; Lemay and Deblois 2002), we are not aware of any studies where ARA was administered in the presence of exogenous ET administration. Protein synthesis data following 24-h stimulation suggest that ET could be involved in both local Ang II production and action in the large arteries. Similar conclusions can be drawn for aldosterone, as ET has been shown to modulate aldosterone production (see above), and the effects of aldosterone on the cardiovascular system, such as fibrogenesis and hypertrophy, can be prevented by treatment with an ETRA (Park and Schiffrin 2001a,b).

3.3 Cardiac Remodeling

As previously reported, Ang II stimulates ET-1 production in cardiomyocytes and fibroblasts (Ito et al. 1993; Gray et al. 1998). Accordingly, ET receptor blockade abolished the hypertrophic effects of Ang II in these cell lines (Ito et al. 1993). This action may be due to either an autocrine or a paracrine mechanism, thus implicating an involvement of fibroblasts (Gray et al. 1998). It is logical to postulate that ET may, at least partially, mediate the cardiac trophic effects of Ang II in pathological conditions that implicate the RAAS. Exogenous Ang II administration does not increase the *in vivo* myocardial content of ET-1 (Barton et al. 1998). Furthermore, from *in vivo* studies it is not clear whether the reduction of left ventricular hypertrophy (LVH) with ETRA is only secondary to a reduction in blood pressure (see Dao and Moreau 1999 for a review). In a recent study, hydralazine was co-administered with Ang II to prevent blood pressure elevation, but an ETRA was not effective in preventing ventricular hypertrophy (Moser et al. 2002). In rats overexpressing the Ren2 gene, cardiac function was enhanced without amelioration of cardiac remodeling and fibrosis (Rothermund et al. 2000). At variance with these findings, two studies showed a pressure-independent effect of an ETRA, the first in the prevention of ventricular hypertrophy in 2K/1C rats (Ehmke et al. 1999) and the second in its regression in Ang II-induced hypertension (Ficai et al. 2001). Overall, the situation is complex and several factors must be considered when interpreting these results, including hemodynamics and the model of LVH used.

4

Renal Interactions

Renal protection by ETRA, both in context of hypertension and renal failure, is quite remarkable and clearly independent of effects on arterial pressure (Rabelink et al. 1998; Moreau and Rabelink 1999). Endothelin seems to be involved in key events leading to chronic renal failure, such as mesangial cell proliferation, extracellular matrix production, and renal fibrosis. When transgenic rats expressing the human renin and human angiotensinogen genes were treated with ETRA, expression of markers of renal inflammation were blunted (Muller et al. 2000). These results are in line with demonstrations that Ang II stimulates renal ET production (Barton et al. 1997, 1998; Alexander et al. 2001), and that ARA decrease glomerular ET levels in a model of renal insufficiency (Dumont et al. 2001). Blockade of ET receptors was found to improve renal function in models of Ang II hypertension (Herizi et al. 1998; Alexander et al. 2001; Riggelman et al. 2001). In addition, the increase in sodium sensitivity associated with chronic exogenous Ang II administration was reported to be mediated by ET_A receptors (Ballew and Fink 2001).

5

Oxidative Stress

Oxidative stress is a consequence of the oxidant properties of free radicals and molecules that are produced mainly by mitochondria, or enzymes such as NAD(P)H [nicotinamide adenine dinucleotide (phosphate), reduced] oxidase, xanthine oxidase or NO synthase (Wallace and Melov 1998). Reactive oxygen species (ROS), apart from their capacity to induce damages to cells and tissues, can impair endothelial function (Heitzer et al. 2001) and are associated with increased blood pressure (Wu et al. 2001). ROS also promote cellular growth, and are suspected of playing important roles in vascular remodeling (Mezzetti et al. 1999). A number of studies have clearly established that Ang II can stimulate superoxide anion production by activating NAD(P)H oxidase (Griendling et al. 1994; Rajagopalan et al. 1996; Touyz and Schiffrin 2001). Thus, it may be postulated that ROS production is likely to play an important role in cardiovascular disorders involving Ang II, such as cardiac and vessel wall hypertrophy or atherosclerosis.

Taking into consideration the aforementioned interactions between Ang II and ET, and the similarities between ET- and ROS-induced vasoconstriction and cell proliferation, it is likely that ROS are closely related either to the production or signaling of ET. Treatment of human aortic or coronary smooth muscle cells with hydrogen peroxide or xanthine/hypoxanthine oxidase (a superoxide anion producer) increased ET synthesis (Kahler et al. 2001; Ruef et al. 2001). This up-regulation of ET production by oxidative stress was also observed in endothelial cells (Saito et al. 2001). Moreover, it was noted that treatment with tempol and vitamin E, two antioxidants, can block the increase of plasma ET levels follow-

ing chronic Ang II administration (Ortiz et al. 2001). ROS thus appear to be important mediators of Ang II-induced ET production.

In various cell types, such as intestinal mucosae (Oktar et al. 2000), VSMC (Wedgwood et al. 2001a,b), and monocytes (Huribal et al. 1994), ET appears to participate in a positive feedback loop where it increases ROS production, and it is increased by oxidative stress. The mechanisms underlying superoxide anion production are still unclear, but several studies suggest that NAD(P)H oxidase can be directly activated by ET (Duerrschmidt et al. 2000; Fei et al. 2000). These data show that in addition to being stimulated by ROS, the ET system can also

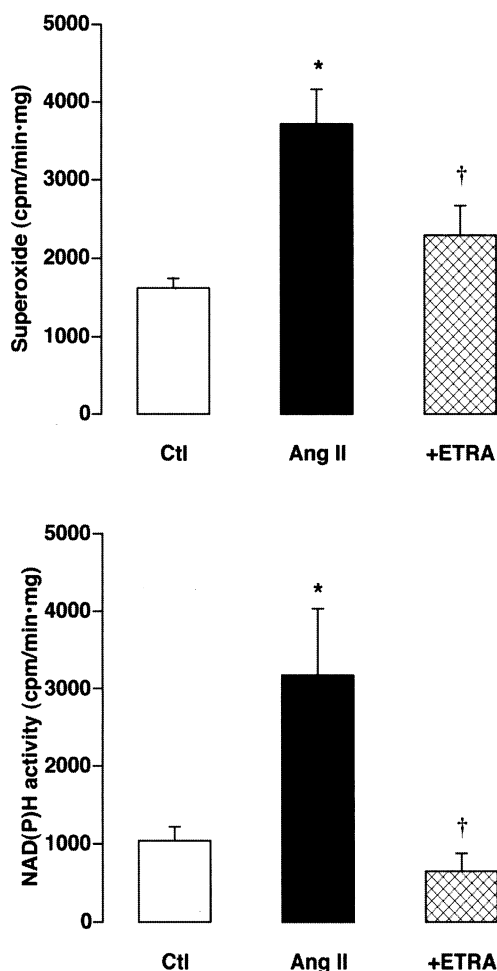


Fig. 3 Upper panel: measurement of superoxide production by lucigenin (5 mM)-enhanced chemiluminescence in the aorta of control (Ctl), angiotensin II (Ang II, 200 ng/kg/min for 12 days), or Ang II in combination with an endothelin receptor antagonist (+ETRA, LU302872 30 mg/kg/day). Lower panel: measurement of aortic NAD(P)H oxidase activity in the same groups, estimated by the superoxide anion formation upon stimulation with NADH (10^{-4} M) after subtraction of basal production. *, $p < 0.05$ as compared to control values; †, $p < 0.05$ as compared to Ang II or ET-1 values

contribute to ROS production by activating similar signaling pathways as Ang II; this suggests a positive interaction (and potential mutual amplification) between the two systems in promotion of oxidative stress.

Unfortunately, most studies investigating the interaction between the RAAS and ET system were performed *in vitro*. Superoxide production by NAD(P)H oxidase in aortic tissues taken from rats continually treated with Ang II (200 ng/kg day) for 12 days, was studied in our laboratory. Superoxide production was found to be greatly enhanced following chronic Ang II treatment (Fig. 3, upper panel). Concomitant *in vivo* treatment with LU302872, a non-selective ET_A and ET_B receptor antagonist, markedly blunted Ang II-induced superoxide anion production, supporting the hypothesis that this process is dependent on local production of ET. In addition, this Ang II-induced activation of NAD(P)H oxidase was prevented by blockade of ET receptors (Fig. 3, lower panel). Although, little is known about the functional interactions between ET, oxidative stress, and Ang II, our latest results suggest a strong and complex association between these three factors in the control of vascular functions.

6

Combination of ETRA with Inhibitors of the RAAS

Considering the participation of ET in some of Ang II *in vivo* action, the combination of an ETRA and ARA does not appear logical. Both antagonists would work on the same pathway and could have additive effects, at best. Accordingly, ACE inhibitors have been shown to decrease circulating ET levels in different pathological conditions, suggesting that blocking the RAAS functionally affects the ET system (Horky et al. 1993; Uemasu et al. 1994; Galatius-Jensen et al. 1996; Dipasquale et al. 1997).

However, the results of several preclinical and clinical studies suggest otherwise. In a detailed hemodynamic study performed in normotensive and transgenic (mRen-2)27 hypertensive rats, Gardiner et al. showed that the acute fall in blood pressure, over 8 h in hypertensive rats, was cumulative when combining treatments with ARA and ETRA: these treated hypertensive rats obtained a 60-mmHg reduction in blood pressure (Gardiner et al. 1995). In SHR and SHR-SP (stroke-prone), the combination of an ARA with an ETRA also showed an additive hypotensive effect (Ikeda et al. 2000). In Dahl salt-sensitive rats, the ARA, which had no effects on its own, amplified the hypotensive effects of the ETRA (Ikeda et al. 2000). In a study that explored the mechanisms whereby ETRA treatment can amplify the response to inhibitors of the RAAS (and vice versa), the authors reported that blockade of ET receptors increased the sensitivity of pressure-dependent renin release at lower perfusion pressures (Berthold et al. 1999). Thus, it appears that RAAS inhibition is more effective in the presence of ETRA than in its absence. In addition to their hypotensive effect, the combined inhibition of the two systems could also limit end-organ damage either indirectly, by favoring their hemodynamic effects, or directly, by blocking the effects of peptides on protein synthesis and/or ROS generation. Perhaps the most striking

demonstration of the benefits of combined therapy comes from the study of Bohlender et al. (2000). They showed, in the hRen/hAgt transgenic rats, that individual treatments (low-dose ARA or therapeutic-dose ETRA) had little effect on pressure and the high mortality rate at 10 weeks (42%), but the combined therapy normalized blood pressure and totally prevented mortality in those rats.

In a model of hypertension-induced LVH in transition to heart failure, the combination of an inhibitor of the RAAS with an ETRA was effective in improving survival, possibly because each peptide plays variable pathophysiological roles during the transition (Iwanaga et al. 2001). In the acute phase following an experimental myocardial infarction (MI), the combined treatment was also superior to individual treatments, in the prevention of fibrosis (Tzanidis et al. 2001). Moreover, long-term combined ETRA and ACE inhibition was shown to more effectively improve the progression of cardiac failure following extensive MI, than monotherapy (Fraccarollo et al. 2002). A favorable cardiac hemodynamic effect was also observed in another study of post MI-induced heart failure rats, in which ETRA was administered acutely on top of chronic ACE inhibition (Qiu et al. 2001). In a model of congestive heart failure induced by rapid atrial pacing in pigs, the combination of an ARA and an ETRA provided the best recovery of LV function (New et al. 2000). In patients with congestive heart failure receiving an ACE inhibitor as standard therapy, the addition of an ETRA improved the hemodynamic profile of patients, suggesting that this combined therapy may provide an interesting alternate treatment (Kiowski et al. 1995; Love et al. 1996; Sutsch et al. 1998).

Several studies focusing on the kidneys have also revealed unexpected benefits for combining the inhibitors of the RAAS and ET system. Benigni et al. reported that an ACE inhibitor and an ETRA have beneficial additive effects, in a model of nephropathy with proteinuria (Benigni et al. 1998). Moreover, in a dog model of renovascular hypertension, the combined therapy of an ARA with ETRA produced additional hypotension, as compared to the treatment with ARA alone (Massart et al. 1998). More recently, the effectiveness of combined therapy to attenuate glomerulosclerosis was confirmed in a low-renin model of partial kidney resection, independent of pressure reduction (Amann et al. 2001). However, albuminuria was not improved by the combined treatment. Along the same lines, this combined therapy was effective in reducing blood pressure, but did not ameliorate renal damage in a low renin model of hypertension (DOCA-salt model) (Pollock et al. 2000). In partially nephrectomized rats, a model of progressive renal injury, the inhibition of the RAAS (by ACE inhibitor or ARA) improved glomerular filtration rate, proteinuria, glomerulosclerosis, and tubular injury; but the additional inhibition of the endothelin system did not produce any additional effect (Cao et al. 2000).

Overall, most of the preclinical data suggest that a combined therapy of RAAS and ET inhibitors can amplify the beneficial effect of each individual treatment; one possible exception is the treatment of certain renal diseases. Although the RAAS requires the ET system for several of its cardiovascular effects, inhibiting both systems appears favorable. Available observations indicate either that the

interaction between the two systems is more complex than anticipated, or that the individual antagonist does not produce full inhibition of their respective system. In that respect, it is noteworthy that only one dose of each antagonist is generally used in most combination studies, and in some circumstances, the dosage was even selected to provide a minimal effect in order to favor an improvement. Although it remains to be established whether combined therapy would be better if one of the two systems was fully inhibited, the current therapeutic trend to decrease the dosage of single drugs in favor of low-dose combinations would support the clinical relevance of this combined therapy.

7

Conclusion

Interactions between the RAAS and ET system can have clinical significance in the evolution of cardiovascular diseases and their respective treatments. The RAAS has profound effects on the cardiovascular system, as exemplified by the numerous clinical applications of ACE inhibitors, ARA and aldosterone antagonists. However, at the vascular and renal levels, endothelial ET, acting mainly through its specific receptors located on VSMC and mesangial cells, seems to mediate many effects of Ang II, such as vasoconstriction, hypertrophy, fibrogenesis, and oxidative stress (Fig. 4). Thus, ETRA may represent a new therapy for

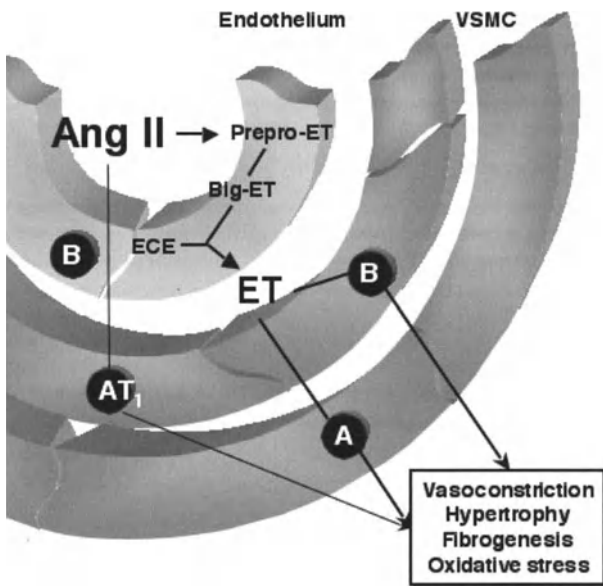


Fig. 4 Schematic representation of angiotensin II (*Ang II*) and endothelin (*ET*) interaction in the vessel wall. Although *Ang II* produces some direct effects, several of its actions are mediated, at least in part, by local *ET* and can be effectively blocked by endothelin receptor antagonists. *A*, endothelin A receptor; *AT*₁, angiotensin II type 1 receptor; *B*, endothelin B receptor

fine-tuning the RAAS at the level of the arteries and kidneys. Considering the rather low antihypertensive efficacy of ETRA and their superior ability to limit end-organ damage, both ETRA and inhibitors of the RAAS could be used most profitably in combination to maximize therapeutic benefits.

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Part 2
Genetics

Genetics of the Human Renin–Angiotensin System

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Abstract Genes of the human renin–angiotensin system have been extensively studied within the last 10 years. A large number of polymorphisms have been described and tested in linkage and association studies in regard to cardiovascular traits such as essential hypertension, coronary disease, and diabetic nephropathy. Gene structure, polymorphisms, and associations are summarised for each gene. Association between polymorphisms of the angiotensinogen and angiotensin I-converting enzyme genes and the corresponding plasma concentrations have been well demonstrated. However, possible relationships with hypertension or end-organ damage are debated. Other surprising findings have been observed such as mutations of the angiotensin type 2 receptor in X-mental retardation. Future studies should use more integrated approaches, based on the genotyping of haplotypes, the measurement of sophisticated phenotypes, the evaluation of gene–gene and gene–environment interactions. As well, genetic investigation of new components of the renin–angiotensin system such as the prorenin receptor, the angiotensin-converting enzyme type 2, and the aminopeptidase A and N genes are necessary. Prediction of drug efficacy and/or of adverse drug reaction on a pharmacogenetic profile will need large, well-designed trials in which patients will be standardised on the drug dosage and metabolism.

Keywords Renin · Angiotensin · Polymorphism · Gene · Hypertension

1 Introduction

The genes of the renin–angiotensin system are probably those that have attracted the greatest attention of researchers working on the molecular pathophysiology of hypertension and cardiovascular diseases. During the last 10 years, considerable progress has been achieved, since the genes encoding all the proteins of the renin–angiotensin system have been cloned in humans and rodents, informative markers and polymorphisms identified, and numerous linkage and association studies performed using different phenotypes. We will not review here the important results that have been generated by experimental transgenic models (reviewed in other chapters of this book) and will focus, instead, on the results obtained on the human genes.

2 The Angiotensinogen Gene

2.1 Gene Structure, Mutations and Polymorphisms

The human angiotensinogen (AGT) gene belongs to the superfamily of serpins and is localised to chromosome 1q42.3. The human angiotensinogen gene contains 5 exons with an organisation that is similar to other serine protease inhibitors (Kageyama et al. 1984; Gaillard et al. 1989).

A large number of single nucleotide polymorphisms (SNPs) have been described in the 5' flanking region, exons, introns and 3' part of the AGT gene (www.ncbi.nih.nlm.gov/SNP). Among them, the coding SNP (M235T) in exon 2 and the G-6A nucleotide substitution at position -6 upstream from the initial transcription start, have been particularly studied. These two polymorphisms occur almost at the same frequency and are in complete linkage disequilibrium (Inoue et al. 1996; Jeunemaitre et al. 1997). Thus, in a given case-control study, the analysis of the M235T polymorphism will be equivalent to that of the G-6A, and vice-versa. Using several constructs and luciferase assays, Lalouel and colleagues were able to show that the G-6A substitution is associated in vitro with an increased expression of the AGT gene, a possible explanation to the association of the M235T polymorphism with increased plasma angiotensinogen (Inoue et al. 1997). Recently, Nakajima et al. (2002) showed a specific interaction between the G-6A substitutions and the nuclear factor YB1. In cotransfection experiments, YB1 reduced basal AGT promoter activity in a dose-dependent manner. Although these observations suggested a possible role for YB1 in modulating AGT expression, this function was thought likely to occur in the context of complex interactions involving other nuclear factors. However, the true in vivo biological effect of this polymorphism may be more complex, since other polymorphisms, C-532T, A-217G, C-18T, A-20C, T+31C, also in linkage disequilibrium with G-6A and M235T (Fig. 1), might play a role in the variation of transcription of the gene (Sato et al. 1997; Jeunemaitre et al. 1999; Paillard et al. 1999; Ishikawa et al. 2001).

The C/A polymorphic site at position -20 is located between the TATA box and the transcription initiation site, within a sequence that can bind the estrogen receptor (Zhao et al. 1999). The orphan receptor Arp-1 also binds to this sequence and reduces in vitro the oestrogen receptor-induced promoter activity (Narayanan et al. 1999). The A/G polymorphic site at position -217 has recently been studied in more details (Jain et al. 2002). The authors showed that the surrounding sequence corresponds to a consensus C/EBP binding site, and that reporter constructs containing the human AGT gene promoter with nucleoside A

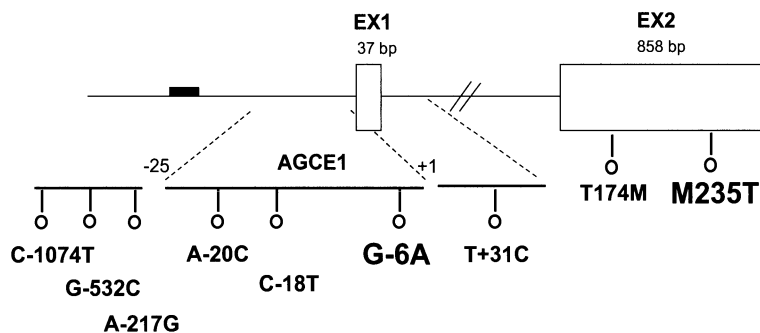


Fig. 1 Main polymorphisms of the human angiotensinogen gene

at -217 have increased basal promoter activity in Hep G2 cells. In addition, they found an increased frequency of the -217 A allele in 186 African-American hypertensive subjects compared to 156 normotensive (0.29 vs 0.19, $p=0.002$).

The ethnic origin has a strong effect on the allele frequency of most of the polymorphisms found at the AGT locus. For example, the 235T allele frequency varies from 40% in Caucasians to 70% in the Asian and 80%–90% in the African-American population and even 93% in Nigerians (Rotimi et al. 1994).

2.2

Relation with Plasma Angiotensinogen

AGT, the renin substrate, is mainly synthesised by the liver and is the unique substrate for renin. In humans, plasma AGT levels are around the K_m of renin, and therefore it is logical to suspect that a chronic state of increased plasma AGT might increase angiotensin I (Ang I) and facilitate hypertension and/or cardiovascular diseases. Its role in human hypertension was suspected in an epidemiological study where a strong correlation was found between plasma AGT concentration and blood pressure (BP) (Walker et al. 1979) and in another study where offspring of hypertensive patients had elevated plasma AGT levels (Fasola et al. 1968). Heritability of plasma AGT has been suggested. Segregation analysis in 685 members of 186 families recruited from a rural community in southwest Nigeria suggested that about 13% of the variance in plasma AGT concentration was due to the recessive gene segregation (Guo et al. 1999).

Among the 15 polymorphisms that we initially identified, two of them leading to amino acid changes, 174 M and M235T, were found to be associated with hypertension and plasma AGT concentration (Jeunemaitre et al. 1992c). This association between plasma AGT level and the M235T genotype was further confirmed in white children (Bloem et al. 1995). In African-American young individuals, Pratt and colleagues showed an association between a haplotype containing the T235 allele and a polymorphism located in the promoter region of the gene (Bloem et al. 1997). In a large sample of the Monica Augsburg cohort, a mild co-dominant and significant increase of plasma AGT concentration was also observed according to the M235T polymorphism (Schunkert et al. 1997). In the Danish general population, homozygosity for both the 235T and T174 allele was associated with a 10% increase in plasma AGT (Sethi et al. 2001). Because of the intra and interassay variability of the plasma AGT measurement and the mild association with the M235T polymorphism, a large number of individuals is required to detect this relation.

To analyse the influence of the M235T polymorphism on the ethinylestradiol-induced increase in plasma AGT concentration and on the resulting generation of Ang I and Ang II in plasma, we compared changes in the circulating renin-angiotensin system after short-term (2 days) and repeated (7 days) administration of 50 μg ethinylestradiol in homozygous normotensive men (TT and MM) (Azizi et al. 2000). In the 7-day study, TT subjects had higher peak plasma AGT concentrations than did MM subjects. In the short term, however, complete

readjustment of the circulating renin–angiotensin system occurred through a decrease in renin release, which blunted the effects of the increase in AGT concentration.

2.3

Relation with Essential Hypertension

With Lalouel's group, we reported the first molecular arguments for a role of the AGT gene in human essential hypertension (Jeunemaitre et al. 1992c). An extensive study was performed in two large series of hypertensive sibships yielding a total of 379 sib pairs (Salt Lake City, Utah, USA and Paris, France) and using a highly polymorphic microsatellite marker at the AGT locus. An excess of AGT allele sharing was found mainly in severely hypertensive sib pairs and in men, suggesting a positive although mild relationship.

Since then, several linkage studies have led to controversial results. Caulfield et al. showed a strong linkage and an association of the AGT gene locus to essential hypertension in a set of British families (Caulfield et al. 1994, 1996). At the other extreme, no evidence for linkage was found in a large European study involving 630 affected sibling pairs, either in the whole panel or in family subsets selected for severity or early onset of disease (Brand et al. 1998). Linkage of the AGT gene to essential hypertension was also found in 63 affected African Caribbean sibling pairs (Caulfield et al. 1995). Similarly, positive albeit modest significant excess of AGT allele sharing was found in 46 extended Mexican American (Atwood et al. 1997). No linkage was found between the AGT locus and hypertension in 310 hypertensive Chinese sibling pairs (Niu et al. 1998).

The association between the M235T polymorphism and essential hypertension has been tested in a large number of case-control studies that have been reviewed recently (Jeunemaitre et al. 1999; Lalouel et al. 2001). A meta-analysis of case-control studies representing 5,493 Caucasian patients showed that the 235T allele was significantly but mildly associated with hypertension [odds ratio (OR): 1.20; 95% CI (confidence interval): 1.11–1.29; $p < .0001$], association which increased in studies with positive family history (OR: 1.42; 95% CI: 1.25 to 1.61, $p < .0001$) (Kunz et al. 1997). Another meta-analysis that included 10,720 white subjects showed a 32% increase in the risk of elevated BP associated with the 235T allele (Staessen et al. 1999). More recently, Sethi et al. studied 9,100 men and women from the Danish general population (Sethi et al. 2001). On multifactorial logistic regression analysis, women homozygous for the 235T allele versus non-carriers had an odds ratio for elevated BP of 1.29 which increased to 1.50 if they were also homozygous for the T174 allele. No significant association was found in men. The analysis of 4,322 subjects of the National Heart, Lung and Blood Institute (NHLBI) Family Blood Pressure Program (FBPP), showed only a weak association between the G-6A polymorphism (Province et al. 2000). In a Japanese population, the analysis of nine polymorphisms at the AGT gene confirmed that the G-6A, T+31C and M235T polymorphisms are in absolute linkage disequilibrium and that the haplotype containing -6A, +31C and 235T was

associated with hypertension but not with plasma AGT in this particular study (Sato et al. 2000). More recently, the analysis of 1,425 subjects of urban populations of Vitoria, Brazil, showed a linear relation between 235T allele number and BP (Pereira et al. 2003). The magnitude of BP variations was about 3–4 mmHg per copy of the 235T allele.

The high prevalence of the 235T allele may explain why no relation between 235T or 174 M allele frequencies and hypertension was observed in a study of African-Americans (Rotimi et al. 1994). In the Japanese population, a significant association between this allele and high BP was found in several separate case control studies (Hata et al. 1994; Iwai et al. 1994; Kamitani et al. 1994; Morise et al. 1995). These differences in allele frequencies might also facilitate false-positive results in case of admixture in case-control studies. Altogether, these results highlight the modest effect of the AGT locus and the difficulty of identifying susceptibility genes by linkage analysis in complex diseases.

2.4

Relation with Other Phenotypes

Other studies have tested the influence of the AGT gene on various pathological conditions, such as coronary heart disease and diabetic nephropathy, for which the reader will find recent reviews (Jeunemaitre et al. 1999; Smithies et al. 2000; Wang and Staessen 2000; Katsuya and Ogihara 2001; Lalouel and Rohrwasser 2001). Several phenotypes probably merit a particular attention.

The first is the possible relationship between AGT gene polymorphisms and body weight. AGT is indeed abundantly expressed in human adipose tissue (Saye et al. 1990) and plasma AGT concentrations have been correlated to BP and body mass index (Eggena et al. 1991; Bloem et al. 1995; Cooper et al. 2000). The analysis of a group of young hypertensive adults on a 3-year follow-up period showed a positive interaction between body weight changes and the G-6A polymorphism (Chaves et al. 2002). Some studies have also suggested that the AGT M235T polymorphism effect could be highly sensitive to environmental context for physical activity (McCole et al. 2002; Rauramaa et al. 2002).

The second is the possible relationship with pregnancy-induced hypertension. Ward et al. (1993) found a significant association between the AGT 235T variant and pre-eclampsia in both Caucasian and Japanese samples. Using another strategy, analysis of the allelic inheritance of the GT repeat in 52 sibling pairs of preeclamptic sisters, Arngrimsson et al. (1993) showed a significant linkage between the AGT locus and preeclampsia in Icelandic and Scottish families. However, other studies found no indication of association or linkage between preeclampsia and the AGT gene, and recent genome-scan studies have reported possible loci on chromosomes 4q (Harrison et al. 1997), 2p (Arngrimsson et al. 1999; Moses et al. 2000) and 9p (Laivuori et al. 2003). Two molecular mechanisms could explain the relationship between AGT polymorphisms and pregnancy-induced hypertension. The first is its association with AGT expression in decidual spiral arteries (Morgan et al. 1997), reduced plasma volume

during the follicular phase of the menstrual cycle (Bernstein et al. 1998), and intra-uterine growth (Zhang et al. 2003). The second is its possible influence on the formation of high-molecular-weight AGT (Gimenez-Roqueplo et al. 1998). Both mechanisms acting together, an increased AGT expression due to the G-6A variant, and an increased proportion of active monomeric form of AGT, could increase the local formation of Ang II, facilitate an hyperplasia of the spiral arteries and thus lead to a reduction of the uteroplacental blood flow.

3

The Renin Gene

3.1

Gene Structure, Mutations and Polymorphisms

The human renin gene is located on the short arm of chromosome 1 (1q32-1q42) (Cohen-Haguenaer et al. 1983). The primary structure of renin precursor was deduced from its cDNA sequence: it consists of 406 amino acids with a pre and a pro segment carrying 20 and 46 amino acids, respectively (Imai et al. 1983; Hobart et al. 1984). The human renin gene spans 12 kb of DNA and contains 8 introns (Miyazaki et al. 1984). The structure of the renin gene is similar to that of pepsinogen, a closely related aspartyl protease.

We identified an informative C/A repeat polymorphism in the 3' part of the gene (Lifton and Jeunemaitre 1993). From pairwise logarithm of differences (lod) score calculations, the sex-averaged recombination fraction between the renin and AGT locus was estimated at 0.26. Several restriction fragment-length polymorphisms (RFLPs) have been located throughout the renin gene: *TaqI* and *BglI* polymorphisms in the 5' region, *HindIII* in the 3' region, and *HinfI* in the first intron (Jeunemaitre et al. 1992b). On the NCBI site, seven SNPs are present at the human renin gene—respectively at exons 1, 2, 3, 9 and 10—but have not all been validated (www.ncbi.nih.nlm.gov/SNP). A systematic screening of the coding sequences of the gene by single-strand conformation polymorphism analysis (SSCP) and direct sequencing allowed us the identification of a synonymous SNP at exon 2 [Thr(ACA)→Thr(ACC) at position 2 of the mature renin], and a polymorphism in intron 4 (T+17int4G). A trinucleotide repeat (CTG)₈ is also present in intron 7, displaying 4 alleles with an average heterozygosity of 0.49 (Fig. 2). In the Japanese population, two novel SNPs have been detected, the one in intron 4 (T+17int4G), and one in exon 9 (G1051A) leading to an amino acid change (Val351Ile) (Hasimu et al. 2003). Our group identified two SNPs close to a strong enhancer element located more than 5 kb upstream of the promoter of the human gene (Germain et al. 1998; Fuchs et al. 2002). These two SNPs are located at positions -5434 and -5312 and seem to influence in vitro the levels of transcription, when the corresponding constructs are transfected in choriodecidual (Fuchs et al. 2002).

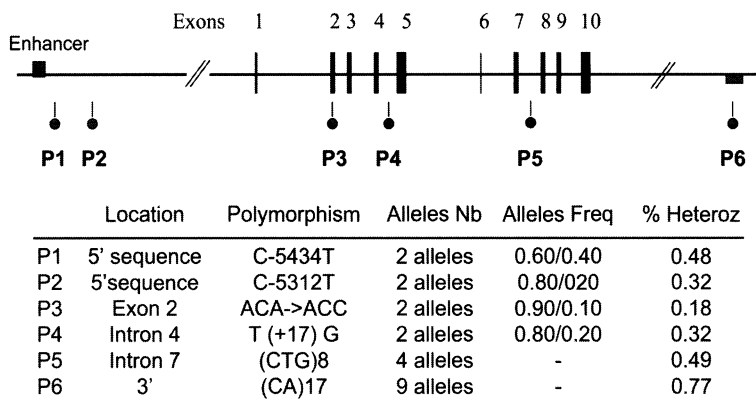


Fig. 2 Main polymorphisms of the human renin gene

3.2

Relation with Plasma Renin

Activation of the renin system depends on the renin-AGT reaction which is the first and rate-limiting step leading to Ang II production. Hypertensive individuals are usually classified according to their renin status. Interestingly, renin levels seem at least partly heritable, as observed by Grim and colleagues (1980) in twins submitted to well-standardised conditions of posture and sodium diet and more recently by Rossi et al. (1999). Our analysis of 175 sibling pairs and trios who participated to a multicentre trial showed very significant correlations of plasma renin levels either on a high- or a low-salt diet. Further analysis of this cohort showed the familial aggregation of low-renin hypertension with twice more low-renin families than expected (10.9% vs 5.5%), in contrast to the normal-renin state, where the observed and expected number of families was similar (61.0 vs 58.6%) (Fisher et al. 2002).

Testing 212 patients with EH and 209 age-matched normotensive subjects, Hasimu et al. (2003) found no difference in three renin polymorphisms either separately, or combining them into haplotypes. However, plasma renin activity (PRA) levels in patients with essential hypertension (EH) with the GG genotype at position 1051 were significantly higher than in subjects with GA and AA genotypes, thus suggesting that the amino acid change (Val351Ile) may affect the enzymatic function of renin or may be in linkage disequilibrium with polymorphisms affecting renin transcription.

A mutation responsible for a syndrome with hyperproreninaemia has been found in a unique Dutch family (Villard et al. 1994). All family members were normotensive and had normal PRA. A point mutation in the last exon of the gene at codon 387 of the preprorenin cDNA introduced a premature stop codon (TGA) in the renin gene sequence. This mutation directs the synthesis of a truncated form of renin, with 20 amino acids deleted from the carboxyl terminus. We found also a mutation on the signal peptide-encoding region of the pre-

profragment of renin (Pro 8 Ala) in a unique family (X. Jeunemaitre, unpublished). No particular biological phenotype could be associated with this mutation.

3.3

Relation with Essential Hypertension

Numerous studies have involved renin to some degree in experimental forms of hypertension. In human, one of the first case-control study was that performed by Soubrier et al. (1990). Renin gene allele and haplotype frequencies of 3 RFLPs were similar in 120 normotensive and 102 hypertensive subjects. To explore further the potential role of the renin gene as a genetic determinant of hypertension, we used the hypertensive sib pairs approach (Jeunemaitre et al. 1992b). Using the same clinical criteria and the same renin gene haplotypes as in the association study, no linkage was found between the renin gene and hypertension, suggesting again that the renin gene does not have a frequent and/or important role in the pathogenesis of essential hypertension.

In a single large Utah human pedigree with high prevalence of coronary disease and hypertension, there was no significant association between the renin RFLPs and BP or plasma renin activity (Naftilan et al. 1989). Interpretation was, however, limited by the very low number of patients studied. In another preliminary report, Morris and Griffiths (1988) compared the renin RFLPs of 29 subjects under antihypertensive treatment with those of 202 adult patients. No association was found between hypertension and the renin gene allele but, again, no definite conclusion could be drawn since only a few hypertensives were studied, clinical data were not available and the renin gene polymorphism was defined by a single and weakly informative RFLP. The most recent study corresponds to that performed in a Japanese population by Hasimu et al. (2003). No statistical difference was observed in the genotype distribution of three polymorphisms between the 212 patients and the 209 age-matched normotensive subjects.

4

The Angiotensin I-Converting Enzyme

Angiotensin I-converting enzyme (ACE) is a zinc metalloprotease whose main functions are to convert Ang I into Ang II, and to inactivate bradykinin. It is assumed that this step of the renin–angiotensin system is not limiting in plasma, and indeed there is no indication that plasma ACE levels are directly related to BP levels. However, the local generation of Ang I and the degradation of a bradykinin might depend on the level of ACE expressed in tissues.

4.1

Gene Structure, Mutations and Polymorphisms

The enzyme consists of two highly homologous and functionally active domains resulting from a gene duplication (Soubrier et al. 1988). There are two ACE promoters, a somatic promoter localised on the 5' side of the first exon of the gene and a germinal, intragenic, promoter located on the 5' side of the specific testicular ACE mRNA (Hubert et al. 1991). The two alternate promoters of the ACE gene exhibit highly contrasting cell specificities, as the somatic promoter is active in endothelial, epithelial and neuronal cell types, whereas the germinal promoter is only active in a stage-specific manner in male germinal cells (Howard et al. 1990). It is thought that plasma ACE concentration reflects the level of the synthesis of the somatic enzyme.

The most studied is an insertion/deletion (I/D) of a 287-base pair DNA fragment in the intron 16 of the gene, corresponding to an Alu sequence. Soubrier's group also identified eight new polymorphisms in 95 healthy nuclear families, most of them being in strong linkage disequilibrium with the I/D polymorphism (Villard et al. 1996). More recently, Rieder and colleagues performed a complete genomic scan of the ACE gene (24 kb) from 11 individuals (Rieder et al. 1999). They identified 78 varying sites in 22 chromosomes that resolved into 13 distinct haplotypes. Among these polymorphisms, 17 are in absolute linkage disequilibrium with the ACE I/D polymorphism, producing two distinct and distantly related clades.

4.2

Relation with Plasma ACE Levels

From a geneticist point of view, plasma ACE concentration is an interesting marker as it varies markedly between individuals (from 1 to 8 at the extremes of the distribution) but remains remarkably constant when measured repeatedly in a given subject (Alhenc-Gelas et al. 1991). This important variability is due, in large part, to a major genetic effect, as shown by Cambien et al. (1988). In a family study where there was an intrafamilial resemblance between plasma ACE levels, they estimated that this effect accounting for approximately 30% and 75% of the ACE variance in parents and in offspring, respectively.

The role of the ACE gene in the genetic control of plasma ACE has been clearly established. In their seminal observation of 80 healthy subjects, Rigat and colleagues showed that the serum ACE concentration of DD subjects was almost twice as high as that observed in patients homozygous for the I allele, whereas heterozygous patients were intermediate (Rigat et al. 1990). Like for serum ACE, T lymphocyte ACE levels are significantly higher in patients homozygous for the D allele than in the other subjects (Costerousse et al. 1993). Another study combining segregation and linkage analysis in 98 healthy nuclear families showed that the ACE I/D polymorphism is only a neutral marker in strong linkage disequilibrium with the putative functional variant (Tiret et al. 1992). After

adjustment for the I/D polymorphism, all polymorphisms of the 5' group remained significantly associated with ACE levels, which suggested the existence of two quantitative trait loci (QTL) acting additively on ACE levels accounting for 38% and 49% of the ACE variance in parents and offspring, respectively. The authors suggested that the causal variant should be located within the 3' part of the gene. Despite these efforts, the causative variant responsible for the increase in ACE expression has yet to be found, which might foreshadow the difficulty of identifying causal molecular variants in complex traits.

A rare missense mutation (Pro1199Leu) in the stalk region of the mature ACE protein has been identified in eight Dutch families, in which plasma ACE values exceeded fourfold the upper limit of normal (Kramers et al. 2001). All affected individuals were heterozygous for the mutation which is supposed to lead to an alteration in the juxtamembrane region of ACE, thus in a more efficient cleavage of the protein. The physiology of the renin–angiotensin system and BP were not altered in affected individuals, indicating that in the presence of similar amounts of membrane-bound ACE, the higher extracellular concentration of ACE is of no clinical significance (Kramers et al. 2001).

4.3

Relation with Essential Hypertension

The observation that plasma ACE levels are under the direct control of an ACE gene variant rapidly made the ACE I/D polymorphism one of the most popular markers tested in cardiovascular diseases. An interrogation of the PubMed database with “angiotensin I-converting enzyme insertion/deletion polymorphism” retrieves 652 publications. We will only summarise here some of the main findings using the ACE gene as a candidate gene for human essential hypertension and cardiovascular disorder.

One association study comparing a normotensive and a hypertensive Australian population with two hypertensive parents, showed an association of hypertension with ACE gene polymorphism (Zee et al. 1992). This finding was interpreted as due to an over risk of cardiovascular events in hypertensive patients carrying the D allele (Morris et al. 1994). Harrap et al. (1993) investigated the distribution of the ACE I/D gene polymorphism in young adults with contrasting genetic predisposition to high BP (Watt et al. 1992) (“four-corners approach”): young adults with high BP and two parents with high BP did not show any significant difference in the I/D allele frequencies when compared with adults of the same age but with low BP and no genetic predisposition to high BP. Other association studies were also negative (Higashimori et al. 1993; Schmidt et al. 1993). In a large series of hypertensive sib pairs from Utah, we found no evidence of linkage between hypertension and a growth hormone gene polymorphic marker in complete linkage disequilibrium with the ACE gene (Jeunemaitre et al. 1992a). Taken together, these results suggest that the ACE gene does not play a major role on BP variance in these populations.

Some positive results suggest, however, that the ACE locus might influence BP variability in a sex-specific manner. In a logistic regression analysis of 3,095 participants in the Framingham Heart Study (O'Donnell et al. 1998), the adjusted odds ratios for hypertension among men for the DD and DI versus II genotypes were 1.59 and 1.18, respectively, whereas no effect was observed in women. Positive results were also reported by Fornage et al. in the analysis of a large population-based sample of 1,488 siblings having a mean age of 15 years and belonging to the youngest generation of 583 randomly ascertained three-generation pedigrees from Rochester, MN (Fornage et al. 1998). In sex-specific analyses, genetic variation in the region of the ACE gene explained as much as 35% of the interindividual BP variation, again in males but not in females. Finally, Julier et al. (1997) conducted an affected sib-pair analysis in French and U.K. families and explored the region of chromosome 17q 23–32, based on the location of the ACE locus and on the QTL observed in rat on the homologous region of chromosome 10. Significant evidence of linkage was found near two closely linked microsatellite markers, D17S183 and D17S934, that reside 18 cM proximal to the ACE locus in the homology region.

4.4

Relation with End-Organ Damage

A very large number of reported and unreported studies have been performed and have been reviewed (Cambien and Soubrier 1995; Butler 2000; Danser and Schunkert 2000; Rieder and Nickerson 2000; Wang and Staessen 2000). The ACE I/D polymorphism has been associated with coronary heart disease, including myocardial infarction, stable and unstable angina pectoris, restenosis following percutaneous coronary angioplasty and stenting, left ventricular hypertrophy, cardiac insufficiency, peripheral artery disease, stroke and Alzheimer disease, proteinuria, diabetic nephropathy and retinopathy, human performance to exercise, aggravation of the course of IgA nephropathy and of polycystic kidney disease, and a variety of other diseases.

Of course, when such an attractive polymorphism is available, it is quite simple for researchers and clinicians to set up a case-control study and perform a statistical test to see whether a difference exists between each group, even if the a priori hypothesis is not substantiated by a strong pathophysiological background. Thus, among all the studies performed (>700), it is likely that a large number of false positive studies exist, due to publication bias and to selection criteria that do not fulfil those suggested for “high-quality” association studies (Bogardus 1999; Sharma and Jeunemaitre 2000). If well-performed, a meta-analysis can also help in the estimation of the strength of the impact of a given polymorphism on a given trait. In that regard, it is remarkable to observe that meta-analyses of the effect of the ACE I/D polymorphism on myocardial infarction found no or very mild association when analysed on several thousand patients, with mainly a publication bias for the small and positive studies (Fig. 3) (Agerholm-Larsen et al. 2000; Keavney et al. 2000). A similar observation has

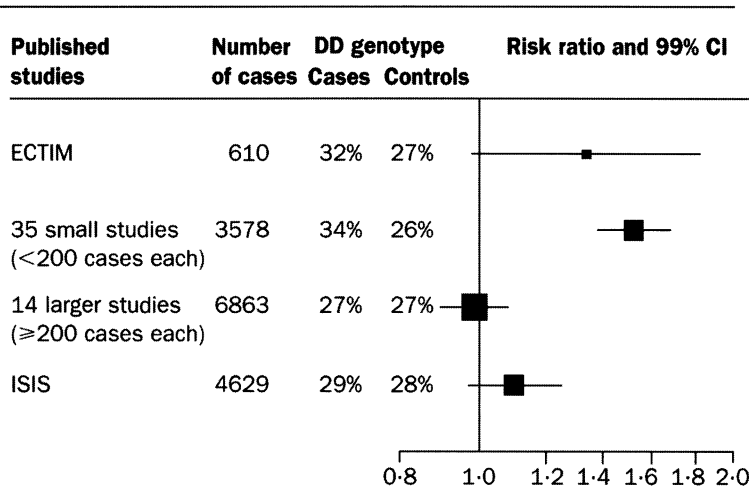


Fig. 3 Association between the human ACE gene and myocardial infarct: a meta-analysis. *CI*, confidence interval; *ECTIM*, Etude Cas-Temoin de l'Infarctus du Myocarde; *ISIS*, International Studies of Infarct Survival. From Kearney et al. (2000)

been made by meta-analyses of its association with coronary restenosis (Agema et al. 2002; Bonnici et al. 2002). A greater impact of the polymorphism might occur on ischemic stroke (Sharma 1998) and on diabetic nephropathy (Fujisawa et al. 1998). On the whole, it is also important to consider that associations might vary across gender or ethnic groups, or different socio-ecological settings, and that very few studies consider the potential gene–gene and gene–environment interactions.

Probably one of the most innovating findings has been the association between the I allele of the ACE I/D polymorphism with enhanced endurance performance in elite distance runners, rowers and mountaineers (Montgomery et al. 1997, 1999). The nature of the gene–environment interaction between ACE I/D polymorphisms and physical training, an overview of recent findings and a discussion of possible underlying mechanisms, have been reviewed by these authors (Woods et al. 2000).

5 The Angiotensin II Type 1 Receptor

Ang II receptors which mediate all the biological and physiological effects of the renin–angiotensin system are also candidate genes for essential hypertension. Most of the peripheral and central actions of Ang II, including vasoconstriction, facilitation of sympathetic transmission, modification of vascular and cardiac structure, renal salt and water retention, are mediated by the angiotensin type 1 (AT₁) receptor (AT1R). The AT1R belongs to the family of seven-transmembrane domain, G protein-coupled receptors. It is inserted into the plasma mem-

brane of Ang II target, vascular smooth muscle, renal vasculature and mesangial, adrenal and brain cells.

5.1

Gene Structure, Mutations and Polymorphisms

The human gene has been cloned and is located on chromosome 3q21-3q25 (Furuta et al. 1992). It consists of at least five exons and spans more than 55 kb of genomic DNA (Guo et al. 1994). All the coding sequence is encoded by the last exon, the first four encoding 5' untranslated sequences, with multiple transcription initiation sites. There are at least four distinct alternatively spliced transcripts, whose relative abundance varies from one tissue to another one. Interestingly, a long AT1R isoform has been recently described, that has diminished activity for Ang II (Martin et al. 2001). Modifications of the relative abundance of the long and short isoforms of the AT1R might allow for a fine tuning of Ang II responsiveness in given circumstances or in a given tissue.

Several polymorphisms have been detected at the AT1R locus. An informative CA repeat polymorphism maps within 15 kb downstream the 3' end of the coding sequence (Davies et al. 1994). The interrogation of the human SNP database (www.ncbi.nlm.nih.gov/SNP) shows the presence of more than 20 SNPs, not all being validated nor their frequency determined. No polymorphism has been found to change the coding sequence of the receptor. The two SNPs at the AT1R gene that have been extensively used in the literature are those initially identified by Bonnardeaux et al. (1994). They consist of a non-synonymous change within the coding sequence (T573C) and a nucleotidic change in the 3' untranslated region (+1166A/C) whose frequency is 0.50–0.55 and 0.30–0.35 in Caucasian subjects. Other diallelic polymorphisms have been identified in the 3' untranslated region of the gene (+575T/C, +1062A/G, +1517G/T) in strong linkage disequilibrium (Bonnardeaux et al. 1994; Rolfs et al. 1994). Erdmann et al. performed a systematic screening of 2.5 kb of the 5' flanking region of the gene in a sample of 150 healthy subjects (Erdmann et al. 1999). They found eight polymorphic sites, six of them being in strong linkage disequilibrium but not with the +1166A/C polymorphism. Poirier et al. (1998) screened the first four exons and 2.2 kb in the 5' flanking region of the AT1R gene by SSCP and sequencing. Seven polymorphisms were detected in the 5' region at positions –1424, –810, –713, –521, –214, –213 and –153 upstream from the transcription start (<http://genecanvas.idf.inserm.fr>). All were in complete (or nearly complete) linkage disequilibrium. More recently, Antonellis et al. (2002) developed a technique for rapid identification of polymorphisms in long stretches of genomic DNA and analysed the AT1R gene. They genotyped 18 polymorphisms spanning the 60.5-kb AGTR1 locus, with an average spacing of 3.2 kb and an average minor allele frequency of 24%.

5.2

Relation with Essential Hypertension

The first study to investigate the relationship between the AT1R gene and essential hypertension was that performed by Bonnardeaux et al, with both an affected sibling pair approach and a case-control study (Bonnardeaux et al. 1994). There was no evidence for linkage between the microsatellite marker CA at the gene locus and essential hypertension in 267 hypertensive sib pairs from 138 pedigrees. The case-control study performed on a panel of 206 hypertensive subjects and 298 normotensive individuals was mainly negative, except for the 1166C allele, which was more frequent in hypertensive (0.36) than in normotensive subjects.

Other negative results were obtained by other groups. Castellano et al. (1996) examined a sample of 212 subjects randomly selected from a general population in Northern Italy. No statistically significant differences among AT1R A/C1166 genotypes were observed for ambulatory BP, left ventricular mass or carotid artery wall thickness. Several polymorphisms at the promoter region of the AT1R gene were tested by Zhang et al. in a large Caucasian population-based sample (Zhang et al. 2000). None of these polymorphisms showed evidence for association with hypertension. Negative results were obtained by two groups in Japan (Kikuya et al. 2003; Ono et al. 2003). The analysis of the A1166C polymorphism in 3,918 subjects recruited from the Suita study showed similar frequencies of the C allele (0.08), this frequency being markedly lower than in Caucasian subjects (Ono et al. 2003). The analysis of 802 subjects of the Ohasama study found no statistical difference in ambulatory BP according to the genotype, as soon as it was adjusted for confounding variables (age, sex, BMI) (Kikuya et al. 2003).

However, some studies found a positive association between essential hypertension and the A1166C polymorphism. Wang et al. (1997) analysed a well-characterised group of 108 Caucasian hypertensive subjects with a strong family history and 84 controls, in Australia. In this limited series, the 1166C allele was more frequent in hypertensives (0.40) than in controls (0.26). In the Finnish population, the sib pair analysis of 329 hypertensive individuals of 142 families gave a positive multipoint lod score of 2.9 (Kainulainen et al. 1999). Interestingly, a genome-wide scan performed in 47 families with two affected siblings and additional family members, showed the AT1R locus as the most significant locus in the Finnish population (Fig. 4) (Perola et al. 2000). However, no particular mutation or polymorphism was identified.

In a recent analysis of 218 Caucasian hypertensive patients and sib pairs, we found no direct relation with basal BP levels but a positive relationship between the acute BP response to Ang II and the AT1R locus (Vuagnat et al. 2001). Significant familial resemblances in the Ang II-induced systolic and diastolic BP response were observed, and the AT1R gene could participate to its genetic determination. However, no significant association was found between the A1166C polymorphism and the BP and aldosterone responses to infused Ang II in 116 male normal volunteers (Hilgers et al. 1999). Spiering et al. studied the reactivity

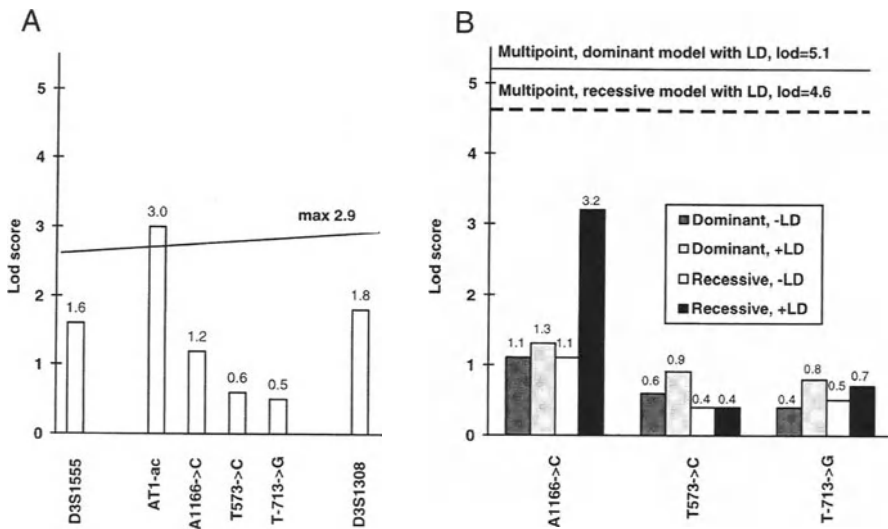


Fig. 4A, B Linkage and association between the AT₁ receptor gene and hypertension. In **A**, the line represents maximal logarithm of differences (lod) score obtained in the multipoint sib-pair linkage analyses of the six markers. In **B**, the lod scores have been obtained using different models in a subgroup of nonobese sib pairs. *Bars* represent two-point lod scores between hypertension and each marker. (From the Finnish study in Perola et al. 2000)

to infused Ang II of 42 subjects with essential hypertension, after 7 days of high-sodium diet (Spiering et al. 2000). They found that renal plasma flow, glomerular filtration rate and renal vascular resistance were more pronounced in patients with the CC genotype compared to those with the AA genotype. However, the changes of plasma renin, aldosterone and atrial natriuretic peptide (ANP) were not dependent on the genotype. It is interesting to observe that the effect of the genotype on the renal response to infused Ang II is probably dependent on the gender with a significant effect in males but not in females (Vuagnat et al. 2001; Reich et al. 2003).

5.3 Relation with End-Organ Damage

Taking into account the multiple actions of Ang II on multiple organs such as heart, vessels and brain, a large number of association studies have been performed to test the possible association of the AT1R gene polymorphisms and end-organ damage. It is difficult to summarise them in a short paragraph. On the whole, the polymorphisms that have been analysed are often unique in a given study, and/or tested on a too limited sample to be really contributive.

The A1166C polymorphism has been associated to aortic stiffness (Benetos et al. 1996; Lajemi et al. 2001), left ventricular mass (Osterop et al. 1998), coronary vasoconstriction (Amant et al. 1997), increased response to Ang II in iso-

lated human arteries (Henrion et al. 1998; van Geel et al. 2000) and myocardial infarction in interaction with the ACE I/D polymorphism (Tiret et al. 1994). A positive association between the T573C polymorphisms and microalbuminuria was also found in a group of young adults with essential hypertension, the TT genotype being suggested as an independent protective factor for microalbuminuria (Chaves et al. 2001). An increased frequency of the 1166CC genotype was observed in a Polish group of 430 patients on dialysis compared to 260 healthy controls, with a significantly shorter time from the onset of renal disease to end-stage renal disease (ESRD) in patients with C allele compared to those with AA genotype (Buraczynska et al. 2002).

In a recent attempt to test the association between this polymorphism and cardiovascular events, Hindorff et al. (2002) analysed 800 African Americans and 1,372 random participants of the Cardiovascular Health Study. Although a marginal interaction was observed in the white treated participants with increased risk of congestive heart failure and stroke, on the whole the A1166C polymorphism was not associated with BP control or cardiovascular events on the entire populations. Finally, no relation was found between AT1R density in platelets—a possible biological intermediate phenotype—and the A1166C genotype (Paillard et al. 1999).

6

The Angiotensin II Type 2 Receptor

Since its discovery and molecular characterisation, the type 2 Ang II (AT₂) receptor (AT2R) has been enigmatic with respect to signalling pathways and function. These aspects are developed in another chapter of this book. Evidence has now emerged that Ang II exerts actions through the AT2Rs, which are directly opposed to those mediated by the AT1R. The AT2R appears to act as a modulator of complex biological programmes involved in embryonic development, cell differentiation, tissue protection and regeneration, as well as in programmed cell death (Gallinat et al. 2000). A number of studies suggest a role of AT(2) receptors in brain, renal, and cardiovascular functions and in the processes of apoptosis and tissue regeneration (Unger 1999).

6.1

Gene Localisation, Structure and Polymorphisms

The human AT2R gene was first isolated from a human genomic DNA library of leukocytes (Tsuzuki et al. 1994). The gene is organised in three exons but the entire coding region is contained in the third exon (Martin and Elton 1995) and encodes a 363 amino acid protein which is highly homologous to the sequences of the rat and mouse protein. Although they exhibit a similar binding affinity for Ang II, the AT1R and AT2R share only a 34% identity at the amino acid level. The AT2R is located on chromosome X in both human and rat species, the human AT2R gene being assigned to Xq22 (Koike et al. 1994).

6.2

Relation with Urogenital Abnormalities

Nishimura et al. (1999) reported that mice inactivated for the AT2R gene have phenotypes that remarkably resemble human congenital anomalies of the kidney and urinary tract. In the same study, the authors suggested that, in humans, a polymorphism could be a risk factor for the development of anomalies of the kidney and urinary tract (CAKUT). A substantial fraction of the human population (about 30%–50%) carry a A-to-G transition at position –1332 in intron 1 (A-1332G) in the lariat branchpoint motif of this intron, that might perturb the mRNA splicing efficiency. Nishimura et al. (1999) found a strong association between this polymorphism and the incidence of CAKUT: 10 of 13 Caucasian patients (77%) and 17 of 23 (74%) Caucasian German males with ureteropelvic junction stenosis or atresia, being hemizygous for this A-to-G transition, compared to an allelic frequency of 42% in controls.

However this association has been disputed. Hiraoka et al. (2001) compared the frequency of the intron 1 polymorphism in 66 Japanese boys with CAKUT and controls. The frequency of the A–G transition was not different between the control population (31 of 102, 30%) and the patients with CAKUT [23 of 66 (35%)]. Similar negative results were obtained by Yoneda et al. (2002) who found no evidence for the implication of the AT2R gene in the pathogenesis of primary familial vesicoureteral reflux. These authors evaluated the incidence of A-1332G transition in 82 male and 110 female patients and unaffected family members. The incidence of A-1332G transition was similar in male and female patients with primary familial vesicoureteral reflux and controls. Moreover, the transmission/disequilibrium test revealed no significant distortion of genotype transmission from mother to children.

6.3

Relation with Cardiovascular Diseases

Since the AT2R might oppose the vasoconstrictor and antinatriuretic effects of Ang II mediated through the AT1R, several genetic studies have investigated whether variations within the AT2R might play a counterregulatory protective role in the regulation of BP and in cardiovascular disorders such as neointima formation after vascular injury, cardiac hypertrophy and myocardial infarction.

In humans, only few association studies have been performed. Our laboratory was not able to find significant difference in a set of hypertensive and controls (M. Giacché, X. Jeunemaitre, unpublished results). Schmieder et al. analysed BP, left ventricular structure, plasma Ang II and aldosterone concentrations in 120 young male subjects (Schmieder et al. 2001). Presence of the A allele of the intronic +1675 G/A polymorphism was associated with an increase left ventricular mass index in hypertensive subjects (Schmieder et al. 2001). More recently, Herrmann et al. analysed the same polymorphism in two large cohorts of 1,968 individuals (the Glasgow Heart Scan, GLAECO and Glasgow Heart Scan Old, GLAOLD stud-

ies) with echocardiographically and electrocardiographically assessed left ventricular measurements (Herrmann et al. 2002). In both studies, the genotype frequencies were similar in hypertensive and non-hypertensive individuals (0.50). In the GLAOLD study and in females only, the AT2R +1675A allele was more common in females with episodes of coronary ischaemia and myocardial infarction. In the same cohort, the +1675A allele was associated with left ventricular hypertrophy in males only. However, these results could not be replicated in the GLAECO cohort, giving weak credibility for these genotype/phenotype interactions.

6.4 Relation with X-Mental Retardation

Whereas AT1Rs are abundant in a number of adult tissues, AT2Rs are mainly expressed during embryonic life and their expression declines rapidly after birth and is then restricted to few organs and mainly in some brain areas. The presence of high levels of AT2Rs within the neonatal brain supports the suggestion that this receptor has a role in development and possibly in cognitive function via its involvement in axonal regeneration (Lucius et al. 1998). This possible role was also suggested by the attenuated exploratory behaviour and anxiety-like behaviour of AT2R-deficient mice (Hein et al. 1995; Ichiki et al. 1995; Okuyama et al. 1999).

A recent human study further supports a role for the AT2R in brain development and cognitive function (Vervoort et al. 2002). These authors analysed a *de novo* balanced translocation 46,X,t(X;7)(q24;q22) in a female patient with moderate mental retardation. They mapped the X-chromosome breakpoint and demonstrated by RT-PCR that the AT2R gene was silenced in this patient, possibly through a position effect. The authors then screened a series of 33 males with X-linked mental retardation (MR) and a large cohort of 552 unrelated male patients with MR of unknown cause. Eight of the 590 unrelated male patients with MR were found to have sequence changes in the AGTR2 gene, including 1 frameshift and 3 missense mutations (Fig. 5). Although these results have not

- **Balance X; 7 translocation in a female patient with mental retardation (MR)**
- **8 / 590 patients with MR have AT2R mutations**

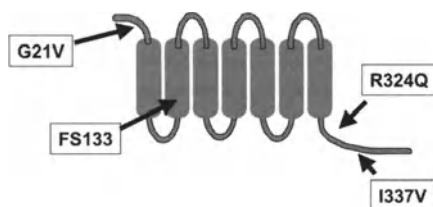


Fig. 5 Mutations of the AT₂ receptor gene and X-linked mental retardation

been replicated yet, they indicate that the AT2R might contribute to regulate central nervous system functions, including behaviour.

7

Future Directions

With the notable exception of few monogenic forms of cardiovascular diseases for which the molecular basis has been often already elucidated, there is no indication of the number of genetic loci involved in these complex traits, the frequency of deleterious alleles, their mode of transmission, and the quantitative effect of any single allele. Because of their aetiologic heterogeneity, it is likely that no single biochemical or genetic marker will help the clinician in the management of most of the patients. How can we progress in the comprehension of the involved molecular pathways, and in the possible application of genetic markers? Some of the avenues that could be explored are indicated below.

7.1

Test Gene–Gene and Gene–Environment Interactions

7.1.1

Gene–Gene Interactions

It is important to note that most of the studies performed so far have been conducted in a very simple design, by testing one by one the role of each gene (sometimes of only one polymorphism) of the renin–angiotensin system. However, it is recognised that the situation is much more complex, involving a multitude of genes controlling the susceptibility to cardiovascular diseases, acting in an additive or interactive manner together with environmental factors. A more integrated approach needs to be designed in which several genes and some environmental factors could be tested with or without a priori specific hypothesis.

In that regard, the first attempts that have been made by several authors to test the interaction between renin–angiotensin system genetic polymorphisms are of interest. For example, interactions between the A1166C polymorphism of the AT1R gene with the ACE I/D polymorphism has been found to explain a genetic susceptibility to increased diastolic BP (Henskens et al. 2003) and myocardial infarction in interaction (Tiret et al. 1994). A study performed by Staessen and colleagues (2001) showed the combined effect of the ACE I/D, the alpha-adducin Gly460Trp and the aldosterone synthase C-344T polymorphisms on BP level. Lovati and colleagues (2001) found an interaction between the AGT M235T polymorphism and the ACE I/D polymorphism for the occurrence of end-stage renal disease in diabetic patients. Such an interaction for the progression of renal insufficiency in patients with IgA nephropathy has been also discussed (Pei et al. 1997; Frimat et al. 2000). Although these studies face the problem of stratification and thus of increased probability of false positive results,

their replication in other well-performed and powerful studies might help to better estimate the role of the RAS polymorphisms in these complex traits.

7.1.2

Gene–Environment Interactions

As mentioned above, functional polymorphisms exist at the renin–angiotensin system. Nevertheless, their effect on hypertension and cardiovascular diseases has been highly disputed. One of the reasons for this is probably that the phenotype itself has not been determined with enough precision nor the polymorphisms tested in non-standardised environmental conditions (especially posture, salt diet, therapy). If we admit, as it is probably true, that these polymorphisms exert their effect only in certain conditions, a “stress-the-genotype” approach might help us to unravel their true pathophysiological influence. One of the best example of this approach involves the studies performed by Montgomery and colleagues to evaluate the effect of the ACE I/D polymorphism on left ventricular mass in response to exercise (Montgomery et al. 1999, 2002). The strategy was to select subjects homozygous for the I or D allele from a large number of military recruits and precisely phenotype left ventricular mass before and after a 10-week physical training period. In the several studies performed, left ventricular mass increased with training, but with DD men showing roughly threefold greater growth than II men. When indexed to lean body mass, left ventricular growth in II subjects was essentially negligible.

Some studies have also suggested that the AGT genotype might influence the BP response to non-pharmacological therapy, especially be a marker of salt-sensitivity. In the Trials of Hypertension Prevention Phase II (TOHP-II), both sodium reduction and weight loss were tested in randomised trials as BP-lowering strategies. Participants were typed for the G-6A polymorphism. In the usual care group, the AA genotype was associated with a higher 3-year incidence of hypertension compared to GA or GG genotypes. In the salt reduction and weight loss intervention groups, this AA genotype was also associated with a larger reduction in BP compared to GA and GG genotypes (Hunt et al. 1998). The same polymorphism G-6A was tested with the BP response to the Dietary Approaches to Stop Hypertension (DASH) diet (Svetkey et al. 2001). After 8 weeks, net systolic and diastolic BP response to the DASH diet was significantly greater in individuals with the AA genotype compared to those with the GG genotype. Undoubtedly, the use of these “stress-the-genotype” approaches to explore gene-environment interactions might be the key to understanding the complex effects of the renin–angiotensin system gene polymorphisms.

7.2

Analysis of Other Components of the Renin–Angiotensin System

7.2.1

The Prorenin Receptor

Up to now, Ang II has been believed to be the main, if not exclusive, effector of the renin–angiotensin system through binding to its receptors AT₁ and AT₂. The recent demonstration and the cloning of a functional receptor of prorenin might change this view (Nguyen et al. 2002). The highest level of expression of this receptor is observed in heart, brain, placenta, kidney and eye. Immuno-histological studies of heart and kidney show that it is localised in vascular structures, associated to smooth-muscle cells in coronary and renal artery (Nguyen et al. 2002). This receptor might have cellular effects per se on vascular smooth-muscle cells, independent of Ang II. It might represent an essential mean to capture renin and prorenin from the circulation and to concentrate renin and prorenin in tissues, especially at the interface with endothelial cells. In this context, the search of functional genetic polymorphisms will be of importance regarding their potential impact on the modulation of smooth muscle cell tone and of coronary artery function (Nguyen et al. 2003).

7.2.2

The ACE2 Gene

The identification of an enzyme similar to ACE, called ACE2, adds further complexity to the system. The ACE2 gene was initially cloned from a human cardiac cDNA library (Donoghue et al. 2000). ACE has a single catalytic domain, whereas somatic ACE has two catalytic domains. The phylogenetic analysis clearly shows that they evolve by duplication of a single-domain ACE-like protein, before the divergence of insect and mammalian lineages (Cornell et al. 1995). ACE2 transcripts are found mainly in vascular endothelial cells of the heart and kidney and in testis.

The ACE2 gene encodes for a carboxypeptidase that is able to convert Ang I to angiotensin 1-9. Since angiotensin 1-9 has no known effect on blood vessel and can be converted by ACE in angiotensin 1-7, which is a blood vessel dilator, it has been suggested that ACE2 might prevent the formation of the vasopressor Ang II. The recent work, published in *Nature* (Crackower et al. 2002) tends to demonstrate the pathophysiological importance of this enzyme. The authors were able to show that, in the salt-sensitive Sabra hypertensive rat strain, there is a markedly decrease expression of the ACE2 gene, although no mutation was found in the coding sequence of the gene. In addition, targeted disruption of the ACE2 gene in mice resulted in a cardiac contractility defect, suggesting its role as an essential regulator of heart function in vivo. If this role is confirmed, the molecular analysis of the human ACE gene in a variety of cardiovascular diseases will be important.

7.2.3

The Aminopeptidases A and N

Among the main bioactive peptides of the brain renin–angiotensin system, angiotensin Ang II and Ang III exhibit the same affinity for type 1 and type 2 Ang II receptors. In the murine brain renin–angiotensin system, Ang II is converted by aminopeptidase A (APA) into Ang III, which is itself degraded by aminopeptidase N (APN). Both peptides, injected intracerebroventricularly, cause similar increases in vasopressin release and BP (Reaux et al. 1999). Recent pharmacological studies using specific APN and APA inhibitors have demonstrated the predominant role of brain Ang III in the control of vasopressin release and BP (Reaux et al. 2001). These genes are therefore attractive candidates for human hypertension.

7.3

Pharmacogenetics

The increasing knowledge of polymorphisms in genes encoding for proteins that are drugs targets, the uncertainty of the individual efficacy and tolerance of these drugs, has fuelled a new molecular discipline, pharmacogenetics. It is expected that pharmacogenetics will help clinicians gauge drugs efficacy and tolerance and will help to give the “right medicine for the right patient” (Roses 2000).

In addition to polymorphisms in drug metabolising enzymes, there are cases where the response to drug treatment might be correlated with gene variants involved in the drug’s mode of action. The renin–angiotensin system is a good example of such possibility, since functional polymorphisms exist as well as drugs blocking or stimulating the system. Although promising, only few therapeutic studies have been correctly conducted in patients according to renin–angiotensin genotypes. We will just take the example of the ACE I/D polymorphism. Its relationship with cough, the major side-effect of ACE inhibitors, is uncertain (O’Toole et al. 1998). Some studies found an increased frequency of the I allele in coughers (Lee and Tsai 2001), others an increased frequency of the D allele, still others no difference in genotype frequencies (Zee et al. 1998). It is therefore likely that this polymorphism does not explain this side-effect, even though it may be part of a particular combination of susceptibility variants belonging to several genes related to the renin–angiotensin system and kallikrein–bradykinin system.

The analysis of the efficacy of ACE inhibitors led also to controversial results. A pharmacokinetic interaction was observed in at least two studies. Todd et al. (1995) showed that the ACE I/D polymorphism might predict residual ACE activity after administration of a single dose of enalapril. Similarly, Ueda et al. found that compared to the II subjects, subjects with the DD genotype had a greater and longer effect of a unique dose of enalaprilat (Ueda et al. 1998). Despite this interaction, reports of BP decrease after chronic administration of an ACE inhibitor have been controversial (O’Byrne and Caulfield 1998).

Interaction with BP responses to hydrochlorothiazide according to the ACE I/D genotype have also been analysed. In a study of 376 patients with essential hypertension, Schwartz and colleagues (2002) found a significant interaction between the effects of the ACE genotype and gender on the responses of both systolic and diastolic BP to hydrochlorothiazide (HCTZ) 25 mg daily. However, the effects of the genotypes were opposed in men and women. Sciarrone et al. (2003) evaluated the BP response to HCTZ in 87 never-treated individuals with mild essential hypertension, according to ACE gene I/D and alpha-adducin Gly460Trp polymorphisms. They found that patients carrying at least one I allele of ACE and one 460Trp allele of alpha-adducin had the largest mean BP (MBP) decrease with treatment, the effect of the combination of genotypes being additive but not epistatic.

Obviously, prediction of drug efficacy and/or of adverse drug reaction with a pharmacogenetic profile will need larger, well-designed trials in which patients will be standardised on the drug dosage and metabolism (Rioux 2000).

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Knockout Models of the Renin–Angiotensin System

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Abstract Angiotensin II, generated from its precursor angiotensinogen by renin and angiotensin-converting enzyme (ACE), mediates its biological effects via two different classes of G protein-coupled receptors, termed angiotensin II AT₁ receptors and AT₂ receptors. Transgenic mouse models have greatly advanced our understanding of the specific functions of the individual parts of the renin angiotensin aldosterone system (RAAS). Recently, all components of the RAAS have been deleted by homologous recombination in the mouse genome. This review summarizes the *in vivo* significance of the available knockout mouse lines of the RAAS. While most of the classical functions of the RAAS are mediated via AT₁ receptor, recent *in vivo* evidence suggests that AT₂ receptors may antagonize the cardiovascular effects of AT₁ receptor activation. Most importantly, AT₂ receptors are required to inhibit the growth-promoting effects of AT₁ receptors in vascular smooth muscle cells and in cardiac myocytes. Targeted deletions of angiotensinogen and ACE genes have extended our knowledge of the embryonic functions of the renin angiotensin system. Most recently, a novel angiotensin-converting-enzyme (ACE2) has been cloned and deleted by homologous recombination in mice. Thus, the experimental findings from transgenic animal models offer new

insights into the physiological regulation of the RAAS and help in the development of novel therapeutical strategies for treating human diseases.

Keywords Transgenic mice · Gene targeting · Angiotensin receptors · Angiotensin · Converting enzyme · Renin

1 Introduction

Angiotensin II (Ang II) is generated by serial cleavage of angiotensinogen in two enzymatic steps (Fig. 1): First renin cleaves angiotensin I (Ang I) from angiotensinogen, then an angiotensin converting enzyme (ACE) removes two amino acids to generate the active octapeptide angiotensin II. Ang II exerts its effects via two classes of G protein-coupled receptors, termed AT₁ and AT₂ with most of its classical actions mediated via the AT₁ receptor. Two subtypes of AT₁ receptors, AT_{1A} and AT_{1B} have been identified in rodents, but humans have only a single AT₁ receptor gene.

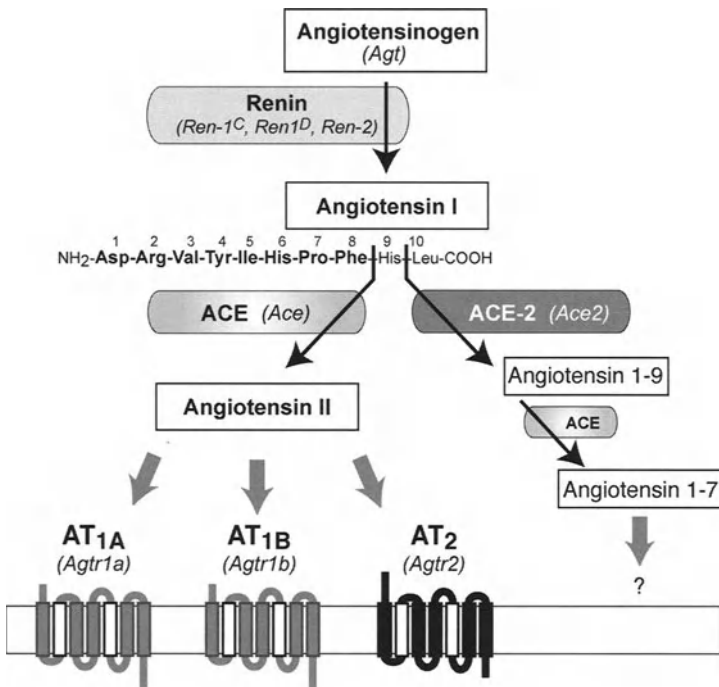


Fig. 1 Components of the renin–angiotensin system in the mouse. Angiotensin II is generated by serial cleavage of angiotensinogen by by renin and angiotensin-converting enzyme (ACE). In the mouse, angiotensin II activates three different G protein-coupled receptors, AT_{1A}, AT_{1B}, and AT₂. A homolog of ACE, termed ACE-2, was recently identified to convert angiotensin I to angiotensin 1-9, which is further cleaved to angiotensin 1-7 by ACE. The receptor for angiotensin 1-7 has not been identified yet. Murine gene symbols are given as *italicized abbreviations*

In this chapter, we summarize recent progress in the molecular physiology and pharmacology of the renin–angiotensin system which was derived from mouse models carrying targeted gene deletions (“knockout mice”). Until recently, all components of the renin–angiotensin system which had been identified were targeted for deletion in mice. Mostly, these models are constitutive “knock-outs” of the entire gene leading to the complete absence of the respective protein. However, a few modifications of the standard gene-targeting technique were used to generate gene duplications (e.g., 1–4 copies of the angiotensinogen gene; Kim et al. 1995), or tissue- or endothelial-specific deletion of ACE expression (Esther et al. 1996; Cole et al. 2002). The major phenotypes of mice with targeted deletions of the renin–angiotensin system are summarized in Fig. 2 and in Table 1.

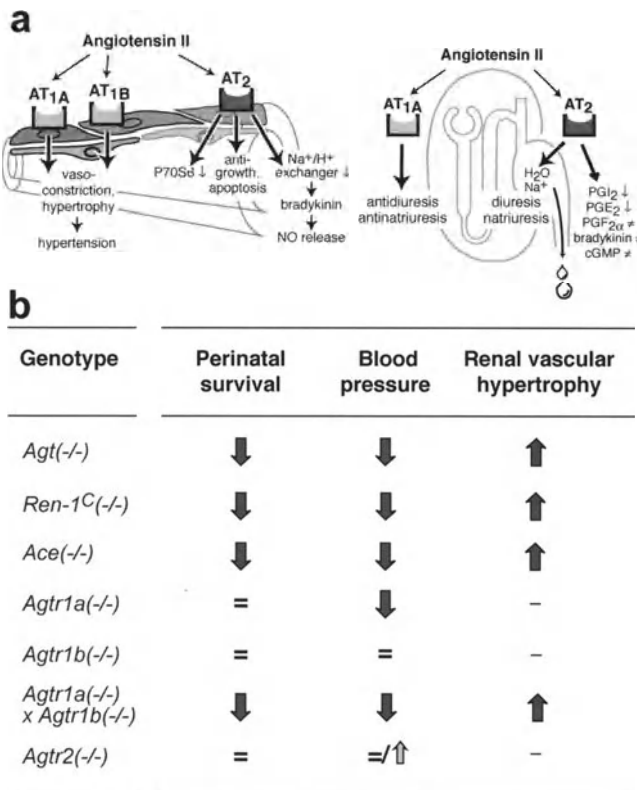


Fig. 2a, b Effects of gene deletion of components of the renin–angiotensin system on survival, blood pressure, and renal development. **a** Vascular and renal functions of AT₁ and AT₂ receptor subtypes derived from studies in gene-targeted mice. **b** Overview of renal, vascular, and mortality phenotypes in mice with deletions in the renin–angiotensin system. While deletion of the genes for angiotensinogen, ACE, and renin-1^C show largely overlapping phenotypes in these systems, the function of angiotensin receptor subtypes are mostly complementary (=, similar to wild-type; -, not detectable). References for the observed phenotypes are given in the text and in Table 1

Table 1 Phenotypes of mice with targeted deletions of the genes of the renin–angiotensin system

Protein	Genetic model	Phenotype	Reference(s)
Angio-tensinogen	<i>Agt</i> (-/-)	Hypotension; kidney: vascular dilatation and hypertrophy, tubular atrophy and interstitial fibrosis; postnatal lethality (before weaning) Diuresis Ang-independent increase in aldosterone after Na ⁺ restriction Delayed repair of injured blood–brain barrier Smaller infarct size after ligation of middle cerebral artery Decreased metabolic efficiency in <i>Agt</i> (-/-) mice, decreased weight gain after high fat diet	Tanimoto et al. 1994; Kim et al. 1995; Niimura et al. 1995 Kihara et al. 1998 Okubo et al. 1997 Kakinuma et al. 1998 Maeda et al. 1999 Massiera et al. 2001
Renin	1-2-3-4 <i>Agt</i> copies <i>Ren-1^D</i> (-/-)	8 mmHg blood pressure increase per gene copy Defect in granule synthesis in juxtaglomerular cells	Kim et al. 1995 Clark et al. 1997
	<i>Ren-1^C</i> KO	Hypotension, increased urine and drinking volume, renal defects	Yanai et al. 2000
ACE	<i>Ren-2</i> KO <i>ACE</i> (-/-)	Normal development Hypotension, no blood pressure response to Ang I, enhanced hypotension after bradykinin	Krege et al. 1995; Esther et al. 1996
	<i>ACE.2</i> (-/-)	Developmental defects of the kidney (atrophic tubules, dilated and thickened arteries) Decreased male fertility	Esther et al. 1996; Hilgers et al. 1997 Esther et al. 1996
	<i>ACE.3</i> (-/-)	Deletion of membrane anchor of ACE (“tissue ACE KO”) → hypotension, renal defects Normocytic anemia	Esther et al. 1997 Cole et al. 2000
ACE-2	<i>Ace2</i> (-/-) <i>Ace</i> (-/-) <i>xAce2</i> (-/-)	Switch of transcriptional control to albumin promoter (“endothelial ACE KO”) → 87-fold increase in hepatic ACE expression, normal blood pressure, normal plasma Ang II Decreased cardiac contractility Normal cardiac function	Cole et al. 2002 Crackower et al. 2002
	AT _{1A} receptor <i>Agtr1a</i> (-/-)	Hypotension Decreased pressure response to Ang II Decreased blood pressure response to central Ang II Reduced effective plasma and extracellular fluid volume No cardiac hypertrophy after Ang II infusion	Ito et al. 1995; Sugaya et al. 1995 Oliverio et al. 1997 Davisson et al. 2000 Cervenka et al. 1999 Harada et al. 1998b

Table 1 (continued)

Protein	Genetic model	Phenotype	Reference(s)
AT _{1B} receptor	<i>Agtr1a</i> (-/-)	Normal cardiac hypertrophy after aortic constriction Normal infarct size after coronary artery ligation, lower incidence of arrhythmias, improved survival, decreased remodeling Normal degree of neointima formation Attenuated antglomerular basement membrane nephritis Blunted renal damage after proteinuria Increased activity and drinking periods with intact circadian rhythm	Hamawaki et al. 1998; Harada et al. 1998b Harada et al. 1998a,b; Nakamura et al. 2002 Harada et al. 1999a Hisada et al. 2001 Suzuki et al. 2001 Mistlberger et al. 2001
	<i>Agtr1b</i> (-/-)	Normal development, normal blood pressure Blunted dipsogenic response to central Ang II	Chen et al. 1997 Davisson et al. 2000
	<i>Agtr1a</i> (-/-) x <i>Agtr1b</i> (-/-)	Hypotension, renal defects	Oliverio et al. 1998a; Tsuchida et al. 1998
AT ₂ receptor	<i>Agtr2</i> (-/-)	Normal development Low penetrance defect in urinary tract Normal blood pressure Moderately increased blood pressure Rightward shift in pressure diuresis and natriuresis curves Hypertension after deoxycorticosterone and salt-loading Altered baroreflex sensitivity Increased vasopressor effects, vascular hypertrophy, increased vascular P70S6 kinase phosphorylation Normal cardiac function at rest Normal cardiac hypertrophy after aortic banding, increased coronary artery hypertrophy and perivascular fibrosis No cardiac hypertrophy after Ang II infusion or aortic banding Normal infarct size and post-infarct remodeling after coronary artery ligation Increased neointima formation Attenuated susceptibility to azoxymethane-induced colon tumorigenesis Impaired drinking behavior in response to water deprivation, reduced locomotor activity Anxiety-like behavior, increased sensitivity to pain	Hein et al. 1995; Ichiki et al. 1995 Nishimura et al. 1999 Hein et al. 1995 Ichiki et al. 1995 Gross et al. 2000b Gross et al. 2000a Gross et al. 2002 Hein et al. 1995; Ichiki et al. 1995; Akishita et al. 1999; Brede et al. 2001 Brede et al. 2001; Gross et al. 2001 Akishita et al. 2000b Senbonmatsu et al. 2000; Ichihara et al. 2001 Xu et al. 2002 Akishita et al. 2000a; Wu et al. 2001 Takagi et al. 2002 Hein et al. 1995; Ichiki et al. 1995 Okuyama et al. 1999; Sakagawa et al. 2000

2 Angiotensinogen

The first gene of the renin-angiotensin system which was deleted in embryonic stem cells and in mice was the angiotensinogen gene (*Agt*) (Tanimoto et al. 1994; Kim et al. 1995; Niimura et al. 1995). As angiotensinogen is the only (known) substrate for the generation of Ang II, *Agt*(-/-), mice were indeed completely deficient in Ang II. This resulted in a marked hypotension and blood pressure was reduced from 100 mmHg in wild-type mice to 67 mmHg in *Agt*(-/-) mice (Tanimoto et al. 1994). In studies with mice carrying 1-4 copies of the *Agt* gene, blood pressure was increased by approximately 8 mmHg per gene copy with plasma angiotensinogen levels ranging between 35% (single *Agt* gene copy) and 145% (4 copies) of the values detected in normal mice with two *Agt* copies (Kim et al. 1995).

One of the most important initial findings in angiotensinogen-deficient mice was the fact that animals without a functional renin-angiotensin system were born alive and did not show any major developmental defects at birth (Tanimoto et al. 1994; Kim et al. 1995; Niimura et al. 1995). However, within 3 weeks after birth, most of the *Agt*(-/-) mice died and only very few animals survived until adulthood. These adult *Agt*(-/-) mice developed severe vascular abnormalities in the kidney. Maturation of the glomeruli was delayed, interlobular and afferent arterioles had thickened walls, juxtaglomerular cells were hyperplastic, and interstitial fibrosis and inflammation developed together with cortical thinning and tubular atrophy. These structural defects were associated with functional renal impairment and a marked inability of *Agt*(-/-) mice to concentrate urine (Kihara et al. 1998). Histologically, the vascular lesions in kidneys from *Agt*(-/-) mice resembled those found in hypertensive nephrosclerosis, where increased expression of transforming growth factor (TGF)- β was implicated in the pathophysiology. Indeed, the endothelial cells of hypertrophic renal arteries from *Agt*(-/-) mice showed elevated expression of TGF- β , indicating that altered growth factor expression may be responsible for the vascular defects in these mice (Niimura et al. 1995).

However, the precise mechanism of renal vascular hypertrophy in *Agt*(-/-) remains to be elucidated. Normally, vascular hypertrophy is associated with hypertension and results from pressure- or Ang II-mediated proliferation of vascular smooth muscle cells. In contrast, *Agt*(-/-) mice were hypotensive and were completely deficient in Ang II. While medial thickening with concentric hypertrophy is characteristic for hypertensive arteries, *Agt*(-/-) vessels showed non-concentric medial thickening with luminal dilatation, suggesting that renal vascular hypertrophy differs from hypertension-induced hypertrophy (Nagata et al. 1996). It is unclear at present whether the vascular defect is due to a deficiency in Ang II signal transduction or due to decreased blood pressure and lowered renal perfusion. In order to address this question, Matsusaka and colleagues generated chimeric mice which were made up by cells with intact and disrupted genes for the AT_{1A} receptor (Matsusaka et al. 1996). These chimeric mice had

normal blood pressure and did not show any signs of hypertrophy in juxtaglomerular cells or afferent arterioles, indicating that the lowered blood pressure may indeed be important for the vascular defects in *Agt(-/-)* mice.

As angiotensin II is an important stimulus for the release of aldosterone from the adrenal cortex, the concept of an integrated “renin–angiotensin–aldosterone system” has been suggested to indicate that aldosterone mediates the long-term effects of the renin–angiotensin system on blood pressure by retaining Na^+ and water. Studies in *Agt(-/-)* mice showed that a powerful non-angiotensin mechanism exists (at least in mice) to increase circulating aldosterone levels after Na^+ restriction even in the absence of angiotensin II (Okubo et al. 1997). During Na^+ restriction in mice, suppression of K^+ , but not angiotensin, led to a marked attenuation of hyperaldosteronism, suggesting that an angiotensin-independent mechanism exists to increase aldosterone during low salt diet (Okubo et al. 1997).

Several studies were performed in *Agt(-/-)* mice which extended the previously known functions of the renin–angiotensin system. In the brain, angiotensinogen was required for normal repair of injury to the blood–brain barrier (Kakinuma et al. 1998). In contrast, *Agt(-/-)* mice had a smaller ischemic core and larger penumbra after surgical induction of a brain infarct (Maeda et al. 1999), indicating that inhibition of angiotensin formation may prolong the therapeutic window for treatment of cerebral infarcts. Furthermore, angiotensinogen seems to be involved in the control of body fat mass by a combination of effects on lipogenesis and locomotor activity (Massiera et al. 2001).

3 Renin

Renin is the rate-limiting enzyme in the biosynthesis of Ang II—the majority of circulating renin is secreted from the juxtaglomerular cells of the kidney. Mice differ from other mammals as some mouse strains have two renin genes, *Ren-1^D* and *Ren-2* (129, DBA mice) while other mice have only one renin gene, *Ren-1^C* (C57BL/6, BALB/c). The second renin gene resulted from a gene duplication on chromosome 1, which presumably occurred 3–10 million years ago, after the speciation of the mouse (Dickinson et al. 1984). The murine renin genes are highly homologous, but they give rise to distinct proteins with different sites for post-translational glycosylation. Glycosylation may be physiologically essential for renin storage in juxtaglomerular cells, as *Ren-1^D(-/-)* mice did not have electron-dense secretory granules in juxtaglomerular cells despite the synthesis of renin-2 in these cells (Clark et al. 1997).

Mice deficient for renin-1^C (Yanai et al. 2000) displayed the same perinatal mortality, renal abnormalities, and hypotension as those mice lacking angiotensinogen (Tsuchida et al. 1998) or ACE (Krege et al. 1995; Carpenter et al. 1996) (see below). *Ren-1^D(-/-)* mice showed hypotension in females, whereas blood pressure was normal in male knockout (KO) mice. In contrast, genetic deletion of the *Ren-2* gene did not result in any obvious phenotype: *Ren-2(-/-)* mice

were viable and healthy and they had normal blood pressure despite an increase in active plasma renin (Sharp et al. 1996). At present, only few data from gene-targeted mice suggest that Ang I may be generated by a non-renin pathway: *Ren-1^C(-/-)* mice did not show the impaired blood-brain barrier function which was observed in *Agt(-/-)* mice, suggesting that renin may be dispensable for the blood-brain barrier function of the renin-angiotensin system (Yanai et al. 2000).

4 Angiotensin-Converting Enzymes (ACE, ACE-2)

Mice deficient in the angiotensin-converting enzyme (ACE) showed the expected hypotension as they failed to generate the vasopressor angiotensin II from endogenous or exogenous Ang I (Krege et al. 1995; Esther et al. 1996). The second function of ACE, i.e., breakdown of bradykinin, was also diminished, as *ACE(-/-)* mice exhibited an exaggerated hypotensive response to infusion of bradykinin (Krege et al. 1995).

ACE(-/-) mice showed developmental defects of the kidney which were similar to the reported effects of perinatal pharmacologic inhibition of the renin-angiotensin system (Guron and Friberg 2000). In analogy to the *Agt(-/-)* mice, *ACE(-/-)* mice had atrophic and dilated kidney tubules, some kidneys were hydronephrotic and all intrarenal arteries including afferent arterioles were dilated and thickened (Hilgers et al. 1997). The defect of *ACE(-/-)* mice to concentrate urine may not only be related to structural defects of the kidney, but may also be caused by altered expression of renal transporter proteins: in mice lacking ACE, decreased expression of the aquaporin APQ1 water channel, the 117-kDa urea transporter UT-A1, and the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter could be detected (Klein et al. 2002).

Surprisingly, male *ACE(-/-)* mice had reduced fertility (Carpenter et al. 1996; Esther et al. 1996) which is in contrast to the observation in angiotensinogen-deficient mice, which showed normal reproductive behavior. Male mice deficient in somatic ACE retain testis ACE and are fertile, thus demonstrating that somatic ACE is not essential for normal male fertility (Hagaman et al. 1998). These results suggest that testicular ACE may process a non-angiotensin substrate which is necessary for reproduction.

ACE is a membrane-bound enzyme, which may be released into the interstitial fluid or into the circulation after proteolytic cleavage. In order to investigate the physiological significance of tissue-bound versus circulating ACE, mice carrying a selective deletion in its membrane anchoring domain have been generated to eliminate all tissue-bound ACE. These mice were termed "*ACE.2(-/-)*" mice (Esther et al. 1997). Surprisingly, these mice developed the same defects (reduced male fertility, hypotension, inability to concentrate urine) as mice with a complete deletion of ACE (Esther et al. 1997). In these "tissue ACE KO" mice, circulating ACE levels were 34% of normal. Thus, tissue-ACE is essential for maintenance of blood pressure and for renal structure and development. In ad-

dition, *ACE.2(-/-)* mice had normocytic anemia with elevated plasma erythropoietin levels, indicating that Ang II stimulates erythropoiesis (Cole et al. 2000). These data may explain clinical observations that ACE inhibitors may reduce hematocrit values in some patients.

Another mouse strain, termed “*ACE.3(-/-)*”, was generated to substitute transcriptional control of somatic ACE transcription from the ACE promoter to the albumin promoter (Cole et al. 2002). Switching the promoter in *ACE.3(-/-)* mice resulted in complete absence of ACE in the lung, aorta, or any vasculature (“endothelial ACE KO”). However, the albumin promoter caused a 87-fold increase in hepatic ACE expression. Surprisingly, blood pressure, plasma Ang II levels and renal structure and function were normal in these mice. Usually one would assume that the lung contains enough ACE to entirely convert blood Ang I to Ang II during a single transit (Ng and Vane 1967), but the data from *ACE.3(-/-)* mice suggest that endothelial ACE is not essential to maintain normal blood pressure. Heterologous expression of ACE in the liver is sufficient to substitute for endothelial ACE. Subjecting this mouse strain to models of cardiovascular injury should be an important step to delineate the role of endothelial ACE.

Among the greatest surprises of recent research in the renin–angiotensin system was the identification of a novel homolog of the angiotensin-converting enzyme, ACE-2 (Donoghue et al. 2000; Tipnis et al. 2000). This homolog of ACE was first cloned from human heart failure ventricle and from lymphoma cDNA libraries. ACE-2 cleaves the C-terminal Leu from Ang I to generate Ang 1-9, which is further converted to Ang 1-7 by “classical” ACE. As outlined in Fig. 1, ACE-2 differs from ACE as it does not generate Ang II or degrade bradykinin. ACE-2 is not inhibited by classical ACE-inhibitors, including captopril and lisinopril. ACE-2 shares 40% amino acid identity and 61% similarity with human ACE (Tipnis et al. 2000), thus similar to renin-2, ACE-2 may be the result of a gene duplication.

When compared with the widespread expression pattern of ACE, ACE-2 is much more restricted in its tissue distribution with primary sites of expression in endothelial cells of coronary and intrarenal vessels, in renal tubules, and in the testis. Until very recently, the function of ACE-2 was completely unknown. In an elegant series of experiments, Crackower and colleagues demonstrated that ACE-2 is essential for the development of cardiac function (Crackower et al. 2002).

The pathophysiological relevance of ACE-2 is highlighted by the fact that the rat ACE-2 gene was mapped to a region of the X chromosome that has previously been implicated in several rat models of high blood pressure (Crackower et al. 2002). Mice with a targeted deletion in their *Ace2* gene had normal blood pressure, but cardiac contractility was severely depressed and circulating Ang II levels were elevated. The precise mechanism of this phenotype remains unclear at present. However, crossing of *Ace2(-/-)* mice with mice lacking “classical” ACE rescued the cardiac phenotype, indicating that altered Ang II generation may be responsible for the pathomechanism in *Ace2(-/-)* mice.

5 Angiotensin II AT₁ Receptors

At present, two types of G protein-coupled receptors have been cloned which mediate the biological effects of angiotensin II, type 1 receptors (AT₁) and type 2 receptors (AT₂). Whereas most species, including humans, have only one AT₁ receptor gene, two AT₁ genes which arose by gene duplication (*Agtr1a*, *Agtr1b*) have been identified in mice and rats, termed AT_{1A} and AT_{1B} receptors. All three angiotensin receptor genes were deleted in mice, revealing separate and distinct functions for these receptors.

Most of the classical functions of Ang II, vasoconstriction, hypertension, aldosterone release, and cardiovascular hypertrophy, are mediated by AT₁ receptors in mice. These functions are predominantly controlled by the AT_{1A} receptor. Mice deficient in AT_{1A} had significantly lower resting blood pressure and a blunted pressure response to infusion of Ang II (Ito et al. 1995; Sugaya et al. 1995). However, AT_{1B} receptors contribute to blood pressure control as Ang II infusion could still elicit moderate hypertensive responses in *Agtr1a*(-/-) mice (Oliverio et al. 1997). In contrast, mice lacking AT_{1B} receptors had normal blood pressure at rest and a normal response to Ang II, indicating that the AT_{1A} receptor is the major receptor involved in blood pressure regulation (Chen et al. 1997).

AT_{1A} and AT_{1B} receptors have similar signal transduction pathways as Ang II induced transient increases in intracellular Ca²⁺ concentration in vascular smooth muscle cells cultured from AT_{1A}-deficient mice (Zhu et al. 1998). However, both AT₁ receptor subtypes may have distinct physiological functions, as the blood pressure response to centrally administered Ang II was completely mediated by AT_{1A} receptors, whereas the dipsogenic response to central Ang II was mediated via the AT_{1B} receptor (Davisson et al. 2000). Mice lacking both AT_{1A} and AT_{1B} receptors showed greater lowering of blood pressure than single receptor-deficient mice (Oliverio et al. 1998a; Tsuchida et al. 1998). The mechanism of hypotension in AT₁ receptor-deficient mice may be related to a reduction in effective plasma and extracellular fluid volume rather than a decrease in peripheral vascular resistance, as arterial hypotension could be rapidly restored in *Agtr1a*(-/-) mice by infusion of isotonic saline (Cervenka et al. 1999).

5.1 AT₁ Receptors and Cardiac Hypertrophy

Cardiac AT₁ receptors play an important role in the regulation of left ventricular contractility and hypertrophic remodeling. In the murine heart, AT_{1A} receptors predominate over the AT_{1B} subtype. In *Agtr1a*(-/-) mice, only very little AT₁ receptor mRNA and Ang II binding sites remained, probably reflecting a very low density of AT_{1B} receptors (Harada et al. 1998b). Infusion of Ang II did not induce *c-fos* gene expression (Harada et al. 1998c) or cardiac hypertrophy in *Agtr1a*(-/-) mice (Harada et al. 1998b). In contrast, *Agtr1a*(-/-) mice still de-

veloped cardiac hypertrophy after left ventricular pressure overload, indicating that AT_{1A}-mediated signaling is not essential for the development of pressure overload-induced hypertrophy (Hamawaki et al. 1998; Harada et al. 1998b). A similar response was found in isolated cardiac myocytes from AT_{1A}-deficient mice in vitro, which showed a normal degree of hypertrophy to mechanical stretch (Kudoh et al. 1998). However, it remains to be determined whether cardiac AT_{1B} receptors may compensate for the loss of AT_{1A} receptors to induce left ventricular hypertrophy.

In contrast to the situation after cardiac pressure overload, AT_{1A} receptors played a role in early and late adaptation to experimental myocardial infarction in mice. While the infarct size did not differ between *Agtr1a(-/-)* and control mice after occlusion of the left coronary artery, the incidence of premature arrhythmic beats was significantly reduced during the reperfusion phase in *Agtr1a(-/-)* mice (Harada et al. 1998a) and survival was also greater in *Agtr1a(-/-)*, mice which was attributed to improved systolic and diastolic function and decreased left ventricular remodeling (Harada et al. 1999b; Nakamura et al. 2002). Similar to the situation in the heart, the AT_{1A} receptor by itself was not essential for neointima formation after vascular injury of the common carotid artery (Harada et al. 1999a).

5.2

Renal AT₁ Receptor Function

Agtr1a(-/-) mice exhibit hydronephrosis due to defective development of the urinary peristaltic machinery during the perinatal period (Miyazaki and Ichikawa 2001). Mice which were lacking AT_{1A} and AT_{1B} receptors showed impaired growth and marked renal abnormalities (Oliverio et al. 1998a; Tsuchida et al. 1998) which were similar to the alterations observed in *Ace(-/-)* and *Agt(-/-)* mice (see above). Despite the hypotension of *Agtr1a(-/-)* mice, renal blood flow and perfusion was normal in these mice and residual renal vasoconstriction could be induced by infusion of Ang II (Ruan et al. 1999). The kidneys of *Agtr1a(-/-)* mice developed normally and showed only mild signs of mesangial expansion and juxtaglomerular cell hypertrophy (Oliverio et al. 1998b). Micro-puncture experiments revealed that the tubuloglomerular feedback response was completely absent in *Agtr1a(-/-)* mice (Schnermann et al. 1997). AT_{1A} receptors may also play an important role in the control of expression of renal tubular transporters and channels (Brooks et al. 2002). In experimental models of renal injury, deletion of the AT_{1A} receptor attenuated the development of antiglomerular basement membrane nephritis (Hisada et al. 2001) and blunted the tubulointerstitial damage after chronic proteinuria (Suzuki et al. 2001).

5.3

Brain AT₁ Receptors

Several functions of Ang II in the central nervous system were investigated in receptor-deficient mice. Deletion of the AT_{1A} receptor attenuated the increase in plasma vasopressin levels after dehydration (Morris et al. 2001), indicating that AT_{1A} receptors are involved in the central regulation of fluid and electrolyte balance.

In the mouse, AT₁ receptors are normally expressed in the suprachiasmatic nucleus, the essential regulator of circadian rhythmicity. Mean daily activity and drinking periods were increased in *Agtr1a*(*-/-*) mice, but their overall circadian rhythm remained intact (Mistlberger et al. 2001).

Occlusion of the middle cerebral artery was used to induce focal cerebral ischemia. In this model, *Agtr1a*(*-/-*) mice showed a smaller lesion area of energy failure and a significantly larger penumbra (Walther et al. 2002). Cultured *Agtr1a*(*-/-*) neurons were more susceptible to cell damage in vitro than wild-type neurons (Walther et al. 2002).

6

Angiotensin II AT₂ Receptors

Despite high levels of AT₂ receptor expression in the developing embryo, genetic deletion of the AT₂ receptor gene was not associated with gross morphological defects or decreased survival (Hein et al. 1995; Ichiki et al. 1995). However, the development of the kidney and urinary tract showed structural defects with very low penetrance in AT₂ KO mice, resembling a human congenital anomaly of the kidney and urinary tract (CAKUT syndrome) (Nishimura et al. 1999).

Some dispute remains with respect to the significance of AT₂ receptors for blood pressure regulation and cardiovascular hypertrophy under pathological situations. While initial reports showed that one strain of *Agtr2*(*-/-*) mice had normal blood pressure (Hein et al. 1995) the other strain had slightly elevated blood pressure at rest (Ichiki et al. 1995). This discrepancy has not been resolved yet, but it may be related to the fact that the two *Agtr2*(*-/-*) strains were generated on different genetic backgrounds [FVB/N (Hein et al. 1995) and C57BL/6 (Ichiki et al. 1995)]. While in the initial publications variable genetic contributions of the 129 strain—from the embryonic stem cells used to generate the *Agtr2* gene deletion—were present, later studies employed mice which were crossed back onto FVB/N or C57BL/6 backgrounds (Ichiki et al. 1995; Siragy et al. 1999a; Gross et al. 2000a; Brede et al. 2001; Ichihara et al. 2001). In addition to genetic backgrounds, environmental conditions may affect the blood pressure response in AT₂-deficient mice. When blood pressure was recorded by telemetry, Gross and coworkers did not detect differences in initial blood pressure between wild-type and *Agtr2*(*-/-*) mice (Gross et al. 2002). However, after several cycles of low- and high-salt diet followed by administration of deoxycorticosterone (DOCA), blood pressure remained higher in *Agtr2*(*-/-*) mice after return-

ing to a normal Na⁺ chow. The importance of renal AT₂ receptors was demonstrated by the fact that pressure diuresis and natriuresis curves were shifted rightward in *Agtr2*(*-/-*) mice. Thus at similar renal perfusion pressures, normal mice excreted three times more Na⁺ and water than mice lacking AT₂ receptors (Gross et al. 2000b). In addition, *Agtr2*(*-/-*) mice were more susceptible to develop hypertension after DOCA-salt loading than normal mice (Gross et al. 2000a). In addition to the renal effects of AT₂, altered baroreceptor reflex sensitivity may be important for the cardiovascular function of *Agtr2*(*-/-*) mice (Gross et al. 2002a).

One remarkable finding in *Agtr2*(*-/-*) mice was the increased sensitivity to the vasopressor effects of Ang II (Hein et al. 1995; Ichiki et al. 1995). Isolated aortic ring segments as well as muscular arteries from *Agtr2*(*-/-*) mice showed increased vasoconstrictor responses to Ang II (Fig. 3), norepinephrine and K⁺ depolarization (Akishita et al. 1999; Brede et al. 2001). Some reports have sug-

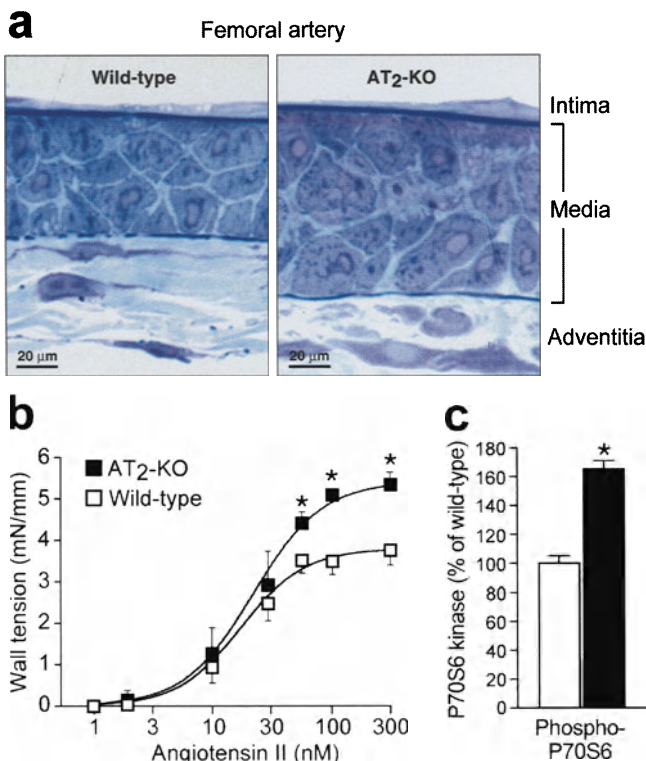


Fig. 3a–c Vascular hypertrophy and increased P70S6 kinase in AT₂ receptor-deficient mice. **a** Longitudinal sections through femoral arteries show enlarged vascular smooth muscle cells and thickened media in specimens from AT₂-deficient mice as compared with wild-type control. **b**, **c** Maximal vasoconstriction induced by angiotensin II (**b**) and abundance of the phosphorylated (i.e., active) form of the ribosomal P70S6 kinase (**c**) were significantly greater in isolated artery segments from AT₂-deficient mice than from wild-type animals. (Reproduced with permission from Brede et al. 2001)

gested that upregulation of vascular AT₁ receptors (Tanaka et al. 1999) or increased levels of circulating ACE (Hunley et al. 2000) may be responsible for the enhanced vascular reactivity of *Agtr2(-/-)* mice. Histological analyses of different blood vessel types demonstrated that *Agtr2(-/-)* mice developed media smooth muscle cell hypertrophy which was associated with increased phosphorylation of the ribosomal P70S6 kinase (Brede et al. 2001). These findings are in line with the observation that AT₂ activation promotes vascular differentiation during development and contributes to vasculogenesis (Yamada et al. 1999). Additional studies suggest that AT₂ may counteract the hypertrophic effects of AT₁ receptor activation in the vasculature in vivo: After aortic constriction, *Agtr2(-/-)* mice developed greater coronary arterial thickening and perivascular fibrosis than wild-type control mice (Akishita et al. 2000b). After vascular injury, the neointimal lesion and smooth muscle cell proliferation were greater in *Agtr2(-/-)* mice compared with normal mice (Akishita et al. 2000a; Wu et al. 2001). Treatment with an AT₁ receptor antagonist decreased neointima formation to a smaller extent in *Agtr2(-/-)* than in control mice, indicating that AT₂ receptor activation after pharmacological AT₁ receptor blockade is important for the beneficial effect of the AT₁ antagonist on neointima growth (Wu et al. 2001).

In blood vessels, murine AT₂ receptors may negatively regulate ischemia-induced angiogenesis through activation of apoptotic processes (Silvestre et al. 2002). In a mouse model of surgically induced hindlimb ischemia (femoral artery ligation) to study angiogenesis, microangiographic vessel density and laser Doppler perfusion showed significant improvement in *Agtr2(-/-)* mice when compared with wild-type mice. AT₂ receptor activation also induced apoptosis in other cell/tissue types, including embryonic fibroblasts (Li et al. 1998) and vascular smooth muscle cells after intima injury of the femoral artery (Suzuki et al. 2002).

6.1

Cardiac AT₂ Receptors

Cardiac function under normal resting conditions was not affected by deletion of the AT₂ receptor gene (Brede et al. 2001; Gross et al. 2001). The contribution of AT₂ receptor signaling to cardiac hypertrophy remains a matter of debate, as some conflicting data have been reported recently. Whereas pressure overload or chronic infusion of Ang II failed to induce left ventricular hypertrophy in one *Agtr2(-/-)* mouse strain (Senbonmatsu et al. 2000; Ichihara et al. 2001), another report did not find any effect of *Agtr2* gene deletion on cardiac hypertrophy after aortic constriction (Akishita et al. 2000b).

After experimental myocardial infarction by ligation of the left coronary artery, infarct size and postinfarct remodeling did not differ between control and AT₂-deficient mice (Xu et al. 2002). In contrast to wild-type mice, *Agtr2(-/-)* mice did not benefit from treatment with an AT₁ receptor antagonist after myocardial infarction. These data demonstrate that—at least in mice—AT₂ receptors

play an important role in the therapeutic effect of AT₁ receptor antagonists (Xu et al. 2002). Compensatory changes may occur in the absence of AT₂ receptors, e.g., upregulation of AT₁ receptor gene expression has been reported in *Agtr2(-/-)* hearts (Gross et al. 2001), in the adrenal medulla and zona glomerulosa (Saavedra et al. 2001a), in kidney glomeruli (Saavedra et al. 2001b), and in arteries (Tanaka et al. 1999).

In *Agtr2(-/-)* mice, infusion of angiotensin II for 7 days produced a marked and sustained increase in systolic blood pressure and a significant reduction in urinary sodium excretion compared with control mice (Siragy et al. 1999a). *Agtr2(-/-)* mice had low basal levels of renal interstitial fluid bradykinin and cyclic guanosine monophosphate (cGMP), an index of nitric oxide production, compared with wild-type mice (Siragy et al. 1999a). In addition, *Agtr2(-/-)* mice had increased renal vasodilator prostanoids, including higher renal interstitial fluid 6-keto-prostaglandin (PG)F_{1α} (a stable hydrolysis product of prostacyclin) and PGE₂ levels than did wild-type mice (Siragy et al. 1999b). Treatment of mice with indomethacin increased blood pressure to hypertensive levels in *Agtr2(-/-)* mice but was without effect in control mice (Siragy et al. 1999b). Absence of the AT₂ receptor led to vascular and renal hypersensitivity to angiotensin II, including sustained antinatriuresis and hypertension. These results strongly suggest that the AT₂ receptor plays a counterregulatory protective role mediated via bradykinin and nitric oxide against the antinatriuretic and pressor actions of angiotensin II.

Agtr2(-/-) mice were also used to investigate tumor growth and metastasis. Surprisingly, mice lacking AT₂ receptors are less susceptible to develop colon tumors after exposure to azoxymethane (Takagi et al. 2002). Azoxymethane is activated to a toxic metabolite in the liver by the cytochrome P450 isoenzyme CYP2E1. While basal CYP2E1 levels were similar in wild-type and in *Agtr2(-/-)* mice, azoxymethane caused more robust downregulation in wild-type mice than in AT₂-deficient animals.

6.2 AT₂ Receptors and Brain Function

Both angiotensin receptor subtypes may also have distinct roles in structural development of the central nervous system. While *Agtr2(-/-)* mice had a higher cell number in certain brain areas (cortex, hippocampus, amygdala, thalamus), *Agtr1a(-/-)* animals had a lower cell number in other brain regions (lateral geniculate and medial amygdaloid nucleus) (von Bohlen und Halbach et al. 2001). The functional relevance of these findings is unclear at present. However, subtle differences in several behavioral parameters were observed between *Agtr2(-/-)* and wild-type mice: *Agtr2(-/-)* animals had impaired drinking behavior in response to water deprivation (Hein et al. 1995), they showed reduced exploratory behavior and locomotor activity (Hein et al. 1995; Ichiki et al. 1995), they exhibited anxiety-like behavior (Okuyama et al. 1999), and were more sensitive to pain stimuli (Sakagawa et al. 2000).

7 Conclusions

Targeted deletion of the genes of the renin-angiotensin system has provided substantial novel information about the physiological function and pathophysiological significance of the individual components of this system. While some targeting experiments have confirmed previous data and sometimes long-established concepts (e.g., hypotension in ACE- or angiotensinogen-deficient mice), other results were quite unexpected [e.g., increased pressure sensitivity of AT₂-deficient mice, cardiac dysfunction of *Ace2*(-/-) mice]. Experimental evidence from gene-targeted mice has helped to identify that the two principal angiotensin receptor types, AT₁ and AT₂, have opposing roles in the control of cardiovascular growth and hypertrophy. Furthermore, data from mice have even provided evidence that AT₂ receptors mediated part of the beneficial therapeutic effect of pharmacological AT₁ receptor antagonists.

The power of mouse molecular genetics was also highlighted after the unexpected cloning of the homolog ACE-2 of the angiotensin-converting enzyme. As classical ACE inhibitors did not influence enzymatic activity of the newly identified ACE-2 enzyme, targeted deletion of the mouse gene encoding for ACE-2 was the most straightforward approach to rapidly identify the physiological relevance of this enzyme.

To date, only mouse models with constitutive gene deletions of the renin-angiotensin system have been reported. As the genes are not only inactive at the time of the experiments but throughout embryonic and postnatal development until the experimental time point, mechanisms of genetic and functional compensation have to be considered. Indeed, several reports have demonstrated that AT₁ receptor expression is upregulated in AT₂-deficient mice. In this situation, careful experiments are required to distinguish whether the observed phenotypes are due to AT₂ receptor deletion or whether they are mediated via enhanced AT₁ receptor signaling.

Despite the fact that genetic mouse models have unraveled novel concepts about the renin-angiotensin system, many questions remain unanswered at present. The pathophysiological role of AT₂ receptors and of ACE-2 warrants further study, the receptor(s) required to mediate the biological, angiotensin-dependent functions of ACE-2 are still unknown, and phenotypes of gene-targeted mice in other organ systems (kidney, brain, skin, etc.) may guide the development of novel therapeutic strategies.

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Transgenics of the RAS

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Abstract The introduction of transgenes into the genome by microinjection of DNA constructs in zygotes is a powerful technology to characterize the function of genes in rodents. It allows overexpression and—by the use of antisense constructs—also attenuation of gene expression in rats and mice. Transgenic technology has been extensively used to study the functions of the renin-angiotensin system (RAS). Most transgenic rat or mouse models overexpressing renin or

angiotensinogen became hypertensive. When the human RAS components were used for the generation of transgenic animals, both renin and angiotensinogen had to be present for angiotensin synthesis and hypertension development due to the species-specificity of the RAS. Transgenic rats with reduced angiotensinogen in the brain are hypotensive and accordingly mice with enhanced angiotensin-II generation in the central nervous system exhibited increased blood pressure, supporting an important role of the central renin-angiotensin system in cardiovascular control. Enhanced angiotensin II generation exclusively in the kidney of transgenic mice also led to hypertension. Transgenic animals with cardiac overexpression of RAS components were prone to cardiac hypertrophy and fibrosis. When the angiotensin-II generating enzyme chymase was expressed as transgene in the vascular wall, hypertension and smooth muscle cell proliferation was elicited. Hypertension was also observed in mice overexpressing angiotensinogen only in fat tissue. Furthermore, transgenic technology was employed to define DNA elements in the promoters of the renin and the angiotensin-converting enzyme (ACE) genes, which are responsible for correct tissue specificity and regulation of their expression. Thus, transgenic technology has been extremely useful to analyze the regulation and the physiological functions of the circulating and particularly the tissue-bound RAS. Novel methods of gene suppression such as RNA interference will render this technique even more utilitarian for this purpose.

Keywords Transgenic rat · Transgenic mouse · Tissue-specific promoter · Microinjection · Renin · Angiotensinogen · Angiotensin

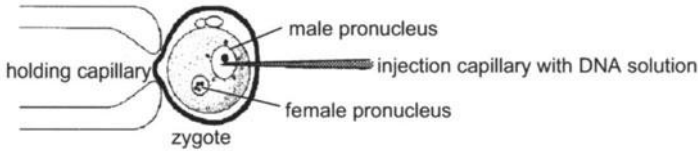
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Transgenic Technology

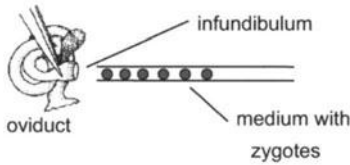
Transgenic technology was established for the mouse more than 20 years ago. Most of the experiments since then have also been performed in mice (reviewed in Palmiter and Brinster 1986; Hanahan 1989; Mockrin et al. 1991; Franz et al. 1997). However, the technique has also been extended to other mammals, such as the rat (Mullins et al. 1990; Hammer et al. 1990; Ganten et al. 1992; Matsumoto et al. 1995). The most common method to produce transgenic mammals is the microinjection of DNA constructs into the paternal pronucleus of a fertilized oocyte (Fig. 1). The injected zygotes are implanted into the oviduct of foster mothers and brought to term. For mice and rats, 5%–20% of the offspring have integrated the transgene into their genome and pass it to their offspring, thereby establishing a transgenic line. Mostly, several copies of the foreign DNA are integrated at one site in a chromosome. The expression of the transgene, however, does not only depend on the copy number but also on the chromosomal environment of the integration site and is therefore not absolutely predictable.

In most of the cases it is intended to overexpress the gene of interest. Ubiquitous overexpression of genes is achieved by linking cDNAs to strong viral promoters with little tissue-specificity or by the use of large genomic constructs containing most or all regulatory elements of the gene, which leads to an in-

1. Microinjection of DNA into zygotes



2. Transfer of injected zygotes into the oviduct of a foster mother



3. DNA-analysis of offspring

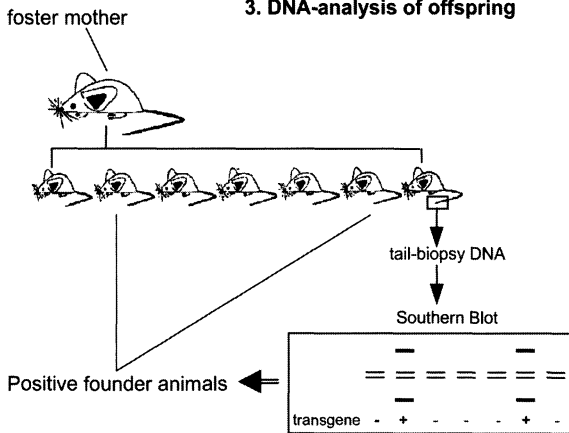


Fig. 1 Scheme for the generation of transgenic animals. About 100 copies of the DNA of interest are injected into a pronucleus of a fertilized oocyte (1). The injected oocytes are transferred into the oviduct of a pseudopregnant foster mother (2). The genomic DNA of the offspring is analyzed, e.g., by Southern blot, and transgene-carrying founder animals are identified (3) and bred to generate the transgenic lines

creased copy number of the gene in the genetically modified animal and to a higher expression with conserved tissue specificity. In order to overexpress a gene in a specific organ or cell type, the respective cDNA is fused to a tissue-specific promoter, and this construct is used for the production of transgenic animals. When oncogenes like the SV40 T antigen are expressed in a specific cell type of transgenic animals, the cells are transformed and can eventually be taken in permanent cell culture. Furthermore, inducible promoter systems are available, which allow for the control of transgene expression by the application

of substances to the genetically modified animals, e.g., ecdysone (No et al. 1996), tetracyclines (Kistner et al. 1996), or indole-3 carbinol (Kantachuvesiri et al. 2001).

The transgenic expression of genes for proteins like luciferase, β -galactosidase (lacZ), green fluorescent protein (GFP), or chloramphenicol acetyltransferase (CAT), which can easily be visualized and quantitatively assessed, allows for the analysis of promoter regions important for tissue-specificity and inducibility of transcription of a gene.

The microinjection technique has also been applied to downregulate gene expression in mice and rats. The method used is the expression of antisense RNAs in transgenic animals by the microinjection of constructs in which a part of the cDNA of interest has been fused in opposite orientation to a strong promoter. This results in the synthesis of an RNA complementary to the transcript from the gene of interest. The two RNAs will form a duplex in cells, which blocks translation of the mRNA. Despite the fact that the ablation of gene expression by antisense RNA is never complete and sometimes even fails (Jaquet et al. 1996), animal models with physiological alterations have been successfully developed using this method (Katsuki et al. 1988; Munir et al. 1990; Pepin et al. 1992; Matsumoto et al. 1995; Schinke et al. 1999; Beggah et al. 2002).

Because of the complexity of the cardiovascular system and its regulation, the functional analysis of genes involved in its regulation has been grossly limited to whole organism models. Therefore, transgenic technology has also been of highest importance for characterizing the role of the renin-angiotensin system (RAS) in cardiovascular regulation and in the etiology of diseases. The expression of all RAS components has been altered by genetic manipulation in rats and mice. In particular, the physiological functions of angiotensin II, locally generated in tissues such as brain, heart, and kidney, have been the focus of transgenic research.

2 **Ubiquitous Overexpression**

2.1 **Rodent Angiotensinogen**

The first transgenic model with alteration in angiotensinogen expression was a mouse overexpressing the rat gene under the control of the mouse metallothionein promoter (Ohkubo et al. 1990). Although exhibiting high levels of circulating angiotensin II, these mice were normotensive. Our group has generated a transgenic mouse with the rat angiotensinogen gene under the control of its own regulatory sequences (Kimura et al. 1992). These animals [TGM(rAO-GEN)123] exhibit marked transgene expression in brain and liver and high levels of circulating angiotensinogen and angiotensin II, that cause high blood pressure (Kimura et al. 1992) and the typical signs of end-organ damage also

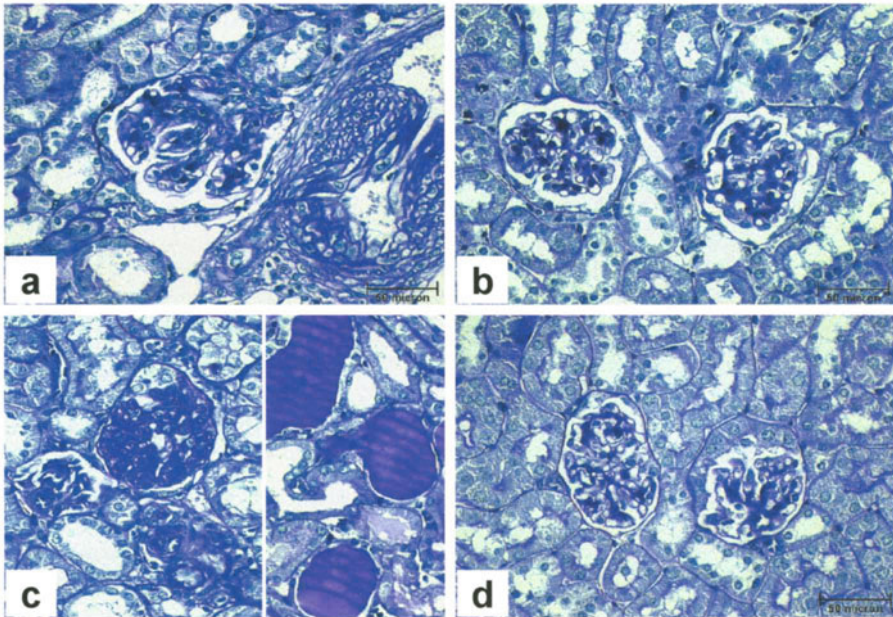


Fig. 2a–d Kidney damage in mice with modified angiotensinogen expression. Periodic acid–Schiff staining of kidney sections from angiotensinogen-knockout (**a**), wild-type (**b**), TGM(rAOPEN)123 (**c**), and TGM(rAOPEN)123 mice on a knockout background (**d**). TGM(rAOPEN)123 (**c**) show the typical signs of tubular and glomerular damage also observed in hypertensive patients, e.g., hyalin depositions. When kidney expression of angiotensinogen is blunted by ablation of the endogenous gene (**d**) the damage is hardly detectable. Angiotensinogen-deficient mice (**a**) show thickening of the elastic vascular lamina and of the glomerular basement membrane as described previously. (Niimura et al. 1995)

observed in human hypertensives, such as cardiac hypertrophy and renal fibrosis (Fig. 2) (Kang et al. 2002).

2.2 Human Angiotensinogen and Human Renin

All rodents transgenic for human angiotensinogen remain normotensive even though some of them exhibit very high levels of the human protein in plasma (Takahashi et al. 1991; Ganten et al. 1992; Yang et al. 1994). The same is true for all animals carrying the human renin gene as transgene (Fukamizu et al. 1989; Ganten et al. 1992; Sigmund et al. 1992; Thompson et al. 1996; Sinn et al. 1999a). These findings corroborate previous biochemical studies showing that human renin and angiotensinogen do not interact with their rodent counterparts (Oliver and Gross 1966). Because of this species specificity of the enzyme-substrate reaction, only double transgenic mice and rats carrying both the human renin and angiotensinogen genes as transgenes become hypertensive (Fukamizu et al. 1993; Merrill et al. 1996; Thompson et al. 1996; Bohlender et al. 1997; Sinn et al.

1999a). These “humanized” rodent models independently developed by several groups are useful to test human-renin inhibitors, which because of the species specificity cannot be tested in normal rodents (Ganten et al. 1992).

Transgenic rats expressing the two human RAS genes develop a fulminant form of malignant hypertension and die at about 7 weeks of age. Because of that, they have become an important model to study the pathophysiology of end-organ damage caused by hypertension and high levels of angiotensin II in tissues (Luft et al. 1999). The work of Luft et al. has shown that angiotensin II acts in concert with mineralocorticoids, since treatment with the mineralocorticoid receptor antagonist spironolactone blunts cardiac hypertrophy and fibrosis and rescues the animals (Fiebeler et al. 2001). Furthermore, the double transgenic rats have furthered the concept that the immune system is intimately involved in the pathogenesis of hypertensive renal damage probably via the activation of nuclear factor (NF)- κ B by angiotensin II (Mervaala et al. 1999). Inhibitors of NF- κ B such as PDTC and immunosuppressing agents such as dexamethasone, cyclosporine A, and aspirin abolish kidney damage without much effect on blood pressure (Mervaala et al. 2000; Müller et al. 2000, 2001).

Interestingly, when female transgenic mice and rats with the human angiotensinogen gene are bred with male animals carrying the human renin gene, they develop hypertension during pregnancy, which is completely cured after delivery (Takimoto et al. 1996; Bohlender et al. 2000). Thus, such animals may also be relevant models for specific forms of pregnancy-induced hypertension.

2.3

Mouse Renin

The most frequently used transgenic model for the functional analysis of the RAS has been a rat carrying the murine renin gene, *Ren-2* [TGR(mREN2)27] (Mullins et al. 1990). The same transgene containing all exons and introns as well as 5.3-kb upstream and 9.5-kb downstream sequences had been tested before in transgenic mice (Mullins et al. 1989) and in both species it is highly expressed in a variety of tissues including kidney, adrenal gland, and brain. Despite relatively low levels of circulating angiotensin II, TGR(mREN2)27 rats develop severe hypertension already starting at early age (5 weeks after birth) reaching 240 mmHg at 12 weeks of age (Lee et al. 1991, 1996; Kreutz et al. 1998). In some genetic backgrounds or after duplication by homozygosity breeding, the transgene even causes malignant forms of hypertension with a high mortality rate at the same age (Whitworth et al. 1994; Lee et al. 1996; Kantachuvesiri et al. 1999). Also in the surviving heterozygous TGR(mREN2)27, hypertension is accompanied by end-organ damage as evidenced by a marked cardiac hypertrophy and typical signs of hypertensive renal damage such as tubulointerstitial fibrosis, glomerulosclerosis, and albuminuria (Bachmann et al. 1992; Böhm et al. 1995; Böhm et al. 1996). The animals have been very useful for elucidating the role of angiotensin II in the etiology of end-organ damage. Even very low levels of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor

AT₁ antagonists—which do not lower blood pressure significantly—blunt the development of cardiac hypertrophy and renal damage in this model (Böhm et al. 1995, 1996). These results explain the extreme effectiveness of these drugs for organ protection in hypertensive and diabetic patients (Brown and Vaughan 1998). Obviously, angiotensin II exerts blood pressure-independent effects on target organs. Concordantly, alterations in Ca²⁺ handling and adrenoceptor signaling have been observed in cardiac cells of TGR(mREN2)27 similar to the ones diagnosed in heart failure patients (Zolk et al. 1998).

The adrenal gland is the organ with the highest transgene expression in TGR(mREN2)27 (Mullins et al. 1990; Zhao et al. 1993). The high renin activity in this organ disturbs steroidogenesis and leads to increased concentrations in particular of mineralocorticoids in the plasma of the animals (Sander et al. 1992; Peters et al. 1993; Sander et al. 1994). This effect may participate in the etiology of the hypertension observed in the transgenic rats, since suppression of adrenal steroidogenesis by dexamethasone normalizes blood pressure (Djavidani et al. 1995).

The hypertension developed by TGR(mREN2)27 may also partly be of neurogenic origin. These animals generate up to tenfold more angiotensin II in the central nervous system than control rats (Senanayake et al. 1994). When they are anesthetized by chloralose-urethane, blood pressure drops to normal, arguing in favor of this central angiotensin generation as cause for their hypertension (Diz et al. 1998). Furthermore, when the central angiotensin generation in TGR(mREN2)27 is blunted by crossbreeding with transgenic rats exhibiting a brain-specific deficiency in angiotensinogen [TGR(ASrAOGN), see Sect. 3.4], a significant reduction in blood pressure is observed (Schinke et al. 1999).

Interestingly, TGR(mREN2)27 exhibit significant perturbances of the circadian rhythm of blood pressure. While in normal rats blood pressure is highest in the active phase, which is at night, the transgenic rats show highest blood pressure levels at noon (Lemmer et al. 1993). The same shift in blood pressure acrophase is observed after chronic angiotensin II infusion into normal rats (Baltatu et al. 2001). Thus, it may be caused by enhanced angiotensin II levels in peripheral tissues. However, the central RAS also is involved in this shift, since TGR(ASrAOGN) are resistant to the influence of chronic angiotensin II infusion [(Baltatu et al. 2001), see Sect. 3.4].

Furthermore, TGR(mREN2)27 rats contain drastically enhanced prorenin levels in plasma, which may also cause the cardiovascular phenotype of this animal model. This protein is probably mainly released from the adrenal gland, since adrenalectomy reduces its levels to normal (Bachmann et al. 1992; Tokita et al. 1994). In a different transgenic rat model, Veniant et al. (1996) have shown that prorenin, when expressed in the liver of transgenic rats by using the α 1-antitrypsin promoter, reaches similarly high circulating levels as in TGR(mREN2)27. As a consequence, these rats show a comparable degree of cardiovascular hypertrophy as TGR(mREN2)27, probably because prorenin may be activated in peripheral tissues. The recent discovery of a renin (and prorenin) receptor with

signaling function may help to explain the physiological actions of elevated circulating prorenin (Nguyen et al. 2002).

Again using the *Ren-2* gene, a transgenic rat model with inducible hypertension has been developed. To this purpose, the promoter of the cytochrome P450 enzyme CYP1A1 was employed which can be induced mainly in the liver by treatment of the animals with a xenobiotic drug, indole-3 carbinol (Kantachavesiri et al. 2001). After application of the drug, the upregulated prorenin and renin levels lead to an increase in blood pressure and vascular injury.

Recently, a novel method to express a transgene in a defined tissue was applied to generate a comparable model (Caron et al. 2002). A chimeric mRNA of mouse *Ren-1^C* and *Ren-2* was integrated by homologous recombination into a site between the apolipoprotein C3 and A1 gene of the mouse, a region which is strongly influenced by a liver-specific enhancer. As expected, the gene is exclusively expressed in the liver leading to high levels of circulating renin and prorenin that is not controlled by the physiological renin regulators. The animals exhibit significantly increased blood pressure, water intake, and urine output and develop all signs of end-organ damage in kidney and heart similar to the above-mentioned models.

3

Brain-Specific Models

3.1

Human Angiotensinogen and Human Renin

Transgenic mice with overexpression of the human RAS only in the brain become hypertensive (Morimoto et al. 2001, 2002a). Interestingly, it is not important from which cell type the two proteins, human renin and angiotensinogen, are produced. Combined synthesis of both RAS components in neurons by the use of the synapsin-I promoter or in astrocytes employing the promoter for the glial fibrillary acidic protein (GFAP) equally causes hypertension and increases water and salt intake (Morimoto et al. 2002b). In these models, the high blood pressure can be reduced by intracerebroventricular injection of the angiotensin II receptor AT₁ antagonist, losartan, suggesting that the brain RAS is a major determinant of hypertension. Part of the effect seems to be mediated by the release of vasopressin, since intravenous injection of a V1 receptor antagonist attenuates the hypertensive phenotype (Davisson et al. 1998; Morimoto et al. 2002b). In some of the models but not in all, ganglion blockade also affects blood pressure, indicating that enhanced sympathetic outflow may also be involved in the central angiotensin action (Davisson et al. 1998; Morimoto et al. 2002b).

3.2

AT_{1A} Receptor

Mice overexpressing the AT_{1A} receptor in most neurons, by using the neuron-specific enolase promoter, remain normotensive but are very sensitive to intracerebroventricular injections of angiotensin II and losartan, indicating that blood pressure in this model is balanced by the baroreflex (Lazartigues et al. 2002).

3.3

Angiotensin II

Recently, a transgenic mouse was presented with eight times more angiotensin II in the brain but normal levels in the circulation (Lochard et al. 2003). The peptide is liberated during secretion from an artificial chimeric protein (Methot et al. 1997) expressed under the control of the GFAP promoter. Also these animals are hypertensive.

3.4

Antisense RNA Against Angiotensinogen

The already-mentioned transgenic rat model, TGR(ASrAOGEN), has provided numerous insights in the functionality of the brain RAS. These rats carry a transgene expressing an antisense RNA against angiotensinogen specifically in astrocytes of the brain under the control of the GFAP promoter (Schinke et al. 1999). This causes a reduction of local angiotensinogen levels by 90% and reduced central angiotensin generation (Huang et al. 2001) without affecting the circulating RAS. The rats are slightly hypotensive and excrete increased amounts of diluted urine (Fig. 3). This mild diabetes insipidus is probably caused by reduced vasopressin levels in the circulation, again supporting a central involvement of brain angiotensin II in vasopressin secretion.

Probably due to decreased central angiotensin levels, TGR(ASrAOGEN) exhibit an increased expression of AT₁ receptors in most brain areas beyond the

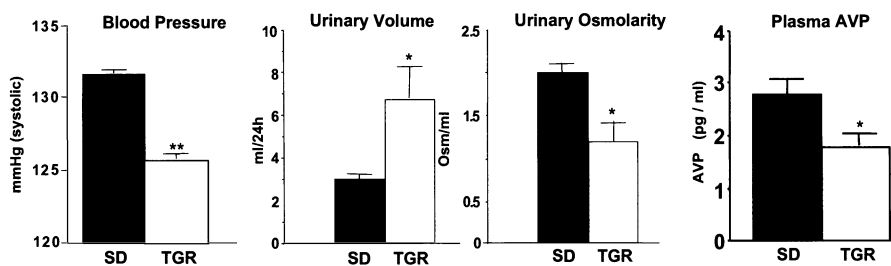


Fig. 3 Phenotype of TGR(ASrAOGEN). TGR(ASrAOGEN) (*TGR*) exhibit reduced blood pressure and excrete a high amount of urine with low osmolarity, probably due to lowered plasma levels of vasopressin (*AVP*) compared to Sprague-Dawley control rats (*SD*)

blood-brain barrier, which causes an augmented drinking response to intracerebroventricularly injected angiotensin II (Monti et al. 2001).

Furthermore, TGR(ASrAOGEN) show alterations in short-term and long-term blood pressure variability and an increased baroreflex sensitivity due to an imbalance of the parasympathetic and sympathetic nervous system (Baltatu et al. 2001). Together with the findings that TGR(mREN2)27 (Borgonio et al. 2001) and mice expressing the human RAS (Davisson et al. 1998) exhibit a decreased baroreflex sensitivity, these data characterize central angiotensin as a relevant moderator of the baroreflex.

As mentioned above, central angiotensin is also significantly involved in cardiovascular rhythm control. Renin overexpression in TGR(mREN2)27 (Lemmer et al. 1993), as well as low-dose peripheral infusions of angiotensin II in normal rats (Baltatu et al. 2001) cause an inversion of the circadian blood pressure rhythm. This effect of increased peripheral angiotensin II is absent in TGR(ASrAOGEN) (Baltatu et al. 2001). Thus, peripheral angiotensin II requires central angiotensin II as a mediator of the rhythm shift. Since only the rhythm of blood pressure but not of heart rate is altered by angiotensin II, the peptide does not appear to affect the main oscillator in the suprachiasmatic nucleus, but seems to affect its output pathways or its synchronization with peripheral oscillators in cardiovascular organs.

Since the pineal gland is considered to regulate circadian rhythms, we studied the content of melatonin and its indole precursors in pineals of TGR(ASrAOGEN) rats (Baltatu et al. 2002). The levels of these indoles were significantly decreased in TGR(ASrAOGEN). Moreover, the mRNA for the first enzyme in melatonin synthesis, tryptophan hydroxylase, was significantly reduced in TGR(ASrAOGEN) rats in contrast to the transcript of the *N*-acetyltransferase gene which was unaffected. These results demonstrate that a local pineal RAS exerts a tonic modulation of indole synthesis by influencing expression and activity of tryptophan hydroxylase.

4

Kidney-Specific Models

In addition to the brain, locally generated angiotensin in the kidney is also relevant for blood pressure regulation. Sigmund et al. (Davisson et al. 1999; Ding et al. 2001) recently showed that overexpression of human angiotensinogen in the kidney, in the presence of ubiquitously expressed human renin without spillover of angiotensin II into the circulation, leads to hypertension. The importance of local angiotensin generation in end-organs has been confirmed in a hybrid mouse model carrying a rat angiotensinogen transgene on an angiotensinogen knockout background (Kang et al. 2002). These animals became hypertensive due to the exclusive expression of rat angiotensinogen in the liver and brain. However, due to the absence of local angiotensinogen synthesis in the kidney and heart, end-organ damage was attenuated (Fig. 2). Thus, the local synthesis of angiotensin II is pivotal for a full development of end-organ damage.

5 Heart- and Vessel-Specific Models

5.1 Angiotensinogen and Angiotensin II

Mice overexpressing angiotensinogen only in the heart remained normotensive but nevertheless developed cardiac hypertrophy, indicating that the local formation of angiotensin II induces cardiac damage independent of blood pressure elevation (Mazzolai et al. 1998, 2000). By contrast, transgenic mice that generate angiotensin II from the aforementioned chimeric protein (Methot et al. 1997) exclusively in the heart do not develop hypertrophy unless spillover of the peptide into the circulation raises blood pressure (van Kats et al. 2001). Cardiac fibrosis is, however, detected in all angiotensinogen-transgenic mice, independent of hypertension, suggesting a direct effect of cardiac angiotensin II on this parameter.

5.2 Angiotensin-Converting Enzyme

Transgenic rats overexpressing ACE predominantly in the heart have been produced (Tian et al. 1996). In spite of very high levels of ACE activity in the heart, there are no morphological alterations of this organ unless it is pressure-overloaded by aortic banding. This treatment results in a significantly higher hypertrophic response in the ACE-transgenic rats than in control animals, supporting the important role of angiotensin II in this process.

5.3 AT₁ Receptor

The α -myosin heavy-chain promoter has been employed to overexpress AT₁ receptors in the heart of transgenic animal models (Hein et al. 1997; Paradis et al. 2000; Hoffmann et al. 2001). However, the phenotypes of the transgenic animals generated were markedly different. Mouse models exhibit a drastic cardiac hypertrophy and die within several days (Hein et al. 1997) or months (Paradis et al. 2000) of age. Despite high levels of AT₁ receptors in the heart (Rosenkranz et al. 1997; Hoffmann et al. 2001), the transgenic rats appear absolutely normal unless the heart is pressure- or volume-overloaded by aortic banding or aortocaval shunt, respectively, which, as for the ACE-transgenic rats, elicit a more pronounced hypertrophy than in control animals (Hoffmann et al. 2001). The difference between the models might be related to a differential sensitivity of mouse and rat hearts for angiotensin II effects that may be linked to a high level of uncoupled AT₁ receptors in the transgenic rats (Rosenkranz et al. 1997).

5.4

AT₂ Receptor

When the AT₂ receptor is overexpressed in the heart, the resulting transgenic mice show no obvious morphological alterations but they are less sensitive to angiotensin II-induced blood-pressure elevation and less susceptible to remodeling of the left ventricle after myocardial infarction, indicating that the AT₂ receptor counteracts the AT₁ receptor (Masaki et al. 1998; Sugino et al. 2001; Yang et al. 2002). The possible mechanism of AT₂ action involves activation of kinins, as has been shown in another transgenic mouse model overexpressing the AT₂ receptor in vascular smooth muscle cells (Tsutsumi et al. 1999). These animals did not increase blood pressure after angiotensin II infusion due to a counter-regulatory hypotensive action of the transgenic AT₂ receptor via bradykinin and NO.

5.5

Chymase

Vascular smooth muscle overexpression of rat vascular chymase, an enzyme that like ACE metabolizes angiotensin I to II, caused hypertension and smooth muscle cell proliferation in transgenic mice (Ju et al. 2001). The authors used a tetracycline-regulated transgene (Kistner et al. 1996), allowing them to switch on and off transgene expression by doxycycline application. These animals underscore the possible importance of chymases for pathophysiological angiotensin II generation.

6

Fat-Specific Models

Adipose tissue is a major site of angiotensinogen expression. In order to analyze the role of this protein in fat tissue, Massiera et al. (2001) generated a transgenic mouse overexpressing angiotensinogen in adipocytes using the aP2 promoter. The resulting animals showed adipocyte hypertrophy, enlarged total fat mass, and hypertension, the latter probably being caused by a spillover of angiotensinogen into the circulation. Thus, angiotensinogen generation in fat may be physiological relevant for adipocyte development and regulation but may also represent a significant source for circulating renin substrate.

7

Testis-Specific Models

The only RAS component studied by transgenic technology in testis was ACE. Besides the promoter responsible for endothelial expression of the enzyme, a second promoter in intron 13 of the gene drives expression of the testis isoform of ACE. Male knockout mice for ACE are sterile, indicating an essential role of

this isoform (Krege et al. 1995). Sen et al. have studied this role by rescuing testicular ACE expression in knockout mice by introducing a transgene with a sperm-specific promoter linked to the cDNA coding for the testis isoform of ACE (Ramaraj et al. 1998). When the endothelial isoform is introduced in the same way, no restoration of male fertility is observed (Kessler et al. 2000). However, the exact function of the testis isoform of ACE in male fertility is still unresolved.

8

Promoter Studies

8.1

Renin

Transgenic experiments have shown that DNA sequences in the promoter as well as in transcribed regions of the *Ren-2* gene are necessary for correct tissue specificity of expression. Transgenic mice with 5.3 kb (Mullins et al. 1989) or 2.5 kb (Tronik et al. 1987; Tronik and Rougeon 1988) of the promoter region and all exons and introns, or with a 4.6-kb promoter fragment and SV40 T antigen as a reporter gene, express the transgene correctly in the kidneys, reproductive organs, and submandibular and adrenal glands; and transformed renin-producing cells have been developed from these mice (Sigmund et al. 1990a,b,c, 1991; Jacob et al. 1991). Animals with only 2.5 kb of the promoter and the same reporter gene, however, show ectopic expression and do not develop renin-producing tumors (Sola et al. 1989). Thus, there appears to be some redundancy of tissue-specific elements in the promoter and in the transcribed region, since 2.5 kb of promoter are only sufficient for correct expression in concert with all exons and introns, while longer 5'-flanking regions are independent of other parts of the *Ren-2* gene. Comparable results have also been published for the *Ren-1^D* gene, since 5 kb of the promoter leads to correct expression in transgenic mice, predominantly in the kidney in the presence of the entire transcribed region but not when fused to a CAT reporter gene (Miller et al. 1989).

Furthermore, the human renin gene is expressed in a partially ectopic pattern in transgenic animals. Gene regulation is inadequate in transgenic mice (Fukamizu et al. 1989; Sigmund et al. 1992; Thompson et al. 1996; Yan et al. 1998a; Sinn et al. 1999b) and rats (Ganten et al. 1992; Bohlender et al. 1997) if only up to 3 kb of upstream sequences are included in the construct. While the kidney is the major tissue of expression, other organs such as the spleen and testis, contain considerable amounts of transgenic mRNA. However, when constructs are used with more than 25 kb of 5'-flanking sequences, transgene expression is largely confined to the kidney and to a lesser extent to the lung and other organs which also express renin at low levels in humans (Yan et al. 1998a,b; Catanzaro et al. 1999; Sinn et al. 1999a). Furthermore, bilateral nephrectomy depletes circulating human renin and prorenin in these mice, as expected, while animals with shorter transgene constructs paradoxically show in-

creased plasma prorenin levels after the operation (Yan et al. 1998a). These differential sources of circulating renin may also be responsible for observed differences in blood pressure after coexpression of human angiotensinogen in human renin transgenic animals. Animals with short transgene constructs become hypertensive (Fukamizu et al. 1993; Merrill et al. 1996; Thompson et al. 1996; Bohlender et al. 1997) and mice with long promoter regions develop either only slightly elevated blood pressure or even remain normotensive (Catanzaro et al. 1999; Sinn et al. 1999a). This indicates that the normal downregulation of the human renin transgene by elevated blood pressure occurs only in animals harboring long promoter regions.

Recently, Fuchs et al. (2002) showed that 12 kb of the upstream region, but neither 2.8 kb nor 5.8 kb, are sufficient to drive correct tissue-specific expression of the human renin gene. In their transgenic mice, the lacZ reporter gene is expressed in juxtaglomerular cells and in a striped pattern in renal arteries, but surprisingly they also detected stripes of transgenic lacZ activity and endogenous mouse renin immunoreactivity in arteries outside the kidney, e.g., in the aorta. Furthermore, classical stimulators of renin synthesis such as two-kidney, one-clip hypertension and salt depletion increase the expression of the lacZ transgene even more pronounced than the endogenous renin gene, indicating that all DNA elements necessary for tissue specificity and upregulation of human renin gene expression reside within the 12-kb fragment employed in the lacZ construct.

Accordingly, angiotensin II infusion in human renin transgenic mice with only a 900-bp upstream region results in a paradoxical upregulation of human renin expression, while mouse renin is expectedly suppressed (Keen and Sigmund 2001).

8.2 Angiotensin-Converting Enzyme

Using lacZ as a reporter gene in transgenic mice, the group of Bernstein et al. has restricted the testis-specific promoter of ACE to 91 bp in intron 13 of the gene (Langford et al. 1991; Howard et al. 1993). However, the testis-specific elements could not yet be determined (Zhou et al. 1996; Esther et al. 1997).

9 Conclusions

Transgenic technology in concert with gene targeting (see the previous chapter, by Brede and Hein, this volume) has been extremely useful to determine the physiological functions of the RAS. In particular, the analysis of tissue RAS relies mainly on these techniques since pharmacological approaches cannot be easily targeted to specific organs. When novel methods of gene suppression such as RNA interference (Tuschl 2002) will be available for use in transgenic models, transgenic technology will become even more utilitarian for this purpose.

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Gene Therapy and the Renin–Angiotensin System

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Abstract The success of gene therapy requires specific targets. For the potential treatment of cardiovascular disease by gene therapy, the renin–angiotensin system offers targets that are known to be effective based on pharmaceutical drugs that have targeted angiotensin-converting enzyme (ACE) angiotensin type I receptors (AT₁R). Other targets include renin and angiotensinogen. It is clear that lowering overactive amounts of angiotensin II has positive effects on reducing high blood pressure, decreasing left ventricular hypertrophy, and lowering the risk of heart attacks, stroke, and kidney failure. To inhibit the RAS system we have used antisense (AS) inhibitors which can be delivered either as oligonucleotides and used like small molecule drugs or in vectors with a sequence of DNA in the antisense direction. This review summarizes the effects of these two approaches on reducing high blood pressure. Gene therapy with antisense offers long-lasting, highly specific inhibition of gene products such as the components of RAS when their overactivity needs to be reduced in order to ameliorate disease.

Keywords Gene therapy · Angiotensin-converting enzyme (ACE) · Angiotensin type I · Renin · Angiotensinogen · Antisense (AS) · Oligonucleotides

1 Introduction

The renin–angiotensin system (RAS) is important in blood pressure regulation, volume regulation, and vascular tissue growth. Angiotensin II (Ang II), an octapeptide, is the active peptide of the system. It is formed from angiotensin I (Ang I) by angiotensin-converting enzyme (ACE). Ang I is formed from angiotensinogen (AGT) by renin. Ang II is the ligand for Ang II type 1 receptors (AT_1R) and Ang II type 2 receptors (AT_2R). In addition, angiotensin II metabolites, Ang III, Ang IV, and Ang 1–7, are active and may have independent receptors (Fig. 1). All the components of RAS are present in the brain and in the periphery; however, renin levels in the brain are very low. Both the brain and blood-borne RAS are important for blood pressure regulation. The brain RAS is also involved in drinking, salt intake, the baroreflex, and hormonal release from the paraventricular nucleus (PVN). The peripheral tissue RAS is involved in cardiac hypertrophy and hyperplasia. AT_1 receptors have been shown to mediate blood pressure and the growth effects of Ang II (Benetos et al. 1996; Chung and Unger 1999; Kurland et al. 2002; Unger 2002). The role of the AT_2 receptor is still uncertain, although it has been implicated in apoptosis and has effects opposing those of the AT_1 receptor (De Paepe et al. 2001, 2002; Schmieider et al. 2001; Unger 2002). However, mice with AT_1 receptors, but lacking AT_2

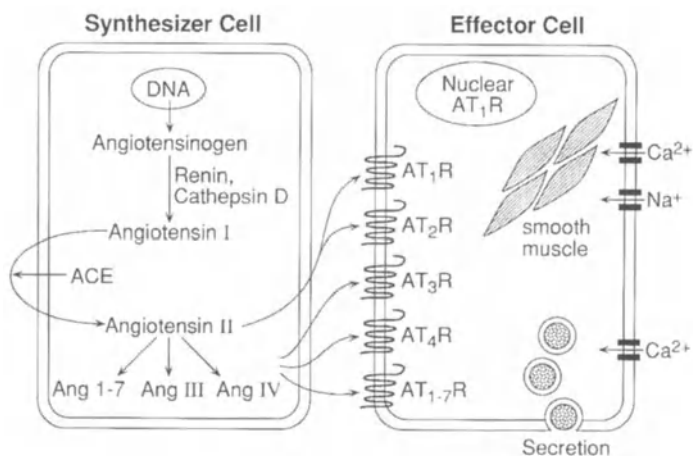


Fig. 1 The renin–angiotensin system (RAS) at the cellular level. In the brain and other tissues, all evidence points to the system being formed by a paracrine mechanism involving cells with genes for synthesis of angiotensin II (Ang II) and cells for uptake of metabolites through different, specific receptors. As angiotensin-converting enzyme (ACE) is an ectoenzyme, its action on the conversion of Ang I to Ang II is extracellular. Understanding of the specific functional roles of these receptors and metabolites is the key to specific gene targeting for cardiovascular gene therapies

receptors, did not develop hypertrophy in response to Ang II infusion (Ichihara et al. 2001), suggesting that the relationship between the two receptor types is more complex.

The rate-limiting step in the RAS cascade is the conversion of AGT to the decapeptide, Ang I. Increases in AGT production have been shown to effect blood pressure both in man and in experimental animals (Walker et al. 1979; Kim et al. 1995; Bloem et al. 1997). Transgenic mice that produce high levels of Ang II are hypertensive (Ohkubo et al. 1990; Fukamizu et al. 1993; Merrill et al. 1996; Davisson et al. 1999; Morimoto et al. 2001; Stec et al. 2002), and hypertensive rat models have increased levels of Ang II (Morton and Wallace 1983; Phillips and Kimura 1986, 1988; Morishita et al. 1992; Navar et al. 1995). Spontaneously hypertensive rats (SHR) also have an increased density of Ang II receptors (Gutkind et al. 1988; Brown et al. 1997).

Human genetic studies have shown that the AGT gene is linked to hypertension. In French and Utahan populations, the AGT 235T variant is more frequent in hypertensives than in controls (Atwood et al. 1997; Corvol and Jeunemaitre 1997; Niu et al. 1998; Jain et al. 2002). The ACE gene insertion/deletion variant has also been implicated, but the association of this gene with hypertension may depend on both ethnicity and gender (O'Donnell 1998; Agerholm-Larsen et al. 2000). There is also some evidence for the involvement of AT₁ receptor gene polymorphism involvement in human hypertension and arterial stiffness (Bonnardeaux et al. 1994; Benetos et al. 1996; Kurland et al. 2002).

The pressor effects of circulating Ang II have been known since the 1930s (Phillips and Schmidt-Ott 1999) and ACE inhibitors are a preferred class of drugs used to treat high blood pressure. Both ACE inhibitors and the newer AT₁ receptor antagonists decrease left ventricular hypertrophy (LVH) and hypertension (Chung and Unger 1999; Phillips 2001a,b; Unger 2002). However, these drugs have to be taken daily, and despite being effective in controlling hypertension, only 27% of patients with hypertension take the drugs consistently (Phillips 2000, 2001a,b). A shocking 73% of patient with hypertension do not comply with their drug treatment (Kaplan 1998). Cardiovascular disease is the leading cause of death in the United States and Europe, and WHO estimates that 17 million people worldwide die of cardiovascular diseases every year (www.americanheart.org). Hodgson and Cai reported that the cost of hypertension was \$108.8 billion in the U.S. in 1998, and the American Heart Association estimated the direct and indirect costs of cardiovascular diseases to be \$329 billion in the U.S. in 2002 (Hodgson and Cai 2001). Clearly, new treatments for hypertension are needed. We propose that a gene therapy approach would offer several advantages that may increase compliance: First, the treatment would be long-lasting (weekly, month or longer); and second, the high specificity of gene targeting would produce few side effects.

One approach is to target the mRNA of components involved in hypertension. Even though hypertension is a multifactorial disease, inhibition of RAS remains a promising strategy, as pharmacological depression of the system is known to decrease blood pressure. We propose antisense inhibition of well-doc-

umented drug targets such as ACE, AGT, and AT₁R. Preclinically, we have tested antisense oligonucleotides (AS-ODN), plasmids and viral vectors to decrease levels of components of the RAS (Tables 1 and 2) (Phillips et al. 1994; Kagiya et al. 2001; Phillips 2001a,b).

Table 1 Gene therapy for hypertension: AS against brain RAS vasoconstrictor genes

Target gene	Construct	Route of delivery	Animal model	Max Δ BP (mmHg)	Duration of effect	Reference(s)
AT ₁ receptor	AS-ODN	ICV	SHR	-45	7 days	Gyurko et al. 1993, 1997; Piegari et al. 2000
AT ₁ receptor	AS-ODN	ICV	CIH	-35	4 days	Peng et al. 1998
AT ₁ receptor	AS-ODN	ICV	2K1C chronic	-20	>5 days	Kagiya et al. 2001
AT ₁ receptor	AAV	ICV	SHR	-40	>9 weeks	Phillips et al. 1997
AGT	AS-ODN	ICV	SHR	-35	n.d.	Gyurko et al. 1993; Wielbo et al. 1995; Kagiya et al. 1998
AGT	AS-ODN	ICV	CIH	-40	2 days	Peng et al. 1998
Renin	AS-ODN	ICV	SHR	-20	3 days	Kubo et al. 2001
AGE 2	Decoy ODN	ICV	SHR	-30	7 days	Nishii et al. 1999

Table 2 Gene therapy for hypertension: AS against peripheral RAS vasoconstrictor genes

Target gene	Construct	Route of delivery	Animal model	Max Δ BP (mmHg)	Duration of effect	Reference(s)
AT ₁ receptor	AS-ODN	IC	CIH	-35	n.d.	Peng et al. 1998
AT ₁ receptor	AS-ODN	IV	2K1C acute	-30	>7 days	Galli and Phillips 2001
AT ₁ receptor	LNSV	IC in 5-day-old rats	SHR	-45	>120 days	Iyer et al. 1996; Lu et al. 1997
AT ₁ receptor	LNSV	IC in 5-day-old rats	60% fructose	-20	>2 weeks	Katovich et al. 2001
AGT	AS-ODN	Portal vein	SHR	-20	4 days	Tomita et al. 1995
AGT	AS-ODN	IV	SHR	-30	7 days	Wielbo et al. 1996; Makino et al. 1998, 1999; Sugano et al. 2000
AGT	P AS-AGT	IV	SHR	-20	8 days	Tang et al. 1999
AGT	AAV	IC in 5-day-old rats	SHR	-25	6 months	Kimura et al. 2001
AGE 2	Decoy ODN	Portal vein	SHR	-20	6 days	Morishita et al. 1996

n.d., Not determined.

2

Antisense Oligonucleotides

AS-ODNs consist of short DNA sequences of 12–20 bases that are complementary to the mRNA producing the protein of interest. They bind to the mRNA and prevent translation of the specific protein encoded in the mRNA. To prevent degradation while in the circulation, the oligonucleotides are phosphorothioated, or otherwise modified, to increase stability. AS-ODNs can be solely administered, but delivery in liposomes, liposomes coupled to Sendai virus, or in carrier molecules increases uptake and prolongs the effect of AS-ODN (Morishita et al. 1993; Hughes et al. 1994; Dzau et al. 1996; Makino et al. 1999; Clare et al. 2000; Zhang et al. 2000, 2001; Fillion et al. 2001). In vitro experiments have shown that AS-ODNs enter the cell and the nucleus (Li et al. 1997).

3

Plasmid Vectors

Full-length antisense mRNA can be manufactured in plasmid vectors under the control of a promoter (Mohuczy and Phillips 2000). Theoretically, plasmids should be effective for a longer duration than AS-ODNs, but practically, the difference is negligible. Although uptake efficiency is an issue, recent studies using liposomes and receptor-mediated uptake have shown adequate effects of antisense mRNA (Tang et al. 1999; Merdan et al. 2002). Plasmid vectors have the potential to express antisense mRNA in specific cell types, if cell-specific promoters or cell-specific delivery systems are used (Zhang et al. 2002). Thus, they would be advantageous when transient expression is required.

4

Viral Vectors

Viral vectors containing cDNA in the antisense orientation, can potentially integrate into the genome and express antisense mRNA for components of RAS. Our results show long-term attenuation of hypertension and cardiac hypertrophy (Tables 1–3) (Phillips et al. 1997; Mohuczy and Phillips 2000; Kimura et al. 2001; Phillips 2001a,b). The adeno-associated virus (AAV) was used because it is safe, stable, long acting, and appropriate for gene therapy in adult models. Retroviruses preferentially infect dividing cells and integrate randomly into the genome (Mohuczy and Phillips 2000; Phillips 2001a,b). They have been used in infant models that study the development of hypertension (Lu et al. 1997; Wang et al. 2000; Metcalfe et al. 2002). However, they are not suitable for adult gene therapy. Lentiviruses (based on HIV, SIV, or FIV) can infect non-dividing cells and have a large carrying capacity; however, they integrate randomly and thus may disrupt other genes and cause mutagenesis (Hauswirth and McInnes 1998; Mohuczy and Phillips 2000; Sinnayah et al. 2002). Adenoviruses have very good uptake and do not integrate into the host genome. They show high levels of short-term expression. Their major disadvantage, as a long-term therapy, is the

Table 3 AS to RAS: effects on growth

Target gene	Construct	Route of delivery	Animal model	Effect studied	Magnitude	Reference
AT ₁	LNSV	IC in 5-day-old rats	TGR mRen2	Hypertrophy	90% decrease	Pachori et al. 2002
AGT	AS-ODN	IV	SHR	Hypertrophy	60% decrease	Makino et al. 1999
AGT	AS-ODN	IV	SHR	Media of aorta	32% decrease	Sugano et al. 2000
AGT	AAV	IC in 5-day-old rats	SHR	Hypertrophy	About 25% decrease	Kimura et al. 2001
ACE	AS-ODN	Into injured artery	SD balloon catheter injury	Neointima formation	Injured control: 0.24 mm ² AS treated: 0.1 mm ²	Morishita et al. 1992

immune and inflammatory responses they elicit (Hauswirth and McInnes 1998; Mohuczy and Phillips 2000; Phillips 2001a; Sinnayah et al. 2002). AAV is, therefore, the viral vector of choice for human use as it does not cause an immune response. It may integrate into the genome when it is modified and certainly has very long-lasting effects. The wild-type AAV does not cause any known diseases, and cannot proliferate without a helper virus. It is likely the safest of viral vectors. The primary disadvantage of the AAV vector is its small carrying capacity. AAV can only accommodate 4.4 kb, and thus the number of promoters, enhancers and length of AS-mRNA is limited. In addition, the deletion of the *rep* sequence removes site-specific integration of the vector (Hauswirth and McInnes 1998; Mohuczy and Phillips 2000; Phillips 2001a,b). Nevertheless, one of the advantages of antisense approach is that it is not necessary to have a full-length DNA, and therefore, shorter AS-DNA sequences can be used effectively in AAV

5 RAS Gene Therapy

We began targeting AT₁R and AGT with AS-ODNs in 1993, as gene therapy for hypertension (Gyurko et al. 1993; Phillips et al. 1994; Sinnayah et al. 2002). In the past 10 years, all the components of the RAS have been targeted for gene therapy and antisense inhibition. Some studies have aimed to understand the mechanisms of actions in RAS; other investigations have studied cardiovascular changes, hypertension, myocardial dysfunction, and the growth effects of the RAS; and still others have studied the hormones of RAS and behavior.

6 AT₁ Receptors

Using antisense oligonucleotides in an intact animal showed that AT₁R antisense injected into the brain lateral ventricle of SHR decreased blood pressure by about 25 mmHg within 24 h, and caused a 16%–40% decrease in AT₁ receptors in the PVN and organum vasculosum lamina terminalis (OVLT) (Ambuhl et al. 1995; Gyurko et al. 1997). These results have subsequently been corroborated by studies from our laboratory, as well as other researchers (Piegari et al. 2000; Sinnayah et al. 2002). AT₁R AS-ODN applied to the central RAS can also attenuate blood pressure in non-genetic models of hypertension. These include the surgical model, two-kidney-one-clip (2K1C), and the environmental model, cold-induced hypertension (CIH) (Peng et al. 1998; Kagiya et al. 2001). The data are summarized in Table 1. In addition to the effect on blood pressure, spontaneous drinking, Ang II, and isoproterenol-induced drinking is decreased by the AT₁R antisense (Meng et al. 1994; Sakai et al. 1995; Peng et al. 1998). Saline-induced (i.e., osmotic) drinking is not affected (Sakai et al. 1995). In early studies, AS-ODN applied to the peripheral circulation failed to elicit a response. When mixed with liposomes, uptake of AT₁-AS-ODN into peripheral organs increased, blood pressure decreased by 25–35 mmHg, and AT₁Rs in kidney and arteries, in both CIH and 2K1C hypertensive rats, also decreased (Peng et al. 1998; Galli and Phillips 2001). The data are summarized in Table 2. AT₁-AS-ODN administered prior to ischemia–reperfusion also protected against myocardial dysfunction (Yang et al. 1998). The effects of AT₁-AS-ODN are transient, lasting for about a week, with the maximum effect seen after 2–3 days.

Viral vectors, on the other hand, enabled very long-term expression of antisense. We have used AAV to deliver AT₁AS mRNA both to the central and the peripheral system of SHR, and obtained attenuation of hypertension by approximately 25 mmHg (see Table 1). The reduction in blood pressure lasted at least 9 weeks (Phillips et al. 1997) and we recorded normalization of blood pressure in double transgenic mice for up to 6 months. These mice have a gene for human renin and another for human angiotensin, and therefore, they constantly overexpress Ang II and are hypertensive. AAV delivery of AS to AT₁R dramatically reduces blood pressure within a few days, and the effect persists for as long as the mice were tested. Our colleagues have used a lentiviral vector (LNSV) to deliver AT₁AS mRNA to 5 day-old SHR, and obtained similar results (see Table 2) (Iyer et al. 1996; Lu et al. 1997; Wang et al. 2000). The attenuation of hypertension and of hypertrophy persisted in the offspring of treated rats. This is probably the result of postnatal age number = “Sec8” (Metcalf et al. 2002; Pachori et al. 2002). We have found that AAV vectors enter the germ line only if the viral vectors are given at a very early age (0–5 days). At this time the blood–testis barrier is open. After postnatal day 5 or 6, rats injected with AAV do not transmit AAV to their offspring.

7

Angiotensinogen

The earliest studies targeting AGT in the brain showed a substantial, up to 40 mmHg, blood pressure decrease in SHR and a decrease of hypothalamic AGT (Wielbo et al. 1995; Gyrko et al. 1993). Subsequent research has confirmed that intracerebroventricular (ICV) injections of AGT-AS-ODN decrease blood pressure in SHR. Injections into the PVN did not affect blood pressure, although it did decrease vasopressin release (Kagiyama et al. 1998). The CIH and 2K1C hypertensive models also respond to AGT-AS-ODN ICV treatment with a decrease in blood pressure (Peng et al. 1998; Kagiyama et al. 2001). The data are summarized in Table 1. The drinking response in CIH animals is also attenuated (Peng et al. 1998). In a normotensive rat, the drinking response to renin and isoproterenol is attenuated by ICV AGT-AS-ODN injection, while the drinking responses to carbachol, Ang II and water deprivation are unaffected (Sinnayah et al. 1997a,b). Early studies report that AS-ODN injected into the peripheral circulation had no effects, likely due to the failure to reach target organs in sufficient amounts. We compared the effects of naked AS-ODN and liposome encapsulated AS-ODN directed against AGT, on blood pressure, AGT and AngII concentration, and hepatic uptake. We found that naked AS-ODN was without effect, whereas liposome-encapsulated AS-ODN lowered blood pressure in SHR. This was accompanied by lower peripheral AGT and Ang II in the liver after injection (Wielbo et al. 1996). Similar results were obtained when AS-ODN was coupled to carrier molecules that targeted delivery to the liver (see Table 2) (Tomita et al. 1995; Makino et al. 1998; Sugano et al. 2000). In addition to effects on protein levels and blood pressure, AGT-AS-ODN also attenuated hypertrophy of the heart and aortic smooth muscles (see Table 3) (Makino et al. 1999; Sugano et al. 2000).

AGT-AS-ODN attenuated hypertension for 4–5 days (Tomita et al. 1995; Makino et al. 1998). When a full-length AGT cDNA was inserted into a plasmid under control of CMV promoter, and injected with liposomes into SHR, the blood pressure decrease lasted for 8 days (Tang et al. 1999). The same construct delivered by AAV to 5-day-old SHR caused a delay in hypertension development, attenuated hypertrophy, and reduced the degree of hypertension for at least 6 months (Kimura et al. 2001).

8

Angiotensin-Converting Enzyme

AS-ODN directed against ACE mRNA have been used to reduce the vascular ACE concentration and formation of neointima after balloon catheter injury (Table 3) (Morishita et al. 2000). ACE-AS-ODN have also been shown to improve cardiac performance after ischemia–reperfusion injury (Chen et al. 2001).

ACE is present at low to moderate levels in large areas of the brain, and in high levels in the NTS, and is likely to generate Ang II locally (Phillips and

Kimura 1999). Increasing ACE levels in the brain, by transfection with a plasmid containing the human ACE gene, caused an increase in blood pressure, heart rate, Ang II, and ACE levels that lasted for 2 weeks (Nakamura et al. 1999). Unpublished results from our lab testing three different sequences of ACE-AS-ODN showed a decrease of 15–25 mmHg in blood pressure in hypertensive SHR.

9 Renin

Renin-AS-ODN decreased blood pressure by about 20 mmHg for 2 days, when injected into the lateral ventricle of SHR. Expression of renin mRNA was also suppressed by this treatment (Kubo et al. 2001). So far, no systemic studies with renin-AS-ODN have been performed. A study with β_1 -AS-ODN showed that renin release was inhibited and blood pressure decreased for up to 1 month in SHR after a single injection of the ODN delivered in liposome (Zhang et al. 2000).

10 AT₂ Receptors

AT₂-AS-ODN infused into the kidney of uninephrectomized normotensive rats, increased blood pressure by about 20 mmHg throughout the infusion period (Moore et al. 2001).

11 Discussion

The use of gene therapy to correct genetic abnormalities and to treat diseases is becoming more relevant, clinically. At least one AS-ODN has been approved by the FDA to treat cytomegalovirus retinitis (de Smet et al. 1999; Orr 2001), and AAV-RPE65 has been used to restore sight in a canine model of blindness (Acland et al. 2001). Adenovirus is being used in phase I and phase II clinical trials in cancer patients (Lamont et al. 2000; Reid et al. 2001; Teh et al. 2001; Freytag et al. 2002; Harvey et al. 2002). AS-ODNs and viral vectors have been introduced into both the periphery and central system of the brain. Peripheral administration is more likely to be the more clinically acceptable therapy. The aforementioned preclinical studies have demonstrated that AS to AGT, AT₁, and ACE can successfully lower blood pressure in hypertensive rats, and attenuate hypertrophy in adult animals when administered systemically. The decrease in blood pressure varies between 15 and 40 mmHg (Tomita et al. 1995; Wielbo et al. 1996; Peng et al. 1998; Makino et al. 1999; Tang et al. 1999; Kimura et al. 2001). While these effects are highly advantageous clinically, it is probably impossible to achieve a “cure” for hypertension with AS-ODN because the mechanism of antisense inhibition is a competition between copies of mRNA and the

amount of ODN delivered to cells. This means that antisense treatment can be effective by reducing overactive receptors or hormones but not interfere with normal physiology. Clearly, there is a dose-dependent effect. We found a correlation between AGT-AS plasmid dose and blood pressure, and there was also a large decrease in blood pressure following AGT-AS-ODN and carrier protein targeting of the liver (Makino et al. 1999; Tang et al. 1999). Another factor favoring the dose-dependant mechanism is that pharmacological drugs, both ACE inhibitors and AT₁R antagonists, are able to normalize blood pressure alone, although some new approaches have tried combinations.

The next step, clinically, is toxicology tests and phase I, I₁ and III trials. In the case of viral vectors, the next step is to ensure that they do not cause adverse effects over a very long time, such as immune responses or tumorigenesis. To this end, we are developing gene switches to turn vectors on and off as needed without resorting to drugs (Phillips et al. 2002). AS gene therapy has the potential of providing extended protection against hypertension, cardiovascular disease, and a multitude of other chronic diseases. Here we have reviewed its use on the RAS as a target system in hypertension and cardiovascular disease, but clearly any target with a known DNA sequence is a target for antisense inhibition.

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Part 3
ANG Receptors

AT₁

AT₁ Receptor Molecular Aspects

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Abstract In the past 12 years, cloning of the AngII AT₁ receptor cDNA and gene have elucidated the primary structure of the protein, but also the structure of the mRNA and gene in different species. Using this cDNA as a tool, the different motifs and sequences involved in functions of the receptor has been established. The binding of AngII implicates several sequences and amino acids of the extra-cellular loops, the N-terminus and upper segments of transmembrane domains (TM)4 to TM7. In contrast, the binding site of nonpeptide inverse agonist losartan is composed of polar residues of TM2 to TM7, and is deeply buried in the lipid bilayer. After agonist binding, the receptor is activated by a conformational

change involving residues of the TMs. In the active state, sequences of intracellular loops two and three and of the C-terminus, which are adjacent to the TM, interact with the G protein to activate it. Sequences of the C-terminus are also involved in activation of other signalling pathways, such as the Jak-STAT pathway. In parallel, seryl residues of the C-terminus are phosphorylated and interact with β -arrestins, resulting in receptor internalization.

Keywords G protein-coupled receptor · AT₁ receptor · Angiotensin II · Site-directed mutagenesis · Ligand binding · Activation · Phosphorylation · Internalization

1 Introduction

The first cloning of the angiotensin II (AngII) AT₁ receptor in 1991 elucidated the primary sequence of the protein and was the starting point of extensive analysis of the structure function relationships of this G protein-coupled receptor (GPCR). The first part of this chapter will review the molecular aspects of the AT₁ receptors, including the structure of the gene(s), mRNAs and protein with their known species differences.

The receptor sequences and amino acids involved in ligand binding, activation and the G protein coupling, signalling, regulation and trafficking of the AT₁ receptor, have been extensively investigated using site-directed mutagenesis of its cDNA and expression of the mutated recombinant proteins. This investigation has produced a precise map of the structural molecular determinants of the different receptor functions. They are reviewed in the second part of this chapter.

Some conformational and mechanistic hypotheses derived from this mutagenesis work and computer modelling are also summarized, despite the absence of structural three-dimensional (NMR, crystallography) data concerning this receptor.

2 Structure of the AT₁ Receptor Gene, mRNA and Protein

2.1 Some History

The presence of AngII binding sites in target tissues such as adrenal cortex (Glossmann et al. 1974) and vascular smooth muscle cells (Gunther et al. 1980) was first demonstrated in the 1970s, when radiolabelled iodinated AngII was first synthesized. These experiments demonstrated the presence of membrane-bound receptors with some pharmacological differences from one tissue to another, but there was no definitive evidence at this time for different molecular species. Despite the preliminary biochemical characterization of the protein,

attempts to purify the receptor were essentially unsuccessful due to loss of AngII-binding properties, after solubilization of the tissues. The first clues on the protein structure came from functional studies demonstrating, using guanosine nucleotide analogues, that the AngII receptor belongs to the protein superfamily of the GPCRs (Wright et al. 1982).

The development of new pharmacological tools in the late 1980s has permitted the identification of two AngII receptor types:

- The AngII AT₁ receptor, which is the standard functional receptor for AngII, binds with high-affinity imidazolic compounds such as Dup753 (losartan) and is sensitive to reducing agents such as dithiothreitol (DTT) (Chiu et al. 1989).
- The AngII AT₂ receptor, a newly identified membrane-bound receptor, binds L-spinacine derivatives (PD123319) (Chiu et al. 1989) and AngII pseudo-peptidic analogues (CGP42112A) (Whitebread et al. 1989) with high affinity, is insensitive to DTT, and is expressed in adrenal medulla and myometrial cells and fetal mesenchymal tissues.

The next step in the elucidation of the AT₁ receptor structure was the cloning of the receptor cDNA. Concurrently, two American labs (Murphy et al. 1991; Sasaki et al. 1991) identified and sequenced the cDNA for, respectively, the bovine adrenal and the rat vascular AT₁ receptors. Their successful strategies used the screening, by AngII binding, of expression cDNA libraries divided into pools and expressed in COS cells. Later, it was demonstrated that there are two subtypes (called AT_{1A} and AT_{1B}) of the AT₁ receptor in rodents (Sandberg et al. 1992) but not in other species, including humans.

2.2

Structure of the Gene

Identification of the genomic sequences encoding the AT₁ receptor followed shortly after the cloning of their cDNAs. Among mammalian species, the gene structure has general conserved features. The sequence coding for the protein is contained in a single exon, which also contains part of the 5' and 3' untranslated sequences. However, the gene contains additional 5' or 3' untranslated exons, whose number and location vary from one species to another.

The human gene, which is almost 50 kb long and located on the chromosome 3q22, consists of four 5' untranslated exons followed by a fifth exon, which contains the receptor open reading frame (Guo et al. 1994; Curnow et al. 1995). Exons 2, 3 and 4 are short (from 58 to 157 bp) and can be alternatively spliced producing eight potential different mRNA species of very similar sizes (Fig. 1).

The two rat AT₁ receptor genes are located on different chromosomes: AT_{1A} on chromosome 17 and AT_{1B} on chromosome 2. The rat AngII AT_{1A} receptor gene consists of four exons and is more than 84 kb in size (Langford et al. 1992; Takeuchi et al. 1993). Exons 1 and 2 are 5' untranslated exons, exon 3 contains

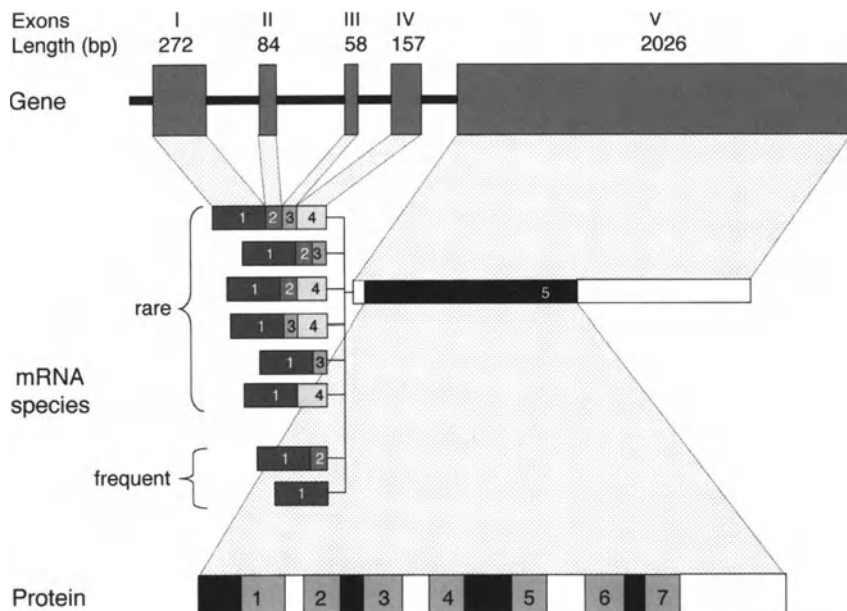


Fig. 1 Structure of the human AT₁ receptor gene, mRNAs and protein. The *upper panel* represents the structure of the gene with the exons (grey boxes numbered I to V) separated by the introns (black lines). Correspondence of each exon with the mRNA sequence or of mRNA with the protein are indicated by *light grey area*. The *middle panel* represents the different mRNA species. They all contain exon 1 (dark grey) and exon 5 (white for the non coding sequence and black for the coding sequence), but exon 2 (grey), 3 (light grey) and 4 (very light grey) are alternatively spliced. The open reading frame codes for the AT₁ protein (359 amino acids, *lower panel*) with its transmembrane domains (grey), extracellular (black) and intracellular (white) sequences

the coding sequence and exon 4 corresponds to 1 kbp of 3' untranslated sequence. The rat AngII AT_{1B} receptor gene has a similar structure, but only three exons have been described so far: the two 5' untranslated exons and exon 3 coding for the protein (Guo and Inagami 1994). This gene is greater than 15 kb in length.

2.3

Structure of the mRNAs

The gene structure predicts eight potential AT₁ receptor mRNA species in humans, depending on the alternative splicing of exons 2, 3 and 4. These different mRNA species have been experimentally identified by RT-PCR (Curnow et al. 1995). However, Northern blot analysis identifies only one band for human AT₁ receptor mRNA, corresponding to a sequence 2.4 kb in length. This apparent discrepancy can be easily explained since exons 2–4 are very short, and therefore the different mRNA species are undistinguishable on Northern blot. The most abundant mRNA species are those containing exons 1 and 5 or 1, 2 and 5.

The functional and expressive differences of these alternatively spliced mRNAs are not known. Exon 1 presents a very complicated GC rich, double-stranded secondary structure, which could be involved in the regulation of translation. Exon 2 contains minicistrons, which may also modify the receptor translation rate. Finally, the mRNA species, in which exon 4 is spliced, may produce a receptor with an amino terminal extension of 32–35 residues, encoded by the end of exon 3 and the beginning of exon 5. The expression of this modified receptor has not been unambiguously demonstrated *in vivo*, and there is no evidence that this first methionine in exon 3 is used as an alternative translation start point of the protein. In addition, the differences in the functions of this potential alternate AT₁ receptor are completely unknown.

Similarly, exon 2 and 4 of the rat AT_{1A} receptor gene are also alternatively spliced. The alternative splicing of exon 4, which contains 1 kb of 3' untranslated sequence, explains the presence of two mRNA species of 2.3 and 3.3 kb on Northern blot (Takeuchi et al. 1993).

The shorter mRNA is expressed in all target tissues of AngII, whereas the 3.3-kb species is abundantly expressed in smooth muscle cells, but not in neurons. It is not known if these various mRNA species have differences in their translation efficiency or in their stability, which would justify a regulation in the proportion of their expression.

2.4

Structure of the Protein

The cloning of the AT₁ receptor cDNA and its sequencing have shown that the receptor is a 359-amino-acid-long integral transmembrane glycoprotein. This has seven stretches of 20 to 26 mostly hydrophobic amino acids, which are assumed to form alpha helices integrated in the lipid bilayer of the cell membrane (Murphy et al. 1991; Sasaki et al. 1991). These seven transmembrane domains (TMs) delineate an extracellular NH₂ terminal segment and an intracellular COOH terminal segment and are connected by three extracellular and three intracellular loops (Fig. 1). This initial characterization was recently completed with the identification of a short eighth hydrophobic alpha helix (14 amino acids) in the COOH-terminal segment of the receptor, which is assumed to be in close contact with the inner face of the lipid bilayer. Like many other GPCRs, the AT₁ receptor is devoid of any N-terminal signal sequence, since its first TM (TM1) fulfils this function and allows proper membrane insertion of the protein.

Further analysis of the primary sequence of the AT₁ receptor identifies several specific features of this protein:

- Four extracellular cysteinyl residues in the primary sequence. Two of them are located in the first (Cys¹⁰¹) and second (Cys¹⁸⁰) extracellular loops and are thought to form a disulfide bridge, which is well conserved in other GPCRs. The two additional cysteines are in the N-terminus segment (Cys¹⁸)

and the third (Cys²⁷⁴) extracellular loop and may form an additional disulfide bridge, which may explain the specific structural sensitivity of this receptor to reducing agents such as dithiotreitol (DTT). There is one cysteine in each of TM2, TM3 and TM4 and two in TM7, but whether they form disulphide bridges or not is unknown. The intracellular segments do not contain cysteinyl residues, except the C-terminal segment, which contains a distal cysteine, which might be palmitoylated and therefore attached to the lipid bilayer, transforming the C-terminus segment in a fourth intracellular loop. Interestingly enough, this last cysteine is present in the rodent AT_{1A} subtype but not in the AT_{1B} subtype, suggesting a difference in the functions or regulation of the two AT₁ subtypes in rodent.

- After the alpha helix VIII, the COOH terminal segment has a sequence extremely rich in seryl and threonyl residues (12 out of 24 amino acids between residues 326 and 349). This cluster of serines and threonines is a potential site of phosphorylation, which regulates the receptor functions.
- The comparison of the AT₁ sequence with those of other GPCRs reveals the conservation of numerous residues in the different TMs, including several prolines (Pro¹⁶², Pro²⁰⁷ and Pro²⁵⁵) and tryptophanes (Trp¹⁵³ and Trp²⁵³). These residues may play a role in the general architecture of the receptor. In addition, a canonical sequence ¹²⁵Asp Arg Tyr¹²⁷ (DRY) sequence is identified in the proximal part of the second intracellular loop. This sequence is the signature of the specific subclass 1 of the GPCR superfamily.

In addition to these structural features of the protein, analysis of the AT₁ receptor primary sequence indicates that it is a glycoprotein. Three potential asparagine-linked glycosylation sites (⁴Asn-Ser-Ser, ¹⁷⁶Asn-Ile-Thr and ¹⁸⁸Asn-Ser-Thr) are located on the NH₂-terminal segment (1 site) and second extracellular loop (2 sites). Site-directed mutagenesis of each of these sites reveals that they are all linked to oligosaccharides, the absence of which does not modify the pharmacological and signalling properties of the AT₁ receptor. However, the unglycosylated form of the AT₁ receptor, resulting from the combined mutation of the three sites, has a major defect in biosynthesis and membrane trafficking (Lanctot et al. 1999).

2.5

Phylogenic Aspects of the AT₁ Receptor

Since the first cloning of the rat AT_{1A} receptor cDNA, the amino acid sequences of AT₁ receptors have been identified in several mammalian species and also in birds and reptiles. The sequence identity between the AT_{1A} and AT_{1B} receptors is 95% in the rat and 94% in the mouse (Fig. 2). The sequence identities of the rat AT_{1A} receptor with other mammalian AT₁ receptors range between 99% (mouse AT_{1A}) and 92% (bovine AT₁) and these comparisons include rabbit, pig, dog and human sequences. Other AngII receptors have been identified in birds (turkey and chicken) and *Xenopus laevis*, but the absence of binding of specific

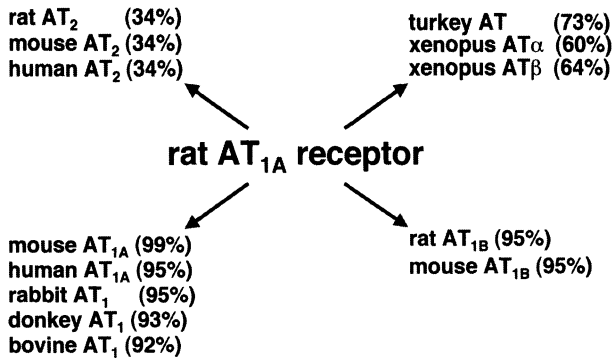


Fig. 2 Amino acid identities of the rat AT_{1A} receptor sequence with other angiotensin II receptors

AT₁ or AT₂ ligands to these receptors explains that they were called AT receptors, despite a canonical coupling to Gq protein and phospholipase C. The sequence identities of these receptors with the rat AT_{1A} receptor are between 73% and 60% at the amino acid level. These sequence homologies are rather high compared to that existing between rat AT_{1A} and AT₂ sequences, which is only 34%. However, this last homology is higher than those with other GPCRs for peptide ligands (25%–30%) or bioamine ligands (20%) or with the mas oncogene (23%), which was for a time considered as a potential angiotensin receptor.

3 Structure–Function Relationships

The cloning of AngII receptors cDNA from various species was the starting point for an extensive work of characterization of its structural features and their involvement in functional aspects of the AT₁ receptor. Site-directed mutagenesis of the AT₁ receptors allowed the identification of many amino acid sequences involved in the ligand binding, activation, G protein coupling, internalization and desensitization of the AT₁ receptors.

3.1 The Ligand Binding Site

The renin–angiotensin system, and the AT₁ receptors in particular, are key targets for drug discovery. Both peptide and non-peptide AT₁ agonists and antagonists are known and their binding sites have been well studied using site directed mutagenesis. Among the mutations altering the ligand-binding capacities of the receptor, it is difficult to discriminate those directly involved in the interaction from those participating in the architecture of the binding pocket and those required for the general architecture of the receptor. It is generally accepted that the docking of the agonist AngII stabilizes a conformational state of the recep-

tor, which allows the initiation of intracellular signalling events. The interactions between the peptide and the exposed residues are more important in the stabilization of the hormone–receptor complex, whereas the interactions with buried amino acids are required for the first steps of the receptor activation switches to occur.

3.1.1

AngII Binding Site

The octapeptide AngII (*Asp*¹-*Arg*²-*Val*³-*Tyr*⁴-*Ile*⁵-*His*⁶-*Pro*⁷-*Phe*⁸) has a high-affinity interaction with the AT₁ receptor ($K_d \approx 1$ nM). This relies on multiple contacts between the amino acid side chains of the peptide, and residues primarily located in the extracellular regions (amino terminus and the three extracellular loops; Fig. 3) (Hjorth et al. 1994). AngII docking also involves polar and charged residues located in the upper parts of the receptor TMs. For example, it is now accepted that C-terminal *Phe*⁸ of AngII interacts with Lys¹⁹⁹ in the upper part of TM5 (Underwood et al. 1994; Noda et al. 1995; Yamano et al. 1995). AngII seems to adopt a hairpin conformation for its docking to the receptor (Matsoukas et al. 1994; Nikiforovich et al. 1994), and there might be a salt-linked triad between Lys¹⁹⁹ and the carboxyl group of *Asp*¹ and *Phe*⁸ of AngII (Joseph et al. 1995a). In addition, Trp²⁵³ (TM6) stabilizes the ionic bridge formed between Lys¹⁹⁹ and *Phe*⁸ of AngII and *Asp*¹ of the peptide interacts with His¹⁸³ in the second extracellular loop of AT₁ (Yamano et al. 1995). Asp²⁸¹, at the junction between the third extracellular loop and TM7, has been identified as a major docking point for AngII through its charge interaction with *Arg*² (Feng et al. 1995). Data and modelling studies suggest that *Arg*¹⁶⁷ might be an important contact point with *Tyr*⁴ (Yamano et al. 1995). This residue might also disrupt the hydrogen bonding between Asn¹¹¹ in TM3 and Tyr²⁹² in TM7, by competing with Tyr²⁹². This would permit the latter to interact with *Asp*⁷⁴ in TM2 during receptor activation (Joseph et al. 1995a,b). *Phe*²⁵⁹ and *Asp*²⁶³ in TM6 might provide a docking site for *His*⁶ of the peptide (Yamano et al. 1995). AngII binding also involves a residue in the outer part of TM3, Lys¹⁰² (Monnot et al. 1996) and others located in TM7 and more deeply buried in the plasma membrane, Asn²⁹⁵ (Schambye et al. 1994) and *Phe*³⁰¹ (Hunyady et al. 1995b). However whether these residues are in direct contact with the peptide, or are involved in intramolecular interactions that define the intramembrane binding pocket for the ligand, is not known. The four cysteinyl residues that form the two extracellular disulphide bridges, Cys¹⁸-Cys²⁷⁴ and Cys¹⁰¹-Cys¹⁸⁰ (Ohyama et al. 1995) are essential for the general architecture of the receptor and therefore for AngII binding, which is suppressed when these residues are mutated.

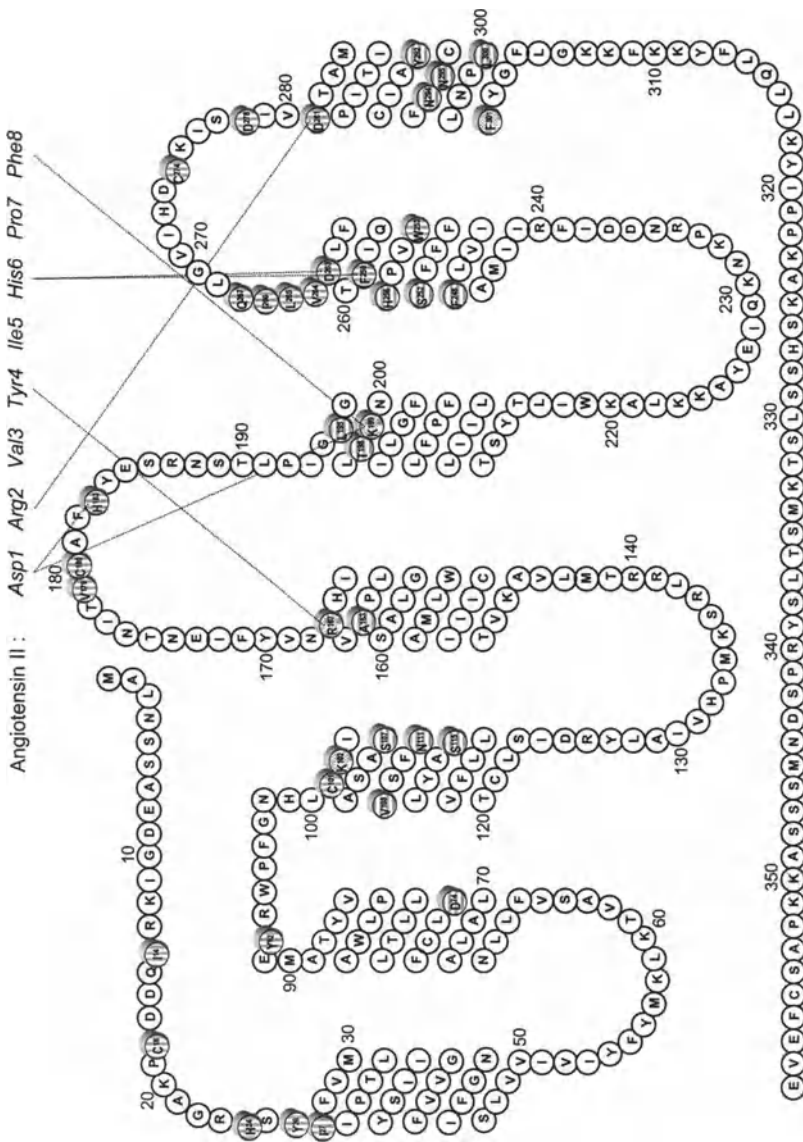


Fig. 3 AT₁ molecular determinants involved in the binding of the peptide agonist angiotensin II (circle with grey vertical stripes), the nonpeptide antagonist losartan (greyed horizontal stripes), or both of them (checkered pattern). AngII sequence is shown at the top, and the possible interactions with various AT₁ residues are indicated with dotted lines

3.1.2 Non-peptide Ligand Binding Site

Early attempts to develop therapeutic agents able to block the AngII receptor were complicated by the peptidic nature of antagonists, such as saralasin, which are devoid of oral activity. Based on imidazole derivatives first described in 1982 (Furukawa et al, US patent 4,340,598), nonpeptide AT₁ antagonists were

synthesized (Timmermans et al. 1993; Goodfriend et al. 1996). The first of this series to reach the clinic, losartan, was followed by a large number of AT₁ antagonists that were orally active.

Identification of the losartan binding site was facilitated by the functional comparison of mammalian AT₁ and amphibian AngII receptors. Both present similar signal transduction mechanisms, but drastically differ in their binding of nonpeptide ligands such as losartan. The amino acids involved in losartan binding to the mammalian AT₁ were identified by analysing the binding properties of rat AT₁ mutant receptors in which the nonconserved residues had been substituted by their *Xenopus* counterparts (Ji et al. 1994). Most of these mutants preserved their affinity for AngII or for the peptide antagonist [Sar¹,Ile⁸]AngII, suggesting that the general conformation of the receptor was unaltered by such replacements. These residues are mainly located in the TMs of the receptor (Val¹⁰⁸ in TM3, Ala¹⁶³ in TM4, Thr¹⁹⁸ in TM5, Ser²⁵² in TM6, Leu³⁰⁰ and Phe³⁰¹ in TM7). Other amino acids have been identified in the various TMs, as shown in Fig. 3 (Bihoreau et al. 1993; Schambye et al. 1994; Noda et al. 1995; Monnot et al. 1996; Balmforth et al. 1997a; Hoe and Saavedra 2002). The residues known to be part of the losartan binding site in the mammalian receptor have been transferred into an amphibian receptor creating a "gain of function" mutant that binds the nonpeptide ligand with an affinity comparable to the rat AT₁ receptor (Ji et al. 1995).

These findings demonstrate that losartan binds to a site defined by amino acids located quite deeply in the membrane-spanning region of the receptor. These residues are mainly distinct from those involved in AngII binding, but all are located in a defined area lying between TM3, 5, 6 and 7 of the receptor. This binding site is similar to the intramembrane binding pocket of GPCRs for smaller ligands, such as catecholamines and acetylcholine.

By comparing the amino acids involved in their respective binding sites, it can be seen that AngII and nonpeptide analogues have common determinants on the receptor such as Lys¹⁰², Ser¹⁰⁵, Arg¹⁶⁷ and Lys¹⁹⁹ (Grolewski et al. 1995; Noda et al. 1995; Yamano et al. 1995). The case of Asn²⁹⁵ appears to be more complex, since its substitution by a series of residues indicates its involvement as a direct contact point with the non peptide ligands of the antagonist type (losartan) but not of the agonist type (L-163,491, L-163,313) (Hunyady et al. 1998).

In summary, similarly to the AngII binding site, it is difficult to discriminate between residues directly involved in a molecular interaction with losartan and those involved in the general architecture of the binding site. The present picture of the ligand-binding sites shows for AngII the involvement of some transmembrane residues and mostly extracellular sequences, whereas smaller ligands such as losartan interact with a transmembrane binding pocket. The smaller ligands need less energy than peptides to achieve high affinity binding and the extracellular regions of the receptor provide additional contact points, stabilizing the binding of the peptide ligands. This fact is not limited to the AT₁ receptor, but applies for other peptide and protein hormones (Gershengorn and Osman 2001).

3.2 Receptor Activation

Several models based on allostery have been suggested to explain the process by which a ligand binding to a receptor leads to a conformational change, which results in intracellular signalling. According to these models, a receptor such as AT₁ exists in at least two interconverting conformations, one active and one inactive, and undergoes spontaneous isomerization between these two forms. In the absence of ligand (basal conditions), the proportion of receptors in the activated state is generally low and the basal conditions can be considered as inactive. This applies to the AT₁ receptor; however, for several individual GPCRs, such as muscarinic receptors, the basal conditions are associated with a high level of signalling activity and the receptor is considered to be constitutively active (Burstein et al. 1997). In the presence of AngII, the equilibrium is shifted towards this active state which allows efficient coupling to the intracellular signalling partners such as the Gq protein. This is due either to a change in the conformation of the receptor induced by AngII or, more probably, to a better affinity of AngII for the active conformation, which is then stabilized. This definition of the agonist, which has a greater affinity for the active state of the receptor, has been extended to other pharmacological compounds. Among the standard antagonists, it has been possible to distinguish the true antagonists, which have a similar affinity for the active and inactive states of a receptor and the inverse agonists, which have a greater affinity for the inactive states. These later compounds are able to shift the equilibrium towards the inactive state when the receptor is in a spontaneous active state, whereas a true antagonist is always neutral for this equilibrium but is a competitor for agonists.

Information from receptor mutants blocked in either the inactive or the active conformations are precious in understanding the activation process of the receptor. Inactivating mutations were the first to be reported. Mutations of polar residues of TM2 (Asp⁷⁴), TM3 (Ser¹¹⁵), TM5 (Tyr²¹⁵) and TM7 (Tyr²⁹²) (Bihoreau et al. 1993; Marie et al. 1994; Hunyady et al. 1995a; Monnot et al. 1996) have been shown to result in inactive receptors, unable to activate the G protein without greatly affecting their agonist-binding properties (Fig. 4). These residues appear to be involved in the activation process of the receptor. Some of these residues (Asp⁷⁴ and Tyr²¹⁵) are conserved among the GPCRs, suggesting that they could be involved in the activating conformational switch leading to the intracellular coupling to the G proteins. Some modelling experiments suggest a possible interaction between Asn⁷⁴ and Tyr²⁹² that would stabilize the active conformation of the receptor (Joseph et al. 1995a).

A NPX₂₋₃Y motif, located at the C-terminal end of TM7 and very conserved among GPCRs, was identified as functionally important in the aminergic GPCRs. The tyrosyl residue is important for receptor internalization (Barak et al. 1994) and the whole sequence participates in the transmission of a signal from agonist-induced conformational changes in the ligand-binding region (Donnelly et al. 1994). The role of this sequence ²⁹⁸Asn-Pro-Leu-Phe-Tyr³⁰² in

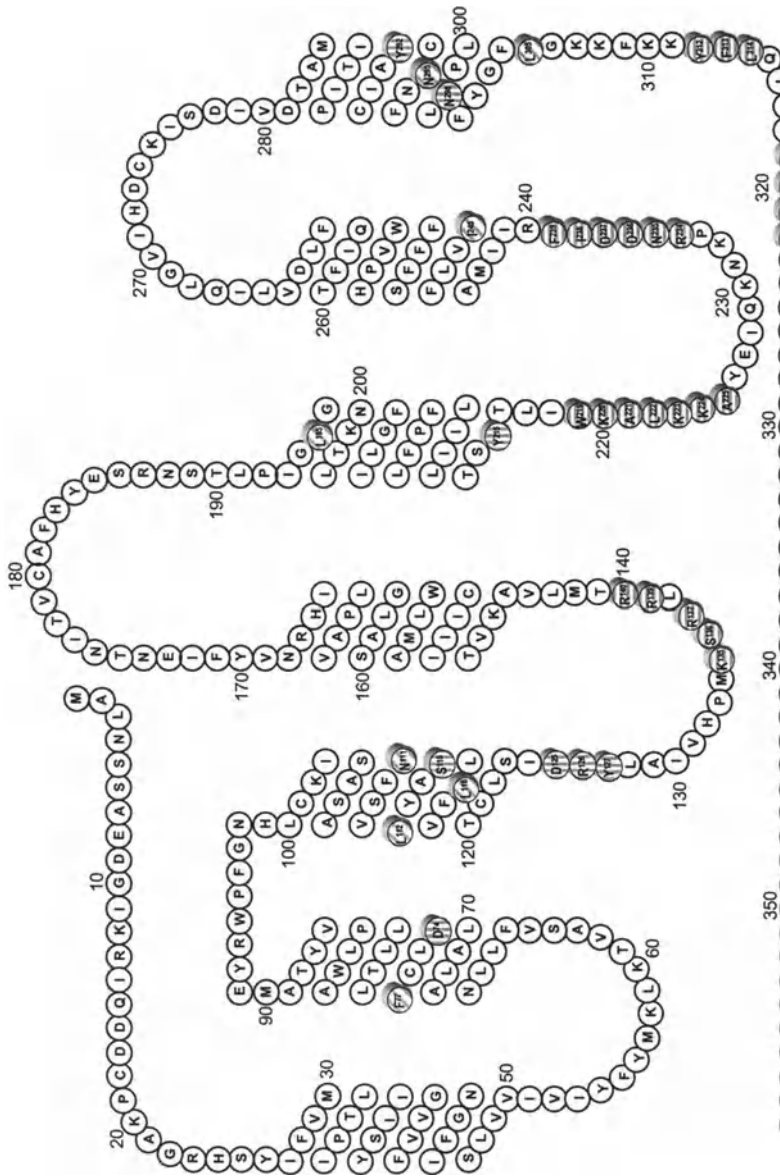


Fig. 4 AT₁ molecular determinants of activation and coupling to the signalling pathways. Residues involved in the receptor activation (circle filled with grey diagonal stripes)/inactivation (grey vertical stripes) process are located mainly in the transmembrane domains. Residues implicated in the coupling to the Gαq protein (background horizontal stripes) and to the Jak2/STAT1 and PLCγ pathways (background checkered pattern) are shown in the IC2, IC3 and C-terminal segment of the receptor

the AT₁ receptor was analysed similarly (Hunyady et al. 1995b), demonstrating that the mutation of Tyr³⁰² does not impair ligand-induced internalization of the receptor. However, this mutation and the replacement of Pro²⁹⁹ reduce—and the mutation of Asn²⁹⁸ suppresses—the G protein-coupling of the receptor and the agonist-induced production of second messengers, demonstrating the role of this motif in receptor activation.

Beside loss-of-function mutations of the AT₁ receptor, gain-of-function mutants have also been identified and are informative for our understanding of the receptor activation mechanisms. First identified by site-directed mutagenesis of the adrenergic receptor (Cotecchia et al. 1990; Kjelsberg et al. 1992), such active mutations have also been found as natural mutations involved in the pathophysiology of several endocrine diseases such as thyroid toxic adenoma (Parma et al. 1993), precocious puberty and chondrodysplasia (Schipani et al. 1995).

Defined as mutations responsible for a permanent and agonist-independent activity of the receptor, higher than that of the wild-type receptor, these gain-of-function mutations are also called constitutively active mutations (CAM). These CAMs are potent pharmacological tools for discriminating true antagonists from inverse agonists, since only the latter are able to inactivate such mutants. In addition, apparent affinity and efficacy of (partial) agonists are better for these mutants blocked in their active state than for the wild-type receptors. The study of such receptors has enriched our understanding not only of GPCR activation mechanisms but also of GPCR pharmacology.

For the AT₁ receptor, *in vitro* studies on constitutively active mutants have implicated Asn¹¹¹ in TM3 (Goblewski et al. 1997; Feng et al. 1998; Miura et al. 1999). Mutation of Asn²⁹⁵ into Ser²⁹⁵ in TM7 has been shown to increase the basal activity of the receptor and a direct interaction between Asn¹¹¹ and Asn²⁹⁵ has been suggested (Balmforth et al. 1997a). However, mutation of this residue in alanine or aspartic acid does not lead to a constitutive activation of the receptor (Perlman et al. 1997; Hunyady et al. 1998).

More recently, the exhaustive cartography of the residues involved in AT₁ receptor activation has been established by identifying a repertoire of CAM after screening a randomly mutated AT₁ cDNA library using a functional test based on the higher sensitivity of these mutants to partial agonists (Parnot et al. 2000). Sixteen mutants with a higher sensitivity to the partial agonist CGP42112A were identified and among these seven showed a significantly higher basal inositol phosphate production (Phe⁷⁷Tyr in TM2, Asn¹¹¹Ser, Leu¹¹²His and Leu¹¹⁸His in TM3, Leu¹⁹⁵Pro in TM5, Ile²⁴⁵Thr in TM6 and Leu³⁰⁵Gln). The mutants should be useful for molecular modelling of AT₁ activation and should help researchers to define the role of the mutated residues in the activation mechanism. The amino acids involved are not conserved among GPCRs, suggesting that if there are common mechanisms for activation of GPCRs, they do not imply highly conserved amino acid motives. As for many CAM receptors identified to date, these residues are mainly located in transmembrane regions, confirming that TM movements play a key role in the transition between the inactive and active states. A striking cluster of mutations was found in TM3, around Asn¹¹¹, indicating that this helix plays an important role in activation.

A proposed model of GPCR activation predicts that rigid body movement of TM3, 6 and 7 induces conformational changes in the cytoplasmic loops that permit G protein interaction with the agonist-activated receptor (Gether and Kobilka 1998). AT₁ mutagenesis results, where many residues involved in receptor activation are located in the TMs, seem to confirm this idea. Mutations of residues

or motives located in the intracellular regions of the receptor have also been shown to impair the receptor coupling. However, for these amino acids it is difficult to determine if, like the transmembrane mutations, they participate in the general structural architecture of the receptor's active conformation or if they represent a direct contact point with the intracellular partner.

3.3

Interaction with G Proteins

The binding of AngII to the AT₁ receptor has been shown to trigger several intracellular signalling pathways. AT₁'s main partner is the Gq/11 protein, which activates the phospholipase C- β (PLC- β). PLC- β generates the second messengers inositol (1,4,5) trisphosphate (IP) and diacylglycerol (DAG), which allow the release of calcium from intracellular stores and activate protein kinase C, respectively. The major interaction of the receptor is with the N- and C-terminal domains of the α -subunit of Gq. Several cytosolic molecular determinants of the AT₁ receptor have been shown to interact with the G α q protein (Fig. 4). In the second intracellular loop (IC2), in addition to the extremely conserved ¹²⁵DRY¹²⁷ motif at the junction with TM3, Ohyama et al. have shown that the mutation of five other residues (Lys¹³⁵, Ser¹³⁶, Arg¹³⁷, Arg¹³⁹ and Arg¹⁴⁰) prevents the receptor from activating the G protein (Ohyama et al. 1992). The third intracellular loop (IC3) is considered as a major determinant in GPCR coupling selectivity and efficiency. The replacement of residues 219–225 and 234–237 of the AT₂ IC3 domain with the corresponding residues of the AT₁ receptor results in an efficient coupling to the Gq protein (Wang et al. 1995). Conversely, the replacement in AT₁ of residues 234–240 by their AT₂ homologues suppresses the Gq coupling of the receptor (Conchon et al. 1997). The coupling of the AT₁ receptor to the heterotrimeric G proteins is also dependent on the proximal region of its carboxyl-terminal cytoplasmic region. More specifically, site-directed mutations of individual amino acids of the hydrophobic cluster Tyr³¹², Phe³¹³, Leu³¹⁴ produce receptors that were insensitive to the guanosine triphosphate (GTP) analogue GTP γ S and were unable to stimulate IP production after stimulation by AngII, demonstrating the absence of G protein coupling. Moreover, a purified peptide containing the wild-type sequence (residues 306–320) was able to directly stimulate the binding of GTP γ S to purified G proteins, whereas similar peptides containing mutations of Tyr³¹², Phe³¹³ or Leu³¹⁴ could not (Sano et al. 1997). It seems therefore that the Gq-protein binding site of the AT₁ receptor consists of a complex spatial arrangement of several determinants from the IC2, IC3 and C-terminus segment.

AT₁ receptors have also been reported to interact with other G proteins, such as G_i-proteins. Peptides derived from the sequence of the amino-terminal half of IC3 (residues 216–231) or from the C-terminus (residues 306–320) stimulate the binding of GTP γ S to purified Gi1, Gi2 and Go proteins (Shirai et al. 1995). More recently, a mutation of TM4 abolished the Gq coupling to AT₁ (no IP production after AngII stimulation), but revealed the coupling to another, as-yet-

unidentified G protein, since the AngII binding was still sensitive to GTP analogues (Feng and Karnik 1999).

3.4 Interactions with Other Signalling Molecules

Recently, the AT₁ receptor has been shown to activate cellular tyrosine kinases including Src, Fyn and Pyk2 (Sayeski et al. 1998). The binding of AngII to AT₁ also activates the intracellular tyrosine kinase Jak2, leading to tyrosine phosphorylation and nuclear translocation of the transcription factor signal transducer and activator of transcription (STAT1) (Marrero et al. 1995). Despite some data indicating that the Jak2/STAT1 pathway is activated via the coupling to Gq protein in vascular smooth muscle cells (Frank et al. 2002), it is generally accepted that Jak2 associates independently from G proteins with the AT₁ receptor and the interacting sequence ³¹⁹Tyr-Ile-Pro-Pro³²² (YIPP) is located in the carboxyl-terminus segment of the receptor (Fig. 4) (Ali et al. 1997). This interaction is independent of AT₁ tyrosine phosphorylation since the mutation of all the intracellular tyrosine residues for phenylalanine does not change its co-immunoprecipitation with Jak2 and STAT1. The YIPP motif has also been implicated in the recruitment of phospholipase C (PLC)- γ 1, via its Src-homology 2 (SH2) domains, in an AngII and tyrosine phosphorylation-dependant manner (Venema et al. 1998). However, the tyrosine phosphorylation of AT₁ is still controversial (Smith et al. 1998), which might cast a slight doubt on the final interpretation of these results.

The proximity of the ³¹⁹YIPP³²² motif, which interacts with the Jak2-STAT1 complex and maybe the PLC γ and the ³¹²Tyr-Phe-Ile³¹⁴ motif which is part of the G protein binding site, suggests either a competition between these signalling molecules or their interaction with different active conformations of the AT₁ receptor. The interaction of Jak2 and Gq with two different active conformations of the AT₁ receptor is suggested by two pieces of experimental data:

- A receptor with the Asp⁷⁴ in TM3 mutated for a Gln, which can no longer activate the Gq protein, is still able to activate the Jak/STAT pathway (Doan et al. 2001).
- Conversely, the deletion of the ³¹⁹YIPP³²² motif indicates that it is not necessary for Gq protein coupling (Thomas et al. 1995b).

In summary, the intracellular sequences of the AT₁ receptor are involved not only in the interactions with G proteins but also with other signalling proteins. The recent cloning of a novel protein (ATRAP), which interacts with the C-terminus of the AT₁ receptor and apparently inhibits the growth properties of AngII, is another example of these interactions with multiple signalling pathways (Daviet et al. 1999; Cui et al. 2000).

3.5 Regulation of AT₁ Receptor Activity

Receptor desensitization is a complex phenomenon leading to decoupling from G proteins, down-regulation and insensitivity to ligands. This phenomenon is associated with receptor phosphorylation, interactions with regulatory proteins and internalization. It has been extensively demonstrated in the model of the β_2 -adrenergic receptor that the active conformations of the receptor allow not only G protein coupling but also phosphorylation by specific G protein receptor

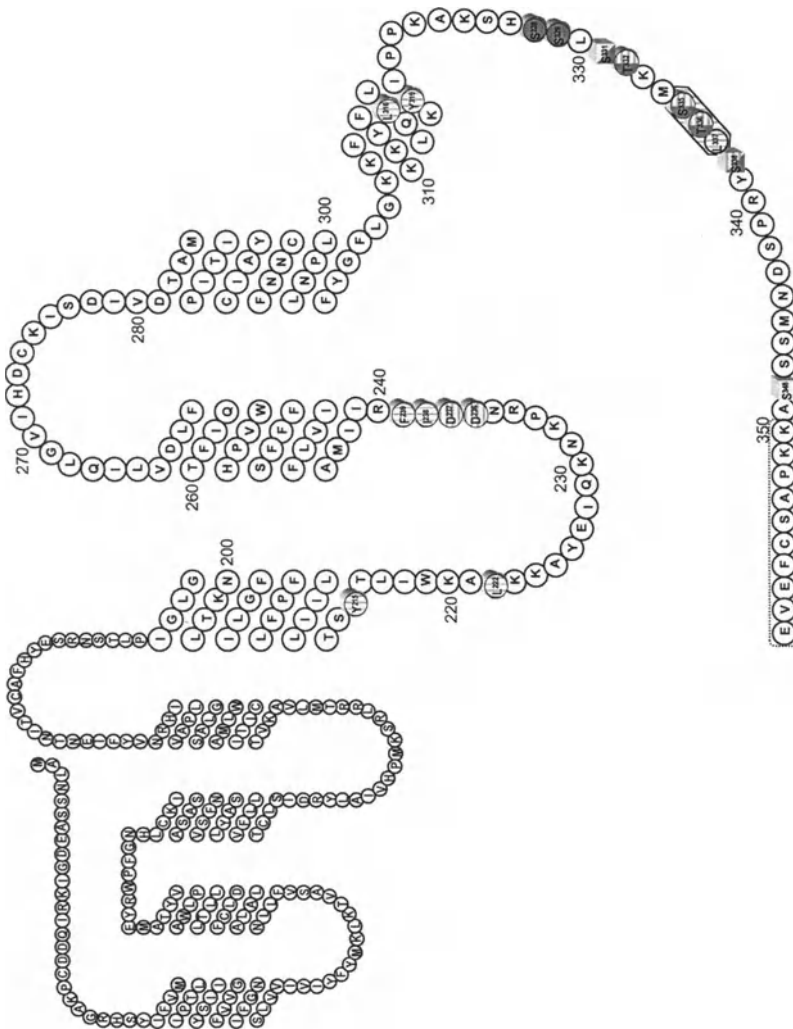


Fig. 5 AT₁ molecular determinants involved in the regulation of the receptor activation and its internalization. The C-terminal region of the receptor presents a high concentration of sequences and residues implicated in receptor phosphorylation [by PKC (circle filled with horizontal stripes) and GRK (cube)], interactions with partners of its trafficking [β -arrestin (filled grey circle) and rab5 (area within dotted line)] and more generally in its desensitization and internalization (circle filled with grey vertical stripes) processes

kinases (GRKs) and by second messenger activated kinases [protein kinases A and C (PKA, PKC)]. The phosphorylated receptor can then interact with cytoplasmic proteins, called arrestins, which prevent association between the receptor and the G protein, leading to desensitization. The binding of arrestin to the activated receptor might also serve to target the latter to clathrin-coated pits for internalization from the cell surface. Studies to identify determinants involved in the regulation of AT₁ receptor activity have focused on the carboxyl-terminal cytoplasmic segment which seems to concentrate a lot of information in less than 50 residues (Fig. 5).

3.5.1

Phosphorylation

The phosphorylation of AT₁ receptors has been shown to be agonist-, time- and dose-dependent in transfected cells and target tissues (Oppermann et al. 1996; Balmforth et al. 1997b; Smith et al. 1998). The AngII-stimulated AT₁ receptor phosphorylation occurs on serine and threonine residues by specific kinases (GRK) and by PKC. The involved GRKs differ from one tissue to another since GRK2, 3 and 5 have been shown to phosphorylate the receptor in HEK293 cells, (Oppermann et al. 1996), but not in Chinese hamster ovary (CHO) cells (Tang et al. 1998).

The phosphorylation sites of the AT₁ receptor for these different serine/threonine kinases have been investigated. Three potential PKC phosphorylation sites (Ser³³¹, Ser³³⁸, Ser³⁴⁸) are located in the carboxyl-terminal segment of the receptor and apparently all of them are utilized to some extent following homologous (AngII) and heterologous [phorbol myristoyl acetate (PMA)] stimulation (Qian et al. 1999). There is no consensus sequence for phosphorylation via GRK, but it is usual to observe acidic residues next to the seryl or threonyl residue they phosphorylate. The sequence ³³²Thr-X-X-Ser-Thr-X-Ser³³⁸ in the middle of the AT₁ receptor C-terminus domain seems to be a major site of phosphorylation by GRK, since the mutation of the serines and threonines to alanines in this sequence results in a drastically decreased level of AngII-stimulated receptor phosphorylation (Thomas et al. 1998; Qian et al. 2001). However, this sequence is not flanked by acidic residues. Asp²³⁶ and Asp²³⁷ located in the third cytoplasmic loop may play this role, since their mutation largely impairs AngII-stimulated GRK-mediated phosphorylation of the receptor (Olivares-Reyes et al. 2001).

3.5.2

Phosphorylation and Regulation of AT₁ Receptor Activity

This major GRK phosphorylation site belongs to a larger sequence of the AT₁ receptor C-terminus extremely rich in seryl and threonyl residues (10 of these residues between amino acids 326 and 348). This sequence is involved not only in receptor phosphorylation but also in its desensitization (i.e. the amplitude of the signalling response to repeated agonist stimulations). Progressive C-termi-

nal truncations of the receptor show that deletion after residue 328, but not after residue 336, produces a receptor with an amplification of AngII-induced intracellular signalling and an absence of heterologous desensitization (Conchon et al. 1998). Even if this amplified signal of the truncated receptor is not observed by all authors (Tang et al. 1998), all agree on the role of the sequence Ser³²⁸ to Ser³⁴⁸ in homologous and heterologous desensitization of the receptor.

3.5.3

Phosphorylation and Activation of the AT₁ Receptor

The increased phosphorylation of the AT₁ receptor after AngII binding suggests that, as for many other GPCRs, the active conformations of the receptor are the substrate of the kinases. However, this interpretation has not been confirmed for the AT₁ receptor by experimental data. Several lines of evidence seem to indicate that phosphorylation of AT₁ requires conformational states at least partly distinct from the active states responsible for signalling:

- Gain-of-function mutants of the AT₁ receptor (Asn¹¹¹Ala, Asn¹¹¹Gly), which activate constitutively the Gq protein signalling pathway, show a reduced basal and AngII-stimulated level of phosphorylation.
- AngII analogues, such as [Sar1,Ile8]AngII, do not induce G protein coupling of the wild-type receptor, but induce its phosphorylation. (Thomas et al. 1998, 2000).
- Partial agonists, such as [Sar1,Ile4,Ile8]AngII, do not induce wild-type receptor coupling to G protein but induce its phosphorylation. Conversely, they activate the G protein coupling but do not induce phosphorylation of constitutively active mutants of the AT₁ receptor.

This implies that the receptor does not have to be in an active state to be phosphorylated, and some active conformational states of the receptor are not subjected to phosphorylation, even after AngII stimulation. Altogether, these results indicate that the molecular switches required for phosphorylation and G protein-mediated signalling are partially distinct.

3.5.4

AT₁ Phosphorylation and β -Arrestin Binding

By interacting with the receptor, the β -arrestins play a major role in uncoupling the receptor from the G protein signalling pathways, in its internalization via the clathrin-coated pits and also in the scaffolding of new signalling complexes. Indirect evidence of an interaction of β -arrestins 1 and 2 with the AT₁ receptor has been given by confocal microscopy imaging. AngII is able to recruit enhanced green fluorescent protein (EGFP)-tagged versions of these proteins to the plasma membrane of cells expressing the AT₁ receptor. The β -arrestins are then internalized in endosomal vesicles together with the receptor (Oakley et al.

2000). Direct evidence of physical interaction between AT₁ and β -arrestin1 and 2 was obtained by co-immunoprecipitation experiments (Luttrell et al. 2001; Qian et al. 2001).

The structural requirements for β -arrestin interaction with AT₁ have been investigated. The crucial role of the AT₁ C-terminus in this interaction has been demonstrated through several experiments:

- An AT₁ mutant, truncated after Lys³²⁵ produces a receptor that can still reach the active state, but which is no longer phosphorylated and does not interact with β -arrestin1.
- After agonist-stimulation, β -arrestin can still be co-immunoprecipitated from endosomes with a chimeric receptor consisting of the β_2 -adrenergic receptor with the carboxyl terminal tail of the AT₁ receptor but not with the wild-type β_2 AR (Anborgh et al. 2000).

The serine/threonine cluster (³²⁸Ser-Ser-X-Ser-Thr-X-X-Ser-Thr-X-Ser³³⁸) in the central region of the C-terminus is a major molecular determinant of β -arrestin binding. Indeed, mutation of the four distal serines and threonines of this cluster into alanines results in a mutant receptor which is poorly phosphorylated and presents an AngII-induced interaction with β -arrestin reduced by 80%. Receptors mutated at the four proximal or three distal Ser/Thr residues can still translocate the β -arrestin to the plasma membrane, but in a less stable complex which dissociates rapidly (Oakley et al. 2001). A mutant in which the three PKC phosphorylation sites have been mutated (Ser331, 338 and 348) has a reduced phosphorylation but normal interaction with β -arrestin1. Altogether, these data indicate that it is not the active conformation, but the phosphorylation of the AT₁ receptor by GRKs, which is the crucial event for its interaction with β -arrestin (Qian et al. 2001).

3.6

Internalization and Trafficking

After activation, G protein coupling and phosphorylation, the AT₁ receptor becomes rapidly internalized ($t_{1/2} \sim 5$ min) and then slowly recycled ($t_{1/2} \sim 1$ h). This internalization is involved in the desensitization/resensitization process and also probably in the activation of some signalling pathways (Sorkin and von Zastrow 2002). In addition, several studies suggest that internalization of the ligand-receptor complex may participate in the sequestration of AngII in some target tissues from the circulation (van Kats et al. 1997). This intracellularly sequestered AngII could be released later for autocrine/paracrine functions, or might bind cytoplasmic or nuclear AngII receptors (Booz et al. 1992; Sugiura et al. 1992).

3.6.1

Molecular Determinants of Internalization

The receptor sequences involved in AngII-induced AT₁ internalization have been extensively studied by site-directed mutagenesis. Internalization motifs have been identified in the third intracellular loop (IC3) and in the C-terminal domain.

The role of the C-terminus in the internalization process has been clearly demonstrated by the deletion of this segment, which drastically impairs the receptor internalization (Hunyady et al. 1994; Balmforth et al. 1995; Thomas et al. 1995a). Further analysis of the carboxyl terminus has identified two short segments that are necessary for receptor internalization (Hunyady et al. 1994, 1995a,b, 1996; Balmforth et al. 1995; Thomas et al. 1995a,b; Conchon et al. 1998; Tang et al. 1998).

- The proximal sequence is the ³¹⁶Leu-Leu-Lys-Tyr³¹⁹ motif, located closer to the plasma membrane. The substitution of Leu³¹⁶ alone gives the strongest internalization impairment phenotype described for a single point mutant (Thomas et al. 1995a). This leucine is part of a dileucine motif, which has been involved in internalization of several membrane receptors, among which the β_2 -adrenergic receptor.
- The distal sequence consists of the amino acid triplet ³³⁵Ser-Thr-Leu³³⁷ in the middle of AT₁ carboxyl terminus, which has been identified as playing an important role in receptor endocytosis, maybe via phosphorylation and interaction with the β -arrestins.

The role of these different sequences, in either receptor interactions with the endocytic machinery or in the general conformation required for internalization, has not been clearly established.

Recently, AT₁ has been shown to interact with rab5, a well-known protein involved in intracellular trafficking. This interaction is not necessary for receptor internalization but for its further redistribution into larger vesicles. The last ten residues of the AT₁ C-terminal tail are involved in this interaction (Seachrist et al. 2002).

3.6.2

Internalization and Activation

Whether the internalization is strictly dependent on the active conformations of the receptor has been investigated and experimental data clearly demonstrate that activation and internalization of the receptor have different conformational requirements:

- A receptor mutant, Asp⁷⁴Asn, which does not couple to the Gq protein is normally internalized after AngII stimulation.

- The peptidic antagonist [Sar1Ile8]AngII elicits a strong and rapid internalization after binding to the receptor (Conchon et al. 1994).
- Some of the truncated mutants have impaired internalization but can still efficiently activate the Gq-protein.

3.6.3

Phosphorylation, β -Arrestin Binding and Receptor Trafficking

While the activation of the AT₁ receptor is not a prerequisite for its internalization, the phosphorylation of the receptor, followed by the binding of β -arrestin, seems to be necessary. Usually, GPCR binds β -arrestin, which acts as an adaptor protein linking the receptor to the coated pit machinery, involving clathrin- and dynamin-dependant processes. Several experimental observations indicate that the involvement of arrestin in AT₁ internalization is a controversial issue:

- Several dominant negative (DN) mutants of β -arrestin (β -arrV^{53D}) and dynamin (K^{44A}) have marginal effects on AT₁ receptor internalization (Zhang et al. 1996).
- The overexpression of GIT1 and Arf6, two factors involved in clathrin-coated vesicle budding from the plasma membrane, is without effect on AT₁ internalization, whereas it impairs endocytosis of the β_2 -adrenergic receptor (Claing et al. 2000).
- Several mutants of the C-terminal region of AT₁ are still normally internalized but have a drastically reduced capacity to form a stable complex with β -arrestin after AngII binding (Oakley et al. 2001).
- As already mentioned, the binding of [Sar1,Ile4,Ile8]AngII, which induce receptor phosphorylation and its interaction with β -arrestin1, surprisingly does not induce AT₁ internalization (Qian et al. 2001).

In direct contrast, a series of recent studies argues in favour of a β -arrestin-, dynamin- and clathrin-dependent mechanism of AT₁ internalization. DN mutants of both arrestin (β -arr1V53D and β -arr1-349) and dynamin inhibit AngII-stimulated internalization (Werbonat et al. 2000; Gaborik et al. 2001). In addition, the phosphorylation mutants described above show a defect not only in β -arrestin interaction but also in internalization (Qian et al. 2001). Despite some contradictory results, the correlation between β -arrestin binding and internalization of the AT₁ receptor is now accepted by a majority of authors and agrees with the clear demonstration of a physical interaction between the two proteins.

Altogether, these data indicate that receptor phosphorylation and β -arrestin binding and internalization share some molecular determinants, but that some conformational states of the receptor can be specific to each of them. These observations lead to the conclusion that the receptor exists in a repertoire of active conformations, each one more or less specific of one or several functions: coupling to various signalling pathways, desensitization, phosphorylation, internalization, etc.

In conclusion, the elucidation of the structure of the gene and the primary sequence of the AT₁ receptor has opened the way to the understanding of the molecular mechanisms responsible for its different functions. A large number of receptor mutants has allowed the dissection of the different functions and interactions of the receptor with other proteins and the identification of the amino acids involved. However, the absence of specific information on the three-dimensional structure of the receptor does not allow a clear comprehension of the molecular mechanisms of its activation. In addition, there is no doubt that many other, yet-unidentified proteins are able to interact with the inactive or active conformations of the receptors. Discovering these proteins is one of the future challenges of AT₁ molecular research.

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AT₁ Receptor Interactions

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Abstract Angiotensin II and antagonist-AT₁ receptor interactions have traditionally been studied on isolated tissues in organ bath experiments. Cells that express endogenous or transfected AT₁ receptors are now also increasingly used to this end. Angiotensin II and angiotensin III interact with the AT₁ receptor in a similar fashion. Other naturally occurring fragments like angiotensin IV or angiotensin-(1-7) display only low affinity for the receptor and the latter even acts as an antagonist. Combination of such structure-activity relationship and receptor mutation studies led to a more complete picture of the molecular events that take place during receptor activation. At least two steps take place: a pre-activation step, in which constraining intramolecular interactions within the receptor are broken by Arg² of angiotensin II, and a subsequent activation step in which the C-terminal side of the hormone plays an essential role. The outcome is a conformational change in the receptor that promotes its interaction with G proteins. Interaction with nonpeptide, biphenyltetrazole antagonists is also a multi-step process, and a “two-state, two-step” model is proposed in which the initial attraction to the receptor is fairly similar for all antagonists and in which a more stable, tight binding antagonist-receptor complex can be formed subsequently. This explains the often-mentioned distinction between “surmountable” and “insurmountable” antagonists in, e.g. vascular smooth muscle contraction experiments. This tight binding state is unrelated to receptor internalisation, but mutation studies reveal the implication of Lys¹⁹⁹. These recent observations comply with the increasing awareness that AT₁ receptors and other G protein-

linked receptors may adopt a number of agonist- and antagonist-bound conformations and/or states, each with its own characteristic properties.

Keywords AT₁ receptor · Angiotensin fragments · Antagonists · Insurmountable · Interaction mechanisms · Cell lines · Receptor mutants

Abbreviations

ACE	Angiotensin-converting enzyme
Ang	Angiotensin
CHO-hAT ₁ cells	Chinese hamster ovary cells expressing the human AT ₁ receptor
GPCR	G protein-coupled receptor
L	Ligand (antagonist)
N ¹¹¹ G mutant	AT ₁ receptor mutant in which Asn ¹¹¹ is substituted by Gly
R, R', R*, R ₁	Receptor in stable inactive-, preactivated-, fully active and tight antagonist-binding states, respectively
TM	Transmembrane-spanning helix

1

Introduction

The octapeptide angiotensin (Ang) II is well known for its hypertensive effect and its ability to stimulate cardiac remodelling. Receptors of the AT₁ subtype play a major role in these processes (de Gasparo et al. 2000). Prevention of the hypertensive and trophic actions of Ang II has proved to be among the most successful strategies for the treatment of hypertension and congestive heart failure. To this end, Ang-converting enzyme (ACE) inhibitors have been introduced to decrease the plasma level of Ang II and, during the last decade, a fairly large number of nonpeptide antagonists have been developed to selectively block the AT₁ receptor (Timmermans 1999a,b; Unger 1999). This chapter deals with pharmacological and molecular aspects of the binding of agonists and antagonists to the AT₁ receptor. Traditionally, such interactions have been tested on isolated tissues in organ bath experiments. Cells that endogenously express the AT₁ receptor and cell lines that have been transfected with the gene encoding such receptor are also increasingly used to this end (Vanderheyden et al. 1999). Compared to isolated tissues, they allow the investigation of ligand-receptor interactions by radioligand binding and by measuring receptor-evoked responses under similar experimental conditions. This allows an objective comparison of both sets of data. Transfected cell systems offer additional advantages. They provide a homogenous receptor system, the untransfected parent cells can serve as a control for the detection of receptor-unrelated phenomena, and mutation studies allow the exploration of the role of specific amino acids in the binding and functional properties of the receptor. The physiological relevance of such intact cell experiments may be questioned, since the AT₁ receptors are often ex-

pressed at high density and in a foreign cellular environment (e.g. Chinese hamster ovary cells expressing the human AT₁ receptor, CHO-hAT₁ cells). Fortunately, however, the functional behaviour of the AT₁ receptors in such cell lines is remarkably similar to that in more complex *in vitro* experimental systems like vascular smooth muscle preparations.

2

Interaction with Naturally Occurring Angiotensin Fragments

Although Ang II has long been considered to represent the end product of the renin-angiotensin system, there is accumulating evidence that this system encloses additional effector peptides with diverse functions. In this respect, shorter Ang II fragments such as Ang III (deletion of the N-terminal amino acid), Ang IV [deletion of the two G protein-coupled receptor (GPCR) amino acids] and Ang-(1-7) (deletion of the C-terminal amino acid) have been found to accomplish central, cardiovascular and renal actions as well (Reaux et al. 2001) (Fig. 1). Similar to Ang II, Ang III is able to act both via AT₁ and AT₂ receptors. Both peptides elicit similar physiological effects, such as stimulation of aldosterone secretion, vasoconstriction and dipsogenic activity. They also bind with similar affinity to the AT₁ receptor and are equally potent agonists for this re-

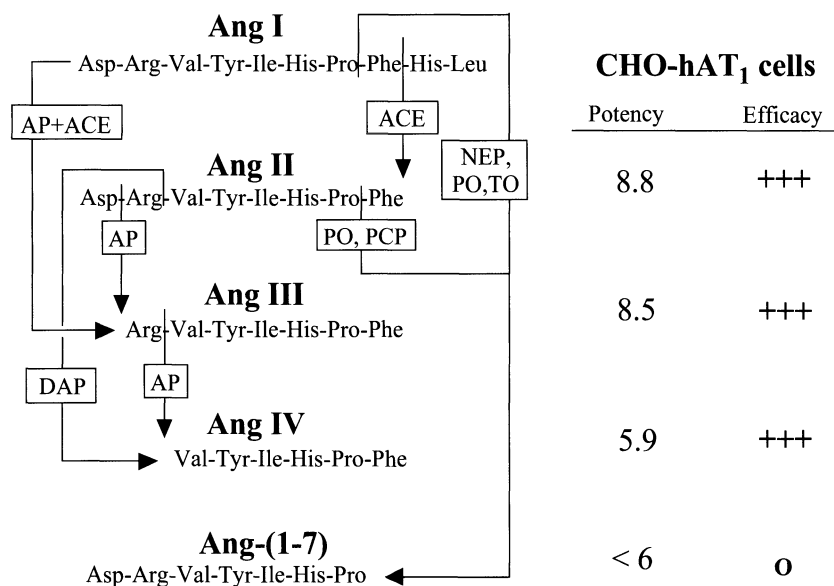


Fig. 1 *Left panel*: overview of the chemical structure and the enzymes involved in the synthesis of Ang II and its different fragments. Abbreviations: ACE, angiotensin-converting enzyme; AP, aminopeptidase; DAP, dipeptidyl aminopeptidases; PO, prolyl oligopeptidase; PCP, carboxypeptidase; NEP, neprilysin; TO, thimet oligopeptidase. *Right panel*: potency (in pEC₅₀ for agonists and pIC₅₀ for antagonists) and efficacy (+++, full agonism; 0, antagonism) of Ang II and its different fragments for inducing inositol phosphate production in CHO cells expressing human AT₁ receptors. (Le et al. 2002, 2003)

ceptor (Le et al. 2002) (Fig. 1). Interestingly, Ang III was shown to be even more effective than Ang II in stimulating the firing rate of certain neurons (Harding and Felix 1987). There is now also good evidence that the modulation of the pressor response and vasopressin release by centrally administered Ang II in rats depends on its prior conversion to Ang III (Zini et al. 1996; Reaux et al. 2001). These findings support the hypothesis that Ang III might be the predominant effector peptide in the brain (Harding et al. 1986).

Ang IV is also able to stimulate the AT₁ receptor as a full agonist, albeit with much lower potency when compared to Ang II and Ang III (Pendleton et al. 1989a; Le et al. 2002) (Fig. 1). This interaction provides a likely explanation for some of the physiological effects elicited by high, micromolar concentrations of this angiotensin fragment and for the ability of AT₁ receptor-selective antagonists such as losartan to block such effects. Yet, most of the physiological effects of Ang IV are already observed at nanomolar concentrations and some of them, such as its facilitating role in memory acquisition and retrieval, are of potential therapeutic interest (Wright et al. 1999; Kramár et al. 2001). The involved cellular targets acquired the status of "AT₄ receptors" (de Gasparo et al. 2000; Mustafa et al. 2001). Very recently, compelling evidence has been presented that at least some of them may be related to a membrane-associated aminopeptidase formerly known as the insulin-regulated aminopeptidase/oxytocinase (Albiston et al. 2001).

Ang-(1-7) has been reported to elicit physiological effects in the vasculature, the CNS and the kidney (Ferrario and Iyer 1998). In this respect, it even emerged that ACE inhibitors may exert their cardiovascular effects not only by blocking Ang II formation but also by promoting the accumulation of Ang-(1-7) (Luque et al. 1996). The effects of this angiotensin fragment have often been investigated in systems which also contain AT₁ receptors and/or AT₂ receptors and, in this respect, it is of interest that some of them could be blocked by the AT₁ antagonist losartan (Yamada et al. 1998; Potts et al. 2000). However, AT₁ receptor interactions do not offer a plausible explanation for such effects. It has been consistently found that Ang-(1-7) displays very low affinity for the AT₁ receptor and even that it acts as an antagonist (Mahon et al. 1994; Bouley et al. 1998; Le et al. 2002) (Fig. 1). Hence, alternative pathways and/or cellular recognition sites need to be invoked to explain the effects of Ang-(1-7).

3

Molecular Aspects of AT₁ Receptor Activation

Molecular cloning studies established that the AT₁ receptor belongs to the GPCR superfamily and, as such, it contains seven transmembrane-spanning α -helices (Hunyady et al. 2001) (Fig. 2). It is primarily coupled through G proteins of the G_{q/11} family to the activation of phospholipase C and calcium signalling. This second messenger system mediates smooth muscle contraction, aldosterone secretion, and the control of ion transport in renal tubule cells (de Gasparo et al. 2000). Whereas only one AT₁ receptor subtype is expressed in the human, two

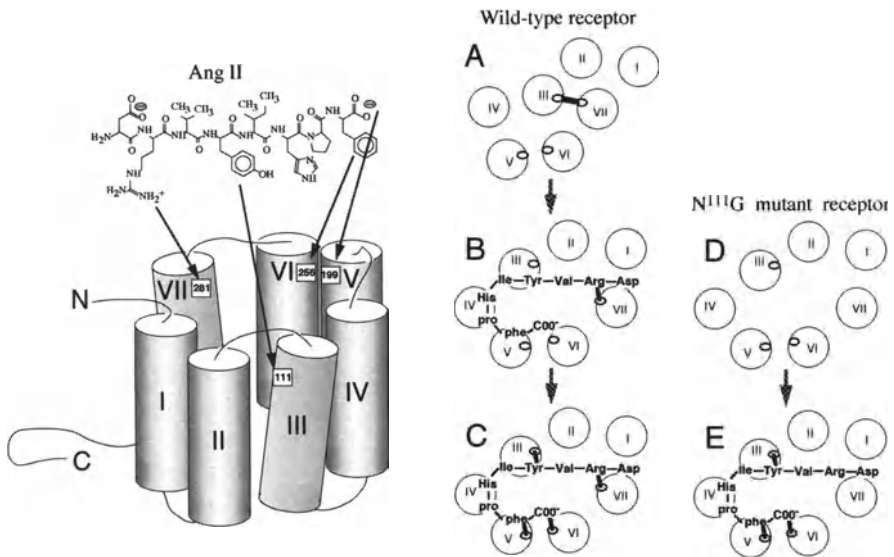


Fig. 2 *Left panel:* side view of the Ang II–AT₁ receptor interaction with reference to crucial parts of both molecules. (According to Hunyady et al. 2001 and Le et al. 2002). Both molecules are separated for the sake of clarity. *Roman and Arabic numbers* refer to transmembrane (TM) helices and amino acid residues of the receptor, respectively. *Right panel:* top view of the different steps involved in the Ang II–AT₁ receptor interaction. (According to Le et al. 2002). *A–C:* wild-type receptor. *A:* the basal state of the receptor is stabilised by the interaction of Asn¹¹¹ with TM VII. *B:* Arg² of Ang II interacts with Asp²⁸¹ of the receptor to relax the receptor into a pre-activated state (R') and to provide optimal binding of carboxy-terminal portion of Ang II. *C:* full receptor activation due to additional interactions with Tyr⁴, Phe⁸ and the terminal carboxyl group of Ang II. *D and E:* N¹¹¹G receptor mutant. *D:* the basal state of the receptor is constitutively activated (and similar to the R' state of the wild-type receptor) due to the absence of a Gly¹¹¹-TM VII interaction. *E:* full receptor activation by Ang II without the need of Arg²

subtypes (i.e. AT_{1A} and AT_{1B} receptors) with similar pharmacological properties but with different anatomical distribution have been found in rodents like the rat (Kakar et al. 1992).

Structure–function relationship studies have led to the proposal that the side chains of Arg², His⁶, and Pro⁷ along with the charged carboxyl-terminus of Ang II are important for its binding to the AT₁ receptor. In addition, Tyr⁴ and the aromatic group of Phe⁸ are important for receptor activation (Noda et al. 1995,1996; Miura et al. 1999; Hunyady et al. 2001) (Fig. 2). This explains why Ang IV and Ang-(1-7) display low affinity for the receptor and why the latter is an antagonist. On the other hand, Asp¹ of Ang II does not appear to be involved in AT₁ receptor binding and activation since Ang II and Ang III display the same characteristics. Site-directed mutagenesis studies in which single amino acids of the AT₁ receptor are substituted by less reactive ones (e.g. glycine and alanine) suggest that extracellular regions of the receptor play an important role in the binding of Ang II (Hunyady et al. 1996) (Fig. 2, left). This is consistent with the general belief that peptide and glycoprotein hormones bind to the ex-

tracellular regions of their GPCRs, as opposed to the binding of small nonpeptide ligands within the central cleft of such receptors.

The outcome of the Ang II binding is a conformational change in the receptor molecule that promotes its interaction with G proteins. Whereas the classic theory of receptor activation assumes that receptors can only adopt a single inactive (R) and active (R*) state, there is accumulating evidence that GPCRs may adopt a range of additional conformations. These may even include states with intermediate activity (R') in addition to the inactive and fully active ones. AT₁ receptor mutation studies provided a major and pioneering contribution to this notion and it is now widely admitted that full activation of this receptor by Ang II proceeds in at least two steps (Noda et al. 1996).

According to these models, the inactive form(s) of the receptor is stabilised by intramolecular bonds between Asn¹¹¹ of its third transmembrane domain (TM III) and amino acid residues present in TM VII (Hunyady et al. 2001) (Fig. 2A). A similar stabilising interaction has also been proposed to occur for rhodopsin, the only true GPCR for which the spatial structure has been elucidated by X-ray diffraction studies (Cohen et al. 1992). When Asn¹¹¹ is substituted by Gly (N¹¹¹G mutation) or by other amino acids with smaller side-chains, such interhelical bonds can no longer take place, and this allows the mutant AT₁ receptor to adopt a more "relaxed" conformation (Fig. 2D). These mutated receptors can still be fully activated by Ang II, but their basal activity is already well above that of the wild-type receptor (a phenomenon referred to as "constitutive activation") (Grobowski et al. 1997). These mutants are regarded to mimic an intermediate, not yet fully activated state (R') of the wild-type AT₁ receptor (Noda et al. 1996; Hunyady et al. 2001) (Fig. 2B). For the wild-type receptor, the disruption of the bonds between Asn¹¹¹ and TM VII is thought to be the first consequence of Ang II binding. Initially, it was advanced that the interaction between Tyr⁴ of Ang II and Asn¹¹¹ of the receptor should play an important role in its pre-activation (Noda et al. 1996). Yet, more recent studies revealed that Arg² of Ang II and Asp²⁸¹ of the receptor might be even more important actors (Le et al. 2002). In the model pictured in Fig. 2, the interaction between these two amino acids will induce a conformational change in TM VII of the receptor, which, similar to the N¹¹¹G mutation, eliminates the constraining interaction between Asn¹¹¹ and TM VII.

In line with the notion that the N¹¹¹G mutation causes a conformational change that is associated with receptor pre-activation, it causes a slight increase of the potency of Ang II and Ang III when compared to the wild-type receptor. Interestingly, the N¹¹¹G mutated AT₁ receptor can be fully activated by Ang IV with a potency that is similar to Ang II and Ang III (Le et al. 2002). This implies that, once the receptor has acquired the preactivated state R', Arg² will no longer play a major role in the binding and full activation of the receptor (Fig. 2E). Instead, the pre-activation is suggested to go along with a conformational change of the receptor to provide optimal binding of carboxy-terminal portion of Ang II (Le et al. 2002). It is this side of the hormone that will take over to produce full receptor activation (to yield R*, Fig. 2C,E). In this respect, the very low

potency and efficacy of Ang-(5-8) to activate the wild-type AT₁ receptor as well as the N¹¹¹G mutant highlights the importance of Tyr⁴ in this part of the activation process (Noda et al. 1996; Miura et al. 1999). Asn¹¹¹ is therefore considered to play a dual role; it stabilises R by interhelical interactions and, once liberated (in R'), it contributes as an "agonist switch" to the formation and/or stabilisation of R* by interacting with Tyr⁴ of Ang II. In addition, evidence has been presented for the terminal carboxyl group of Ang II to form a salt bridge with Lys¹⁹⁹ located in TM V of the receptor. This allows an appropriate positioning of the aromatic side chain of Phe⁸ to undergo hydrogen bonding with His²⁵⁶ in TM VI of the receptor, an interaction which is considered to be of prime importance for the activation process (Noda et al. 1995,1996; Miura et al. 1999). Hence, the Phe⁸ side chain represents the second "agonist switch" of Ang II.

Many synthetic Ang II analogues have been developed by substituting one or more of the original amino acids of Ang II either by other natural amino acids or by synthetic ones. Sarile ([Sar¹,Ile⁸]-Ang II) constitutes a typical example and, in its radioiodinated form, it is widely used for the labelling of AT₁ receptors in radioligand binding studies. Although it has been considered by some to behave as an antagonist, it is able to produce modest activation of the wild-type AT₁ receptor in cell lines (Noda et al. 1996). Such partial agonistic activity also constitutes one of the reasons for the failure of the therapeutic use of saralasin ([Sar¹,Val⁵,Ala⁸]-Ang II) a close analogue of sarile (Pals et al. 1979). Interestingly, sarile has been found to fully activate the N¹¹¹G receptor mutant (Noda et al. 1996). This example illustrates the use of constitutively activated AT₁ receptor mutants to distinguish between true antagonists and weak partial agonists.

4

Interaction with Nonpeptide Antagonists

In the past decade, nonpeptide AT₁ receptor antagonists have been routinely tested for their ability to affect Ang II dose-contractile response curves of vascular smooth muscle preparations. Such organ bath experiments have often been carried out with rabbit aortic rings/strips, a system with very small receptor reserve (Zhang et al. 1993; Robertson 1998). They constitute a major paradigm for comparing the behaviour of different antagonists. Losartan, the prototype of the biphenyl-tetrazole class of antagonists, produces parallel rightward shifts of the Ang II dose-response curve without depressing the maximal response. This behaviour is denoted as "surmountable" (Fig. 3, top). Yet, many structurally related antagonists are also capable of depressing the maximal response. This behaviour is denoted as "insurmountable" (Vauquelin et al. 2001a, 2002). The extent by which the maximal response is depressed is quite variable (Fig. 3, top). For example, it is only partial for irbesartan, valsartan and EXP3174 (the active metabolite of losartan) but almost complete for antagonists such as GR117289, KRH-594 and candesartan (Liu et al. 1992; Robertson et al. 1992; Cazaubon et al. 1993; Criscione et al. 1993; Noda et al. 1993; Mochizuki et al. 1995; Tamura et al. 1997). More recently, such experiments have been repeated with cell lines

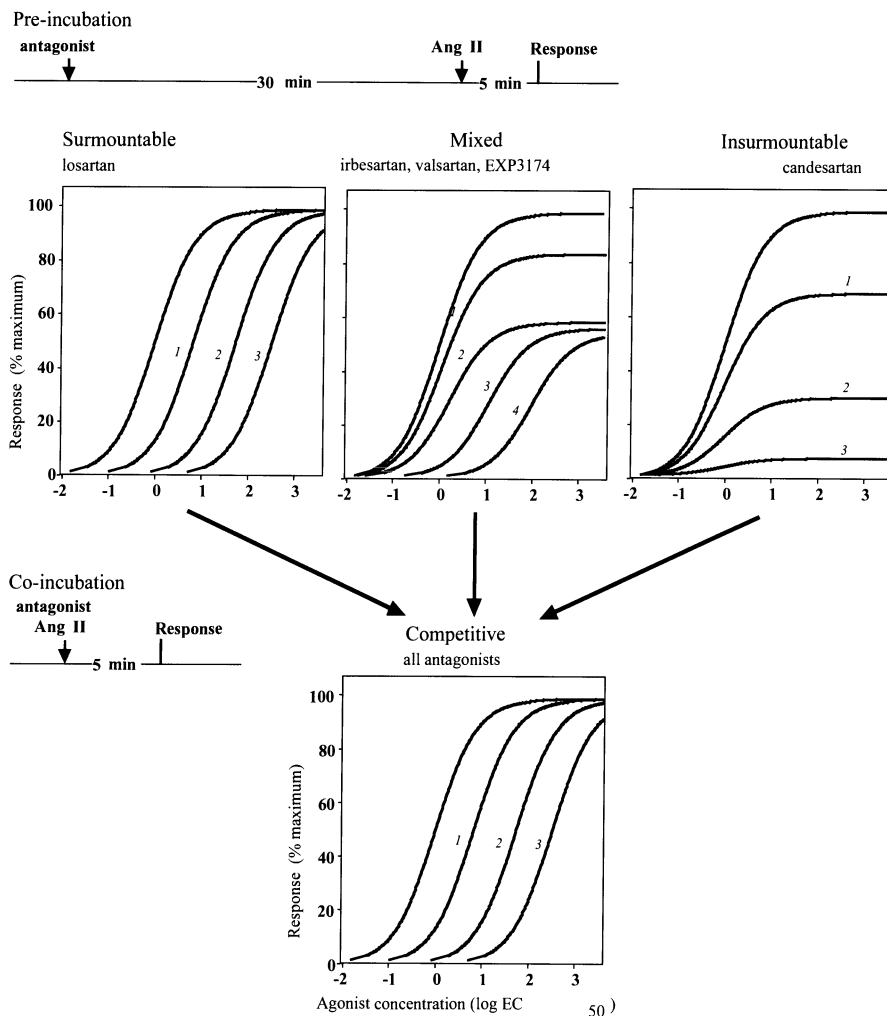


Fig. 3 *Top panel:* theoretical Ang II dose–response curves for antagonist-pretreated AT₁ receptor preparations. *Curve at the left:* no antagonist pretreatment. *Curves with italic numbers:* pretreatment with increasing antagonist concentrations of surmountable, partially insurmountable and nearly fully insurmountable antagonists. (Vanderheyden et al. 1999; Verheyen et al. 2000). *Lower panel:* theoretical Ang II dose–response curves with the same antagonists under co-incubation conditions. (Fierens et al. 1999a; Verheyen et al. 2000)

that endogenously express the AT₁ receptor or that have been transfected with the gene encoding such receptors as CHO-hAT₁ cells. Although these experiments rely on the measurement of receptor-evoked biochemical events within the cell, the provided information is consistent with that obtained by smooth muscle contraction studies, at least when the antagonist is administered ahead of Ang II (Fierens et al. 1999a; Vanderheyden et al. 1999; Verheyen et al. 2000).

Although most AT₁ receptor antagonists possess a biphenyltetrazole moiety (Fig. 5), several of them are structurally different. Such antagonists can also be classified as surmountable (e.g. eprosartan) and insurmountable (e.g. telmisartan and triacid 4-aminoimidazole derivatives) (Wienen et al. 1993; Timmermans et al. 1999b; Vanderheyden et al. 2000a; Hollenberg 2001). Additionally, peptide analogues of Ang II with partial agonistic activity, such as sarile, have also been shown to display insurmountable AT₁ receptor blockade (Pendleton et al. 1989b). Presently, insurmountable antagonism has only been investigated in detail for antagonists of the biphenyltetrazole class. The present discussion will therefore focus on such antagonists.

Insurmountable antagonism does not constitute the sole privilege of the AT₁ receptors either. Similar behaviour has been observed for many other GPCR antagonists, and several theories have been advanced in the past (Vauquelin et al. 2001a, 2002). One major explanation endorses that insurmountable antagonists are non-competitive. The other one takes into account that tissues are invariably pre-incubated with the antagonist before their challenge with the agonist. This second explanation allows competitive antagonists to be insurmountable provided that they bind to the receptor in an irreversible fashion (so that the receptor number is permanently reduced) or that they dissociate sufficiently slowly (so that only a limited number of receptors are liberated before the response is measured) (Kenakin 1987). Co-incubation experiments, in which the agonist and the antagonist are added simultaneously to the receptor, have the potential to provide a clear-cut discrimination between both explanations (Vauquelin et al. 2001a). Indeed, under those conditions, a decrease of the maximal agonist-evoked response is only to be expected in the case of non-competitive antagonism. Whereas such experiments are extremely laborious when dealing with contraction studies, they are commonplace for radioligand binding and functional studies with isolated cells or membranes thereof. In co-incubation experiments, surmountable and insurmountable antagonists were all found to produce parallel rightward shifts of the Ang II dose-response curves in bovine adrenal glomerulosa cells (Criscione et al. 1993) and in CHO-hAT₁ cells (Fierens et al. 1999a; Verheyen et al. 2000) (Fig. 3, lower panel). The same profile also emerges when comparing radioligand binding studies involving pre- and co-incubation experiments (Pendleton et al. 1989b; Hara et al. 1995; De Arriba et al. 1996). Such studies have now clearly established that nonpeptide AT₁ receptor antagonists are competitive with Ang II and even among each other. This also explains the ability of surmountable and insurmountable AT₁-receptor antagonists to counteract each other's effect in a dose-dependent fashion (Wong et al. 1991; Vauquelin et al. 2001b).

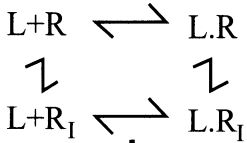
Direct measurements based on the binding of radiolabelled antagonists as well as indirect measurements based on the recovery of receptor functionality in wash-out experiments confirm that surmountable antagonists such as losartan dissociate very rapidly from the AT₁ receptor and that insurmountable antagonists dissociate slowly. For intact CHO-hAT₁ cells, the dissociation half-life of insurmountable antagonists at 37°C was estimated to be 7 min for irbesartan, 17 min for valsartan, 30 min for EXP3174 and 120 min for candesartan (Fierens

et al. 1999b; Vanderheyden et al. 2000b,c; Verheyen et al. 2000). These determinations clearly establish that the blockade by insurmountable antagonists is sufficiently long lasting to impair the accessibility of a proportion of the AT₁ receptor molecules if the ensuing challenge with Ang II only takes place for relatively short time-span. Obviously, the occupancy of the receptors by such antagonists will readjust with time until, ultimately, both the Ang II–receptor and the antagonist–receptor interactions reach equilibrium. Therefore, the insurmountability of slow dissociating antagonists is only “apparent”, and it can theoretically be avoided if the response is measured after a sufficient lag of time. However, such delays are precluded for practical considerations such as the necessity to avoid fading of the response.

Many AT₁ receptor antagonists display a rather complex pattern of insurmountability when examining systems as diverse as muscle strips and transfected cell lines. At low concentrations, they decrease the maximal response to an extent that is dependent on the antagonist in question. When the concentration is further increased, these antagonists still produce rightward shifts of the dose–response curve, but the maximal stimulation will not decline any further. Such mixed insurmountable/surmountable antagonism has also been documented for several other GPCRs (Vauquelin et al. 2002). The simplest explanation would be that the antagonists already liberated of part of the receptor sites during the ensuing agonist exposure. This permits a partial restoration of the response to take place. However, the fast liberation of only part of the antagonist-pretreated AT₁ receptor sites and the much slower liberation of the remainder in washout experiments (Fierens et al. 1999a) cannot be explained by this simple model. Instead, this phenomenon is readily explained when the classical theory of receptor activity with only one thermodynamically stable inactive form of the receptor, R, is extended to include a second inactive form, R₁, which cannot be recognised and stimulated by agonists (Fierens et al. 1999a). Several such “two-state” models have been elaborated over the years according to such a principle (Lucas et al. 1979; de Chaffoy de Courcelles et al. 1986; Liu et al. 1992; Robertson et al. 1996). In their most general formulation (Fig. 4), such models allow R to form fast reversible/surmountable complexes with all antagonists (L). In addition, these models agree on the implication of L.R₁ in insurmountable antagonism and allow an equilibrium between L.R and L.R₁ to take place (Vauquelin et al. 2002). Finally, the proportion between the surmountable and insurmountable antagonist–receptor complexes (i.e. the [L.R]/[L.R₁] ratio) may differ from one antagonist to another. The major differences between the published two-state models reside in the explanations about the precise nature of L.R' and how it mediates insurmountable antagonism.

Insurmountable AT₁ receptor antagonism can obviously be explained by a slow dissociation of the L.R₁ complex. Alternatively, insurmountable antagonism might also be perceived when L.R₁ dissociates swiftly, provided that R₁ is only slowly reconverted into R (Fierens et al. 1999a). According to this latter explanation, there is no strict obligation for the antagonist to remain bound to the receptor to produce a long-lasting effect. In the case of AT₁ receptor antagonists, a

"Two-state" model: general formulation



"Two-state, two-step" model



losartan	100 %	0 %
irbesartan	70 %	30 %
valsartan	50 %	50 %
EXP3174	30 %	70 %
candesartan	5 %	95 %

Fig. 4 Two-state models for insurmountable AT₁ receptor antagonism. The receptor can be stimulated by the agonist when present in the R state/conformation but not when present in the R_I state. In the general formulation (Fierens et al. 1999a), insurmountable antagonism might take place with fast-dissociating antagonist-R_I complexes as well as with fast-dissociating complexes, provided that R_I is only slowly reconverted into R. In the two-step model (Vauquelin et al. 2001a,b), the R_I state is slow-dissociating. Percentages of antagonist-receptor complexes in each state are estimated from inhibition data (Fierens et al. 1999a; Verheyen et al. 2000)

close match has been observed between the dissociation of [³H]-candesartan and [³H]-valsartan from CHO-hAT₁ cells and the recovery of functional receptors in these cells (Fierens et al. 1999b; Verheyen et al. 2000). In other words, the insurmountable effect of such antagonist is directly linked to their receptor occupancy. This supports the first explanation and constitutes a rationale for simplifying the general "two-state" model formulation into the "two-state, two-step" model (Vauquelin et al. 2001a,b) (Fig. 4). In this simplified model, all antagonists initially bind to R to form a fast-dissociating L.R complex. For insurmountable binding, this complex must further be converted into a tight-binding L.R_I complex. Whereas insurmountable antagonists can induce this conversion with various degrees of effectiveness, surmountable antagonists like losartan are unable to do so. Hence, losartan-AT₁ receptor complexes are committed to reside in the fast-dissociating L.R state. Computer simulations were performed to match the "two-state, two-step" model with the experimental behaviour of the antagonists losartan, irbesartan, EXP3174 and candesartan (Vauquelin et al. 2001b). It was found that the initial "attraction" to the receptor (i.e. formation of L.R) is fairly similar for each of the investigated antagonists. The subsequent formation of L.R_I also appears to proceed with the same "ease" for all the insurmountable antagonists but that the stability of this complex is different for each antagonist. Moreover, the model also provides an explanation for the observed proportionality between the experimental [L.R]/[L.R_I] ratio (Fig. 4) and the dissociation rate of each involved antagonist (Vauquelin et al. 2001a).

5

Cellular and Molecular Aspects of Antagonist-AT₁ Receptor Binding

At present, it is not known why the antagonist-AT₁ receptor complexes may adopt distinct states. Differences between L.R and L.R_I may reside at the level of the receptor conformation, its association with other proteins or even its sub-cellular localisation.

Differences in the subcellular localisation of the receptor, with L.R at the cell surface and L.R₁ inside the cell, have been invoked to explain insurmountable AT₁ receptor antagonism. Indeed, receptor internalisation is well known to occur with Ang II. When stimulated by this agonist, the AT₁ receptor has been shown to undergo rapid internalisation into the cell as part of their recycling process (Hein et al. 1997; Hunyady et al. 2001). Initial studies concluded that antagonist-receptor complexes are able to internalise as well (Croizat et al. 1986; Liu et al. 1992; Conchon et al. 1994), but more recently, it emerged that the presented criteria for such internalisation are not adequate in the case of nonpeptide AT₁ receptor antagonists. Instead, work with mutant receptors that had lost their ability to undergo internalisation (Fierens et al. 2001), as well as with green fluorescent protein-conjugated receptors (Hein et al. 1997; Hunyady et al. 2001), rather suggests that antagonist-bound AT₁ receptors always remain at the cell surface.

It is well known that AT₁ receptors and other GPCRs may exist as monomeric and dimeric forms with quite distinct functional properties and that such receptors also acquire a tight agonist-binding conformation when they undergo functional coupling to G proteins (De Lean et al. 1980). It is therefore also plausible that L.R and L.R₁ display differences in their association with other proteins which make part of the membrane or the cellular matrix, or even diffuse freely in the cytosol. At present, still very little is known about this issue, but it could be of great interest since the insurmountable antagonist [³H]-candesartan has been found to dissociate much faster from isolated CHO-hAT₁ cell membranes as compared to the intact cells (Fierens et al. 2002). Thermodynamic characterisation also shows differences for the [³H]-candesartan binding, with an almost completely enthalpy-driven interaction for intact cells as compared to a mixed contribution of both enthalpy and entropy for the membranes. As the most obvious explanations (proteolytic degradation and oxidative phenomena during the membrane preparation) were ruled out, these differences suggest a yet-to-be-disclosed impact of the structural organisation of living cells on the binding properties of AT₁ receptor antagonists (Fierens et al. 2002).

Additional information about the status of L.R and L.R₁ is provided by antagonist structure-binding activity relationship studies and receptor mutation studies. In this respect, it is noteworthy that the synthesis of nonpeptide AT₁ receptor antagonists has been sparked by the discovery that Ang II-mediated vasoconstriction could be antagonised by imidazole-5-acetic acid derivatives such as S-8307, albeit with low potency (Duncia et al. 1990; Timmermans et al. 1993). Starting from such lead compounds, more potent antagonists were designed with the major premises that the carboxyl group of lead and the terminal carboxyl group of Ang II should both point to the same positive charge of the receptor and that the lead requires an additional acidic functionality to mimic the carboxyl group in Asp¹ or the hydroxyl group in Tyr⁴ of Ang II (Fig. 5, top). Further substitutions of S-8307, including the introduction of an anionic tetrazolium led to the development of losartan, the prototype of the biphenyltetrazole-containing sartans (Duncia et al. 1990; Timmermans et al. 1993). The carboxyl group of the lead compounds was replaced by an alcoholic function in

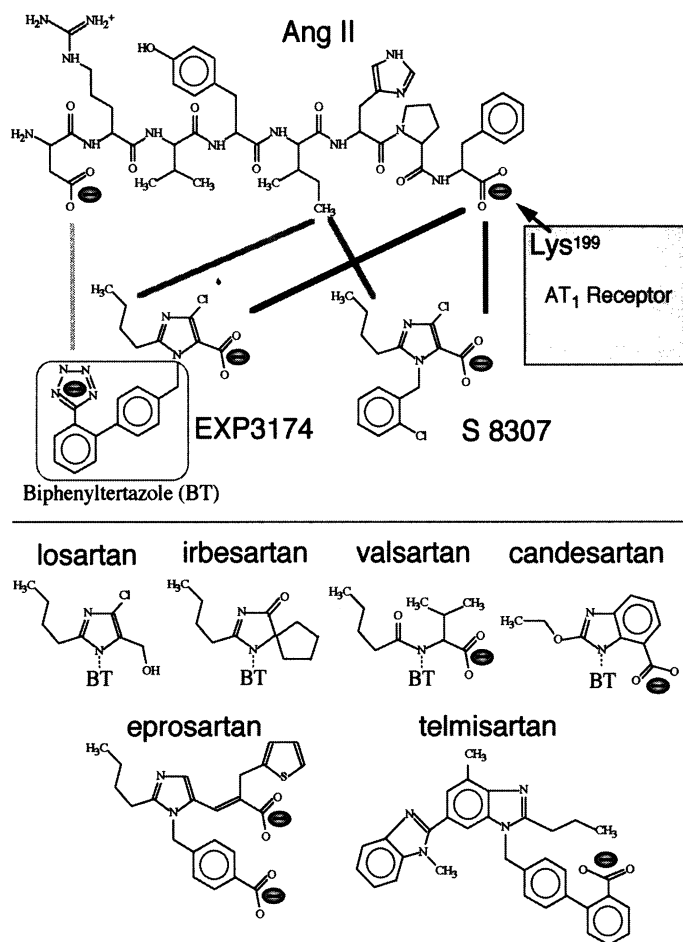


Fig. 5 *Top panel:* supposed structural homologies between Ang II and the nonpeptide AT₁ receptor antagonists S8307 and EXP3174. (According to Timmermans et al. 1993). *Lower panel:* structure of other nonpeptide AT₁ receptor antagonists of the "sartan" class. BT, biphenyltetrazole moiety

losartan but, after oxidative transformation in the liver, this group reappears in EXP3174, the active metabolite of losartan (Wong et al. 1990) (Fig. 5, top). Since losartan is fully surmountable, the carboxyl group of EXP3174 is clearly involved in its insurmountable character. The same distinction can be made between candesartan and its surmountable precursor molecule candesartan cilexetil (Noda et al. 1993; Mochizuki et al. 1995). In the same way, many other AT₁ receptor antagonists with a pronounced insurmountable character appear to possess both a carboxyl group and a tetrazole moiety (Vauquelin et al. 2000). However, the mere presence of a carboxyl group is not sufficient for biphenyltetrazole-containing AT₁ receptor antagonists to be insurmountable. Experiments with candesartan analogues clearly revealed that this group needs to be positioned correctly (Noda et al. 1993).

Taken together, aforementioned structure-binding activity relationship studies are compatible with the following model. Because of the similarity in the initial "attraction" of biphenyltetrazole-containing sartans to the receptor (Vauquelin et al. 2001b), this moiety is likely to play an important role in the formation of L.R. When present, the carboxyl group of such antagonists should not be important for their initial binding, but it should play a major role in the stabilisation of the L.R₁ complex by interacting with basic amino acids that are present in a well-defined binding pocket of the receptor (Vauquelin et al. 2000). Obviously, additional interactions may also be of importance for the stabilisation of L.R₁ and, in this respect, it has been reported that the nature of the alkyl substituent next to the carboxyl group on analogues of UR-7280 may determine whether they are insurmountable or not (De Arriba et al. 1996).

The carboxyl group of insurmountable AT₁ receptor antagonists like EXP3174 is also supposed to mimic the terminal carboxyl group of Ang II (Timmermans et al. 1993) (Fig. 5, top). AT₁ receptor mutation studies support this conclusion. Indeed, Lys¹⁹⁹ is known to be involved in the binding of the carboxy-terminus of Ang II. Its substitution by Gln (an amino acid with a neutral but still polar residue) causes a marked loss of binding affinity for Ang II, similar to that observed when its carboxyl group is replaced by a neutral amide group (Noda et al. 1995, 1996). In contrast, this mutation has only little to moderate effect on the affinity of losartan (Schambye et al. 1994; Noda et al. 1995). In intact CHO-hAT₁ cells, this mutation produces affinity decreases of the nonpeptide antagonists that followed their degree of insurmountability: i.e. about 5-fold for losartan, 10-fold for irbesartan, 18-fold for EXP3174 and 45-fold for candesartan (Fierens et al. 2000). In addition, the dissociation of [³H]-candesartan from the mutated receptor is also appreciably faster than from the native receptor (Fierens et al. 2000). These findings plead in favour of electrostatic bonding between Lys¹⁹⁹ and the carboxyl group of insurmountable antagonists like candesartan and EXP3174 (Fig. 5, top). Yet, whereas such bonding will mediate receptor activation in the case of Ang II and related agonists, it will merely contribute to the stabilisation of a tight binding state of the receptor in the case of insurmountable nonpeptide antagonists.

Yamano et al. (1995) proposed that the anionic tetrazolium substituent of nonpeptide antagonists does not bind to Lys¹⁹⁹. Based on additional mutation studies, these investigators put forward that the tetrazolium moiety might selectively bind to the anionic side chain of Arg¹⁶⁷. However, as the substitution of Arg¹⁶⁷ by Ala abolishes [³H]-Ang II as well as [³H]-candesartan binding to intact CHO-hAT₁ cells (Vauquelin et al. 2001c), the possibility also arises that this amino acid is only important for the structural integrity of the AT₁ receptor. Hence, there is no strict necessity for this amino acid to directly participate in any binding process itself. Based on similar mutation studies, several additional amino acids belonging to TM domains III, IV, V, VI and VII have been found to affect the binding of losartan (Ji et al. 1994). Most of them are positioned within a small distance from each other within a plane that is one or two helical turns below the membrane surface.

6

Inverse Agonism and Multiple AT₁ Receptor Conformations

Whereas Asn¹¹¹-substituted AT₁ receptor mutants exhibit increased affinity for Ang II and related agonists, they have been shown to display decreased affinity for losartan (Monnot et al. 1996; Groblewski et al. 1997). Such mutations are believed to improve the positioning of residues required for agonist binding and bring about a misalignment of the residues required for losartan binding (Hunyady et al. 2001). Recent studies on intact CHO-hAT₁ cells reveal that N¹¹¹G and related mutant receptors display a similar decrease in affinity for losartan, irbesartan, EXP3174 and candesartan (Le et al. 2003). Provided that the wild-type AT₁ receptor can spontaneously adopt a related preactivated state (R'), these findings predict that nonpeptide antagonists have the potential to act as inverse agonists but that this effect should be independent of their degree of insurmountability. So far, there are no direct indications for endogenously expressed AT₁ receptors to undergo such spontaneous pre-activation, but other GPCRs have been reported to do so (Leurs et al. 2000). As Ang IV displays much higher potency for the N¹¹¹G mutant compared to the wild-type receptor (Le et al. 2002), this peptide might constitute a novel powerful tool for the quest for preactivated AT₁ receptors under normal and pathophysiological conditions.

Opposed to this scenario, there is the opinion that agonists do not stabilise pre-existing active states of the AT₁ receptor but that their interaction with the receptor is required to induce receptor activation (Noda et al. 1996; Hunyady et al. 2001). This opens the possibility for different agonists, or even for the same agonist, to cause different conformational rearrangements of the receptor (Thomas et al. 2001). This provides a plausible explanation for the ability of [Sar¹,Ile⁴,Ile⁸]Ang II to promote AT₁ receptor phosphorylation without going through the conformation required for inositol phosphate signalling. It may also explain the fact that multiple forms of signal transduction can participate in the effects of Ang II on cell growth and on the remodelling of cardiac and vascular cells (de Gasparo et al. 2000; Dostal et al. 2000; Eguchi et al. 2000; Haendeler et al. 2000; Hunyady et al. 2001).

Taken together, the current concepts about agonist- and antagonist-AT₁ receptor interaction comply with the increasing awareness that the classical models for receptor activation, in which receptors isomerise between single inactive and active conformations, are over-simplistic (Clark et al. 1999; Onaran et al. 1999). Instead, a number of agonist-bound and antagonist-bound conformations and/or states of the receptor are suspected to occur, each with its own characteristic properties.

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AT₁ Receptor Regulation

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Abstract Clinical and experimental studies have demonstrated that AT₁ receptor expression and regulation play an important role in the pathogenesis of cardiovascular diseases such as atherosclerosis and hypertension. The expression levels of the AT₁ receptor define the biological efficacy of angiotensin II. Many agonists, as for example angiotensin II, growth factors, low-density lipoprotein cholesterol, insulin, estrogen, progesterone, reactive oxygen species, cytokines, nitric oxide, and many others are known to regulate AT₁ receptor expression. Mechanisms of AT₁ receptor regulation include receptor internalization, desensitization, alternative splicing of receptor pre-mRNA, and most importantly modulation of its gene expression by transcriptional and posttranscriptional mechanisms. Posttranscriptional mechanisms predominate AT₁ receptor regulation. Binding of RNA-binding proteins to the 5' and 3' untranslated region of the AT₁ receptor mRNA has been shown to be involved in the regulation of mRNA stability. Recent studies revealed that a region just adjacent to the poly-A tail of the AT₁ receptor mRNA, which has a secondary structure that forms a stem loop, is important for the protein-mRNA interaction. The first identified AT₁ receptor binding protein is calreticulin, which, if phosphorylated, binds to this cognate sequence of the AT₁ receptor mRNA and leads to mRNA destabilization. Signal transduction molecules such as cAMP, reactive oxygen species, MAP kinases, nitric oxide, PI-3 kinase, and others have been shown to be involved in AT₁ receptor regulation by several agonists. The pathophysiological relevance of dysregulated AT₁ receptor expression has been demonstrated in

many *in vitro*, *in vivo* and human studies. Hypercholesterolemia, estrogen deficiency, and hyperinsulinemia are associated with enhanced AT₁ receptor expression and increased oxidative stress, and AT₁ receptor antagonism inhibits pathological cellular processes and the development of endothelial dysfunction and atherosclerotic lesions. AT₁ receptor overexpression very likely represents, among other things, a potential molecular mechanism that links exogenous risk factors to cellular events in chronic vascular disease.

Keywords AT₁ receptor · Regulation · Transcriptional · Posttranscriptional · Agonists · mRNA binding proteins · Hypercholesterolemia

1

Introduction

A series of recent experimental and clinical studies have demonstrated that altered regulation and expression of the angiotensin (Ang) II type 1 (AT₁) receptor plays an important role in the pathogenesis of chronic cardiovascular diseases. The modulation of AT₁ receptor expression levels contributes on the one hand to the adaptation of the renin–angiotensin system to chronic agonist stimulation and serves on the other hand as a possible explanation for the association of various hormonal and metabolic disorders with hypertension and with the development and accelerated progression of atherosclerotic lesions (Peach 1977; Griendling et al. 1993).

2

Regulation of AT₁ Receptor Expression

Back in 1980, it was discovered that the vasoconstriction induced by AngII in resistance vessels is variable (Gunther et al. 1980). Further investigations revealed that AT₁ receptor expression is subject to a negative feedback regulation. Increased levels of AngII reduce AT₁ receptor expression, whereas decreased AngII concentrations enhance AT₁ receptor expression levels (Douglas and Brown 1982; Schiffrin et al. 1984; Griendling et al. 1987; Lassègue et al. 1995). More recently, it has been shown that various agonists other than AngII modulate AT₁ receptor expression. This phenomenon, referred to as heterologous AT₁ receptor regulation, is induced by various growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or fibroblast growth factor (FGF), all of which downregulate AT₁ receptor expression (Nickenig and Murphy 1994). Numerous other factors, including glucocorticoids, aldosterone, forskolin, tumor necrosis factor- α , cytokines, nitric oxide, insulin, low-density lipoprotein (LDL) cholesterol, estrogen, progesterone, sodium chloride, reactive oxygen species, insulin-like growth factor-1, and isoprenaline are known to influence AT₁ receptor expression (Table 1) (Kitamura et al. 1986; Sumners et al. 1986; Douglas 1987; Linas et al. 1990; Kakar et al. 1992; Ullian et al. 1992; Sun and Weber 1993; Tufroet et al. 1993; Lu et al. 1994; Bird et al. 1995;

Table 1 Regulation of AT₁ receptor expression. Various agonists modulate AT₁ receptor expression in different tissues or cell types. Few studies have delineated the participating signal transduction pathways and characterized the mechanism of regulation

Cell/tissue	Agonist	Regulation ↑↓	Mechanism	Signal transduction
VSMC (rat)	IL1 α	↑		
VSMC (rat)	TNF α +IFN γ	↓		
VSMC (rat)	LDL	↑	Posttranscriptional	
VSMC (rat)	AngII, α -thrombin, ATP, forskolin	↓	Posttranscriptional and transcriptional	Phenylarsine oxide
VSMC (rat)	Forskolin, isoproterenol	↓	Posttranscriptional	PKA
VSMC	SNAP (NO donor)	↓	Posttranscriptional	
VSMC	IFN γ	↓		MAP kinase, JAK2
VSMC (rat)	Insulin	↑	Posttranscriptional	Tyrosine kinase, calcium
VSMC (rat)	Estrogen	↓	Posttranscriptional	NO synthase
	Progesterone	↑	Posttranscriptional and transcriptional	PI-3 kinase
VSMC (rat)	Iodoxifene (SERM)	↓	Posttranscriptional	NO synthase
VSMC (rat)	NaCl	↑		
VSMC (rat)	Free radicals	↓	Posttranscriptional	p38 MAP kinase
VSMC (rat)	<i>all-trans</i> retinoic acid (vitamin A)	↓	Transcriptional	
VSMC (rat)	Statins	↓	Posttranscriptional	Geranylgeranylation
VSMC (rat)	EGF	↓	Posttranscriptional and transcriptional	
	FGF	↓		
	PDGF	↓		
Heart (rat)	Dexamethasone	↑		
	Deoxycorticosterone	AT _{1A} ↓		
Cardiac fibroblasts	TNF- α	↑		Inositol phosphat
	IL1 β	↑		
	IL2, IL6	→		
Neuronal cells (WKY and SHR)	Phorbol ester	↑	Transcriptional	
Brain (mouse)	Forskolin	↑		
	Haloperidol	↑		
Astrocytes (rat)	GH	AT _{1A} ↑	Transcriptional	
Pituitary, brain (rat)	Estrogen	↓		
Neuroblastoma cells (mouse)	AngII	↓		
Pituitary	Estrogen	↓	Posttranscriptional	
Adrenal gland		↓		
Uterus		↑		
Bovine zona glomerulosa cells	ACTH	↓		
	cAMP	↓		
	IGF1	↑		
	KCl	↑		
	Cortisol	↑		
	Aldosterone	→		
Mesangial cells (rat)	Glucose	↓		PKC
Mesangial cells (rat)	Dexamethasone	AT _{1A} →, AT _{1B} ↓		
Placenta, trophoblast cells (human)	Progesterone	↓		
	Estradiol	→		

Bruna et al. 1995; Nickenig et al. 1996, 1997a, 1998a,b,c, 2000a,b; Cheng et al. 1996; Kalenga et al. 1996; Sechi et al. 1996; Yang et al. 1996; Nishimura et al. 1997; Sasamura et al. 1997; Ullian et al. 1997; Wang et al. 1997; Ichiki et al. 1998; Amiri and Garcia 1999; Gurantz et al. 1999; Ikeda et al. 1999; Kisley et al. 1999; Krishnamurthi et al. 1999; Müller et al. 2000; Takeda et al. 2000; Bäumer et al. 2001; Wassmann et al. 2001a).

3 Mechanisms of AT₁ Receptor Regulation

Most of the AngII effects are mediated by stimulation of AT₁ receptors. Thus, the number of AT₁ receptors defines the biological efficacy of AngII. There are at least four different aspects involved in AT₁ receptor regulation. First, activation of AT₁ receptors with AngII may evoke internalization of the receptor protein and reduces receptor numbers on the cell surface (Kai et al. 1994). Second, chronic AngII stimulation reduces AngII signaling via protein kinase C-depen-

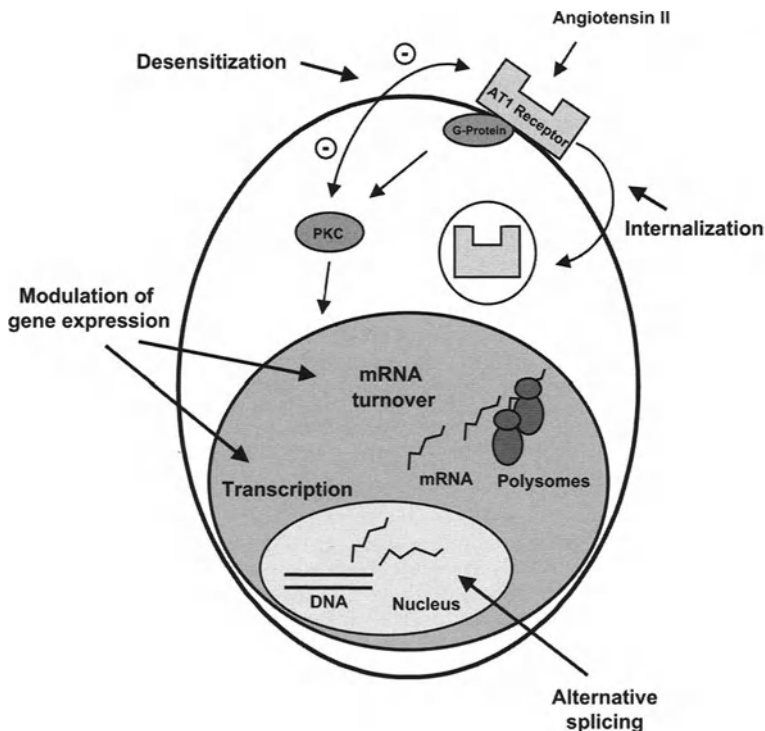


Fig. 1 Principal mechanisms involved in regulation of AT₁ receptor expression. AT₁ receptor expression may be modulated by AngII-induced internalization of the receptor protein or by desensitization of signal transduction pathways downstream of the AT₁ receptor. Furthermore, AT₁ receptor gene expression may be altered at both transcriptional and posttranscriptional levels. Finally, alternative splicing may modulate AT₁ receptor expression

dent pathways, which is referred to as desensitization (Curnow 1996). Third, alternative splicing of the AT₁ receptor pre-mRNA can alter AT₁ receptor protein translation. Fourth, and likely the most important mechanism regulating AT₁ receptors, is modulation of its gene expression (Lassègue et al. 1995; Nickenig and Murphy 1994). These principal mechanisms of AT₁ receptor regulation are summarized in Fig. 1.

Modulations of AT₁ receptor gene expression are generally obvious several hours after agonist stimulation and are sustained for variable periods of time thereafter. Recently, it has been shown that internalization of the AT₁ receptor seems to regulate expression of AT₁ receptor mRNA (Adams et al. 1999).

Gene expression is predominantly modulated by transcriptional and post-transcriptional mechanisms. Among other stimuli, growth factors and AngII are known to reduce the rate of AT₁ receptor mRNA transcription (Lassègue et al. 1995; Nickenig and Murphy 1994). Although numerous consensus sequences were discovered within the promoter region of the AT₁ receptor [e.g., AP-1, SP-1, estrogen-response element, cyclic adenosine monophosphate (cAMP)-response element], the exact mechanisms of transcriptional control of AT₁ receptor mRNA synthesis are poorly understood (Murphy et al. 1992).

4

Posttranscriptional AT₁ Receptor Regulation

In general, the abundance of a particular mRNA transcript and its resulting protein product is not only governed by its transcription rate but also by its half-life (also referred to as mRNA stability). Of relevance for AT₁ receptor regulation has been the finding that numerous agonists that modulate AT₁ receptor expression also affect posttranscriptional processing of its mRNA. Estrogens, AngII, and cAMP-stimulating agents decrease AT₁ receptor expression by stimulating degradation of the AT₁ receptor mRNA (Nickenig and Murphy 1996; Wang et al. 1997; Nickenig et al. 2000b). In contrast, progesterone, LDL, and insulin upregulate AT₁ receptor expression by decreasing its mRNA decay (Nickenig et al. 1997a, 1998a,b, 2000b). The data concerning growth factors such as PDGF are inconsistent. It is well established that these factors reduce AT₁ receptor mRNA transcription rate (Nickenig and Murphy 1994). Experiments following mRNA degradation after blockade of transcription with actinomycin D revealed that growth factors induce degradation of the AT₁ receptor mRNA (Nickenig and Murphy 1994). Other data with recombinant, retroviral approaches have suggested that PDGF may have no effect on AT₁ receptor mRNA stability (Wang and Murphy 1998; Xu and Murphy 2000). These discrepancies are likely due to difficulties in obtaining unambiguous measures of inducible mRNA turnover.

Despite these inconsistencies, there is strong evidence that posttranscriptional mechanisms predominate AT₁ receptor regulation. As is the case for many other mRNAs, this process involves binding of proteins to both the 5' and 3' untranslated region of the AT₁ receptor mRNA. Data from recombinant retroviral AT₁ receptor mRNA species and experiments performed in brain tissue suggest

that proteins interacting with the 5' untranslated region of the AT₁ receptor mRNA are involved in both cAMP- and estrogen-induced modulation of AT₁ receptor regulation (Krishnamurthi et al. 1998, 1999; Wang and Murphy 1998; Xu and Murphy 2000). To date, however, the identity of these proteins remains undefined, as do the precise regions of the AT₁ receptor mRNA involved in binding these proteins.

Several families of RNA binding proteins have been implicated in the regulation of steady-state RNA levels. Packaging, splicing, and nuclear translocation are arranged through actions of nuclear ribonucleoprotein particles (Dreyfuss 1986; Swanson and Dreyfuss 1988; Wilusz and Shenk 1990). Small nuclear RNA-binding proteins are thought to manage further splicing and processing of the 5' cap and the 3' poly-A-tail (Steitz et al. 1983; Konarska and Sharp 1987). In addition, cytosolic RNA-binding proteins have been identified that bind their corresponding mRNA in the 3' untranslated region and that transfer (de)stabilization induced by the respective receptor agonist (Malter et al. 1989; Bohjanen et al. 1991; Brewer 1991; Vakalopoulou et al. 1991; Port et al. 1992). These mRNA binding proteins may bind to distinct mRNA consensus sequences, of which the AUUUA motif has attracted the most attention so far (Malter et al. 1989; Brewer 1991). It was demonstrated that the AT₁ receptor mRNA also binds to RNA-binding proteins and is destabilized in the polyribosomes of vascular smooth muscle cells after stimulation with AngII (Nickenig and Murphy 1996).

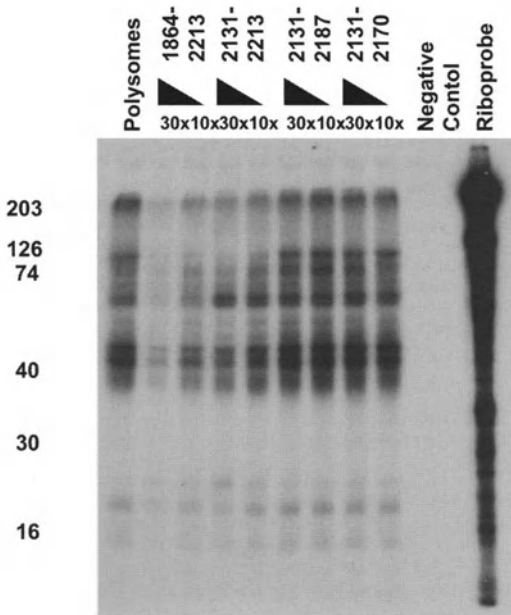
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Mechanisms of Posttranscriptional AT₁ Receptor mRNA Regulation

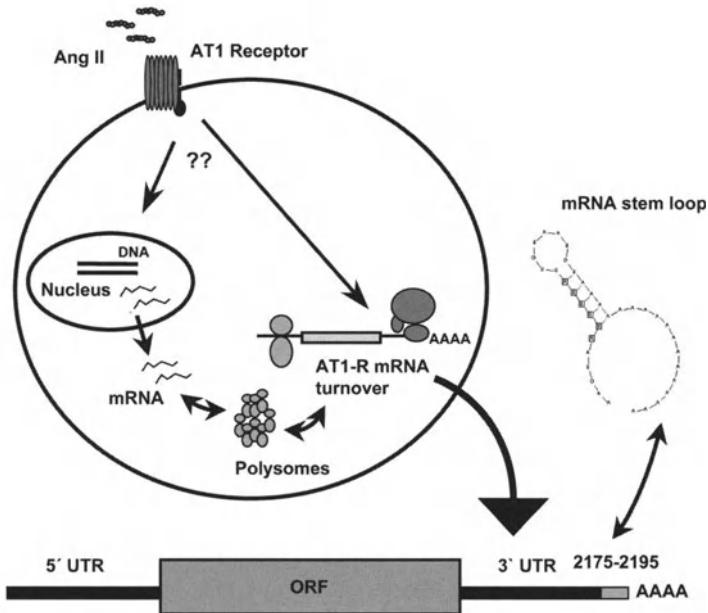
It was recently shown that a family of proteins residing in the polysomal compartment bind to the 3' untranslated region of the AT₁ receptor mRNA (Fig. 2). Detailed analysis revealed that a region between bases 2175–2195 within the AT₁ receptor mRNA, just adjacent to the poly-A-tail, is responsible for the protein-mRNA interaction. Transfection experiments in vascular smooth muscle cells and in vitro decay assays within the polysomal compartment derived from vas-

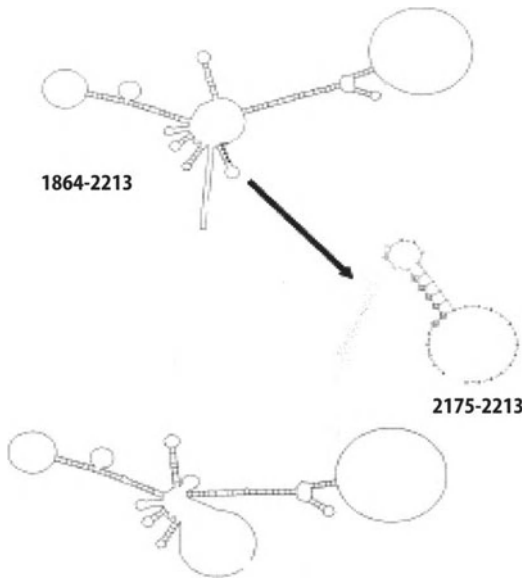
Fig. 2A, B Posttranscriptional regulation of AT₁ receptor expression. **A** UV crosslinking assay of polysomal proteins to the AT₁ receptor mRNA. Representative autoradiogram of the AT₁ receptor mRNA base 1864–2213 crosslinked to polysomal proteins isolated from VSMC. Bands indicate interactions between the labeled mRNA and polysomal proteins. As indicated, specific competition between portions of the AT₁ 3' untranslated region (UTR) and the full-length UTR can be observed. A non-relevant mRNA does not compete. **B** Scheme of posttranscriptional AT₁ receptor expression. Agonists such as AngII (*Ang II*) activate various signaling pathways which involve, for example, reactive oxygen species, nitric oxide, MAP kinases and PI-3-kinase. These events activate/induce proteins residing within the polysomal compartment which bind the AT₁ receptor mRNA in its very 3'UTR at bases 2175–2195. This cognate AT₁ receptor mRNA region forms a stem loop characteristic of mRNA sequences that interact with RNA-binding proteins. This protein-AT₁ receptor mRNA interaction increases or decreases AT₁ receptor mRNA decay, leading ultimately to either up- or downregulation of AT₁ receptor expression. *ORF*, open reading frame; *UTR*, untranslated region

A



B





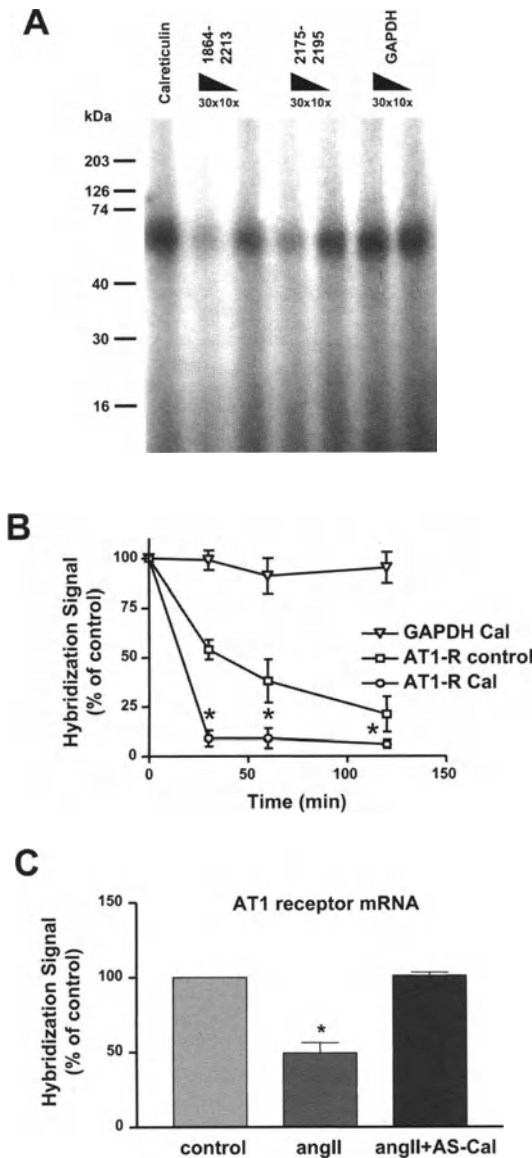
1864-2213 minus 2175-2195

Fig. 3 Computer modeling of AT₁ receptor mRNA. The secondary structures of the wild-type AT₁ receptor mRNA, the AT₁ receptor transcript bases 2175–2195, and an AT₁ receptor mRNA mutant lacking the motif bases 2175–2195 were imaged with the RNAdraw software under standard conditions. The 1864–2213 and the 2175–2213 fragments correspond to the wild-type AT₁ receptor mRNA displaying the stem loop structure. The fragment 1864–2213 minus 2175–2195 shows a mutant in which the stem loop formation is omitted

cular smooth muscle cells (VSMC) demonstrated that protein binding to the bases 2175–2195 of the AT₁ receptor mRNA mediates AT₁ receptor regulation (Fig. 2) (Nickenig et al. 2001). This cognate sequence contains an AUUUUA hexamer which shows intriguing similarities to the β_2 -adrenergic receptor mRNA (Tholanikunnel and Malbon 1997), although the flanking region of this mRNA binding region differs slightly between the genes. The bases 2175–2195 of the AT₁ receptor mRNA are almost completely composed of A and U nucleotides (except for three Gs), a feature that has been shown for most mRNA binding sequences residing in the 3' untranslated region.

Binding of mRNA binding proteins to their corresponding mRNA is profoundly influenced by secondary and tertiary structures of the mRNA. Hairpins

Fig. 4A–C The AT₁ receptor mRNA binding protein calreticulin. **A** Identification of the AT₁ receptor mRNA-binding protein calreticulin. Phosphorylated calreticulin was incubated and crosslinked with the AT₁ receptor riboprobe bases 1864–2213 in the presence of the AT₁ receptor mRNA competitors bases 1864–2213, 2175–2195, and the unspecific mRNA competitor GAPDH (30× and 10× excess over the riboprobe). The binding pattern reveals that calreticulin binds the AT₁ receptor mRNA at bases 2175–



2195 and not the GAPDH mRNA. **B** Calreticulin destabilizes the AT₁ receptor mRNA. Either recombinant phosphorylated calreticulin (*AT1-R Cal*) or the kinase reaction without calreticulin (*AT1-R control*) were added to an in vitro decay assay including the in vitro transcribed AT₁ receptor mRNA. Degradation of AT₁ receptor mRNA was assessed by real-time PCR. As internal control, a GAPDH mRNA was included in the reaction and concomitantly quantified (*GAPDH-Cal*). **C** Cells were transfected with either an insert-less pcDNA3 vector (*control* and *AngII*) or an antisense calreticulin construct (*AS-Cal*). The effect of antisense calreticulin and sense calreticulin construct transfection was monitored by Western blot. Twenty-four hours later, VSMC were incubated with vehicle (*control*) or 1 $\mu\text{mol/l}$ AngII (*angII*) for 4 h. AT₁ receptor mRNA and GAPDH mRNA (not shown) were quantified by real-time PCR. Calreticulin antisense transfection abolishes the destabilizing effect of AngII on AT₁ receptor mRNA

or stem loops formed by the RNA region of interest may interact with neighboring proteins. In the case of the AT₁ receptor, computer modeling showed that the identified AT₁ receptor mRNA binding motif (bases 2175–2195) forms such a stem loop (Fig. 2). That holds true for the entire AT₁ receptor mRNA and also for the isolated 20-base transcript. Deletion of this motif abolishes the stem loop which is in concert with the finding that such a mutated mRNA binds no longer to polysomal proteins, suggesting the importance of secondary structure for protein–mRNA interaction (Fig. 3).

Recently, the first AT₁ receptor mRNA binding protein was identified (Nickenig et al. 2002). Calreticulin, which is known to be involved in intracellular calcium homeostasis and acts as chaperone-like molecule and as receptor for nuclear export, binds, if phosphorylated, to the cognate sequence bases 2175–2195 of the AT₁ receptor mRNA and leads to destabilization of the AT₁ receptor mRNA (Fig. 4). Stimulation with AngII, which causes destabilization of AT₁ receptor mRNA, leads to phosphorylation and thereby to activation of the mRNA binding properties of calreticulin (Nickenig et al. 2002). Phosphorylation causes either a conformational change of calreticulin or activates a preformed binding site of calreticulin which enables the interaction with the mRNA. Furthermore, calreticulin may also bind other protein factors before or while interacting with the target mRNA, it may itself activate RNases which realize the actual AT₁ receptor mRNA decay, or it may induce a change in the tertiary structure of the mRNA leading to a more or less pronounced interference with nucleases. This mechanism of regulation could represent a general mechanism involved in mRNA processing and could be applicable to other genes.

6 Signal Transduction of AT₁ Receptor Regulation

No uniform signal transduction pathway has been defined that inevitably results in modulation of AT₁ receptor expression. In vascular smooth muscle cells, cAMP participates in isoprenaline and possibly in AngII-induced AT₁ receptor downregulation (Lassègue et al. 1995; Wang et al. 1997). Furthermore, superoxide radicals and hydrogen peroxide are involved in AngII-induced AT₁ receptor regulation. The p38 mitogen-activated protein (MAP) kinase mediates free radical-induced AT₁ receptor regulation, whereas p42/44 MAP kinase is presumably involved in insulin-driven AT₁ receptor overexpression (Nickenig et al. 1998a, 2000a). In VSMC, superoxide as well as hydrogen peroxide downregulate AT₁ receptor mRNA expression mediated through posttranscriptional mechanisms (Nickenig et al. 2000a). Again, transcriptional regulation seems to be not of importance. Moreover, nitric oxide mediates estrogen-dependent AT₁ receptor downregulation, whereas phosphoinositide (PI)-3 kinase has been implicated in progesterone-caused AT₁ receptor upregulation (Table 1) (Nickenig et al. 2000b). Thus, various signal transduction pathways have been implicated; however, the detailed cascade between a cell surface impulse and the ultimate modulation of gene expression is only partially understood. Particularly, the steps

immediately upstream of the described AT₁ receptor mRNA binding proteins are not known.

7

Pathophysiological Relevance of Dysregulated AT₁ Receptor Expression

Dysregulated expression of AT₁ receptors may profoundly participate in the development of vascular damage (Nickenig and Harrison 2002). Hypercholesterolemia and in particular elevated LDL cholesterol plasma concentrations play a fundamental role in the pathogenesis of atherosclerosis. Despite a large body of epidemiological evidence, the molecular events leading from hypercholesterolemia to hypertension and atherosclerosis are only partially understood. In recent studies, it has been shown that exposure of vascular smooth muscle cells to LDL markedly augments AT₁ receptor mRNA and protein expression (Nickenig et al. 1997a), and in rabbits with diet-induced atherosclerosis or heritable hyperlipidemia, aortic AT₁ receptor expression is increased twofold (Nickenig et al. 1997b; Warnholtz et al. 1999). Vascular superoxide production is also increased in hypercholesterolemia, which is associated with profound alteration of endothelium-dependent vasodilation. These abnormalities are normalized by blockade of the AT₁ receptor, despite the fact that blood pressure and lipoprotein levels are not changed by this treatment (Warnholtz et al. 1999). Most importantly, development of atherosclerotic lesions can be inhibited by AT₁ receptor antagonism (Warnholtz et al. 1999; Strawn et al. 2000). Subsequent work has provided evidence that hypercholesterolemia increases AT₁ receptor expression in humans (Nickenig et al. 1999). In hypercholesterolemic subjects, AngII infusion produced more than twice the increase in blood pressure as observed in normocholesterolemic subjects. In keeping with this finding, the expression of AT₁ receptors was increased two- to threefold in hypercholesterolemic subjects. Moreover, hypercholesterolemia-associated endothelial dysfunction is improved by AT₁ receptor antagonist treatment in humans (Wassmann et al. 2002a). These findings strongly support the concept that hypercholesterolemia increases AT₁ receptor expression and illustrate how this phenomenon may contribute to the development of atherosclerosis.

On the other hand, hypercholesterolemic patients are frequently treated with 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase inhibitors (statins). Treatment with statins for 4 weeks normalized the pressor response to AngII infusion and completely normalized AT₁ receptor expression (Nickenig et al. 1999). Further experiments in normocholesterolemic spontaneously hypertensive rats and cultured VSMC demonstrated that statins directly downregulate AT₁ receptor expression independent of cholesterol lowering (Wassmann et al. 2001a,b, 2002b). Thus, it may be speculated that the beneficial effects of statins relate not only to cholesterol lowering but also to so-called pleiotropic effects such as AT₁ receptor downregulation.

Many epidemiological studies indicate that premenopausal women have a low incidence of vascular disease but that the risk of cardiovascular events and

the incidence of hypertension increases rapidly after the menopause (Colditz et al. 1987). Observational studies have shown that estrogen replacement therapy may exert beneficial effects on cardiovascular morbidity and mortality, suggesting an important role of estrogens in the pathogenesis of vascular diseases (Mendelsohn and Karas 1999). Estrogen has been shown to have beneficial effects on vascular cells (Mendelsohn and Karas 1999). Recently, it was demonstrated that in ovariectomized rats, AngII-induced vasoconstriction was significantly increased and AT₁ receptor density and mRNA levels were upregulated twofold. Estrogen replacement therapy normalized AT₁ receptor expression in these animals (Nickenig et al. 1998c). Overexpression of AT₁ receptors induced by estrogen deficiency was associated with endothelial dysfunction and increased vascular production of superoxide, and these abnormalities were normalized by either estrogen replacement or AT₁ receptor blockade (Wassmann et al. 2001c). Parallel studies in cultured vascular smooth muscle cells revealed that

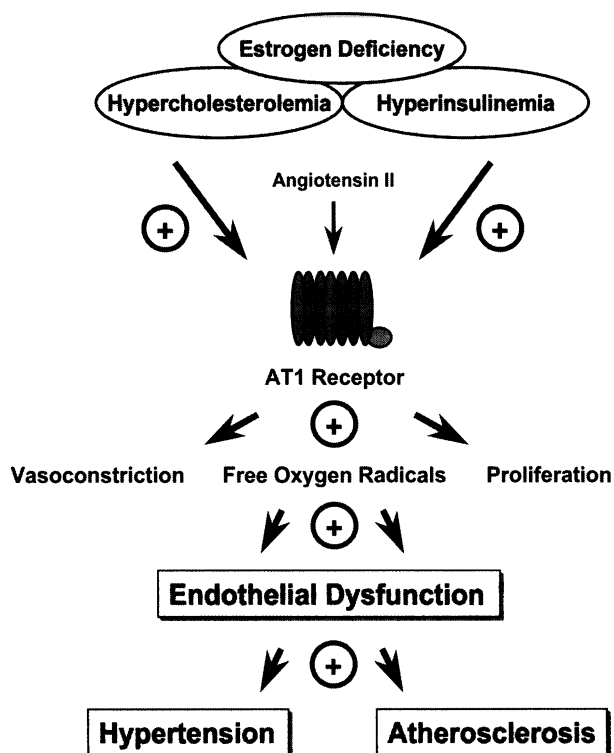


Fig. 5 Interaction of risk factors with AT₁ receptor expression and implications for the pathogenesis of atherosclerosis. Risk factors such as hypercholesterolemia, estrogen deficiency, or hyperinsulinemia may increase AT₁ receptor expression. Increased AT₁ receptor expression results in increased oxidative stress, accelerated growth of vascular smooth muscle cells, and enhanced vasoconstriction. Presumably, AT₁ receptor overexpression is accompanied by upregulation of vascular ACE and potentially other local components of the renin-angiotensin system. These events contribute among other conditions to elevated blood pressure, endothelial dysfunction, and progression of the atherosclerotic process

estradiol causes a downregulation of AT₁ receptor gene expression (Nickenig et al. 2000b). Estrogen-induced downregulation of AT₁ receptor expression could help to explain the association between estrogen-deficiency, hypertension, and atherosclerosis observed in many clinical studies.

Finally, hyperinsulinemia, which is also known to be a risk factor for atherosclerotic diseases, was also found to be associated with profound AT₁ receptor overexpression in VSMC (Nickenig et al. 1998a).

AT₁ receptor regulation very likely represents, among others, a molecular switch connecting traditional risk factors such as hypercholesterolemia, estrogen deficiency, and hyperinsulinemia with hypertension and atherosclerosis (Fig. 5). From this point of view, AT₁ receptor overexpression is one potential molecular mechanism that links a variety of exogenous risk factors to cellular events in vascular disease. Presently, it is not certain if increased activity of other local components of the renin-angiotensin system such as ACE and AngII is concomitantly required, or whether AT₁ receptor overexpression itself is sufficient to propagate vascular dysfunction and lesion formation.

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Angiotensin AT₁ Receptor Signal Transduction

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Abstract The octapeptide hormone, angiotensin II (Ang II) binds to type 1 angiotensin (AT₁) receptors in target tissues and through a number of signal transduction pathways, elicits its effects on blood pressure control and fluid and electrolyte homeostasis. AT₁ receptor antagonists are widely used to treat hypertension and associated cardiovascular and renal disease. In this review, we focus on the signal transduction mechanisms of AT₁ receptors. The list of known effectors through which the AT₁ receptor can signal has dramatically increased over the past 10 years and points to the complexity of receptor signaling to this effector. This complexity, however, may not be surprising given the various actions of Ang II at the cellular level.

Keywords Renin-angiotensin system · Angiotensin AT₁ receptor · Phospholipase C · Adenylate cyclase · G proteins · G protein coupling · Protein kinase · Tyrosine kinase · Mitogen-activated protein kinase (MAPK) · Small G proteins

1 Introduction

Angiotensin II (Ang II) mediates the biological effects of the renin angiotensin system by signaling through its two receptor subtypes (AT_1 and AT_2) in the cell membrane of target tissues. These two receptors play an important role in blood pressure control and fluid and electrolyte homeostasis through modulating the actions of Ang II on vasoconstriction (Griendling et al. 1997), aldosterone secretion (Vallotton 1987), thirst (Fitzsimons 1998), and vascular smooth muscle cell (VSMC) growth (Thomas et al. 1996; Marrero et al. 1997; Xi et al. 1999). Hypotension, hypertension, hyponatremia, and hemorrhage all alter the activity of the renin-angiotensin system. In this review, we focus on the various mechanisms by which AT_1 receptors signal.

2 G Protein Coupling

2.1 Multiple Signaling Pathways

2.1.1 Phospholipase C

The AT_1 receptor belongs to the family of calcium mobilizing G protein-coupled receptors (GPCRs). Upon binding Ang II, the AT_1 receptor stimulates phospho-

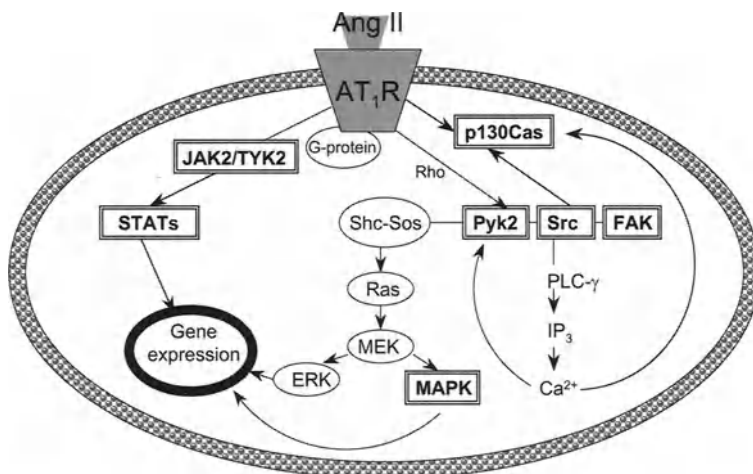


Fig. 1 Multiple AT_1 receptor signaling pathways. Binding of Ang II to the G protein-coupled AT_1 receptor activates PLC, which either leads to the generation of IP₃ with subsequent Ca²⁺ mobilization or generation of diacylglycerol (DAG) with subsequent protein kinase C (PKC) activation. Activation of the AT_1 receptor also leads to activation of the phospholipase A (PLA₂) pathway with subsequent release of arachidonic acid and activation of tyrosine kinase pathways

lipase C (PLC)- β ; hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC then yields inositol 1,4,5-trisphosphate (IP₃). Subsequently, IP₃ binds to its receptor on the sarcoplasmic reticulum, which induces the release of intracellular calcium (Peach and Dostal 1990; Israel et al. 1995) (Fig. 1).

2.1.2

Adenylate Cyclase

The AT₁ receptor also inhibits adenylate cyclase (Catt et al. 1988; Douglas et al. 1990). AT₁ receptors can couple to more than one signal transduction pathway. When rat (r) AT_{1a} receptors were stably transfected into Chinese hamster ovary cells, they not only coupled to the PLC pathway, but in addition, they coupled to inhibition of forskolin-evoked cyclic adenosine monophosphate (cAMP) accumulation (Ohnishi et al. 1992; Teutsch et al. 1992).

2.2

G Protein Types

The AT₁ receptor interacts with the pertussis-insensitive Gq₁₁ class of G proteins (Kai et al. 1996) as well as the Gq_{12/13} family (Macrez-Lepretre et al. 1997). By turning on the α -subunit of the G protein, Ang II-activation of AT₁ receptors stimulates PLC- β ₁ (Schelling et al. 1997). Studies in rat portal vein myocytes using antisense oligonucleotides directed against mRNAs encoding the G protein subunits α , β , and γ suggest that rAT_{1a} receptors activate the PLC pathway by coupling to the G protein heterotrimer, G α_{13} $\beta_1\gamma_3$ (Macrez-Lepretre et al. 1997). However, the rAT_{1a} receptor couples to different G proteins in rat VSMCs. Electroporated antibodies directed against various G protein subunits indicate that the initial receptor-PLC coupling is mediated by G $\alpha_{q11}\beta\gamma$ and G $\alpha_{12}\beta\gamma$ but not by G $\alpha_{13}\beta\gamma$ (Ushio-Fukai et al. 1998). Furthermore, these authors showed that the rAT_{1a} receptor sequentially couples to PLC- β ₁ followed by coupling to PLC- γ . Thus, these studies indicate that AT₁ receptors can couple to different G protein heterotrimers depending upon the cell type and growth stage and that Ang II signaling is temporally controlled.

The binding of guanosine triphosphate (GTP) γ S is influenced by synthetic peptides that represent intracellular cytosolic regions of the AT₁ receptor. These representative synthetic peptides could also activate purified Gi₁, Gi₂, and Go proteins (Shirai et al. 1995). Furthermore, site-directed mutagenesis of the human (h) AT₁ receptor showed that a mutant deficient in coupling to Gi could still retain its ability to bind Gq (Shibata et al. 1996). Taken together, these data suggest the AT₁ receptor can couple to Gi, Go, and Gq.

Most GPCRs possess a number of receptor subtypes, which couple to distinct signaling pathways (Carman and Benovic 1998; Selbie and Hill 1998; Wess 1998). In contrast, many studies suggest that only two AT receptor classes (AT₁ and AT₂) exist. Thus, the finding that AT₁ receptors can signal in different ways

depending on the coupling G protein explains how Ang II can activate multiple signal transduction pathways.

2.3

Determinants of G Protein Coupling

Intracellular loop (IC) 3 of the AT₁ receptor plays a key role in G protein coupling. Site-directed mutagenesis studies demonstrate that residues 219–225 in IC3 are essential for Gq coupling (Wang et al. 1995). Within this loop, Leu²²² in the KALKK amino acid sequence is not only essential for G protein interaction, it also is crucial for signal transduction (Hunyady et al. 1996; Laporte et al. 1998). A leucine in this position (or sometimes another apolar residue) is commonly found in Gq-coupled receptors (Hunyady et al. 1996). Structure–function studies and homology comparisons of AT₁ receptors across species predict that an amphipathic α -helix is formed within this loop. On one side, all the residues are basic while the opposite side contains many uncharged residues. The IC3 of the M3 muscarinic GPCR is also predicted to form an amphipathic α -helix. Evidence that orientation of basic residues within the α -helix is critical for GTPase stimulation and subsequent activation of G proteins has come from mutagenesis structure–function studies and from studies of peptide sequences that correspond to IC3 in GPCRs and which stimulate GTPase activity (Okamoto et al. 1990; Okamoto and Nishimoto 1992; Oppi et al. 1992). This IC3 region is also critical for G protein recognition and coupling to Gi in the muscarinic M3 receptor (Blum et al. 1994; Wess 1998). AT₁ receptor activation of Gq/Gq₁₁-dependent phosphoinositide hydrolysis is controlled by residues in the N-terminus region of IC3 and residues (Ile²³⁸ and Phe²³⁹) in the C-terminal region of IC3 adjacent to the sixth transmembrane domain (TMVI) (Zhang et al. 2000).

IC2 also plays a crucial role in G protein coupling. The highly conserved DRYM amino acid sequence in IC2 is essential for G protein coupling in many GPCRs (Wess 1998) and is also shown to be critical for Gq and Gi coupling in the hAT₁ receptor (Shibata et al. 1996). Furthermore, evidence for the importance of IC2 arises from mapping studies with synthetic peptides. Peptides representing receptor sequences corresponding to the N-terminal region of the IC2 (125–137) inhibited receptor G protein coupling in the rAT_{1a} receptor (Shirai et al. 1995; Kai et al. 1996). Residues in the external third region of TMVI have also been shown to play a key role in signaling without interfering with ligand binding and include residues Val²⁵⁴, His²⁵⁶, and Phe²⁵⁹ in the rAT_{1a} receptor (Han et al. 1998).

G protein coupling in the AT₁ receptor is also influenced by residues in the C-terminal tail. GTP binding to Gi was either stimulated or inhibited depending upon the region of the carboxy terminal cytoplasmic tail that the peptide represented, suggesting Gi and the rAT_{1a} receptor directly interact within this region (Shirai et al. 1995; Kai et al. 1996). Receptor–G protein specificity is apparently controlled by specific sequences in the carboxy tail. Mutagenesis analysis showed that a truncated hAT₁ receptor, which lacked the carboxyl terminal 50

residues coupled to G_q but was unable to couple to G_i (Shibata et al. 1996). There are species differences in the residues that govern the specific determinants of AT₁ receptor G protein coupling. Unlike in the hAT₁ receptor, Tyr³¹², Phe³¹³, and Leu³²⁴ in the rAT_{1a} cytoplasmic tail are essential for coupling to G_q (Sano et al. 1997). In conclusion, multiple contacts within IC2 and IC3, the cytoplasmic tail, and TMVI determine AT₁ receptor binding and activation of G proteins. Furthermore, homology comparisons among GPCRs indicate that residues in similar positions are important in G protein activation in general (Ren et al. 1993).

2.4 Receptor Activation Models

Thus far, there are no X-ray crystallographic structures of AT₁ receptors or any other peptide hormone GPCR. Thus, receptor activation models are commonly based on structure–function studies of mutant receptors. Many of these studies are based on loss-of-function analysis, which is problematic since loss of function could reflect nonspecific effects due to local losses in receptor structural integrity; however, gain-of-function mutagenesis studies and other approaches, such as biochemical mapping, can help in model development.

Mutagenesis studies suggest that Ang II signaling is initiated when Tyr⁴ within the Ang II peptide interacts with Asn¹¹¹ within TMIII of the receptor. This interaction induces a conformational change, which disrupts the interaction between Asn¹¹¹ within TMIII and Tyr²⁹² and Asn²⁹⁵ within TMVII (Marie et al. 1994; Balmforth et al. 1997). This model of receptor activation, implicating TMVII in the initiation site of agonist activation, is similar to models proposed for many GPCRs (Baldwin 1993; Roth et al. 1997). Numerous serine, threonine, and tyrosine residues within the intracellular carboxy tail are phosphorylated in an agonist-dependent manner, and evidence suggests they play a key role in receptor activation, internalization, and desensitization (see Thomas 1999 for an excellent review of the topic).

Commonly, the highly conserved residues within GPCRs play similar roles in G protein activation such as Asp⁷⁴ in TMII (Bihoreau et al. 1993), the DRY amino acid sequence in TMIII (Ohyama et al. 1995) and Tyr²¹⁵ in TMV (Hunyady et al. 1995). Residues in IC3 (Zhang et al. 2000), TMVI (Han et al. 1998), and TMVII (Hunyady et al. 1998) facilitate the rearrangement of helices that lead to receptor activation, while having no effect on ligand binding. While many similarities exist among GPCRs, not all highly conserved sequences regulate the same activities. In the intracellular domain of GPCRs, the NPX_nY sequence is highly conserved. Receptor internalization and resensitization in the β_2 -adrenergic receptor is prevented if the tyrosine in this sequence is substituted with another residue (Barak et al. 1994). In the AT₁ receptor, the corresponding residue in the NPLFY sequence is critical for PLC activation but is not necessary for agonist binding or receptor internalization (Laporte et al. 1996). The residues that determine G protein coupling are not identical to those residues that

govern AT₁ receptor internalization. Ang II-induced receptor endocytosis has been shown to occur in non-signaling mutants of TMII, IC3, and the cytoplasmic tail (Hunyady et al. 1994a,b,c; Thomas 1999).

3

Protein Kinases

Less well understood pathways of AT₁ receptor signaling are through phosphorylation of receptor and non-receptor tyrosine kinases and activation of mitogen-activated protein kinases (MAPK). The fact that these signaling cascades are commonly associated with signaling by growth factors and cytokines, supports the accumulating data suggesting that Ang II plays a pivotal role in mediating long-term growth regulation and inflammatory processes under both physiological and pathophysiological conditions (Hunyady et al. 1994a,b,c; Thomas 1999). In addition to its important role in the control of systemic blood pressure, water, and salt homeostasis, Ang II is a potent growth factor in a number of tissues and cell types (Schnee and Hsueh 2000; Enseleit et al. 2001; Klahr 2001; De Gasparo 2002). To date, the best-defined Ang II-induced tyrosine phosphorylation and MAPK cascade is described in VSMCs. Ang II induces a multitude of actions in these cells that are typical of growth factors and cytokines, including adhesion, migration, synthesis of extracellular matrix, and regulation of long-term cell growth. Abnormal regulation of these pathways has been associated with cardiovascular disease and hypertension (Border and Noble 2001; Deblois et al. 2001; Schiffrin 2002; Unger 2002).

3.1

Tyrosine Kinases

The ability of Ang II to induce tyrosine phosphorylation is still a puzzle, since the AT₁ receptor is a member of the GPCR family and not a member of the tyrosine kinase family of receptors. Apparently, AT₁ receptors do not have intrinsic tyrosine kinase activity. Instead, accumulating experimental data suggest that its tyrosine kinase activity is achieved through activation of both receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases include platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Fujiyama et al. 2001; Saito and Berk 2001). Non-receptor tyrosine kinases include PLC- γ , Src family kinases, focal adhesion kinase (FAK), proline-rich tyrosine kinase (Pyk2), Janus family kinases (JAK), tyrosine kinase (TYK), signal transducers and activators of transcription (STATs), paxillin and p130Cas (Marrero et al. 1996; Berk and Corson 1997; Kim and Iwao 2000; Touyz and Berry 2002).

3.1.1

Receptor Tyrosine Kinases

Another mechanism by which Ang II exerts cell regulatory properties is by activating receptor tyrosine kinases. Accumulating evidence suggests that even though the AT₁ receptor does not have a direct physical association with receptor tyrosine kinases, Ang II-activated AT₁ receptors regulate receptor tyrosine kinase activity by transactivation. Signaling by AT₁ receptor transactivation has been shown for a number of receptor tyrosine kinases, including both Ca²⁺-dependent (e.g., EGF) and independent (e.g., PDGF) receptors (Kalmes et al. 2001; Saito and Berk 2001).

The mechanisms of Ang II-induced receptor transactivation are still poorly understood. Studies suggest that Ang II-activated AT₁ receptors cause cleavage of pro-heparin-bound EGF via metalloproteinases (Haendeler and Berk 2000; Kalmes et al. 2001). The free, heparin-bound EGF then binds to its receptor and becomes autophosphorylated. This process is associated with activation of Pyk2 and Src as well as downstream activation of extracellular signal-related kinase (ERK)1/2 (Eguchi and Inagami 2000; Eguchi et al. 2001). The process of Ang II-induced transactivation of EGF has been reported to be important in VSMC hyperplasia and contraction (Eguchi et al. 2001).

3.1.2

Non-receptor Tyrosine Kinases

- a. PLC- γ . Ang II activation of PLC- γ depends upon phosphorylation of the AT₁ receptor (Fig. 2). Ang II binding to the AT₁ receptor causes phosphorylation of Tyr³¹⁹ in the YIPP motif within the C-terminal intracellular domain of the receptor. The C-terminal region of the phosphorylated receptor is then able to bind to two Src homology 2 domains in PLC- γ 1 (Ali et al. 1997; Venema et al. 1998). Interestingly, evidence also suggests that the same YIPP motif is required for AT₁ receptor binding to the tyrosine phosphatase SHP-2 and to the JAK2 tyrosine kinase; however, the functional significance of these observations remains unknown. Activation of PLC- γ 1 leads to IP₃ production and intracellular Ca²⁺ release (Harp et al. 1997; De Gasparo et al. 2000). The relative contribution of the various PLC isoforms including PLC- γ 1 in AT₁ receptor signaling is not well understood. Species differences in the expression of PLC isoforms exists. In the rat, studies suggest that AT₁ receptors couple to PLC- β , then subsequently to PLC- γ (Marrero et al. 1994; Ushio-Fukai et al. 1998), leading to increased intracellular IP₃ and subsequent Ca²⁺ mobilization as well as tyrosine phosphorylation. Activation of non-receptor tyrosine kinases mediates a number of cellular functions in VSMCs, including cell growth and differentiation (Marrero et al. 1994; De Gasparo et al. 2000).

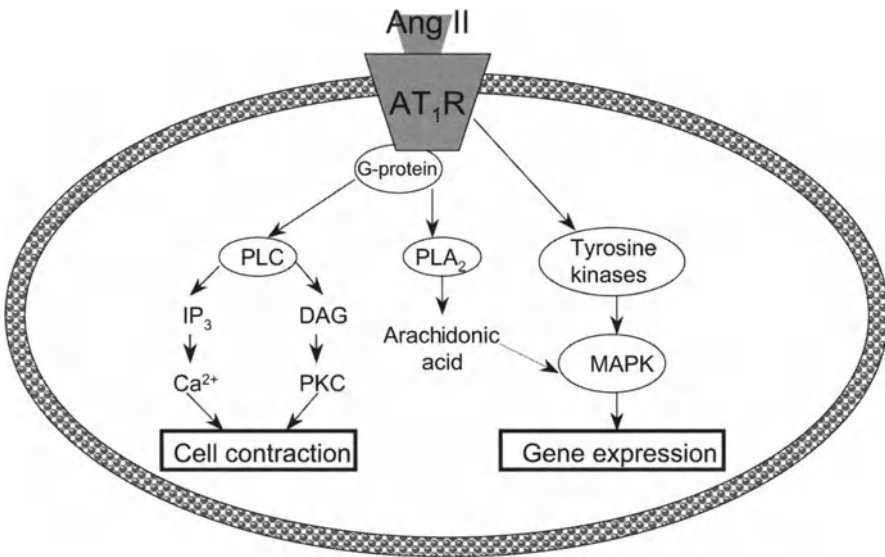


Fig. 2 AT₁ receptor activated tyrosine kinase pathways. Upon binding to the AT₁ receptor, Ang II triggers multiple tyrosine kinase signaling pathways. Ang II activates Src, which in turn regulates PLC- γ activity leading to IP₃ production and subsequent Ca²⁺ mobilization. Ang II activates the Pyk2 pathway, via Ca²⁺-dependent mechanisms, which regulate ERK-dependent signaling pathways. Presumably via Rho GTPases, Ang II activates FAK. Ang II activation of the JAK2/TYK2-STAT pathway regulates gene transcription. Ang II also activates p130Cas via Ca²⁺-dependent mechanisms

- b. Src family kinases. To date, the Src kinase family consists of 14 kinase members. Src-mediated phosphorylation of PLC- γ leads to increased intracellular IP₃ and Ca²⁺ mobilization as well as phosphorylation of the cell adhesion signaling molecule p130Cas (Fig. 2). Evidence suggests Src also plays a role in Ang II-induced activation of FAK and Pyk2 as well as downstream signaling molecules, such as ERK, paxillin, JAK/STAT, and Shc (Erpel and Courtneidge 1995; Touyz and Berry 2002). An Ang II increase in Src-mediated signaling is involved in the regulation of focal adhesion, cytoskeletal reorganization, cell migration, and cell growth (Sayeski et al. 1998; Touyz and Berry 2002), and increased Src-mediated signaling has been implicated in the pathogenesis and development of hypertension (Yamazaki et al. 1999; Touyz and Berry 2002).
- c. FAK and Pyk2. As its name implies, FAK is associated with focal adhesion points between the cell membrane and the extracellular matrix. Among its other roles, Pyk2 provides molecular scaffolding for the docking of signal transduction molecules. FAK is characterized by extracellular matrix-dependent tyrosine auto-phosphorylation, which triggers its interactions with other non-receptor tyrosine kinases, such as Src. These interactions with non-receptor tyrosine kinases lead to further phosphorylation of FAK and subsequent association with Grb2 and the guanosine diphosphate (GDP)-

GTP exchange proteins, Sos and Ras (Fig. 2). Although the direct link between FAK and the AT₁ receptor is still unclear, the potential role for RhoA or other Rho family GTPases has often been implicated. The Ang II-activated FAK cascade plays an important role in the control of cell migration, cell shape, and volume regulation, via phosphorylation of paxillin and talin (Leduc and Meloche 1995; Guan 1997), and overactivation of FAK has been linked to VSMC hypertrophy and atherosclerosis (Taylor et al. 2001; Abbi and Guan 2002). Pyk2 is related to FAK and is also known as Ca²⁺-dependent tyrosine kinase. Studies suggest that Pyk2 associates with GPCRs including the AT₁ receptor and regulates tyrosine kinase-activated cell contraction, migration, and growth (Tang et al. 2000; Rocic and Lucchesi 2001).

- d. JAK/STAT. Ang II-activation of AT₁ receptors leads to the coupling of two members of the JAK family, namely JAK2 and TYK2 and subsequent phosphorylation of several transcription factors in the STAT protein family, including STAT1 α/β , STAT2, and STAT3 (Mascareno and Siddiqui 2000) (Fig. 2). The phosphorylated STAT proteins are then translocated to the nucleus, where they initiate transcription of a number of genes involved in physiological and pathophysiological cell proliferation (Marrero et al. 1998; Meloche et al. 2000). The activation and regulation of the JAK/STAT pathway has been extensively studied over the past few years because of its key role in regulating Ang II-induced cell growth in cardiovascular disease (see Mascareno and Siddiqui 2000; Meloche et al. 2000; Booz et al. 2002 for extensive reviews).
- e. p130Cas. Previous reports suggested that Ang II-activated p130Cas tyrosine phosphorylation is Ca²⁺-, Src- and PKC-dependent; however, emerging experimental data contradict these findings (Sayeski et al. 1998; Touyz and Berry 2002). The precise role of Ang II-activated p130Cas remains unclear. Some studies suggest that this proline-rich protein is involved in the regulation of integrin-mediated cell adhesion through activation of cytoskeletal elements, including FAK, paxillin and α -actin (Takahashi et al. 1998; Ishida et al. 1999) and subsequent involvement in cell proliferation (Ishida et al. 1999; Forte et al. 2002).
- f. Paxillin. An integral component of focal adhesions, paxillin performs as an adapter protein by acting as an anchor in the formation of multiprotein complexes (Bouillier et al. 2001). Ang II-activated paxillin tyrosine phosphorylation, in concert with FAK and Src kinase, is associated with re-organization of the cytoskeletal network, such as formation of new focal adhesions and stress fibers. Some studies suggest that alterations in the pattern of paxillin expression is involved in the pathogenesis of genetic hypertension and cardiac hypertrophy (Sabri et al. 1998; Bouillier et al. 2001).

3.2

Mitogen-Activated Protein Kinases

MAPKs are a family of serine/threonine protein kinases associated with nuclear transduction of extracellular signals in a variety of cell types and under a number of stimuli, including Ang II. Ang II is known to activate three major members of the MAPK family, including ERK1/2, c-Jun N-terminal kinases (JNKs), and p38 MAPK (Touyz and Schiffrin 2000; Touyz and Schiffrin 2001) (Fig. 1). Activation of MAPK is highly complex and involved in mediating several aspects of cell growth and protein synthesis. This cascade begins with phosphorylation by MAPK kinase (MEK), which is under the control of Raf1, both of which regulate ERK1/2 activity. ERK1/2, in turn, translocates to the nucleus where it regulates gene expression by altering the activity of transcription factors through their phosphorylation. In addition to activating ERKs, MAPK is also known to directly regulate the activity of cyclo-oxygenase (COX)-2, Ca²⁺ channels, and the Na⁺/H⁺ exchanger (Robinson and Cobb 1997).

The interaction between Ang II and JNK begins with phosphorylation of JNK/stress-activated protein kinase (SAPK) via the Ca²⁺- and PKC-dependent p21-activated kinase. Once phosphorylated, JNK1 and JNK2 translocate to the nucleus and activate several transcription factors, including c-Jun, ATF-2 and Elk-1 (Schmitz et al. 1998), which subsequently leads to regulation of cell death through apoptosis (Sadoshima et al. 2002). Although studies indicate that Ang II-induced apoptosis is mainly associated with the AT₂ receptor, the AT₁ receptor-mediated JNK/SAPK signaling pathway may also play an important role in promoting cell death.

A third mechanism by which Ang II activates the MAPK signaling cascade is via interaction with p38 MAPK. Similarly to the JNK/SAPK pathway, the Ang II-mediated MAPK signaling cascade is thought to be important in the regulation of apoptosis in the process of cardiac ischemia, hypertrophy, and remodeling (Kim and Iwao 2000; Sharov et al. 2003). The precise mechanisms by which Ang II activates p38 MAPK remain unknown; however, some data suggest this pathway plays an opposing role to Ang II-induced ERK1/2 activation.

4

Small G Proteins

In addition to signaling through activation of multiple heterotrimeric G proteins, tyrosine kinases, and MAPK, Ang II has now been shown to interact with small (21-kDa) guanine nucleotide-binding proteins (also termed small G proteins). So far, five subfamilies of small G proteins have been identified. These include Rho, Ras, Rab, Ran, and adenosine diphosphate (ADP) ribosylation factors. The Rho subfamily comprises RhoA, Rac1, and Cdc42 and is the best characterized with respect to AT₁ receptor signaling (Laufs et al. 2002). RhoA is a cytoplasmic protein coupled to GDP, which becomes activated upon its association with GTP. Following activation, RhoA/GTP interacts with Rho kinase,

which has been implicated in regulating cell contraction via modulating myosin light chain phosphatase activity (Park et al. 2002; Cavarape et al. 2003) and in abnormal Ang II-induced growth regulatory mechanisms in atherosclerosis (Shimokawa 2002). In addition, studies suggest that Ang II regulates cytoskeletal organization, cell growth, and inflammation by activating Rac and consequent involvement with p21-activated kinase and JNK (Laufs et al. 2002; Park et al. 2002).

5

Conclusions

For a long time, it has puzzled many scientists how Ang II could elicit such a wide range of cellular functions. It was initially believed that Ang II-induced signaling pathways were discrete and mainly of short duration. Much evidence now indicates that cross-talk interactions among the myriad of AT₁ receptor signaling pathways mediate the various biological functions of Ang II. This complex interplay of signaling events is triggered by direct receptor activation and also by indirect mechanisms involving tyrosine phosphorylation and MAPK. These signaling pathways are tightly regulated events under normal physiological conditions, and their abnormal regulation has been associated with a number of pathophysiological conditions in almost every tissue and cell type. Much current research focuses on selectively targeting these discrete signaling pathways with the hope of developing new treatment strategies in prevention and treatment of hypertension and cardiovascular and renal disease.

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Sympathetic Interactions of AT₁ Receptors

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Abstract The present chapter deals with the interaction between the renin–angiotensin system (RAS) and the sympathetic nervous system (SNS). Numerous studies have shown that (locally produced) angiotensin II (Ang II), the main effector of the RAS, can enhance sympathetic nervous transmission. Ang II has been shown to enhance ganglionic transmission, to facilitate NA release from synaptic nerve terminals, block NA uptake, enhance NA synthesis, and enhance the postsynaptic effects of noradrenaline. Angiotensin-converting enzyme (ACE) inhibitors and AT₁ receptor blockers have been shown to attenuate the facilitating effect of Ang II on sympathetic neurotransmission. It is generally believed that Ang II exerts its effects on sympathetic neurotransmission through the activation of AT₁ receptors. Several new, non-peptidergic AT₁-selective receptor blockers are now available. The potency of these drugs with respect to the attenuation of the facilitation by Ang II of sympathetic neurotransmission has been the subject in several studies, discussed in the present chapter. In some

studies, the order of potency regarding this sympatho-inhibition by AT₁ blockers differs from the order of potency regarding inhibition of the direct pressor effect of Ang II. These findings suggest differences in affinity of the AT₁ blockers for the pre- and postsynaptic AT₁ receptor. Sympatho-inhibition is a class effect of the AT₁ receptor antagonists. In conditions in which the sympathetic nervous system plays a pathophysiological role, such as hypertension and congestive heart failure, this property may well be of therapeutic relevance.

Keywords Angiotensin II · ATRBs · Prejunctional AT₁ receptor blockade · Sympathetic nervous system · Hypertension · Heart failure

1

Interactions Between the Sympathetic Nervous System and the Renin–Angiotensin System

For several decades it has been well known that the renin–angiotensin system (RAS) and the sympathetic nervous system (SNS) can interact at various levels (van Zwieten and de Jonge 1986; Story and Ziogas 1987; Reid 1992; Saxena 1992). The recognition of the deleterious effects of the activation of both the SNS and the RAS in hypertension and heart failure has generated renewed interest in the reciprocal positive feedback loop between these two regulatory systems.

1.1

Influence of the Sympathetic Nervous System on the Renin–Angiotensin System

It is well known that, via the activation of β_1 -adrenoceptors in the juxtaglomerular apparatus, noradrenaline (NA) can provoke the release of renin from the kidney (Taher et al. 1976). Additionally, at the level of the local or tissue RAS in blood vessels, both renin and angiotensin II (Ang II) can be released through a β -adrenoceptor-mediated mechanism. In the rat isolated perfused mesenteric artery, the β -adrenoceptor agonist isoproterenol induced an enhancement of pressor responses to nerve stimulation (Fig. 1), as well as release of Ang II. These effects could be suppressed by propranolol, captopril, and the peptide AT receptor antagonist [Sar¹-Ile⁸]Ang II (Nakamaru et al. 1986), indicating a β -adrenoceptor stimulation-sensitive local formation of Ang II, which in turn enhances the neuronal NA release and/or the postsynaptic signal transduction of this neurotransmitter. This facilitatory effect of β -adrenoceptor stimulation on the activation of systemic or local RAS might play a role in the pathology of cardiovascular diseases, since in spontaneously hypertensive rats, β -adrenoceptor-mediated activation of the local vascular RAS is enhanced compared to normotensive WKY rats (Kawasaki et al. 1984). Besides animal data, there is sufficient evidence that the interaction takes place in the human situation as well, as shown in the human saphenous vein by Mölderings et al., who demonstrated that saralasin attenuated the facilitatory effect on stimulation-evoked NA release of the β_2 -adrenoceptor agonist procaterol (Mölderings et al. 1988). Furthermore, in the human forearm

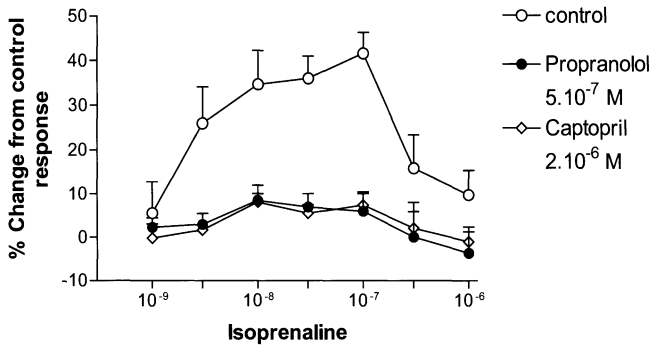


Fig. 1 Facilitation of stimulation-induced pressor responses by β -adrenoceptor stimulation in the rat perfused mesenteric artery. Both β -blockade by propranolol and ACE-inhibition by captopril inhibit the effects of isoprenaline. (From Nakamaru et al. 1986)

of hypertensive patients, isoproterenol-mediated NA overflow was significantly reduced by captopril infusion (Taddei et al. 1991). In conclusion, in a variety of experimental set-ups including human models, a facilitatory, β -adrenoceptor-dependent effect of the SNS on the systemic and the local RAS has been demonstrated which most likely contributes to the pathology of cardiovascular diseases.

1.2

Influence of the Renin–Angiotensin System on the Sympathetic Nervous System

On the other hand, Ang II exerts a marked influence on the sympathetic nervous system. This has been shown on the level of the central nervous system, the adrenal medulla, and the sympathetic ganglia. However, by far the most evidence for the RAS/SNS interaction has been gathered at the level of peripheral sympathetic nerve terminals, both, pre- and postsynaptically (Fig. 2). Functional tissue responses as well as the quantification of released catecholamines have been used to identify and characterize this important interplay and the components involved in its effectuation.

1.2.1

Central Nervous System

In a dog cross circulation study, it was shown that Ang II, infused in the carotid inflow of the cranial circulation, which was vascularly isolated but neuronally connected with its trunk, caused an increase in blood pressure (BP) in both the donor and in the recipient animal (Bickerton and Buckley 1961), pointing to an Ang II-sensitive, central activation of the sympathetic nervous system. It is now becoming clear that a functional RAS is intrinsic to the brain. Central administration of Ang II, in concentrations that are ineffective when administered peripherally, causes an increase in BP and sympathetic outflow as well as a suppression of baroreflex control (Reid 1992; Raizada et al. 1995). Catecholaminer-

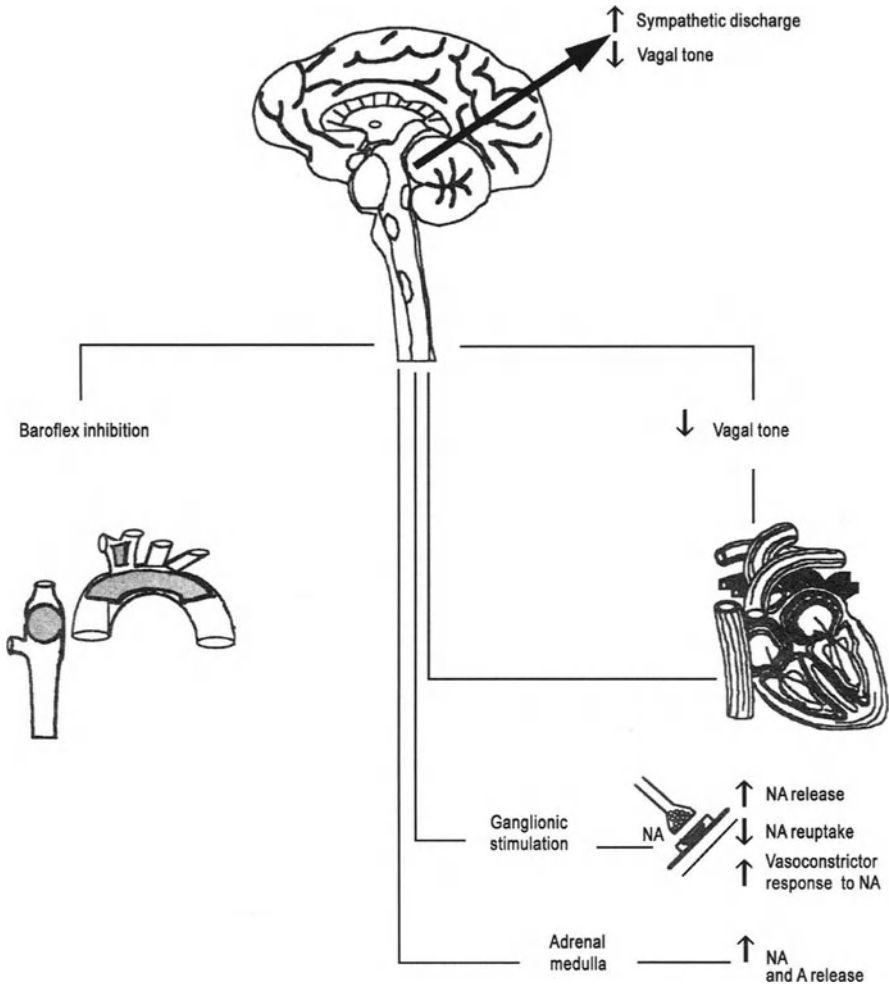


Fig. 2 Sites and mechanisms of interaction between the renin–angiotensin system and the sympathetic nervous system. Abbreviations: NA, noradrenaline; A, adrenaline. (From Grassi 2001)

gic neurons are involved in the central mechanism by which Ang II causes BP increase, since central effects of Ang II can be inhibited by the α -antagonist phentolamine (Jones 1984). Furthermore, in conscious rats, Ang II causes release of NA from the paraventricular nucleus (PVN), which is involved in BP control (Stadler et al. 1992). As with most of the other Ang II-induced effects, these central actions are mediated by the AT₁ receptor subtype. These receptors are also localized in other areas that are involved in BP control such as the nucleus tractus solitarii and the area postrema, whereas AT₁ receptor blockade can inhibit the effects of centrally applied Ang II (Raizada et al. 1995).

1.2.2

Adrenal Medulla

Angiotensin II stimulates the release of catecholamines from the adrenal glands (Peach 1971). A facilitatory effect of Ang II on stimulation-induced adrenaline release was demonstrated in anaesthetized dogs (Foucart et al. 1991). However, the doses needed are high and the physiological relevance of these observations remains uncertain. In pithed rats, adrenalectomy failed to alter the responses to Ang II (Knape 1986) and an infusion of Ang II did not alter plasma adrenaline levels in humans (Mendelsohn et al. 1980).

1.2.3

Sympathetic Ganglia

Injection of Ang II into the blood supply of the caudal cervical ganglia of dogs causes positive chronotropic and inotropic responses. The direct positive inotropic effect of Ang II needed doses that were 60 times higher (Farr and Grupp 1971). In pithed rats, this was shown to be an AT₁ receptor-mediated mechanism (Wong et al. 1990a). Binding sites for Ang II have been identified in rat sympathetic ganglia (Castren et al. 1987). Propranolol was demonstrated to inhibit the tachycardic response to Ang II, suggesting a role of the β -receptor (Finch and Leach 1969; Knape and van Zwieten 1988). Activation of post-ganglionic renal sympathetic nerves by Ang II, mediated by AT₁ receptors, was demonstrated in anaesthetized mice (Ma et al. 2001) (Fig. 3).

1.2.4

Sympathetic Nerve Terminals

In various models, the facilitating effect of Ang II on sympathetic neurotransmission at the peripheral level was demonstrated. This facilitation has been shown to be caused by presynaptic mechanisms, such as an increase of NA synthesis (Roth 1972), an increase of stimulation-induced release of NA (Starke et al. 1977), and the inhibition of its uptake (Panisset and Bourdois 1968; Raasch et al. 2001). The enhancing effect of Ang II on stimulation-induced NA release and vasoconstrictor responses could be antagonized by the peptidergic AT₁/AT₂ receptor antagonist saralasin (Endo et al. 1977; Collis and Keddie 1981) and by various selective AT₁ receptor antagonists such as losartan (Tofovic et al. 1991; Wong et al. 1992; Cox et al. 1996), irbesartan (Christophe et al. 1995), and eprosartan (Ohlstein et al. 1997), but not by the AT₂ receptor antagonists PD123177 or PD123319 (Tofovic et al. 1991; Wong et al. 1992; Brasch et al. 1993). From these experiments, it is generally concluded that Ang II exerts its effects on sympathetic neurotransmission through the activation of AT₁ receptors (Fig. 4).

In addition, an increased responsiveness of vascular smooth muscle to NA (postsynaptic facilitation) has been demonstrated (Purdy and Weber 1988).

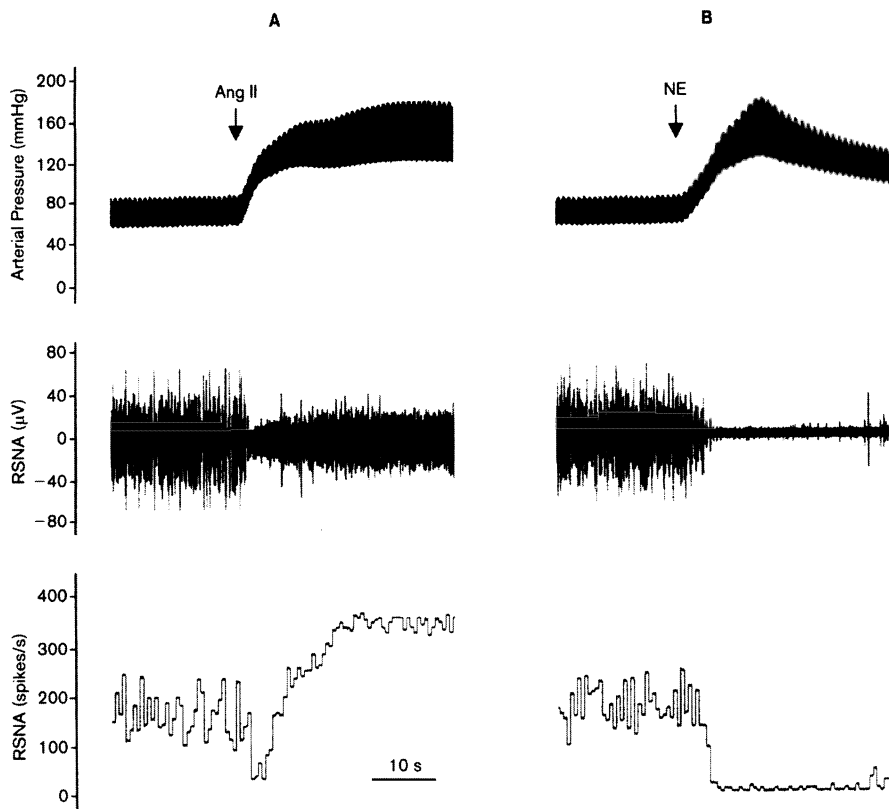


Fig. 3A, B Responses of arterial pressure and renal sympathetic nerve activity (RSNA) to a bolus injection of Ang II (4 ng/g, i.v.) in an anaesthetized mouse. **A** Responses to angiotensin II; **B** Responses to noradrenaline. After an initial inhibition of RNSA, Ang II persistently increases RSNA. An equipotent dose of noradrenaline abolished RSNA. AT₁ blockade by losartan inhibited the Ang II-induced increase in RSNA (not shown). From these data, it is concluded that the BP increase by noradrenaline causes a baroreflex-mediated inhibition of sympathetic outflow, while Ang II—through AT₁-receptors—increases RSNA. (From Ma et al. 2001)

Both the α_1 - and the α_2 -adrenoceptor have been shown to be involved (de Jonge et al. 1982; Richer et al. 1996). In some studies, however, such a postsynaptic facilitation could not be confirmed (Duckles 1981; Seidelin et al. 1991; Moreau et al. 1993a; Yokoyama et al. 1997). It is generally believed that the presynaptic facilitation is quantitatively more important than the postsynaptic enhancement.

Locally produced Ang II can enhance noradrenergic neurotransmission. This has been demonstrated in the guinea pig isolated atria and the rat caudal artery (Ziogas et al. 1984), isolated rat mesenteric arteries (Malik and Nasjletti 1976), kidneys (Boke and Malik 1983), and perfused hearts of rats, guinea pigs, and rabbits (Xiang et al. 1985). Such a local interaction was also demonstrated in the human forearm (Taddei et al. 1991). These studies indicate a significant involvement of the local RAS in the facilitation of sympathetic neurotransmission.

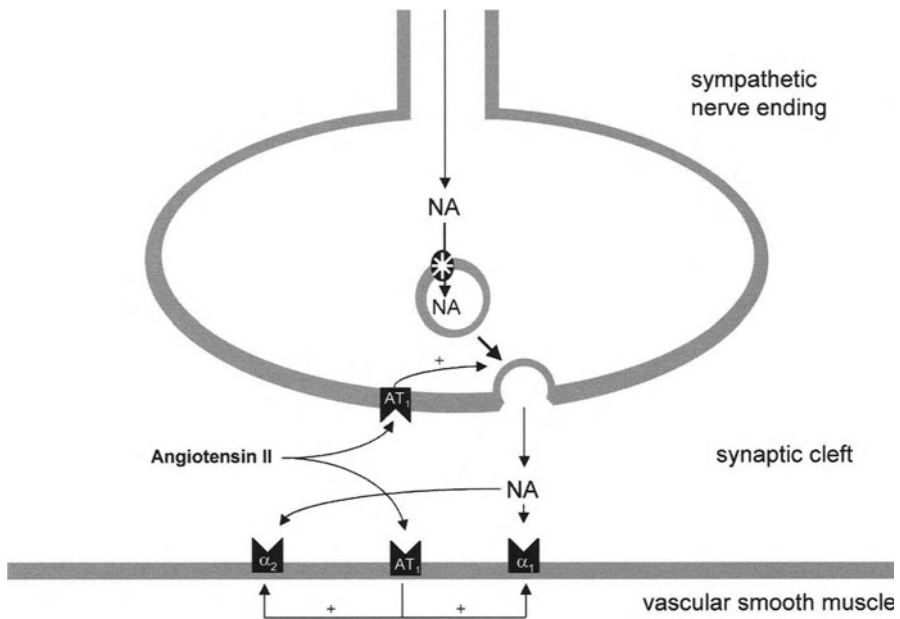


Fig. 4 Facilitatory actions of Ang II at the sympathetic nerve terminal and vascular smooth muscle cell. NA, noradrenaline

In the human forearm, Clemson et al. found that intra-arterial Ang II caused an increase of NA spillover (Clemson et al. 1994). Other authors, however, could not confirm these results (Chang et al. 1995; Goldsmith et al. 1998). In healthy volunteers, Ang II was shown to augment vasoconstriction as elicited by lower body negative pressure (LBNP) (Seidelin et al. 1991). Conversely, chronic angiotensin-converting enzyme (ACE) inhibition had already been demonstrated to diminish the decrements in forearm blood flow caused by LBNP (Morganti et al. 1989)

The mechanism by which Ang II facilitates sympathetic neurotransmission has been sparsely investigated. Ang II, via the AT₁ receptor, activates receptor-linked phospholipase C (PLC), resulting in the hydrolysis of phosphatidyl-inositol-4,5-bisphosphate (PIP₂), producing inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ releases Ca²⁺ from the sarcoplasmic reticulum (SR), but this mechanism appears to have little effect on neurotransmission (Majewski and Musgrave 1995). DAG however, activates protein kinase C (PKC), which through a number of mechanisms plays an important part in transmitter release: (1) by prolonging Ca²⁺ influx, (2) by modulating NA synthesis (through phosphorylation of tyrosine hydroxylase), and (3) by modulation of exocytosis (through phosphorylation of exocytotic proteins) (Verhage et al. 1994).

At the postsynaptic level, the PKC pathway was shown to be involved in the facilitation by Ang II of α-adrenoceptor-mediated responses (Purdy and Weber 1988; Laher et al. 1990).

2 Prejunctional and Postjunctional AT₁ Blockade

Several new, non-peptidergic AT₁-selective receptor blockers are now available. A few studies have recently been published in which the potency of these drugs with respect to the attenuation of the facilitation by Ang II of sympathetic neurotransmission was compared (Ohlstein et al. 1997; Shetty and DelGrande 2000; Balt et al. 2001a,b).

Ohlstein et al. reported that, in the pithed rat model, eprosartan (0.3 mg/kg) attenuated stimulation-induced BP responses, whereas valsartan, losartan, and irbesartan did not (Ohlstein et al. 1997). In earlier reports, higher doses of losartan and irbesartan had already been shown to be effective in blocking the facilitating effect of Ang II on noradrenergic neurotransmission in this model (Wong et al. 1992; Christophe et al. 1995). In the isolated rat mesenteric arteries, Balt et al. found the ranking order of the inhibition the facilitating effects of Ang II on stimulation-induced contractile responses to be telmisartan>irbesartan>losar-

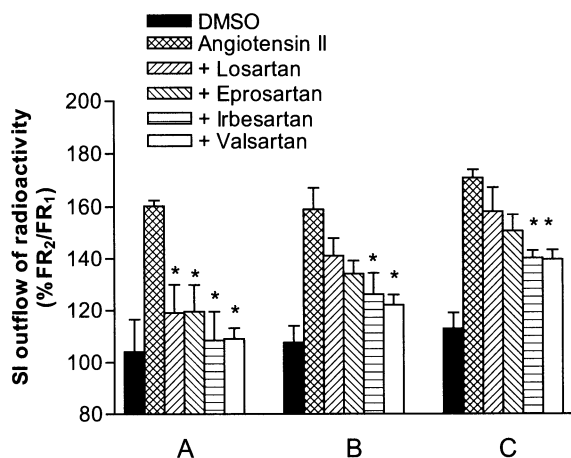
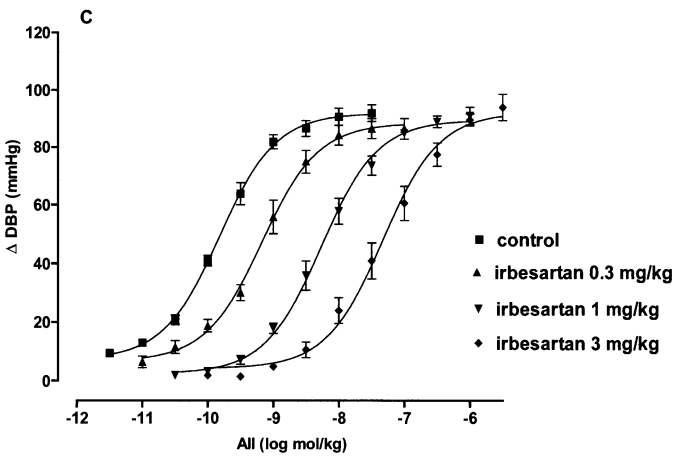
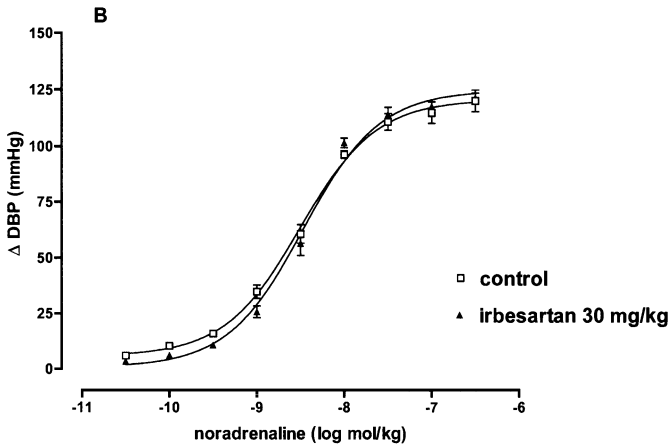
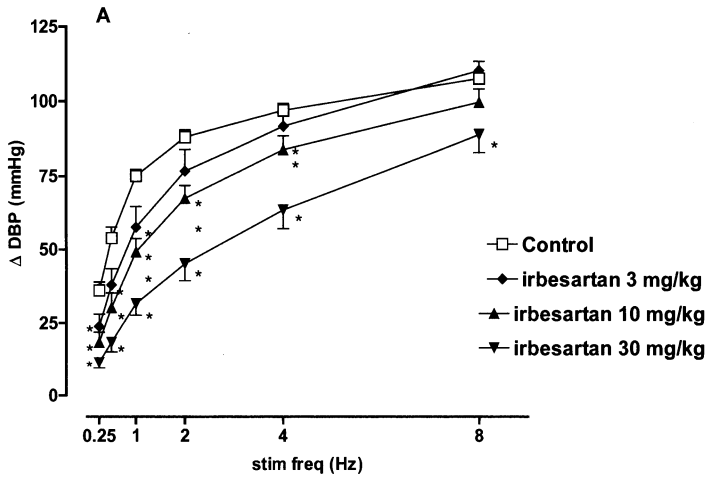


Fig. 5 Effects of losartan, eprosartan, irbesartan, and valsartan (A, 10^{-6} M; B, 10^{-7} M; C, 10^{-8} M) on Ang II (10^{-7} M)-evoked augmentation-induced efflux of radioactive [3H]NA from isolated rat atria. * $p < 0.05$ compared with control Ang II response. (From Shetty and DelGrande 2000)

Fig. 6A–C Effect of AT₁ receptor blockade by irbesartan on: **A** the frequency-response curve induced by electrical stimulation of the spinal cord (T5-L4) * $p < 0.05$ compared to control; **B** the dose-response curve induced by intravenously administered noradrenaline; **C** the dose-response curve induced by intravenously administered Ang II in the pithed normotensive rat. From these data it can be concluded that (1) endogenously generated Ang II can enhance noradrenergic neurotransmission in this model and (2) the facilitation by Ang II is mediated by prejunctional AT₁ receptors. In this model, pre- and postjunctional AT₁ blockade can be compared. See also Fig. 7



tan (Balt et al. 2001a). Nap et al. found telmisartan more potent than losartan and irbesartan regarding sympatho-inhibition in a model in which stimulation-induced NA release from rabbit aortic rings was measured (Nap et al. 2002). In isolated rat atria, in which stimulation-induced NA release was measured, Shetty and DelGrande reported a ranking order of valsartan=irbesartan>losartan=eprosartan (Shetty and DelGrande 2000) (Fig. 5). So, in contrast to Ohlstein's findings, in Shetty and DelGrande's model eprosartan was not significantly more potent than losartan, regarding sympatho-inhibition.

Differences in subtype between prejunctional AT₁ receptors (on sympathetic nerve terminals) and postsynaptic AT₁ receptors (on vascular smooth muscle cells) have been suggested earlier by Guimarães et al. (2001). In the canine isolated mesenteric artery, these authors saw no effect of losartan on Ang II-induced facilitation in doses which clearly inhibited the postsynaptic receptor (Guimarães et al. 2001). Similar findings were published by Balt et al. (2001). In pithed rat, these authors investigated the effects of a number of AT₁ receptor antagonists on (1) stimulation-induced responses, (2) responses to exogenous NA, and (3) responses to exogenous Ang II (Fig. 6A–C, irbesartan as an example). They found that the ranking order of the AT₁ receptor antagonists regarding blockade of the prejunctional AT₁ receptor differed from the ranking order regarding blockade of direct vasoconstrictor effects of Ang II (Balt et al. 2001b). The ratios between ED₂₀ values (as a measure of presynaptic inhibitory dose) and A₂ values (calculated by taking the exponential of the pA₂ values, a measure of the postsynaptic inhibitory dose) differ considerably between the various antagonists (Fig. 7). These findings were confirmed in an in vitro set up: in the rabbit isolated mesenteric artery, eprosartan exhibits sympathoinhibition in

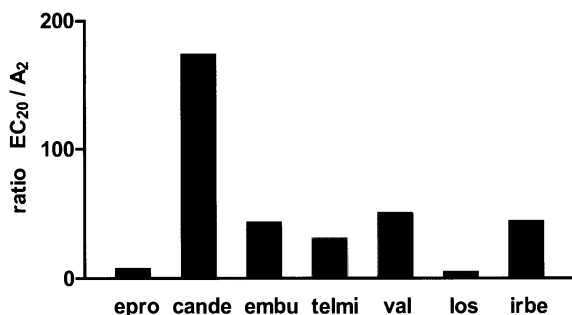


Fig. 7 Ratio between the ED₂₀ values (obtained from stimulation experiments; reflect sympatho-inhibitory potency) and A₂ values (obtained from Ang II experiments; reflect inhibitory potency regarding direct effects of Ang II on the vasculature). ED₂₀: dose which at 2 Hz reduced Δ DBP by 20 mmHg. A₂ values were calculated by taking the exponential of the pA₂ values. Note the substantial differences between the AT₁ blockers studied. The presynaptic effect related with impairment of NA release occurs at relatively low doses for eprosartan and losartan when compared with postsynaptic inhibition of vasoconstriction by Ang II. In contrast, there is a substantial difference between these two dosages for candesartan

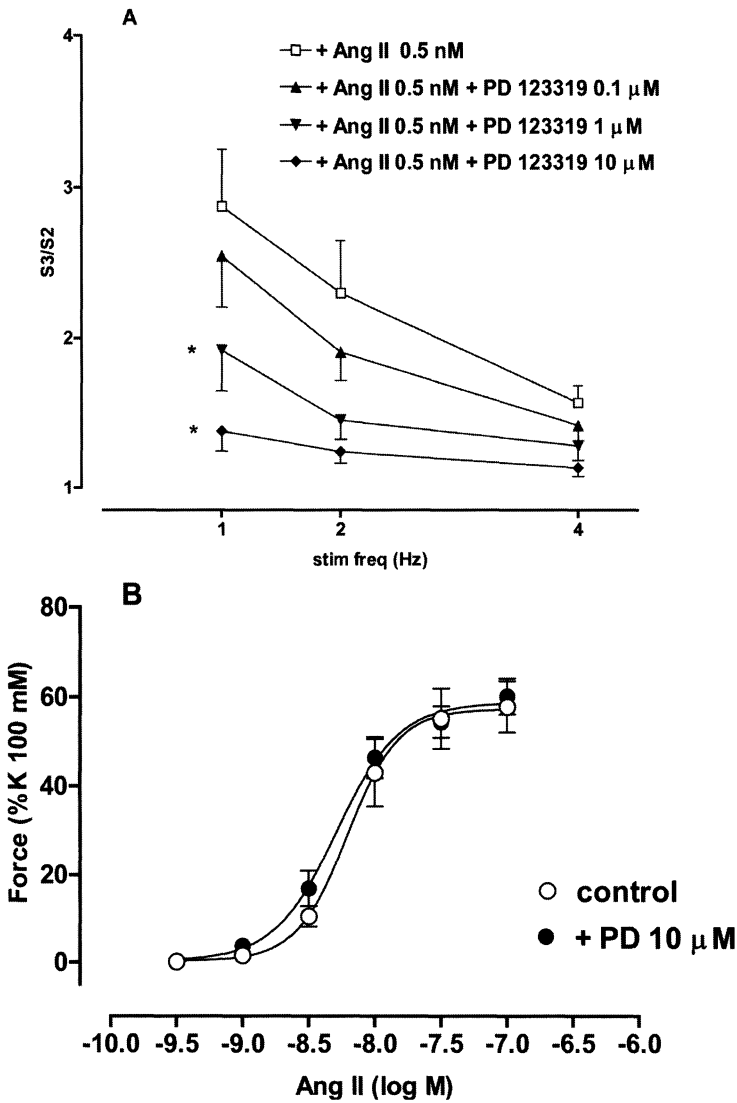


Fig. 8 **A** Inhibitory effects of PD123319 (0.1–10 μM) on the facilitation by Ang II of stimulation-induced contractions. Ang II (0.5 nM) in the presence or absence of PD123319 was added to the organ bath 2 min prior to the third electrical field stimulation (S3). The ratio between forces induced by S2 and S3 (S3/S2) is shown at the ordinate and stimulation frequencies at the abscissa. Values are given as mean \pm SEM. * $p < 0.05$ at each stimulation frequency compared to responses in presence of Ang II (0.5 nM). **B** PD123319 (10 μM) had no effect on the cumulative concentration–response curve for Ang II in the isolated rabbit mesenteric artery. Values are shown as mean \pm SEM

concentrations that inhibit AT₁ receptor-mediated vasoconstriction, whereas candesartan does not (Balt et al. 2002).

However, in another study, performed in the pithed rat, the assumption that prejunctional neuronal and postjunctional vascular AT₁ receptors differ could not be confirmed (Dendorfer et al. 2002). In a set-up in which inhibition of Ang II-induced NA release was measured on the one hand and inhibition of acute increases in BP increase on the other, the order of potency for both pre- and postjunctional inhibitory effects was candesartan > eprosartan > EXP 3174 (the active metabolite of losartan) > irbesartan. Although measuring catecholamine release is a more direct means of sympathetic activity compared to measuring contractile force or BP increase, in that study, the investigators relied on a “tyramine”-like effect of Ang II, whereas in other studies a facilitating role of Ang II on electrically stimulation-induced NA release was investigated.

In the isolated rat caudal artery, the facilitation by Ang II of stimulation-induced contractions as well as NA release could be inhibited by PD123319 (0.1 μM), which at this concentration is assumed to block the AT_{1B}-subtype (Cox et al. 1996). These findings were confirmed in the isolated rabbit mesenteric artery and aorta, in which stimulation-induced contractions and NA efflux was measured. In contrast, PD123319, in concentrations as high as 10 μM, had no effect on direct vasoconstrictor effects of Ang II (Nap et al. 2003; Fig. 8). These findings strongly suggest that the prejunctional AT₁ receptor is of the AT_{1B}-subtype, and the postjunctional AT receptor is not.

However, in humans, the AT₁ receptor is encoded by one gene only, so these findings may not apply to humans. No studies so far have been published in which sympatho-inhibitory properties in humans are compared.

3

Interactions Between the Sympathetic Nervous System and the Renin–Angiotensin System in Hypertension

Both the RAS and sympathetic nervous system have been extensively studied in animal models as well as in human hypertensives. Pharmacological tools targeting various components of both BP regulatory systems have been successfully applied in the treatment of hypertension.

The spontaneously hypertensive rat (SHR) has been used as a model for human essential hypertension. The substantial role of the sympathetic nervous system in the development and maintenance of hypertension in this model is firmly established. Likewise, in human hypertension the etiological role of the sympathetic nervous system has been demonstrated by measurements of regional NA spillover, spectral analysis of heart rate variability, and measurements of sympathetic nerve activity (Esler et al. 1990). The efficacy of ACE inhibitors and AT₁ antagonists in the treatment of hypertension clearly demonstrates the involvement of the RAS in cardiovascular control. In recent years, substantial knowledge has been acquired concerning the role of Ang II in the

development of hypertension and hypertensive target organ injury (Weir and Dzau 1999).

The facilitating effect of Ang II on sympathetic neurotransmission in SHR has been shown to be increased compared to their normotensive controls, Wistar-Kyoto (WKY) rats, despite similar or even lower plasma renin activity (PRA) or plasma Ang II levels (Lokhandwala and Eikenburg 1983; Dang et al. 1999). Possibly, an increased density of AT₁ receptors, as reported in several neuronal tissues of the SHR (Raizada et al. 1995) or a decreased neuronal NA uptake (Kawasaki et al. 1982) could be involved in the enhanced facilitation in the SHR. Part of the evidence concerning this issue is derived from experiments with isolated tissues (Collis et al. 1979; Clough et al. 1982; Kawasaki et al. 1982). In the pithed rat model, Clough et al. demonstrated, using an ACE inhibitor, an increased potentiation of sympathetic neurotransmission by Ang II in SHR compared to WKY both at the prejunctional as well as at the postjunctional level (Clough et al. 1982). These authors postulated that such a mechanism may explain the potent hypotensive action of captopril compared to that in the WKY, where captopril does not lower BP. The same was postulated for AT₁ blockade (Jonsson et al. 1993). Indeed, losartan does not lower BP in the WKY (Wong et al. 1990b). In pithed SHR, selective AT₁ receptor antagonists have been shown to attenuate stimulation-induced diastolic (D)BP responses (Häuser et al. 1998; Moreau et al. 1993b,c).

Recently, Raash et al. demonstrated that chronic ACE inhibition increased cardiac NA reuptake in SHR (Raasch et al. 2001) (Fig. 9). Interestingly, in patients with essential hypertension, a reduced presynaptic NA uptake has been described (Rumantir et al. 2000). It is not clear to which extent the sympatho-inhibitory effects of RAS blockade also apply to humans: some studies in hypertensives demonstrated that both ACE-inhibition and AT₁ blockade reduce plasma NA (Weinberger 1982; Moan et al. 1994). In other studies, no such effect was found (Nicholls et al. 1981; Grossman et al. 1994). We already mentioned the

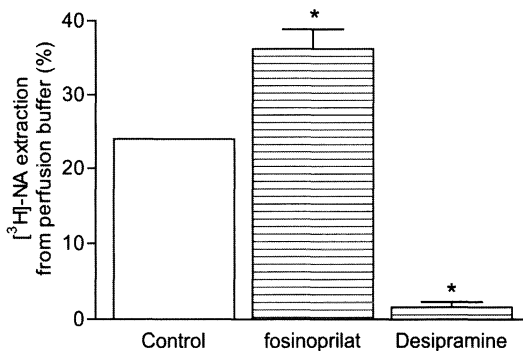


Fig. 9 ACE inhibition by fosinoprilat stimulates uptake of [³H]NA into isolated perfused rat hearts. In contrast, uptake-1 inhibition by desipramine almost abolished [³H]NA-extraction. (From Raasch et al. 2001)

limited value of plasma NA levels. Sakata et al., using an [^{123}I]metaiodobenzylguanidine (MIBG) scintigraphy technique, demonstrated that both chronic ACE-inhibitor therapy as well as AT_1 blockade reduced cardiac sympathetic activity (Sakata et al. 2002). In another study, acute losartan treatment blunted the sympathetic stimulatory effect of cold stress in hypertensive subjects on BP and NA concentrations, without changing baseline plasma NA (Rodriguez-Garcia et al. 2000). On the other hand, chronic ACE-inhibition treatment did not significantly affect sympathetic nerve traffic, measured by microneurography, nor plasma NA in hypertensive subjects (Grassi et al. 1998). Struck et al. demonstrated that antihypertensive treatment with amlodipine caused an increase in muscle sympathetic nerve activity, whereas treatment with valsartan did not (Struck et al. 2002). These authors conclude that AT_1 blockade not only lowers BP, but also changes the relation between BP and sympathetic outflow, in other words it resets the baroreflex set-point toward lower BP values.

4

Interactions Between the Sympathetic Nervous System and the Renin–Angiotensin System in Congestive Heart Failure

Various forms of congestive heart failure are known to be accompanied by activation of the RAS and the sympathetic nervous system (Mancia 1990). Plasma levels of NA have been shown to be increased and heart rate is usually elevated (Thomas and Marks 1978; Cody et al. 1982). NA spillover from the heart is increased as much as 50-fold in heart failure patients (Esler et al. 1997). In studies in which sympathetic nerve traffic was studied with the microneurography technique, the number of sympathetic bursts per minute was higher in patients with heart failure compared to healthy age-matched subjects (Leimbach et al. 1986). Sympathetic stimulation of renin release results in sodium and water retention, ultimately causing congestion. Indeed, sympatho-excitation, reflected by increased levels of plasma NA, is now seen as a risk factor in various, even asymptomatic stages of heart failure (Rector et al. 1987; Mancia 1990; Benedict et al. 1996).

As early as 1962, the activation of the RAS in heart failure was reported (Davis 1962). In 1978, two groups simultaneously reported that blocking the RAS improves cardiac and systemic haemodynamics (Curtiss et al. 1978; Gavras et al. 1978). It combines afterload reduction with diminished retention of salt and volume. Since then, suppression of the activity/influence of the RAS has shown to have favorable effects in experimental heart failure (Pfeffer et al. 1985; Richer et al. 1999) as well as in clinical trials, such as the CONSENSUS-study. (For review, see Brunner-La Rocca et al. 1999.)

In the treatment of heart failure, β -blockers are now more and more recognized as beneficial drugs (Sharpe 1999). Apart from inhibiting the sympathetic nervous system, these drugs inhibit renin secretion by acting on β -receptors in the juxtaglomerular apparatus in the kidney, and also in the vasculature, as described above. Indeed, in patients with heart failure, treatment with a β -blocker

(whether or not in addition to ACE inhibition) reduces Ang II levels (Campbell et al. 2001). Interestingly, in the Val-HEFT trial, a combination of ACE inhibition, AT₁ blockade, and β -blockade, so-called “triple blockade,” significantly increased mortality in heart failure patients (Cohn and Tognoni 2001).

In the CONSENSUS trial, ACE-inhibition treatment was associated with a significant reduction of plasma NA levels (Swedberg et al. 1990). In smaller studies, similar findings were reported with AT₁ blockade by some (Gottlieb et al. 1993;

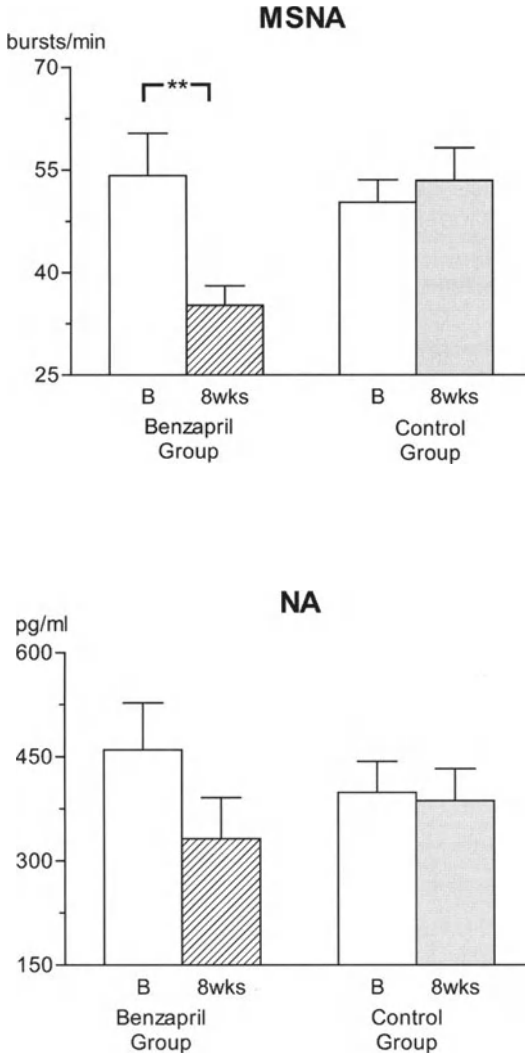


Fig. 10 Bar graphs showing muscle sympathetic nerve activity (MSNA) and plasma NA-values before (*open bars*) and after 8 weeks of benazepril treatment (*hatched bars*) or an 8-week observational period (*shaded bars*) in heart failure treatment. ACE-inhibition significantly reduced MSNA. (From Grassi et al. 1997)

Crozier et al. 1995) but not by all authors (Houghton et al. 1999). In addition, a low dose of an ACE inhibitor was shown to increase parasympathetic activity (Kamen et al. 1997). In patients with heart failure, acute losartan administration increases forearm blood flow by 25%, in contrast to age- and sex-matched controls (Newby et al. 1998). These findings suggest that endogenous Ang II contributes to the maintenance of peripheral resistance. Since there is a sympathetic tone in the forearm under basal conditions, this increased sympathetic tone may be caused by a facilitatory effect of Ang II. In the same study, lower body negative pressure (LBNP) failed to reduce forearm blood flow in heart failure patients, consistent with defective baroreflex control. Additionally, losartan had no effect on the blood flow response to LBNP. Hence, no direct evidence of Ang II-induced facilitation was found. In a pacing-induced heart failure model, chronic AT₁ blockade was shown to reduce renal sympathetic nerve activity, as well as to enhance baroreflex sensitivity (Murakami et al. 1997). In an analogy to this animal model, similar findings were obtained in humans subjected to chronic ACE inhibition (Grassi et al. 1997): in patients with NYHA class II heart failure, treatment with the ACE inhibitor benazepril caused significant reductions in muscle sympathetic nerve activity (see Fig. 10). Plasma NA values were slightly but insignificantly reduced in this study. Additionally, ACE inhibition improved baroreflex restraint on sympathetic drive.

5

Conclusions

In summary, there is an overwhelming body of evidence that the RAS and SNS interact. However, most of the evidence is derived from animal experiments, sometimes far from the clinical setting, using high, unphysiological concentrations of Ang II. Nevertheless, clinical importance of this interaction may follow from the fact that both the RAS and the SNS play a pivotal role in the pathophysiology of hypertension and even more so in heart failure. Consequently, there exists important potential for the positive reciprocal feedback loop between the RAS and the SNS to be involved in the pathophysiology and therapeutic approaches of these conditions.

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Part 3
ANG Receptors

AT₂

Molecular Aspects of AT₂ Receptor

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Abstract The octapeptide angiotensin II (Ang II) binds to two subtypes of receptors, AT₁ and AT₂, that both belong to the superfamily of G protein-coupled receptors (GPCRs). The AT₁ subtype is a classical GPCR in terms of coupling and signaling, and appears to mediate all known physiological actions of Ang II. In contrast, the AT₂ subtype is an atypical receptor that has remained a puzzle since its discovery in 1989 and molecular cloning in 1993. Over the past 10 years, a number of studies have aimed at elucidating the signaling pathways and

functions of the AT₂ subtype. A role for AT₂ receptors has been established during fetal development and in cardiovascular, brain, and renal functions. In most cases, the AT₂ receptor has been shown to counteract the effects of the AT₁ subtype. AT₂ also negatively cross-talks with growth factor receptors and plays a major role in the processes of apoptosis, migration, differentiation, and tissue regeneration. Depending on the cellular model and function examined, the AT₂ receptor activates different signaling pathways, that can be classified into three major types: regulation of protein phosphorylation, activation of phospholipases, and/or regulation of nitric oxide (NO)/cGMP. In the present chapter, we review recent advances on the molecular aspects of the AT₂ receptor: its structural features (functional domains involved in ligand binding and receptor activation), signaling pathways, coupling to G proteins and association with other intracellular partners. We then examine the molecular mechanisms by which AT₂ antagonizes the effects of the AT₁ subtype. Examples of AT₂ receptor gene alterations associated with human diseases such as congenital anomalies of kidney and urinary tract (CAKUT) or X-linked mental retardation, are also discussed.

Keywords G protein-coupled receptor · Structure/function relationship · Signaling pathways · Negative cross-talk · Phosphorylation · Arachidonic acid · Nitric oxide · Cardiovascular · Apoptosis · Migration · Neuronal differentiation · AGTR2 gene alteration

1

Introduction

The first evidence that the octapeptide angiotensin II (Ang II) was able to bind to two distinct receptor subtypes was provided in 1989, in studies showing distinct binding sites for non-peptidic analogs of Ang II in the rat adrenal gland (Chiu et al. 1989a) and different sensitivity of Ang II binding sites to the reducing reagent dithiothreitol (DTT) (Chiu et al. 1989b). These receptor subtypes, first designated as ATA and ATB, are now well-known as type 1 (AT₁) and type 2 (AT₂) receptors. The molecular cloning of cDNAs encoding the AT₁ and AT₂ receptors revealed that both subtypes belong to the superfamily of G protein-coupled receptors (GPCR) organized as single polypeptides that span the cell membrane seven times. AT₁ and AT₂ subtypes thus belong to the same superfamily of receptors and they bind the same ligand; however they exhibit no more than 35% amino acid sequence homology, and they differ in many aspects. The AT₁ subtype appears to mediate most known physiological effects of Ang II and behaves as a classical GPCR with regard to G protein coupling and associated intracellular signaling pathways. In contrast, the AT₂ receptor is an unconventional GPCR; in many cell types, this receptor is not coupled to classical G proteins, its intracellular signaling pathways remain controversial, and its physiological functions are still to be fully characterized. Over the past 10 years, a number of studies have aimed at elucidating the signaling pathways and functions of the

AT₂ subtype. A role for AT₂ receptors has been established in cardiovascular, brain, and renal functions and in the processes of apoptosis and tissue regeneration. In most cases, the AT₂ receptor has been shown to counteract the effects of the AT₁ subtype. In the present chapter, we will focus on the structural features of the AT₂ receptor, the signaling pathways associated with AT₂ receptor activation, and the molecular mechanisms by which AT₂ antagonizes the effects of the AT₁ subtype. Examples of AT₂ receptor gene alterations associated with human diseases will also be discussed.

2 Structural Features of the AT₂ Receptor

2.1 Functional Domains Involved in Ligand Binding

The AT₂ receptor has been distinguished from the AT₁ subtype on the basis of its selective binding to specific synthetic ligands—among them the peptidic CGP 42112 and non-peptidic PD123319 compounds—and its insensitivity to AT₁ receptor antagonists such as losartan. AT₁ and AT₂ receptors share only 35% amino acid sequence identity but they bind Ang II with similar affinity, and the question has been raised whether both receptors bind Ang II through their conserved amino acid residues, or whether they display distinct binding sites to the same ligand.

GPCR binding sites have been shown to differ considerably depending on the type of ligand considered. For instance, binding of β -adrenergic receptors to small molecules like catecholamines involves residues located in the transmembrane regions (tm), whereas receptors for glycoproteins essentially use their large N-terminal portion for ligand binding. In the case of AT₁ receptors, binding to the octapeptide Ang II has been shown to involve amino acid residues located in the tm as well as in the N-terminus.

Initial studies of the AT₂ receptor binding site, using photoaffinity-labeled analogs of Ang II (Servant et al. 1997), revealed that the amino-terminal end of Ang II interacts with the first 30 N-terminal residues of the AT₂ receptor, whereas the carboxyl-terminal end of Ang II interacts with the inner half of the third transmembrane domain (tm3) of the receptor (residues 129–138) (Fig. 1). Further studies of deleted mutants of the AT₂ receptor confirmed the essential role of N-terminal residues in binding to Ang II (Yee et al. 1998). Interestingly, these latter studies also revealed that the N-terminal part of the receptor does not interfere with binding to the synthetic AT₂-selective ligand CGP 42112, indicating distinct binding sites for the natural and synthetic ligands.

The N-terminal portion of the AT₂ receptor contains five potential N-glycosylation sites, at least three of which are utilized (Lazard et al. 1994b). Indeed, the AT₂ receptor is heavily glycosylated; however, enzymatic deglycosylation only has a modest effect on AT₂ receptor binding properties (Servant et al. 1996).

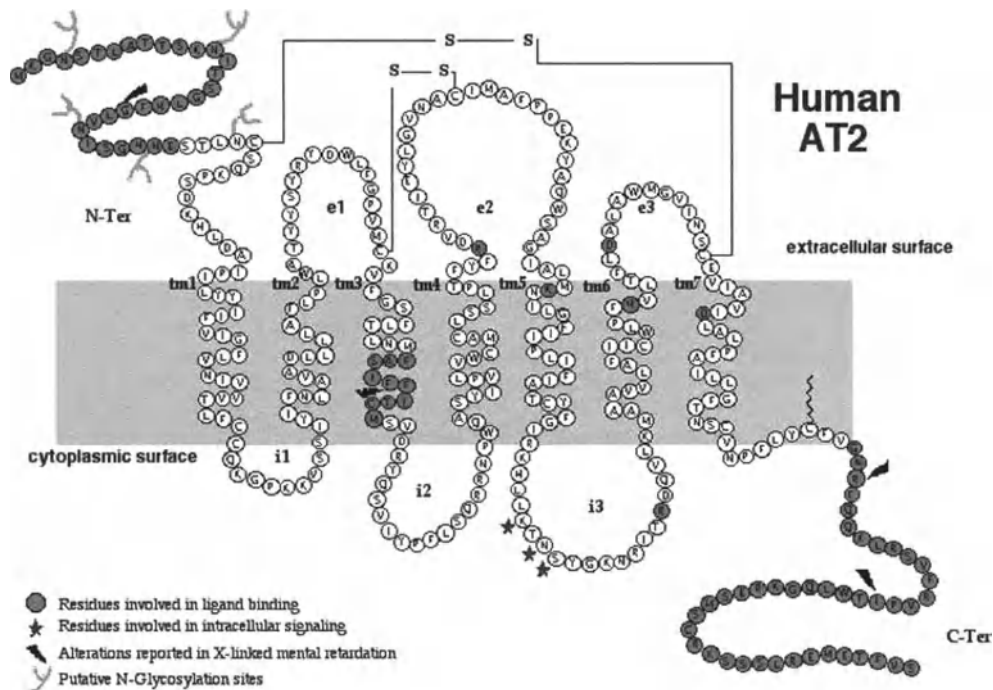


Fig. 1 Structural features of human AT₂ receptor

Site-directed mutagenesis indicated that, as for the AT₁ receptor, charged residues located in the transmembrane domains of the AT₂ receptor play a critical role in binding to Ang II (Fig. 1). Thus, amino acid Lys 215, a conserved residue corresponding to the critical Lys 199 of the rat AT₁ receptor in the tm5, is required for efficient binding of AT₂ to Ang II (Yee et al. 1997; Pulakat et al. 1998). Replacement of Asp 279 in the tm6 by an alanine also leads to a loss of binding to Ang II (Heerding et al. 1998). Residues Arg 182 located at the junction between e2 and tm4 (Heerding et al. 1997; Kurfis et al. 1999) and His 273 in the tm6 (Turner et al. 1999) are required for high-affinity binding to both Ang II and CGP 42112. In contrast, substitution of Asp 297 to Lys in the tm7 resulted in loss of affinity for Ang II (Heerding et al. 1997; Knowle et al. 2001) but retained partial binding to CGP 42112 (Knowle et al. 2001), further indicating that binding sites for Ang II and CGP 42112 are overlapping but not superimposable.

In addition to the N-terminus and tm regions, intracellular portions of the AT₂ receptor, namely the third intracellular loop (i3) and the C-terminal tail, have also been identified as playing a role in ligand binding. This finding was unexpected, as it is generally accepted that cytoplasmic portions of GPCRs are rather involved in intracellular signaling and internalization. Chimeric receptors in which the i3 of AT₂ has been replaced by that of AT₁ were no longer able to

bind Ang II and CGP 42112 (Dittus et al. 1999). Reciprocally, chimeric receptors in which the i3 of AT₁ has been replaced by that of AT₂ showed high-affinity binding to CGP 42112A—but not to PD123319—which was further enhanced in the presence of the AT₂ receptor tm7 domain (Hines et al. 2001b). The role of the C-terminal tail (residues 322–363) in binding and activation of AT₂ was recently examined (Pulakat et al. 2002). A C-terminally deleted mutant of the AT₂ receptor showed reduced affinity to Ang II and unexpectedly showed increased affinity to the peptide analog CGP 42112.

Chimeric receptors in which tm6 and/or tm7 domains of the AT₂ receptor were substituted into the AT₁ receptor revealed functional similarities between the two subtypes of receptors in the domains mediating agonist-dependent activation (Hines et al. 2001a). Further studies combining photo-affinity labeling, site-directed mutagenesis and modeling recently indicated that although poorly homologous in sequence, AT₁ and AT₂ binding sites are superimposable, and revealed that Ang II binds to both receptors in an extended form parallel to the transmembrane regions (Deraet et al. 2002).

2.2

Disulfide Bonds in the AT₂ Receptor

The observation that the AT₂ binding site for a small peptide such as Ang II involves residues located in the N-terminus as well as in the tm6 domain suggested that these regions may be spatially close to each other, possibly via the formation of disulfide bonds. The existence of disulfide bonds in the AT₂ receptor had for a long time remained a puzzle because of the unusual behavior of this receptor in the presence of the reducing reagent DTT. While the AT₁ subtype is inactivated in the presence of DTT, the AT₂ receptor paradoxically shows higher affinity to Ang II in the presence of the reducing reagent (Chiu et al. 1989b). Several cysteines are conserved at similar positions in the sequences of the AT₁ and AT₂ receptors. Site-directed mutagenesis of conserved cysteines in the AT₂ receptor (Feng et al. 2000; Heerding et al. 2001) have confirmed the existence of two disulfide bonds (Cys35–Cys290) and (Cys117–Cys195) that link N-ter to e3 and e1 to e2, respectively, in the same manner as in the AT₁ subtype (Fig. 1). As for the AT₁ subtype, abolishing the (Cys117–Cys195) bond (between e1 and e2) leads to inactivation of the AT₂ receptor. Surprisingly, disruption of the other bond (Cys35–Cys290) that links N-ter to e3, leads to increased binding affinity for the ligands. Analyses of single Cys mutants suggested that a Cys–disulfide bond exchange may occur between the free SH group of Cys(35) or Cys(290) and the (Cys117–Cys195) disulfide bond, resulting in production of an inactive population of receptors through formation of a non-native disulfide bond (Feng et al. 2000). Further analysis of AT₁/AT₂ chimeras suggested to Heerding et al. that the N-ter and e3 regions of the AT₂ receptor may possess latent binding epitopes that are only uncovered after DTT exposure (Heerding et al. 2001).

2.3

Ligand-Independent Activation of the AT₂ Receptor

By comparing the affinity profiles of AT₁ and AT₂ receptors towards a panel of substituted analogs of Ang II, Miura and Karnik suggested that the AT₁ subtype is in a constrained conformation and is activated only when bound to the agonist, whereas the AT₂ receptor is “relaxed,” in that no single interaction is critical for binding (Miura and Karnik 1999). In this respect, the native AT₂ receptor resembles the N111G mutant AT₁ receptor, which is constitutively active. These observations led to the interesting hypothesis that activation of the AT₂ receptor may be ligand-independent, a hypothesis consistent with previous findings that replacement of the i3 loop of the AT₂ receptor by that of the AT₁ subtype led to constitutive activation of AT₁ pathways (Wang et al. 1995). It was indeed shown shortly afterwards that AT₂-mediated apoptosis in cultured fibroblasts, epithelial cells, and vascular smooth muscle cells was regulated by overexpression of the receptor rather than by ligand activation (Miura and Karnik 2000). A ligand-independent effect of the AT₂ receptor was also indicated in recent experiments showing heterodimerization between AT₁ and AT₂ receptors, leading to attenuation of the AT₁ signaling pathways (AbdAlla et al. 2001). These authors showed that AT₂-mediated antagonism of AT₁ receptor activation is attenuated by release of AT₁/AT₂ heterodimers but remains unaffected in the presence of the AT₂ receptor antagonist PD123319. Constitutive activation of the AT₂ receptor may, however, depend on the cell type or signaling pathway considered, since other recent studies indicate that AT₂-mediated activation of tyrosine phosphatase SHP-1 in N1E-115 and transfected COS cells is not ligand-independent (Feng et al. 2002).

2.4

Functional Domains Involved in Intracellular Signaling

As for most GPCRs, the third intracellular domain of the AT₂ receptor has been shown to be a major determinant of receptor activation. Intracellular injection into cultured rat neurons of a 22-amino acid peptide corresponding to the i3 loop of the AT₂ receptor elicited increased opening of potassium channels similar to that produced by AT₂ receptor activation (Kang et al. 1995). Transfection of the same i3 peptide into vascular smooth muscle cells resulted in reduction of serum-stimulated DNA synthesis and cell proliferation as well as a decrease in mitogen-activated protein kinase activity, therefore simulating the effects of AT₂ receptor activation (Hayashida et al. 1996). In both studies, the effect of the i3 peptide was blocked by pertussis toxin, indicating the involvement of a Gi protein.

The role of the i3 loop as a major determinant of AT₂-mediated responses was further established in studies showing that chimeric AT₁ receptors in which the i3 loop was replaced by that of the AT₂ were able to transduce AT₂-specific signals, while keeping their AT₁-selective binding properties (Daviet et al. 2001).

Using site-directed mutagenesis, crucial residues (Lys 240, Asn 242, and Ser 243) located in the intermediate portion of the i3 loop have been identified as being involved in AT₂-induced apoptosis, extracellular signal-regulated kinase (ERK) inhibition and SHP-1 activation in PC12 cells (Lehtonen et al. 1999a). Consistently, AT₂ chimeras containing either the i3 or the i2 loop of the AT₁ receptor were no longer able to associate with, nor activate, tyrosine phosphatase SHP-1 (Feng et al. 2002). Interestingly, loss-of-function of the i3 chimera was restored when the C-terminal tail was additionally replaced by that of AT₁, indicating that all three intracellular portions of the AT₂ receptor participate in the coupling to phosphatase SHP-1 (Feng et al. 2002). Studies by Pulakat et al. recently indicated a negative effect of the C-terminal tail in signaling, as AT₂-mediated reduction of cyclic guanosine monophosphate (cGMP) levels in oocytes was further enhanced by C-terminal (322–363) deletion (Pulakat et al. 2002). The same C-terminally deleted mutant was no longer able to interact with ErbB3, a recently identified partner of the AT₂ receptor (see Sect. 3).

3 Coupling to G Proteins

3.1 Coupling to Gi

The AT₂ receptor clearly belongs to the superfamily of GPCRs; however, coupling of this receptor to classical G proteins still remains a matter of debate. Initial studies performed in pheochromocytoma PC12 cells by Bottari showed that the AT₂ receptor is insensitive to analogs of guanosine triphosphate (GTP) (Bottari et al. 1991) and does not activate a GTPase (Brechler et al. 1994), suggesting that the AT₂ receptor is not coupled to heterotrimeric G proteins. Later studies reported the involvement of pertussis toxin-sensitive Gi proteins in AT₂ signaling pathways, leading to opening of potassium channels (Kang et al. 1994; Huang et al. 1995) and apoptosis (Yamada et al. 1996; Horiuchi et al. 1997), and established that AT₂ is able to interact with G α (i2) and G α (i3) in rat fetus extracts (Zhang and Pratt 1996). More recently, Hansen et al. showed in reconstituted systems that AT₂ is able to trigger rapid activation of purified G α (i) and G α (o) but not of G α (q) and G α (s), as measured by radioactive GTP γ S binding (Hansen et al. 2000). The ability of AT₂ to couple to Gi, but not to other G α proteins, was also demonstrated in recent studies using chimeric G proteins (Sasamura et al. 2000).

Thus, while most GPCRs function by coupling to a large panel of G proteins sometimes simultaneously in the same cell type, the AT₂ receptor seems to be restricted to coupling to G α (i). However, signaling pathways elicited by the AT₂ receptor in a number of cell types remained insensitive to pertussis toxin, indicating coupling to other G proteins, or to G protein-independent pathways. Two studies have indicated coupling of the AT₂ receptor to other G proteins: in neuroblastoma NG108–15 cells, AT₂ has been shown to inhibit the opening of calci-

um channels through activation of a non-identified G protein different from Gi (Buisson et al. 1995). In a recent study, Feng et al. have demonstrated an essential role for $G\alpha(s)$ as a scaffold protein in the constitutive association between AT_2 and tyrosine phosphatase SHP-1, illustrating a novel mechanism for GPCR-induced activation of SHP-1 that is independent of $\beta\gamma$ subunits of heterotrimeric G proteins and does not involve activation of $G\alpha(s)$ (Feng et al. 2002).

3.2

Other Interacting Partners of the AT_2 Receptor

Increasing evidence indicates that in addition to coupling to heterotrimeric G proteins, GPCRs also transduce their activation signals via other intracellular regulatory proteins, most of which often interact with the C-termini of GPCRs (Marinissen and Guntkind 2001; Brady and Limbird 2002). This is also the case for the AT_1 receptor, which interacts via its C-terminal tail with a novel protein designated AT_1 receptor-associated protein (ATRAP) (Daviet et al. 1999). Expression of ATRAP in vascular smooth muscle cells results in enhanced AT_1 receptor internalization and inhibition of AT_1 -mediated activation of signal transducer and activator of transcription (STAT)3, Akt, and cell proliferation, indicating that ATRAP is a negative modulator of the AT_1 pathway (Cui et al. 2000).

In an attempt to identify novel interacting partners of the AT_2 receptor, Knowle et al. have screened a random peptide library using the whole AT_2 receptor as a bait in the two-hybrid system method. A peptide corresponding to the ATP binding site of ErbB3, a member of the epidermal growth factor (EGF) receptor family, has been isolated. The cytoplasmic tail containing the ATP binding site of ErbB3 has been shown to interact with the i3 loop and C-ter portion of the AT_2 receptor (Knowle et al. 2000; Pulakat et al. 2002). Although the consequence of ErbB3 interaction on AT_2 receptor signaling has not yet been established, this finding is particularly relevant in the light of the major role played by the ErbB family in growth and development (Olayioye et al. 2000) and of the inhibitory effect of AT_2 on EGF receptor (EGFR) signaling (Elbaz et al. 2000; Shibasaki et al. 2001; DePaolis et al. 2002).

A similar approach has been used in our laboratory to identify novel interacting partners of the AT_2 receptor. The 52 C-terminal residues of the AT_2 receptor were used as a bait to screen a mouse fetal cDNA library in the two-hybrid system. One novel sequence, designated AT_2 receptor interacting protein (ATIP), was isolated and found to specify a new family of proteins that all interact with the AT_2 receptor, via a conserved coiled-coil domain allowing dimerization (S. Nouet et al., submitted). Transfection of ATIP into eukaryotic cells leads to inhibition of insulin receptor autophosphorylation and growth factor-induced ERK activation, indicating that ATIP simulates AT_2 receptor activation and may be a novel component of growth-inhibitory pathways.

4

Intracellular Signaling Pathways Activated by AT₂ Receptor

The AT₂ receptor is highly expressed at late stages of fetal development and its expression falls abruptly after birth. In the adult, this receptor is found in specific sites—adrenals, kidney, ovary, brain, vasculature—and is upregulated during the process of tissue regeneration following injury. At the cellular level, most documented AT₂-associated signaling pathways are those related to organogenesis and tissue repair, renal function, and neuronal activation. Although differing extensively depending on the cellular model and biological response examined, AT₂ signaling pathways can be classified into three major types: (1) regulation of protein phosphorylation, (2) activation of phospholipases, and (3) regulation of nitric oxide (NO)/cGMP (Fig. 2), that have been found to work in concert in some cell types.

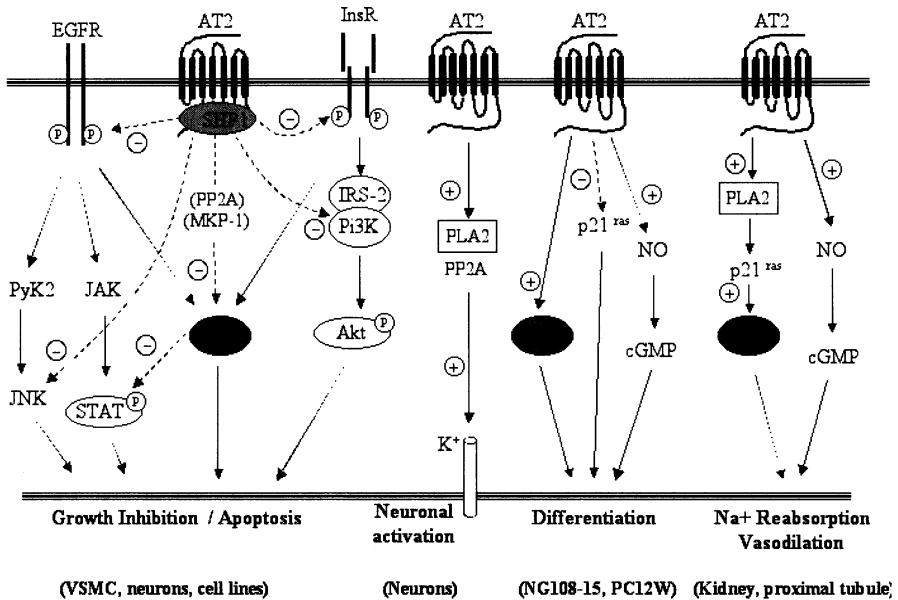


Fig. 2 Major AT₂ receptor signaling pathways

4.1

Cell Proliferation and Apoptosis

It is now well established that the AT₂ receptor inhibits cell proliferation and promotes apoptosis *in vivo* and *in vitro* (for reviews, see Horiuchi et al. 1999b; Nouet and Nahmias 2000; Stoll and Unger 2001). Growth-inhibitory effects of the AT₂ receptor are due, at least in part, to negative cross-talk with growth factor receptor tyrosine kinases and mainly involve activation of phosphatases and inhibition of protein kinases.

4.1.1

Protein Tyrosine Phosphatases

Protein tyrosine dephosphorylation and activation of orthovanadate-sensitive protein tyrosine phosphatases were the first cellular responses that were associated with AT₂ receptor activation (Bottari et al. 1992; Brechler et al. 1994; Nahmias et al. 1995). Consistent with a role on growth inhibition, the AT₂ receptor has been shown to rapidly activate the SH2 domain-containing tyrosine phosphatase SHP-1 (Bedecs et al. 1997), a vanadate-sensitive phosphatase that directly interacts with phosphorylated receptor tyrosine kinases and negatively regulates cytokine and growth factor receptor signaling pathways. A number of studies conducted by M. Horiuchi group have established a pivotal role of SHP-1 in AT₂-mediated ERK inactivation and apoptosis (Lehtonen et al. 1999a; Cui et al. 2001, 2002). In vascular smooth muscle cells (VSMC) from AT₂ transgenic mice, AT₂ induces increased association of SHP-1 with EGFR, leading to inhibition of AT₁-mediated EGFR *trans*-activation and activation of ERK (Shibasaki et al. 2001). AT₂ acting through SHP-1 also mediates inhibition of AT₁-induced c-Jun N-terminal kinase (JNK) activation and c-jun expression (Matsubara et al. 2001) as well as inhibition of the insulin-induced PI3 kinase association with IRS-2 and Akt phosphorylation (Cui et al. 2002). Moreover, SHP-1 appears responsible for Janus kinase (JAK)2 dephosphorylation and termination of the AT₁-activated JAK/STAT cascade (Marrero et al. 1998) and has been shown to participate in AT₂-mediated inhibition of AT₁-induced superoxide formation in human umbilical venous endothelial cells (Sohn et al. 2000).

How does the AT₂ receptor activate SHP-1? In some cell types, AT₂-mediated activation of SHP-1 is sensitive to pertussis toxin, indicating coupling to Gi. Although not firmly demonstrated, it may be possible that AT₂ functions like the somatostatin sst2 receptor that constitutively interacts with SHP-1 in a complex including Gi (Lopez et al. 1997). In other cell types however, AT₂-mediated SHP-1 activation is not sensitive to pertussis toxin. A recent study has demonstrated constitutive association of AT₂ with the inactive form of SHP-1 in a complex containing G α (s)—but not other G α -proteins—independently of $\beta\gamma$ subunits of G proteins. Upon AT₂ receptor stimulation, SHP-1 and Gs still interact but they dissociate from the receptor and SHP-1 becomes activated (Feng et al. 2002).

In addition to SHP-1, two other phosphatases, i.e., protein phosphatase 2A (PP2A) and mitogen-activated protein (MAP) kinase phosphatase (MKP)-1, are involved in AT₂-mediated ERK inactivation and apoptosis. The okadaic acid-sensitive Ser/Thr phosphatase PP2A is involved in inhibition of AT₁-mediated ERK activity in neurons (Huang et al. 1996), and in AT₂-mediated apoptosis in the presence of UV irradiation (Shenoy et al. 1999). The AT₂ receptor also stimulates dual-specificity (Tyr/Thr) MKPs that inactivate ERK by direct dephosphorylation on Tyr and Thr residues. In PC12W cells, AT₂ induces increased expression of MKP-1, leading to inactivation of ERK1 and ERK2 (Yamada et al. 1996) and reduced phosphorylation of the anti-apoptotic protein bcl-2

(Horiuchi et al. 1997). Recently, a role for MKP-3 in Ang II-mediated apoptosis and bcl-2 dephosphorylation and degradation in endothelial cells has also been demonstrated (Rossig et al. 2002). In contrast to SHP-1 whose catalytic activity is increased within minutes, MKP-1 and MKP-3 are transcriptionally activated within hours, suggesting that a relay between different tyrosine phosphatases may be necessary to allow the pro-apoptotic effects of the AT₂ receptor (Nouet and Nahmias 2000).

4.1.2

Inhibition of Protein Kinases

In parallel to tyrosine phosphatase activation, the AT₂ receptor mediates inhibition of protein kinase activity, leading to attenuation of mitogenic signaling cascades. AT₂ interferes at early steps of mitogenic cascades, by inhibiting insulin- and EGF- induced receptor tyrosine kinase autophosphorylation (Elbaz et al. 2000) and EGF receptor *trans*-activation by the AT₁ receptor (Shibasaki et al. 2001; DePaolis et al. 2002). AT₂ also inactivates downstream protein kinases: in addition to its widely described effect on ERK, AT₂ inhibits AT₁-induced JNK activity (Matsubara et al. 2001), and inhibits insulin-induced IRS-2-associated PI3 K and subsequent Akt phosphorylation (Cui et al. 2002). As a consequence of ERK inactivation, AT₂ inhibits the phosphorylation of STAT1, STAT2, and STAT3 induced by several growth factors including AT₁, with no effect on JAK (Horiuchi et al. 1999a). Studies of AT₂ knockout mice have indicated that AT₂ also negatively regulates the phosphorylation of p70S6 kinase in the vasculature—an effect which is not due to inhibition of upstream kinases ERK nor Akt—suggesting a role for AT₂ in the regulation of vascular hypertrophy *in vivo* (Brede et al. 2001).

4.1.3

Ceramides

In PC12W cells, AT₂ induces generation of ceramides, illustrating another pathway that links AT₂ receptor activation with apoptosis (Gallinat et al. 1999). AT₂-mediated accumulation of ceramide in PC12W cells was shown to be due to *de novo* biosynthesis of sphingolipids. This pathway was blocked by pertussis toxin and orthovanadate, indicating the involvement of Gi and phosphotyrosine phosphatases in this process (Lehtonen et al. 1999b).

4.2

Cellular Differentiation

Consistent with its role in nerve regeneration (Lucius et al. 1998), the AT₂ receptor induces morphological differentiation and neurite elongation in neuronal cell lines NG108–15 (Laflamme et al. 1996) and PC12W (Meffert et al. 1996), as well as in granular cells from rat cerebellum (Cote et al. 1999). These morpho-

logical changes are correlated with increased levels of polymerized tubulin and microtubule-associated proteins Tau and MAP2c, which are involved in neurite extension (Laflamme et al. 1996; Stroth et al. 1998; Cote et al. 1999). In PC12W cells, AT₂ downregulates the expression of MAP1B, in contrast to what is seen during nerve growth factor (NGF)-induced differentiation (Stroth et al. 1998). AT₂ stimulation in PC12W also results in reduced expression levels of the middle-sized neurofilament subunit (NF-M), which is decreased in regenerating neurons and in neuronal cultures undergoing apoptosis (Gallinat et al. 1997).

AT₂ receptor signaling pathways leading to neurite elongation in NG108–15 cells include inhibition of GTP-bound p21ras (Gendron et al. 1998), but surprisingly also include a sustained increase in ERK1 and ERK2 activation that is essential for neurite outgrowth (Gendron et al. 1999). Inhibition of p21ras using the dominant negative mutant RasN17 failed to impair the stimulatory effect of Ang II on ERK, suggesting that p21ras is not an upstream component of the AT₂-activated ERK cascade (Gendron et al. 1999). AT₂ also mediates activation of ERK1 and ERK2 in quiescent PC12W cells (Stroth et al. 2000) and AT₂-mediated neurite extension is blocked by the MEK inhibitor PD098059, confirming the essential role of ERK activation in AT₂-mediated differentiation (Stroth et al. 2000). Taken together, these studies tend to suggest that the net cellular effect of the AT₂ receptor may depend on the growth status of the cell. Thus, in cells cultured in the presence of growth factors, AT₂ inhibits ERK activation, thereby leading to inhibition of cell proliferation, whereas in quiescent cells, AT₂ activates ERK to promote neuronal differentiation and neurite extension.

Another signaling pathway linking AT₂ activation to neuronal differentiation involves increased NO production (Cote et al. 1998). In NG108–15 cells, AT₂ induces a rapid increase in NO synthase (NOS) activity and a subsequent increase in intracellular cGMP levels via a pertussis toxin-sensitive Gi protein (Gendron et al. 2002). AT₂-mediated activation of NO/cGMP is independent of ERK activation, and rather represents a parallel and complementary pathway involved in neurite branching.

A role for AT₂ receptors in the differentiation of VSMC has also been suggested by studies of AT₂ receptor knockout mice, showing reduced expression of calponin and h-caldesmon—two constituents of vascular contractile apparatus—in null mice as compared to wild-type mice (Yamada et al. 1999).

4.3 Migration

Cell migration is an essential step during development and tissue repair. The AT₂ receptor has been reported to induce cell migration in microexplant cultures of rat cerebellum (Cote et al. 1999), and to inhibit the migration of AT₂-transfected VSMC plated on laminin (Chassagne et al. 2002). The decreased migration of AT₂-transfected VSMC was correlated with increased synthesis and binding to fibronectin. Addition of GRGDTP peptide, which prevents cell attachment to fibronectin, reversed the inhibitory effect of AT₂ on cell migration,

indicating that VSMC migration is inhibited via synthesis and binding of fibronectin (Chassagne et al. 2002).

In another study conducted on AT₂-transfected VSMC, AT₂ receptor activation was shown to induce a significant increase of collagen synthesis, and this effect was attenuated by treatment with pertussis toxin and Gi antisense oligonucleotides (Mifune et al. 2000). Similar effects were observed in AT₂-transfected mesangial cells, but not in transfected NIH-3T3(AT₂) fibroblasts, further indicating differential effects of AT₂ receptor stimulation in different cellular models (Mifune et al. 2000).

In endothelial cells derived from rat heart (CEC) or bovine aorta (BAEC), AT₂ upregulates the expression of thrombospondin-1 and fibronectin, indicating a role for this receptor in remodeling of endothelial extracellular matrix (Fischer et al. 2001).

4.4 Neuronal Activation

Electrophysiological studies conducted by C. Summers group have shown that in cultured neurons from newborn rat brain, the AT₂ receptor stimulates a delayed rectifier K⁺ current (Martens et al. 1996), leading to increased neuronal firing rate (Zhu et al. 2001). In catecholaminergic neurons in which AT₁ and AT₂ receptors are co-localized, the two subtypes induce opposite effects on K⁺ current, indicating functional negative cross-talk between AT₁ and AT₂ in regulating electrophysiological responses of catecholaminergic neurons (Gelband et al. 1997). AT₂-mediated stimulation of K⁺ current in rat neuronal cells involves a pertussis toxin-sensitive Gi protein and activation of serine/threonine phosphatase PP2A (Kang et al. 1994; Huang et al. 1995). In these cells, AT₂ receptor activation also leads to increased activity of phospholipase A (PLA)₂, generation of arachidonic acid and production of 12-lipoxygenase (12-LO) metabolites (Zhu et al. 1998, 2000). Inhibition of PP2A abolished the stimulatory effects of AT₂ on neuronal delayed-rectifier potassium (K⁺) current but did not affect Ang II-stimulated (3H)-AA release, suggesting that PP2A is a distal event in this pathway (Zhu et al. 1998).

Another signaling pathway involving NO/cGMP production has been shown to link the AT₂ receptor to neuronal activation in PC12W and NG108-15 cells. In these cells, AT₂ negatively cross-talks with N-methyl-D-aspartate (NMDA) receptors, leading to attenuation of NMDA-induced increase in cGMP levels and/or NO release (Schelman et al. 1997).

4.5 Renal Function

In the kidney, the AT₂ receptor counteracts the anti-natriuretic and pressor effects mediated by the AT₁ subtype (Siragy et al. 1999). In conditions of elevated Ang II such as sodium depletion, AT₂ mediates a renal vasodilator cascade in-

cluding generation of bradykinin, NO, and cGMP (Carey et al. 2000), a response partially mediated by neural NOS in addition to other NOS isoforms (Siragy and Carey 1997). A similar AT_2 signaling pathway involving NO and cGMP production has been described in the gastro-intestinal tract (Carey et al. 2000) and AT_2 has also been shown to increase aortic cGMP by a kinin-dependent mechanism in stroke-prone spontaneously hypertensive rats (SHRSPs) (Gohlke et al. 1998).

In isolated microperfused rabbit preglomerular afferent arteriole, activation of the AT_2 receptor causes endothelium-dependent vasodilation, and this effect is mediated by cytochrome P450 epoxygenase metabolites of arachidonic acid (AA) rather than by the NO pathway (Kohagura et al. 2000).

Thus, although triggering different cellular responses, renal and neuronal AT_2 receptors are linked to similar signaling pathways, i.e., regulation of NO/cGMP and AA metabolite production. Extensive studies by J. Douglas group have shown AT_2 -mediated activation of PLA2 and generation of arachidonic acid metabolite in rabbit kidney proximal tubule epithelial cells (Jacobs and Douglas 1996; Harwalkar et al. 1998). Of interest, AT_2 -mediated activation of PLA2 in these cells leads to activation of MAP kinase (Dulin et al. 1998) and p21ras, via a Shc-Grb2-SOS pathway usually associated with receptor tyrosine kinase (RTK) activation (Jiao et al. 1998).

5

Negative Cross-Talk Between AT_2 and AT_1 Receptors

Although showing distinct patterns of expression, the AT_1 and AT_2 receptors have been shown to be expressed within the same cell type. Their relative contribution to the effects of Ang II is therefore of particular importance in the light of the recent utilization of anti- AT_1 receptor antagonists as potent anti-hypertensive drugs. Functional negative cross-talk between AT_1 and AT_2 receptors on growth and hypertrophy, cardiovascular and neuronal functions has been demonstrated (Masaki et al. 1998; Sumners and Gelband 1998; Horiuchi et al. 1999b; Unger 1999; Stoll and Unger 2001). Obviously, negative cross-talk between AT_1 and AT_2 receptors is a consequence of opposite signaling pathways, but another mechanism, i.e., receptor heterodimerization, has recently been shown to participate in AT_2 -mediated antagonism of AT_1 function.

5.1

Opposite Signaling Pathways

The mitogenic AT_1 receptor activates several independent phosphorylation cascades, including MAP kinase (ERK and JNK), JAK/STAT, and PI3-kinase/Akt pathways, which are inactivated following stimulation of the AT_2 receptor. ERK1 and ERK2 seem to be a major target of the AT_1/AT_2 cross-talk, as AT_2 inhibits AT_1 -activated ERK in a variety of cellular models through activation of either SHP-1, PP2A, or MKP-1 phosphatases (Nouet and Nahmias 2000). The ERK cas-

cade was originally shown to be activated by receptor tyrosine kinases. As for other GPCRs, AT₁-mediated activation of ERK at least partly results from *trans*-activation of receptor tyrosine kinases, in particular the EGF receptor (Hackel et al. 1999). The observations that AT₂ inhibits autophosphorylation of insulin receptors and EGF receptors (Elbaz et al. 2000; Shibasaki et al. 2001; DePaolis et al. 2002) and that AT₂ interacts with ErbB3, a member of the EGFR family (Knowle et al. 2000), therefore suggest that RTKs may constitute important cross-points between AT₁ and AT₂ signaling pathways at the level of the membrane. Other cross-points, however, have been identified downstream of membrane receptors, since AT₂ mediates dephosphorylation of STAT1, STAT2, and STAT3 with no effect on JAK activity (Horiuchi et al. 1999a), inhibition of JNK with no effect on Pyk2 (Matsubara et al. 2001), and inhibition of PI3-kinase and Akt with no effect on IRS-1 nor on the insulin receptor (Cui et al. 2002).

5.2

Heterodimerization

Increasing evidence indicates that, like other families of membrane receptors, GPCRs are able to self-assemble in the membrane, and that homo- and heterodimerization is a novel mechanism controlling GPCR activation and regulation (Salahpour et al. 2000). Homodimerization of AT₂ receptors in human myometrium has been suggested by cross-linking experiments even before the cloning of the AT₂ cDNA (Lazard et al. 1994b). Recent studies conducted by the U. Quitterer group (AbdAlla et al. 2001) have demonstrated heterodimerization between AT₁ and AT₂ receptors, leading to attenuation of the AT₁ signaling cascade in PC12 cells, fetal fibroblasts, and in human myometrial biopsies. AT₁ antagonism induced by the AT₂ receptor does not involve sequestration of G α proteins, and is rather mediated by direct receptor interaction independently of ligand binding and signaling by the AT₂ receptor. AT₁/AT₂ heterodimerization, being agonist-independent, is mainly regulated by the expression level of AT₂, unraveling the importance of AT₂ receptor transcriptional and post-transcriptional regulation in the AT₁/AT₂ cross-talk. The AT₂ receptor can thus be considered as a natural antagonist of the AT₁ subtype, illustrating a novel mode of negative communication between the two subtypes of Ang II receptors

6

AGTR2 Gene Alterations Associated with Human Diseases

In agreement with the widely described effect of AT₂ receptor in growth, development, and neuronal activation, two studies have reported alterations of the AT₂ receptor gene (AGTR2) that were associated with human diseases: (1) congenital anomalies of the kidney and urinary tract (CAKUT) and (2) X-linked mental retardation.

6.1

Congenital Anomalies of the Kidney and Urinary Tract

One interesting phenotype of AT₂ receptor-null mice includes anomalies of kidney development that strikingly mimic various features of human CAKUT (Nishimura et al. 1999). Establishment of these anomalies in the AT₂ knockout mice is preceded by delayed apoptosis of mesenchymal cells that surround the urinary tract during ontogeny. Detailed analysis of human AGTR2 gene in male Caucasians revealed a significant association between CAKUT and a nucleotide transition (A1332 to G) in the first intron of the gene. Splicing of AT₂ mRNA resulting from the G allele was found to be abnormal—lacking exon 2—and inefficient, suggesting that a single A/G mutation in the first intron may simulate the effect of AT₂ gene knockout (Nishimura et al. 1999).

The human AGTR2 gene is located on chromosome X (Lazard et al. 1994a) and comprises three exons that span more than 5 kb of genomic sequence (Martin and Elton 1995). The entire coding region of the AT₂ receptor gene is contained in the third exon, the first two exons being untranslated. Studies by Warnecke et al. have revealed the presence of enhancer intronic elements in the first intron of AGTR2 that are necessary for efficient transcription of the gene (Warnecke et al. 1999b). In human heart, exon 2 is alternatively spliced and the major AT₂ transcript is that containing exons 1, 2, and 3. Of interest, exon 2 has an inhibitory effect on reporter gene expression, indicating possible regulation of AT₂ receptor expression *in vivo* by differential splicing (Warnecke et al. 1999a).

Alterations in the AGTR2 gene leading to abnormal transcription of AT₂ mRNA may thus be responsible for developmental anomalies of the kidney. In recent studies, however, no correlation was found between A/G transition in the first intron of AGTR2 and the occurrence of CAKUT in a population of 66 Japanese male patients (Hiraoka et al. 2001) nor between A1332/G transition and the pathogenesis of primary familial vesicoureteral reflux (Yoneda et al. 2002).

6.2

X-Linked Mental Retardation

A recent study has reported alterations in the sequence of the AGTR2 gene in several cases of X-linked mental retardation (MR) (Vervoort et al. 2002). One female patient with a chromosomal (X;7) translocation showed complete extinction of AT₂ receptor mRNA, although the chromosomal breakpoint occurred outside the AGTR2 gene. Further examination of males from families with possible X-linked MR revealed a deletion of one thymidine at nucleotide position 395, causing a frameshift at position Phe133 in the tm3 and resulting in a truncated protein (Fig. 1) in two affected males within the same family and one unrelated patient. In addition, three missense mutations in the coding region of AGTR2: a (Gly21/Val) substitution in the N-ter portion of the AT₂ receptor, and Arg324/Gln and Ile337/Val mutations in the C-terminal portion of the receptor

were detected in seven sporadic patients with MR (Fig. 1). Although the functional consequences of these mutations on AT₂ receptor activation and regulation have not yet been examined, it is interesting to note that all mutations are located in regions that have been shown to be involved in ligand binding or signaling (Fig. 1). These findings are consistent with other studies showing (1) expression of the AT₂ receptor mRNA in developing and adult brain (Nuyt et al. 1999), (2) a role for AT₂ in nerve regeneration, and neuronal differentiation and activation, and (3) a possible role for AT₂ in behavior and cognitive functions (Hein et al. 1995; Ichiki et al. 1995; Okuyama et al. 1999; Braszko 2002), further supporting the hypothesis that the AT₂ receptor may play a role in human brain development and/or trophic maintenance of neuronal connections important for learning and memory.

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AT₂ Receptor of Angiotensin II and Cellular Differentiation

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Abstract Since its discovery, the AT₂ receptor of angiotensin II has been one of the most controversial G protein-coupled receptors. The AT₂ receptor is widely distributed in the fetus, but in most tissues, its expression is dramatically diminished few hours after birth. These observations have led to the hypothesis that this receptor may play an important role during fetal development. During the last decade, many studies have been conducted to elucidate the role of the AT₂ receptor in many different tissues and cell lines. Apart from a well-described action in cell apoptosis, one of the major roles attributed to the AT₂ receptor of angiotensin II is its involvement in cellular differentiation. The AT₂ receptor is involved in differentiation of many tissues. For example, in cells from neuronal origin, activation of the AT₂ receptor was shown to induce neurite outgrowth and elongation, to modulate neuronal excitability, and to promote cellular migration. In steroidogenic tissues, the AT₂ receptor is associated with the

development of the human fetal adrenal gland, where it induces apoptosis and probably cell migration, as well as of granulosa ovarian cells, where it promoted ovulation and oocyte maturation. The AT₂ receptor is also transiently expressed in smooth muscle cells where it may play a role in vasculogenesis, and in adipocytes where it induces production of prostacyclin (PGI₂) involved in preadipocyte differentiation. This chapter describes in detail how the AT₂ receptor of angiotensin II acts on cellular differentiation, with a particular emphasis on neuronal differentiation. Indeed, signaling mechanisms involved in the AT₂ effects are various and can be classified as atypical. For instance, AT₂ receptor activation promotes mitogen-activated protein kinase (MAPK) cascade through a p21^{ras}-independent pathway, and stimulates, in a parallel way, nNOS/cGMP/PKG signaling; both are essential to promote neurite outgrowth.

Keywords AT₂ receptor · Angiotensin · Cellular differentiation · Signaling mechanisms · MAPK cascade · Neurite outgrowth · Fetal adrenal · NG108-15 cells

1

AT₂ Receptor Expression in Fetal Tissues

One of the most remarkable features of the AT₂ receptor is its high level of expression in many fetal tissues (Shanmugam et al. 1995; Tanaka et al. 1995; Breault et al. 1996; Schütz et al. 1996), including the brain (Grady et al. 1991; Millan et al. 1991; Tsutsumi et al. 1991) (see chapter by Nishimura, this volume), suggesting an involvement of the AT₂ receptor in fetal development. Indeed, involvement of the AT₂ receptor has been documented in different models of differentiation such as morphological neuronal differentiation, steroidogenesis in gonads and in adrenal gland, contractility in smooth muscle cells, and prostaglandin production in adipocytes. The most important features of these events will be reviewed in this chapter.

2

AT₂ Receptor and Induction of Neuronal Differentiation

Due to its high level of expression in the neonatal brain and in some area of the adult brain involved in cognition and behavior, it was hypothesized that the AT₂ receptor was important in neuronal development and in the formation and/or maintenance of neuronal connections. Both the development and differentiation of cells of neuronal origin have been extensively studied and were shown to involve four different, essential stages of cellular evolution. These processes include cell growth, cell migration, neurite outgrowth, and synaptogenesis, all of which are controlled by the extracellular environment (hormones, growth factors, neurotransmitters, the extracellular matrix, cell adhesion molecules, and electrical activity).

2.1 Neurite Outgrowth

Over the last decade, evidence has been obtained that binding of angiotensin II (Ang II) to its AT₂ receptor modulates at least one of the above-mentioned criteria for neuronal differentiation, the induction of neurite outgrowth. Ang II-induced morphological changes have been studied in NG108-15 cells (a neuroblastoma-glioma hybrid cell line) and PC12 W cells (cell line derived from rat pheochromocytoma), two cell lines which express only the AT₂ receptor, or in primary cultures of rat cerebellar granule cells, in which the two types of receptors, AT₁ and AT₂ are present (Côté et al. 1999) (Fig. 1E, F and Fig. 3A). Three-day treatment of non-differentiated NG108-15 cells (Laflamme et al. 1996) or PC12 W cells (Meffert et al. 1996) with 100 nM Ang II induces morphological differentiation as characterized by the outgrowth of neurites (Fig. 1A-D).

Neurite extension is initiated at the growth cone and involves several biochemical steps directed towards promoting the assembly of tubulin monomers into microtubules necessary to support the growing neurites (Laferrriere et al. 1997). Several molecules play crucial roles in regulating neurite outgrowth and differentiation. Among these are the microtubule-associated proteins (MAPs), which include MAP1B, MAP2, and tau (Matus 1988; Smith 1994). These proteins promote tubulin polymerization, stabilize microtubules, and exhibit embryonic and adult isoforms, whose differential expression during brain development correlates with the maturation of neuronal circuitry. For example, MAP2 and tau bind to distinct populations of microtubules in adult neurons: MAP2 to somatodendritic microtubules and tau to axonal microtubules (Matus et al. 1981; Binder et al. 1984). In NG108-15 cells, neurite outgrowth induced by Ang II stimulation of the AT₂ receptor is correlated with an increase in the level of polymerized tubulin and in the levels of the microtubule-associated protein, MAP2c (Laflamme et al. 1996). Mediation by the AT₂ receptor may be inferred since these cells contain only AT₂ receptors; these effects are mimicked by CGP 42112 (an AT₂ receptor agonist); they are not suppressed by addition of DUP 753 (an AT₁ receptor antagonist) but are abolished by co-incubation with PD 123319 (an AT₂ receptor antagonist). In rat pheochromocytoma PC12 W cells, similar neurite elongation was observed upon Ang II stimulation, and was also associated with an increase in MAP levels and/or association with microtubules, in particular MAP2 and MAP1B (Meffert et al. 1996) but a decrease in the level of neurofilament middle molecular weight subunit, NF-M (Gallinat et al. 1997; Stroth et al. 1998).

The effects of Ang II, through the AT₂ receptor, on neurite elongation have also been observed in more physiological models, such as postnatal retinal explants (Lucius et al. 1998) and microexplant cultures of cerebellum (which contain both AT₁ and AT₂ receptors) (Côté et al. 1999). In the latter, Ang II treatment, through the AT₂ receptor localized in neurons, strongly increased β III-tubulin polymerization, a neuron-specific tubulin isoform (Matus 1988), and increased expression and association of phosphorylated forms of MAP2 (isoforms a, b and c) and tau (Côté et al. 1999). These findings confirm that AT₂ receptor

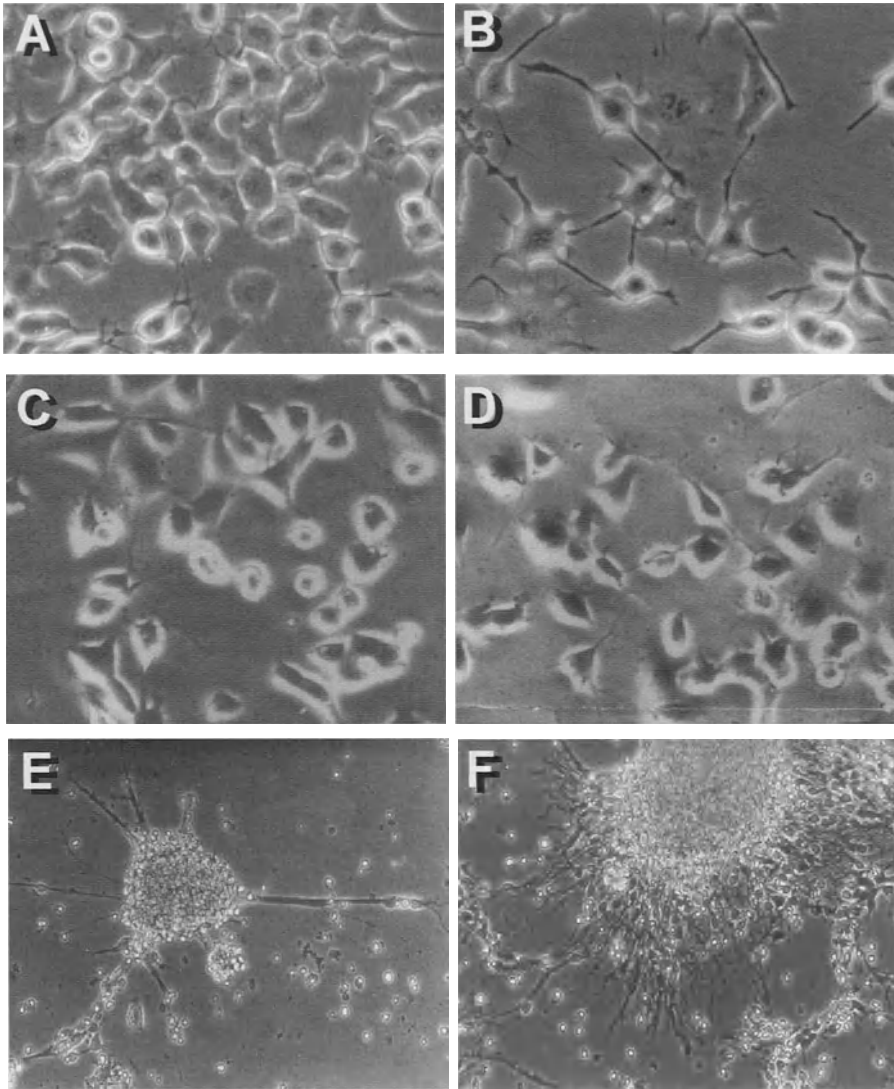


Fig. 1A–F Phase-contrast morphology of angiotensin II-treated cells of neuronal origin. Cells were cultured in media containing 10% fetal calf serum (FCS) for the NG108-15 cells (**A, B**), 0.5% FCS for the PC12 W cells (**C, D**) or neurobasal medium supplemented with B27 for cerebellar microexplants (**E, F**). Cells were cultured in the absence (**A, C, E**) or in the presence of 100 nM Ang II (**B, D, F**) over 3 days for the NG108-15 cells, 5 days for the PC12 W cells and 4 days for cerebellar microexplants. (Reproduced with permission from Gendron et al. 2002; (**A, B**); Stroth et al. 1998 (**C, D**); and Côté et al. 1999 (**E, F**))

stimulation activates all the components involved in the process of neurite elongation. Several developmental studies indicate that increases in tau and MAP expression precede that of tubulin, suggesting that the primary effect of the AT₂ receptor activation may be on tau and MAP2 rather than on tubulin itself.

Thus, alone, AT₂ receptor activation promotes neurite outgrowth and specific phosphorylation/association of MAPs with tubulin. However, in conditions where the AT₁ receptor is also present, such as in dibutyryl cAMP (dbcAMP)-treated NG108-15 cells [a well-known differentiating factor for NG108-15 cells (Hamprecht et al. 1985; Beaman-Hall et al. 1993; Laflamme et al. 1996)], or in cerebellum (Côté et al. 1999), activation of AT₁ receptor inhibits the effect produced by the AT₂ receptor. These observations further illustrate the well-described negative cross-talk interaction between the two types of Ang II receptors (for reviews see Inagami et al. 1999; Unger 1999; Stoll et al. 2001).

2.2 Cell Migration

In addition to elongation, Ang II application in cerebellar microexplants induces a marked cell migration observed at the center of the microexplant moving towards the periphery (Côté et al. 1999) (Fig. 1 E, F). Similar cell migration is observed during AT₂ receptor-induced regeneration of postnatal retinal microexplants (Lucius et al. 1998). These effects were more pronounced in cells treated with Ang II and DUP 753 or in cells treated with 10 nM of CGP 42112. Moreover, incubation with Ang II and PD 123319 blocked the AT₂ receptor-mediated effect. The study by Côté et al. (1999) confirms the hypothesis raised by Jöhren et al. (1998) that the high expression of AT₂ receptors in the inferior olivary–cerebellar pathway may be associated with a role for the AT₂ receptor in neuronal plasticity and cerebellar development. Indeed, neuronal development and differentiation of the cerebellum involve several steps including proliferation (in the ventricular zone), migration (through the ventricular zone to the cortical zone), and finally either neurite extension or apoptosis, once the cells have reached their specific destination (Cambray-Deakin et al. 1987; Komuro et al. 1998). In the cerebellar microexplants, where both neuronal and glial cells are present, AT₂ receptor activation induces not only neurite outgrowth, a process associated with morphological differentiation, but interestingly, cell migration as well. However, although extensively described for other systems (Yamada et al. 1996; Shenoy et al. 1999), the AT₂ receptors did not induce apoptosis in these cerebellar microexplants. Because the decision between apoptosis versus survival depends on the specific combination of local factors, the effect of Ang II observed in microexplant cultures may be due to interaction of AT₂ receptor signalization with factors locally produced by the mixed population of cells present in the microexplants, such as BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin 3) (Segal et al. 1995) or PACAP (pituitary adenylyl cyclase activating peptide) (Basille et al. 1994; Cavallaro et al. 1996).

2.3 Neuronal Excitability

Neuronal differentiation is characterized by a differential expression of ionic channels resulting in a change in the electrical activity. The T-type Ca²⁺ channel

is involved in protein synthesis, growth, and proliferation and is predominantly present at earlier stages and disappears later (Yaari et al. 1987; McCobb et al. 1989; Kostyuk et al. 1993; Schmid et al. 1999). Activation of the AT₂ receptor by Ang II or CGP 42112 reduced the amplitude of the T-type current in non-differentiated NG108-15 cells by an as-yet-unknown mechanism which involved a phosphotyrosine phosphatase and a pertussis toxin (PTX)-insensitive G protein (Buisson et al. 1992; Buisson et al. 1995). Considering the antiproliferative effect of mibefradil, a selective antagonist of the T-type Ca²⁺ channel (Schmitt et al. 1995), it is tempting to postulate that the AT₂-induced blockage of the T-type current could be involved in Ang II-dependent NG108-15 differentiation. The transient K⁺ current I_A and the delayed-rectifier K⁺ current I_{KV} are enhanced by AT₂ receptor activation (Kang et al. 1993). This effect on K⁺ current results in the shortening of both action potential duration and refractory period, leading to an increased firing rate (Zhu et al. 2001). Increased membrane excitability is one of the fundamental characterizations of neuronal development during embryonic and postnatal ages (Fitzgerald 1987; Gao et al. 1998), particularly for the establishment of synaptic transmission (Gao et al. 1998). It is interesting to note that the decrease in the T-type current amplitude and enhancement of K⁺ currents' amplitude, both occurring during differentiation, are mimicked by activation of the AT₂ receptor (for more details, see chapter by Diez in the next volume).

An inhibition of the visual response to flash stimulation was reported in rat superior colliculus (Merabet et al. 1997). This inhibition was mainly attributed to AT₁ receptor but a slight effect was also observed with CGP 42112. Furthermore, activation of the AT₂ receptor depresses glutamate depolarization and excitatory postsynaptic potentials in locus coeruleus (Xiong et al. 1994). However, AT₂ receptors have an excitatory effect on inferior olivary neurons (Ambühl et al. 1992). All together, these results indicate that the AT₂ receptor is able to modulate several different types of currents, but it is not yet known if one common or several signaling pathways are used.

During differentiation, microtubules are characterized by phases of rapid elongation and shortening. This dynamic instability is regulated by phosphorylation–dephosphorylation of microtubule-associated proteins (Maccioni et al. 1995; Sanchez et al. 2000), by environmental cues (Komuro et al. 1998) often targeted at the growth cone level for the axon (Letourneau 1996; Williamson et al. 1996), by calcium transients and electrical activity (Kater et al. 1991; Gomez et al. 2000; Spitzer et al. 2000). All these phenomena are stimulated by the AT₂ receptor.

2.4

Mechanisms Involved in AT₂-Induced Neurite Outgrowth

As indicated above, the association/dissociation of MAPs to microtubules is rapid and dynamic and is dependent on MAPs' phosphorylation states. Each MAP can be phosphorylated on multiple Ser/Thr and Tyr residues by the activation

of different protein kinases such as glycogen synthase kinase (GSK3 β), p42/p44^{mapk}, cyclin-dependent protein kinases (CDKs), cAMP- or calcium-dependent protein kinases (respectively, PKA and PKC) or dephosphorylated by the activation of different phosphatases (PP1, PP2A) (Sanchez et al. 2000). Moreover, MAP-2 is the main substrate for ERK1 and ERK2 while tau is phosphorylated by several kinases, including proline-directed serine/threonine protein kinase (Avila et al. 1994). Over the past 5 years, substantial amounts of data have been obtained, using both biochemical and morphological approaches to determine how Ang II could induce such differentiation processes.

Activation of the AT₂ receptor has been shown to modulate many signaling events including the activation/inhibition of kinases and phosphatases (Horiuchi et al. 1999; Nishimura et al. 1999; Unger 1999) (see chapter by Balt and Pfaffendorf, this volume). In NG108-15 cells cultured in the presence of 10% fetal bovine serum, Ang II (or CGP42112) application induced a time-dependent modulation of tyrosine phosphorylation of several proteins. In particular, Ang II induced a sustained increase in the activities of p42 and p44 MAPK (p42^{mapk} and p44^{mapk}). This delayed-but-sustained activation of p42/p44^{mapk} was shown to be essential for Ang II to promote neurite outgrowth and elongation. Accordingly, when cells were treated with a specific inhibitor for MEK1 (PD98059, 10 μ M), the Ang II-induced p42/p44^{mapk} activity and neurite outgrowth were completely abolished (Gendron et al. 1999). Supporting these observations, Stroth and co-workers (2000) then demonstrated that the Ang II-induced neuronal differentiation of PC12 W cells was also dependent on p42/p44^{mapk} activation. Again, in this model, PD 98059 was sufficient in blocking Ang II's effects on neurite outgrowth and on p42/p44^{mapk} activation (Stroth et al. 2000). Thus, p42/p44^{mapk} plays an important role in the regulation of the mechanisms involved in Ang II-induced neuronal differentiation.

Gendron et al. (1999) demonstrated that, during the course of p42/p44^{mapk} activation by Ang II, p21^{ras} activation was inhibited. Reinforcing these observations, cells transfected with the dominant negative form of the Ras protein, RasN17, exhibit an increased basal state of p42/p44^{mapk} activation, which remains sensitive to Ang II stimulation. These results indicate that p21^{ras} inhibition alone activates p42/p44^{mapk} and that Ang II stimulation further increases this activity, by a p21^{ras}-independent mechanism. Among the alternative pathways reported to activate p42/p44^{mapk} independently of p21^{ras}, the nitric oxide (NO) signaling cascade and the Rap1/B-Raf signaling cassette are the most extensively described. The former is known to be activated by the AT₂ receptor, both in the kidney (Siragy et al. 1996; Siragy et al. 1997; Carey et al. 2000) and in NG108-15 cells (Schelman et al. 1997; Côté et al. 1998). Gendron et al. (2002) have shown that the increase in cyclic guanosine monophosphate (cGMP) was consecutive to a rapid increase in NO synthase (NOS) activity induced by dephosphorylation of neuronal NOS (nNOS). This increased activity did not involve modification in expression of nNOS. This effect, specifically stimulated by Ang II via the AT₂ receptor, is mediated by a PTX-sensitive Gi protein. However, cGMP is not involved in Ang II-induced activation of p42/p44^{mapk}. Indeed, the

blockade of any protein from this signaling cascade (NOS), soluble guanylyl cyclase (sGC), or cGMP-dependent protein kinase (PKG) failed to interfere with the effects of Ang II on the p42/p44^{mapk} cascade of signaling (Gendron et al. 2002). In summary, these investigators stated that the activation of p42/p44^{mapk} cascade is independent of NO production, at least in NG108-15 cells.

As recent observations obtained in developing brain or in neuronal cell lines have indicated that NO could be involved in morphological neuronal differentiation (Peunova et al. 1995; Poluha et al. 1997; Sheehy et al. 1997) and in migration of neurons (Wright et al. 1998), this same group further investigated if NO could be involved in the AT₂ receptor mechanism of action in the induction of morphological neuronal differentiation of NG108-15 cells. Daily application of dbcGMP was sufficient to induce neurite outgrowth, suggesting that NO/sGC/cGMP pathway is involved in the AT₂-mediated differentiation. In agreement with these observations, inhibition of sGC or PKG (LY83583 or methylene blue and KT5823, respectively) impaired Ang II effects on neurite elongation. However, fine morphological comparison between Ang II- and dbcGMP-stimulated NG108-15 cells revealed that cGMP itself was responsible for neurite branching and filopodia formation, while PKG was necessary for elongation. Indeed, preincubation of the NG108-15 cells with KT5823 blocked Ang II-induced elongation, but did not interfere with branching and filopodia formation. Such observations corroborate results obtained by Phung et al. (1999) that cGMP and PKG have distinct effects on differentiation of PC12 cells. Together, these results indicate that p42/p44^{mapk}, NO/cGMP, and PKG have independent but complementary effects in the induction of dynamic neurite outgrowth and neurite branching. Axonal branches may be regulated by a cGMP-dependent pathway. Such collateral branches are important in the establishment and refinement of neuronal connections during development (Gallo et al. 2000).

What remains unclear at this point is the initial events linking AT₂ receptor activation and the increase in p42/p44^{mapk} activity. In a recent study (Gendron et al. 2003), we demonstrated that the activation of the AT₂ receptor rapidly, but transiently, activated the Rap1/B-Raf complex of signaling proteins. In RapN17- and Rap1GAP-transfected cells, the effects induced by Ang II were abolished, demonstrating that activation of these proteins was responsible for the observed p42/p44^{mapk} phosphorylation and for morphological differentiation. In several models—such as PC12 cells—cAMP and cAMP-activated proteins are required for nerve growth factor (NGF) activation of Rap1, as well as for p42/p44^{mapk} (York et al. 1998). To assess whether cAMP was involved in the activation of Rap1/B-Raf and neuronal differentiation induced by Ang II, NG108-15 cells were treated with stimulators or inhibitors of the cAMP pathway. However, dbcAMP and forskolin did not stimulate Rap1 nor p42/p44^{mapk} activities. Furthermore, addition of H-89, an inhibitor of protein kinase A, or Rp-8-Br-cAMPS, an inactive cAMP analog, failed to impair p42/p44^{mapk} activity and neurite outgrowth induced by Ang II. These observations clearly indicate that cAMP, a well-known stimulus of neuronal differentiation, do not participate in the AT₂ receptor signaling pathways in the NG108-15 cells. Therefore, the AT₂ receptor of Ang II ac-

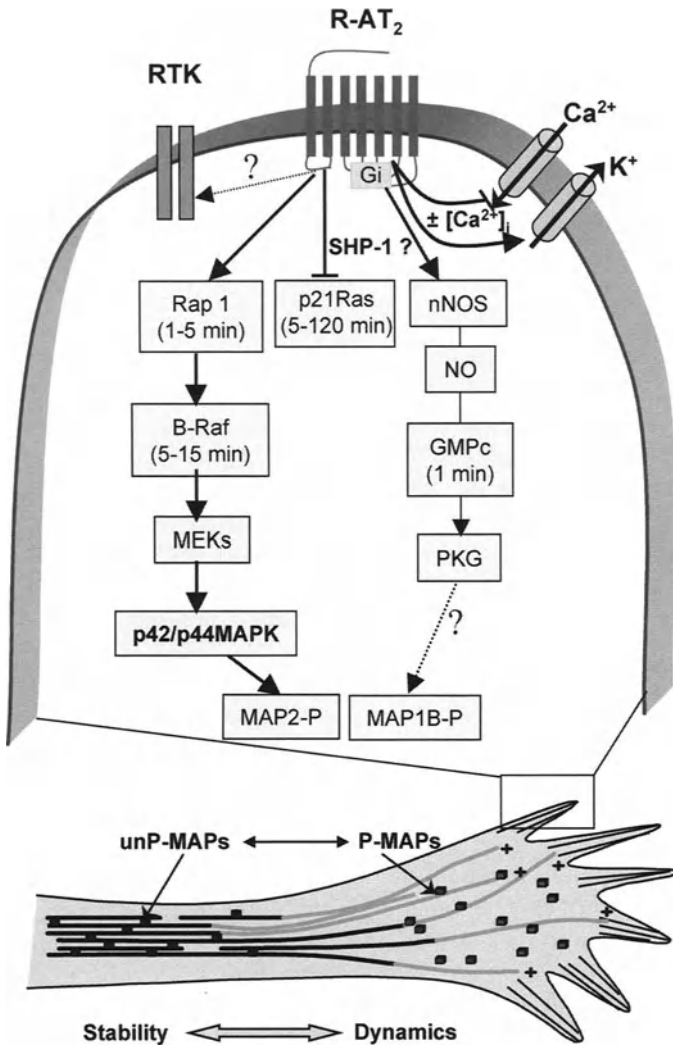


Fig. 2 Schematic representation of the AT₂ receptor signaling mechanisms involved in neurite elongation. The Ang II AT₂ receptor induction of neurite outgrowth and elongation involves at least two pathways. After binding of Ang II, the activated AT₂ receptor rapidly inactivates p21^{ras} but stimulates the Rap1-B-Raf pathway, leading to a delayed p42/p44^{mapk} phosphorylation. A second pathway involving the NO/sGC/cGMP signaling cascade is also required for observable neurite outgrowth. In addition, AT₂ receptor modulates intracellular calcium concentration ([Ca²⁺]_i) by inhibiting Ca²⁺ channels and activating K⁺ channels. Together, these parallel pathways could modulate gene expression and the phosphorylation state of different microtubule-associated proteins (MAP2, MAP1B) to control microtubule stability/dynamics responsible for neurite elongation. *nNOS*, neuronal NOS (nitric oxide synthase); *NO*, nitric oxide; *sGC*, soluble guanylyl cyclase; *MEK*, mitogen-activated protein kinase kinase; *unP-MAPs*, unphosphorylated MAPs; *P-MAPs*, phosphorylated MAPs. (The lower panel is adapted from Gordon-Weeks 1991)

tivates the signaling modules of Rap1/B-Raf and p42/p44^{mapk} via a cAMP-independent pathway to induce morphological differentiation of NG108-15 cells. Thus, how Rap1 could be activated in this model remains unknown.

Several questions still remain to be answered in regards to the AT₂ receptor. However, in agreement with other reported results, some hypotheses could be raised. For example, the effect of Ang II on neuronal differentiation and on stimulation of p42/p44^{mapk} activity are clearly observed when cells are cultured in the presence of serum, suggesting that a growth factor-promoting effect is required in the signaling mechanism of the AT₂ receptor. It is also not yet clear how NOS is dephosphorylated by Ang II; however, a role for SHP-1 could be envisaged (Bedecs et al. 1997; Feng et al. 2002) (see chapter by Balt and Pfaffendorf, this volume). Another alternative may be through interaction between the AT₂ receptor and the bradykinin receptor. Indeed, it is already known that bradykinin mediates AT₂ receptor-induced NO production (Siragy et al. 1996; Gohlke et al. 1998; Searles et al. 1999). In addition, heterodimerization between the bradykinin (B2) receptor and the AT₁ receptors has been demonstrated (AbdAlla et al. 2000). Similar effects could be envisaged in NG108-15 cells, where BK receptors are expressed (Chiang et al. 1989; McIntyre et al. 1993). In addition, the AT₂ subtype has been shown to interplay with members of the EGF-receptors family or with insulin receptors (Elbaz et al. 2000; Knowle et al. 2000).

All these observations concerning AT₂ receptor signaling involved in neurite outgrowth are summarized in Fig. 2. After binding of Ang II, the activated AT₂ receptor induces a sustained activation of p42/p44^{mapk} cascade and an increase in NO and cGMP content. The initial events, at least in NG108-15 cells, involve inactivation of p21^{ras} activity (5–120 min), but a rapid activation of Rap1 (1–5 min). Activated Rap1 then enhances the activity of B-Raf (5–15 min) that in turn stimulates p42/p44^{mapk} phosphorylation (30–60 min). Finally, the return of p42/p44^{mapk} phosphorylation to basal level, occurring much later (seen after 120 min of Ang II treatment), may be under a phosphotyrosine phosphatase activity such as SHP-1, shown to be activated after the AT₂ receptor stimulation. In parallel, the initial effect of AT₂ on the dephosphorylation of the nNOS (thus activation of nNOS) may be subsequent to the activation of the phosphotyrosine phosphatase SHP-1. All these events could occur at the end of the dendrite (or in the growth cone for the axon), where elongation, retraction, and pathfinding are initiated. Together, p42/p44^{mapk}, cGMP, and PKG could regulate gene expression and modulation of the phosphorylation states of different microtubule-associated proteins (MAPs) such as MAP2, tau, and MAP1b.

2.5

Conclusion for the Involvement of AT₂ in Neuronal Differentiation

In conclusion, the results reported to date indicate that AT₂ receptor activation promotes and/or accelerates all the processes involved in morphological differentiation since, its stimulation (1) increases polymerization of the β III-tubulin, the specific neuronal isoform, (2) increases tau expression, thereby increasing

axonal outgrowth, (3) increases MAP2c and MAP1B expression and phosphorylation, promoting polymerization of unstable microtubules, (4) decreases expression of NF-M protein, affecting stability to favor plasticity, (5) stimulates cell migration and, finally, (6) modulates membrane excitability. In addition, AT₂ effects are antagonized by the AT₁ receptor, indicating that AT₁ and AT₂ receptors have opposite actions on neuronal differentiation.

These observations clearly demonstrate the involvement of the AT₂ receptor in the fine tuning of neuronal differentiation and cell migration, two very important events occurring during brain development. Thus, both AT₁ and AT₂ receptor types may participate, together with other environmental growth factors, adhesion molecules and the components of the extracellular matrix, in the differentiation of brain areas expressing these receptors.

Lessons from knockout mice or from neurological disorders reinforce the idea that AT₂ receptor may be important for neuronal development. Indeed, perturbations in exploratory behavior and locomotor activity have been observed (Hein et al. 1995; Ichiki et al. 1995), as well as an anxiety-like behavior (Okuyama et al. 1999). In addition, a decrease in the expression of the AT₂ receptor is observed in areas of the adult brain, implicated in the development of neurological disorders such as Alzheimer disease, Huntington disease, or Parkinson disease (caudate nucleus, putamen and substantia nigra, temporal cortex) (Ge et al. 1996). Finally, recent observations indicate that *AGTR2* mutations are correlated with mental retardation (Vervoort et al. 2002). Together, such observations indicate inappropriate neuronal differentiation and plasticity (in adult) when AT₂ is absent or genetically modified.

3 Differentiation of Steroidogenic Tissues

3.1 AT₂ Receptors in the Adrenal Gland

Among the peripheral tissues which express AT₂ receptors are the adrenal glands in rats, ovine, and human (Aguilera et al. 1994; Shanmugam et al. 1995; Wintour et al. 1998). In the human fetal adrenal gland, immunocytochemical studies revealed that AT₂ receptor labeling is predominant in the fetal zone (identified using P450C17 as a fetal zone marker) and in a population of cells having a radial disposition, which could correspond to the future chromaffin cells, as attested by chromogranin A and dopamine β -hydroxylase reactivities (Breault et al. 1996) (Fig. 3B). Labeling was also present around the central medulla vein.

The human fetal adrenal gland is morphologically and functionally different from both the adult adrenal gland and fetal gland of non-primates. Morphological evidence indicates that the human fetal adrenal gland is a very dynamic organ, in which proliferating cells located at the periphery migrate, then differentiate, and finally undergo senescence in the central part of the gland. The func-

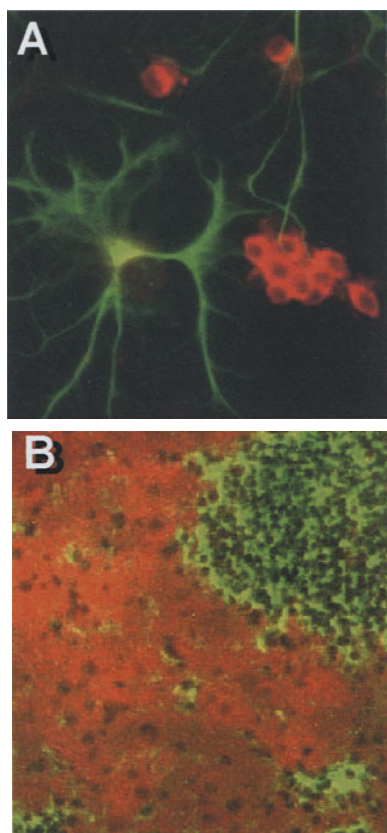


Fig. 3A, B Immunofluorescence localization of AT_2 receptor in cerebellar granule cells and in human fetal adrenal gland. Primary cultures of cerebellar microexplants or frozen sections of an 18-week-old human fetal adrenal gland were processed for immunofluorescence labeling using a primary antibody directed against the AT_2 receptor, and visualized after incubation with a secondary anti-rabbit-coupled antibody. In cerebellar microexplants, AT_2 receptor (labeled with rhodamine, red) was identified in neurons but not in astrocytes (labeled with anti-gial fibrillary acidic protein labeled with fluorescein, green; **A**). In the fetal adrenal gland, AT_2 receptor (labeled with fluorescein, green) was identified in fetal cells and in islets of chromaffin cells (upper right). Visualization of cellular composition has been achieved using Evans's blue solution (orange; **B**). The anti- AT_2 receptor antibody was a gift from Dr. Ian Bird, Department of Obstetrics and Gynecology, University of Wisconsin, Madison, USA

tional role of the fetal adrenal gland is to produce large amounts of DHEA/DHEAS (dihydroepiandrosterone and its sulfated derivative) (from the fetal zone). In contrast, cortisol secretion is absent during the first 15 weeks of gestation, and appears in a zone called transitional zone, visible between the fetal and definitive zones (Mesiano et al. 1997).

Data by Chamoux et al. (1999) indicate that the high expression of AT_2 receptors in the fetal zone may be correlated with the high level of apoptosis present in this area of the adrenal gland during development. Indeed, in human fetal adrenal cells cultured for 24 h, Ang II, via the AT_2 receptor, induced DNA fragmentation and cleavage of the DNA repair enzyme, poly-(ADP-ribose) polymerase (PARP), thus corroborating numerous studies indicating that the AT_2 receptor can induce cell apoptosis (see previous chapter, by Nahmias and Boden, this volume). Furthermore, stimulation with Ang II or CGP 42112 strongly modified the actin network, inducing membrane blebbing and a complete disappearance of the stress fiber network. At that time, actin was localized exclusively along the plasma membrane, with labeling predominant at the base of the bleb formation.

The same group has also shown that the AT₂ receptor overlaps the fibronectin expression pattern in the human fetal adrenal gland (Chamoux et al. 2001), whereas laminin is expressed only at the gland periphery, where the AT₂ receptor is absent, while collagen IV is expressed throughout the fetal gland. Because it is now well accepted that extracellular matrix can induce intracellular signaling or interact with hormonal or growth factor transduction pathways leading to specific cell behaviors such as proliferation, migration, apoptosis, or gene expression (Aplin et al. 1999; Giaccotti et al. 1999), the authors examined Ang II effects on cells cultured on various matrices. Altogether, their results indicate that the extracellular matrix modulates cell behavior and hormonal responsiveness in the human fetal adrenal gland. In particular, collagen IV favors steroid secretion in response to adrenocorticotropin (ACTH) and Ang II stimulation, laminin alone enhances proliferation, and fibronectin favors cell behavior associated with fetal zone cells by enhancing apoptosis and ACTH responsiveness in terms of DHEA/DHEAS secretion.

Interaction between Ang II and matrices is indeed well known (see chapter by Schiffrin, following volume). Chassagne et al. recently confirmed a positive interaction between AT₂ and fibronectin: in this study, stimulation of the AT₂ receptor stopped migration of smooth muscle cells by stimulating the synthesis of fibronectin as well as increasing binding to this matrix (Chassagne et al. 2002). Fisher et al. indicated that activation of the AT₂ receptor enhanced the expression of fibronectin mRNA in endothelial cells (Fischer et al. 2001). These data indicate a strong correlation between fibronectin and AT₂-receptor mediated effects of Ang II in terms of inhibition of proliferation and induced cell apoptosis.

Interestingly, the synergistic effect of the AT₂-receptor/collagen IV on steroid secretion seems to be specific to cytochrome P450C17 activity, since no increase was observed for DHEAS production. This observation is of interest, since DHEA-sulfotransferase activity usually parallels that of P450C17 (Parker et al. 1999). However, in vitro, the effects of collagen IV on Ang II responsiveness are not due to an increase in mRNA expression of the enzyme, but rather to a specific hormonal/environmental regulation of the sulfotransferase and/or lyase activity. In addition, Ang II abrogated expression of the 3- β -hydroxysteroid dehydrogenase (3 β -HSD) mRNA. These results may be compared with the in vivo physiology: P450C17 is largely expressed in the fetal zone, but absent in the periphery, where collagen and laminin are both present. In contrast, 3 β -HSD is present at the periphery where fibronectin is less present. Taken together, these data highlight the intricate regulation of 3 β -HSD onset and P450C17 maintenance in the human fetal adrenal gland, and suggest that AT₂ receptor, together with fibronectin- or collagen-induced events, may influence activity of these enzymes. Interestingly, the G α_{i1-2} and G α_{i3} proteins also exhibit a mirror distribution, with G α_{i3} being localized in fetal cells, as the AT₂ receptor. These results are summarized in Fig. 4, outlining the interactions between hormonal and environmental factors in controlling human fetal adrenal development.

Fibronectin is known to favor proliferation when recognized by its high-affinity integrin receptor $\alpha 5\beta 1$. No typical receptor for fibronectin is expressed in

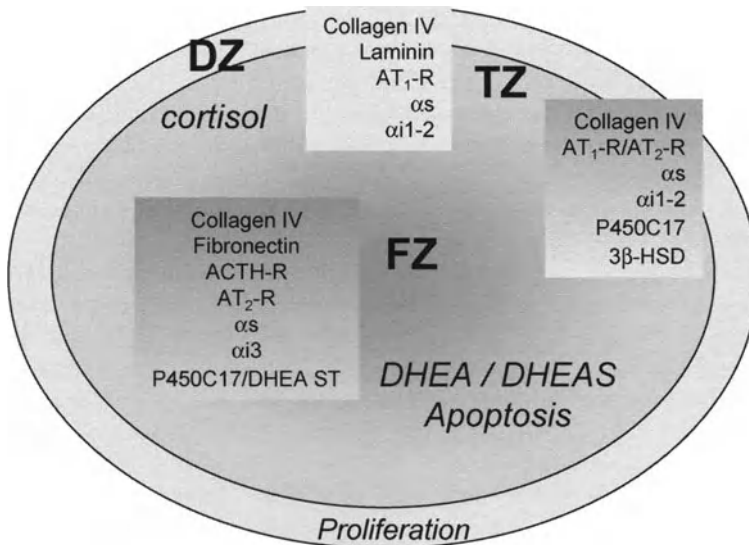


Fig. 4 Summary of the differential distribution of the angiotensin II receptors (AT₁ and AT₂), extracellular matrix components and heterotrimeric G proteins in the human fetal adrenal gland during the second trimester of gestation. Correlation is presented with known steroid production. Collagen IV and Gs are distributed throughout the fetal gland, while laminin is predominant in the definitive zone. Fibronectin is predominant in the fetal zone, as are the AT₂ receptor and the Gα_{i3} protein. DZ, definitive zone; FZ, fetal zone; TZ, transitory zone. (Reproduced with the permission of Chamoux et al. 2002)

the human fetal adrenal gland, but the α₃β₁ integrin, which can serve as a moderate-affinity receptor is present (Kuhn et al. 1994; Chamoux et al. 2001). Recent studies have shown that α₃β₁ disrupts cell–cell adhesion (DiPersio et al. 2000; Kawano et al. 2001), which may be correlated with the migration observed in the fetal zone. The absence of α₅β₁ may also explain why these cells are non-proliferative and are subject to apoptosis. These observations reinforce the notion that extracellular matrix and their receptors/integrins can modulate hormone responsiveness. The AT₂ receptor, which induces various physiological effects, could be particularly sensitive to such interactions.

3.2

Differentiation of Ovarian Granulosa Cells

Autoradiographic studies revealed the intense localization of Ang II receptors present in the granulosa layers of ovaries in rat (Pucell et al. 1991), rabbit (Yoshimura et al. 1996), and human (Johnson et al. 1997). AT₂ receptors are predominantly located in granulosa cells layers of the preovulatory follicles, whereas AT₁ receptors are more concentrated in the thecal cell layers and stroma. Ang II, through AT₂ receptor activation, induced ovulation and oocyte maturation, characterized by meiotic maturation of ovulated ova and follicular oocytes

in the absence of gonadotropin. AT₂ receptor stimulation also stimulated production of prostaglandins (PGE₂ and PGE_{2α}) and estradiol, but not progesterone (Yoshimura et al. 1996). This observation suggests an AT₂ receptor-specific effect on aromatase activity (Yoshimura et al. 1996) or on the passive conversion of Ang II-induced thecal androgen production to granulosa cells estrogen (Pucell et al. 1991). In addition, Johnson et al. (1997), using human granulosa cells, showed that the decrease in progesterone production by activation of the AT₂ receptor was mediated through inhibition of 3β-HSD activity (but did not affect protein expression). In another study, AT₂ receptor expression was discernable only on granulosa cells present in follicle, attaining the tertiary stage of atresia, and representing the advanced process of ovulation and follicle disorganization (Obermuller et al. 1998). At this stage of maturation, the neoexpression of AT₂ receptors counteracts follicle-stimulating hormone (FSH)-stimulated survival signals and induces cell death (Kotani et al. 1999).

These data indicate that AT₂, but not AT₁ receptors for Ang II, play an important role during follicular development and in the ovulatory process. Moreover, as in the adrenal gland, AT₂ receptor activation induces specific steroid production, stimulating pathways involving P450C17 enzyme activity, inhibiting those involving 3β-HSD, and inducing apoptosis as well.

4

AT₂ Receptors in Chromaffin Cells

As mentioned earlier, AT₂ receptors are found in fetal adrenal gland, not only on adrenocortical cells, but also on chromaffin cells (Fig. 3B), as attested by colabeling with chromogranin A and dopamine β-hydroxylase reactivities (Breault et al. 1996). Even if not yet formally demonstrated, a role for the AT₂ receptor in cell migration could be hypothesized, based on observations made in neuronal cells (Lucius et al. 1998; Côté et al. 1999). Indeed, pheochromoblasts originating from the neural crest begin migration throughout the fetal cortex as early as 6 weeks of pregnancy, and progressively colonize the center of the gland leading to the formation of the medulla. Paracrine action of steroids from the fetal cells induce their progressive differentiation into chromaffin cells.

In contrast to most tissues, expression of the AT₂ receptor in chromaffin cells persists in the adult (Martineau et al. 1999; Takekoshi et al. 2000). However, the role of the AT₂ receptor on catecholamine secretion is not yet clearly established. Martineau et al. (1999) found that AT₂ receptor stimulation increases both epinephrine and norepinephrine secretion. Initially, Takekoshi et al. (2001), found an inhibitory effect of the AT₂ receptor; however, more recently, the same group found that CGP42112, as well Ang II, stimulated catecholamine synthesis, through a process involving cGMP-dependent regulation of calcium influx through voltage-dependent Ca²⁺ channels (Takekoshi et al. 2001). The overall proposed hypothesis is that AT₂ stimulation decreases cGMP production, resulting in a reduction of PKG activity which leads to an increase of Ca²⁺ mobilization through voltage-dependent channels, stimulating catecholamine release.

However, the specific target of action, as well as the net effect of AT₂ receptor stimulation on catecholamine secretion, still remains unclear. Because the two types of Ang II receptors are present on chromaffin cells (Ishii et al. 2001), an interaction between the transduction pathways activated by both AT₁ and AT₂ receptors could be envisaged when attempting to explain Ang II action.

5 Differentiation of Smooth Muscle Cells

Yamada et al. (1999) have shown that the AT₂ receptor is transiently expressed during late gestation in the fetal vasculature and that this expression rapidly declines after birth. The authors examined the expression of various differentiation markers for vascular smooth muscle cells in the aorta of wild-type and of AT₂ receptor-null mice. Although there is no change in the expression of α -smooth muscle actin, mRNA levels of calponin and of the high-molecular weight caldesmon (h-caldesmon) are lower in the AT₂ receptor knockout mice than in control, wild-type mice. Their data demonstrated a correlation between time-dependent expression of the AT₂ receptor with that of h-caldesmon and calponin. All three proteins are absent during early development (E13–E15), their expression increases between E18–E20, then declines after 1 day in the neonate. These data suggest that the AT₂ receptor plays a role in the phenotypic differentiation of smooth muscle cells, by acting on cytoskeletal-associated proteins, rather than on actin itself. These proteins, h-caldesmon and calponin, bind actin and inhibit actomyosin ATPase, thus inhibiting contractility. Taken together, the delayed expression of these smooth muscle cells markers suggests that the AT₂ receptor may play some role in vasculogenesis.

6 Differentiation of Adipocytes

Adipose tissue contains differentiated and undifferentiated cells. Preadipocytes contain both AT₁ and AT₂ receptors (Darimont et al. 1994; Mallow et al. 2000). In addition, the AT₂ receptor expression is high in preadipocytes, but is completely lost after differentiation, suggesting a role for AT₂ during cell differentiation (Mallow et al. 2000). Using Ob1771 preadipocyte clonal cell line, Darimont et al. (1994) have shown that Ang II stimulation of AT₂ receptors stimulated the production of prostacyclin (PGI₂), the major metabolite of arachidonic acid in these cells. Through paracrine action, PGI₂ released by these cells is then able to participate in the differentiation of other undifferentiated preadipocytes (Darimont et al. 1994).

7

Conclusion and Perspectives

Altogether, these studies demonstrate that the AT₂ receptors (1) are involved in the modulation of apoptosis and migration, (2) affect expression of specific differentiating markers, such as MAPs in neurons, and (3) affect P450C17 expression in adrenals or caldesmon in smooth muscle cells. Even if initial studies conducted using models of knockout mice for the AT₂ receptor did not reveal major changes in development, more recent studies indicate several perturbations, in addition to the regulation of blood pressure. A role of AT₂ receptor in migration may appear as an important one, since knockout mice for AT₂ receptor present congenital anomalies of the kidney and urinary tract (CAKUT), both in mice and in human, presenting anomalies of the *AGTR2* gene (Nishimura et al. 1999; Miyazaki et al. 2001).

Despite numerous studies, little is known about the initial events associated with the AT₂ receptor, and the overall mechanism of action remains incompletely understood. For many of the cell models described in this chapter, stimulation of the AT₂ receptor induced modifications in cytoskeleton organization. Much of the data in the literature indicate that most of the proteins involved in intracellular signaling cascades are linked to microfilaments or microtubules (Schmidt et al. 1998; Schlaepfer et al. 1999; Nikolic 2002), among which are the heterotrimeric G proteins (Ibarrondo et al. 1995; Côté et al. 1997), the phosphatases SHP-1 (Brumell et al. 1997; Kim et al. 1999), and SHP-2 (Schoenwaelder et al. 2000; Xu et al. 2001), and the tyrosine kinases Src or Pyk2. Of note, a recent study showed that the AT₂ receptor inhibited the kinase Pyk2 (Matsubara et al. 2001). It is now well accepted that extracellular matrix can induce intracellular cell signals or interact with hormonal or growth factor transduction pathways leading to specific cell behaviors such as proliferation, migration, apoptosis, or gene expression (Aplin et al. 1999; Giancotti et al. 1999). Accumulating evidence suggests that the AT₂ receptor activates different signaling pathways, which may be attributed to interactions or associations with cytoskeleton-associated proteins, which together are linked to specific integrins; all are coordinated to imprint specific cell responses according to the nature of the extracellular matrix components.

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AT₂ Renal Aspects

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Abstract Renin–angiotensin system (RAS) plays an important role in the regulation of body fluids and electrolyte homeostasis and blood pressure control. Angiotensin II (Ang II), the major effector hormone of the RAS, functions mainly through stimulation of two angiotensin receptors subtypes, AT₁R and AT₂R. The well-known actions of Ang II have been attributed mainly to activation of various signal–transduction pathways modulated by AT₁R. Further research has led to the identification of the angiotensin subtype-2 (AT₂R) that is clearly distinct from AT₁R in its structure, tissue-specific expression, and signaling mechanisms. In this chapter, we review the different aspects of AT₂R including its development and structural features, its signaling pathways, and its localization in different tissues. The main focus of this review is on the AT₂ receptor

locations and actions within the kidneys, and the different ways that AT₁R interacts with AT₂R. While on one hand AT₁R activation stimulates vasoconstriction, vascular cell hypertrophy and hyperplasia, sodium retention, stimulation of reactive oxygen species (ROS) production, and induction of inflammatory, thrombotic, and fibrotic processes, and under some conditions can lead to apoptosis in some cell types, on the other hand, AT₂R has vasodilatory, antigrowth, and apoptotic actions that opposes AT₁R actions. In the kidneys, AT₂R promotes vasodilation and natriuresis and plays a role in blood pressure (BP)-lowering effect of the AT₁R blockers. This vasodilatory action of AT₂R has been shown to be through bradykinin (BK)-/nitric oxide (NO)-dependent pathways. Besides reviewing the potential physiological actions of AT₂R in the kidneys, we also shed some light on the potential roles played by AT₂R in several disease processes affecting the kidneys, such as glomerular diseases, diabetes mellitus, and in congenital anomalies of the kidney and urinary tract.

Keywords AT₂ receptor · AT₁ receptor · Kidney · Nitric Oxide · Bradykinin

1

Introduction

Renin-angiotensin system (RAS) plays an important role in the regulation of body fluids and electrolyte homeostasis and BP control. Although RAS was originally regarded as a circulating system, most of its components are localized in tissues, indicating the existence of a local tissue RAS as well (Dzau 1988; Navar 1999). All the components of RAS, including renin, the substrate angiotensinogen, angiotensins, the enzymes involved in the synthesis and degradation of angiotensins, as well as angiotensin receptors are present in the kidney.

Angiotensin II (Ang II), the major effector hormone of the RAS, functions mainly through stimulation of two angiotensin receptors subtypes, AT₁R and AT₂R. These cellular receptors have been cloned, pharmacologically characterized, and localized at various tissues. The angiotensin subtype-1-receptor (AT₁R) is widely distributed throughout the body and mainly in the vasculature, heart, adrenal glands, kidneys, and nervous and endocrine systems.

The well-known actions of Ang II have been attributed mainly to activation of various signal-transduction pathways modulated by AT₁R. However, the discovery of the highly selective, peptide and non-peptide ligands such as CGP42112A and PD123319 has led to the identification of the angiotensin subtype-2 (AT₂R) (Timmermans et al. 1993). AT₂R is clearly distinct from AT₁R in its structure, tissue-specific expression, and signaling mechanisms.

The Ang II receptors can be distinguished according to inhibition by specific antagonists. AT₁R is selectively antagonized by biphenylimidazoles such as losartan, whereas tetrahydroimidazopyridines specifically inhibits AT₂R (Ardaillou 1999). AT₂R is also identified by being selectively activated by CGP42112A. This is a hexapeptide analog of Ang II, which at low dose may act as an AT₂R agonist, but may also inhibit the AT₂R and AT₁R at higher concen-

trations (Criscione et al. 1990). The availability of the radiolabeled forms of the highly selective AT₂R ligands (Whitebread et al. 1991; Heemskerk et al. 1993) made direct localization of AT₂R a practical possibility.

Drugs that antagonize AT₁R cause marked elevation of the levels of plasma Ang II, which in turn activates the AT₂R. With the increasing usage of AT₁R blockers, elucidation and understanding of AT₂R-mediated physiological actions will have important pharmacotherapeutic implications.

2

AT₂ Development and Structural Features

The AT₂R is a seven-transmembrane receptor comprising 363 amino acids, with a molecular mass of approximately 41 kDa. It has low amino acids sequence homology (around 32%–34%) with AT₁R (Kambayashi et al. 1993; Mu Koyama et al. 1993; Table 1).

The AT₂R protein consists of five potential N-glycosylation sites in the extracellular N-terminal domain and 14 cysteine residues. The second intracellular loop consists of a potential protein kinase C phosphorylation site (Griendling et al. 1996). The third intracellular loop is essential for AT₂R signal transduction via mitogen-activated protein (MAP) kinase inactivation (Lehtonen et al. 1997). The AT₂R expression is species-dependent. In general, humans have a much higher level of expression of AT₂R than rodents. In humans, AT₂R expression can be equal to, or even exceed that of AT₁R, but the functional significance of the AT₂R in humans remains unknown (Carey et al. 2000). The gene for AT₁R is located on chromosome 3 and exists as a single copy of the X-chromosome in

Table 1 General properties of AT₂

Structure	363 amino acids 7 transmembrane
Molecular mass	41 kDa
Chromosomal localization	X-chromosome
Selective ligands	PD123319, PD123177, CGP42112-A
Signal transduction	Activation of phosphatase Inhibition of kinases Closing T-type Ca ⁺⁺ channel Opening delayed rectifier K ⁺ channel Stimulation of cGMP/NO Activation of phospholipase A ₂ Activation of STAT-1
Action	Generation of ceramides Pressure diuresis and natriuresis Vasodilatation Apoptosis Blood pressure reduction ??? Protective role in D.M. complication ??? Role in glomerular disease

???, There is some information to support a protective role but not enough to accept it as a fact.

human, rat, and mouse. The genes encoding the AT₂R are localized at xq22-q2 in human (Koike et al. 1994), chromosome xq3 in rat (Tissir et al. 1995), and chromosome x in mouse (Hein et al. 1995), with 99% amino acid residue sequence identity between rat and mouse, and 72% amino acid residue sequence identity between rat and human. While in some species AT₁R is expressed in two subtypes, genomic analyses have indicated that there is only one subtype to the AT₂R gene family (Mukoyama et al. 1993; Ichiki et al. 1995). While a 4.5-kb AT₂ cDNA was cloned from rat PC12 W cells and rat fetuses (Kambayashi et al. 1993), an AT₂ genomic 4.4-kb DNA fragment was cloned from mice (Ichiki et al. 1994). The AT₂R genomic DNA of all three species, humans, rats, and mice, consists of three exons with an uninterrupted coding region being confined in the third exon (Tsuzuki et al. 1994). This confinement of the entire coding system in one exon allowed the AT₂ coding sequence, or fragments containing more extensive sequence, to be cloned.

The expression of the AT₂R gene is dependent on growth state. The AT₂R gene is expressed ubiquitously at very high level in the fetus but then declines soon after birth in many, but not all, tissues. While it decreases to undetectable levels in the skin, the decline stops at certain levels in the heart and adrenal gland. Moreover, while AT₂R is expressed in uterine myometrium under non-pregnant conditions, it declines during pregnancy to return to nonpregnant levels after partition (de Gasparo et al. 2000). Furthermore, in special cells such as PC12 W cells (Kijima et al. 1995), R3T3 cells (Horiuchi et al. 1995), or mesangial cells (Goto et al. 1997), AT₂R expression is markedly increased when a confluent quiescent state is reached.

Several conditions were shown to modulate the expression of AT₂R. AT₂R expression is increased in adults after tissue injury, myocardial infarction, vascular injury, heart failure, and after sodium depletion (Siragy and Bedigian 1999). In contrast, Ang II, norepinephrine, insulin-like growth factor, basic fibroblast growth factor, and transforming growth factor beta (TGF) all decrease AT₂R expression (Carey et al. 2000b). Several cell lines express the AT₂R, but not the AT₁R, such as PC12 W, R3T3, and some lines of N1E115.

3

AT₂ Signaling

Ang II mediates its effects by acting directly through its receptors and via cross-talk with intracellular signaling cascades of other vasoactive agents, growth factors, and cytokines. Although the exact signaling pathways and the functional roles of AT₂R are not fully elucidated, these receptors may antagonize, under physiological conditions, the AT₁R-mediated actions (Ciuffo et al. 1998; Yamada et al. 1998).

Several signal transduction elements for AT₂R have been described. The growth-inhibitory effects of the AT₂R are reported to be at least partly mediated by the activation of protein tyrosine phosphatase, which results in the inhibition of AT₁R-activated mitogen-activated protein MAP kinase (Horiuchi et al. 1999).

In neuronal cells cultured from neonatal rat hypothalamus and brain stem, serine/threonine phosphatase 2A (PP2A) activation and subsequent extracellular signal-regulated kinase (ERK) inactivation through AT₂R have been reported (Huang et al. 1996). In the heart of transgenic mice overexpressing cardiac AT₂R, ERK activity was noted to be decreased, suggesting that ERK inactivation by the AT₂R has a physiological role *in vivo* (Masaki et al. 1998). Other signaling pathways, such as closing of a T-type Ca⁺⁺ channel (Buisson et al. 1995), opening a delayed rectifier potassium channel (Kang et al. 1995), elevation in cyclic guanosine monophosphate (cGMP), stimulation of particulate guanylate cyclase, and stimulation of NO production have been proposed as signal transduction elements for AT₂R (Siragy and Carey 1996; Tsutsumi et al. 1999; Siragy et al. 2000). *In vivo* studies in the kidney have shown that Ang II can activate phospholipase A2 via AT₂R, resulting in the release of arachidonic acid and its metabolites, which in turn can activate Ras kinase and MAP kinase. (Dulin et al. 1998; Jiao et al. 1998).

Seebach et al. explored the signal transducer and activator of transcription (STAT) pathway as a possible mechanism of Ang II signaling in fetal human mesangial cells. These studies showed that AT₂R mediated the Ang II activation of STAT1, which may be an important signaling pathway for Ang II-induced cellular responses (Seebach et al. 2001). Another signaling pathway involving apoptosis through generation of ceramides was suggested by Gallinat et al. (1999).

4

AT₂R Localization

AT₂R is expressed in abundance in mesenchymal tissues of the developing fetus, such as the uterus, the adrenal medulla, and specific brain regions, suggesting neuronal and developmental roles for the AT₂R (Matsubara 1998). However, in almost all tissues there is a rapid regression to low levels or even disappearance of expression of the AT₂R in the early postnatal period. Arce et al. showed high expression of AT₂R in 2-week-old rat hindbrains, which was absent in the adult rat, suggesting a probable role of these receptors in neuronal development (Arce et al. 2001). In adults, AT₂R were demonstrated in rat kidney (Zhuo 1992; Aguilera 1994; Ozono et al. 1997), rat heart (Sechi et al. 1992; Wang Z-Q et al. 1998), rat brain (Tsutsumi and Saavedra 1991), and rat adrenal glands (Shanmugam 1995). Other tissues that showed AT₂R expression were uterine myometrium, ovary, and pancreas (Matsubara 1998).

Using binding studies and quantitative autoradiography, AT₂ expression in adult Sprague-Dawley (SD) rat aorta, has been localized through out the aortic wall (Vinswanathan et al. 1991). Similar results were demonstrated in Wistar-Kyoto (WKY) and spontaneous hypertensive (SHR) rats (Song et al. 1995). Both AT₁R and AT₂R were expressed in endothelial cells derived from SHR coronary arteries in an 80:20 ratio. (Stoll et al. 1995).

Immunohistochemistry staining showed that rat AT₂ expression varies with vessel size. It is more abundant in endothelial and vascular smooth muscle cells

(VSMC) in micro-vessels than in large vessels (Nora et al. 1998). Tsutsumi et al. failed to identify AT₂R expression in the endothelium of aorta from wild-type (WT) or transgenic mice overexpressing the AT₂R (Tsutsumi et al. 1999). Taken together, these results suggest that demonstration of the AT₂R in endothelial cells depends on species, size of vessels, and experimental conditions.

AT₂R is also expressed in both medial and adventitial layers of the rat mesenteric vasculature with predominant localization in the adventitial layer (Bonnet et al. 2001). In the heart, immunostaining for AT₂R was positive in the myocardium and coronary vessels throughout the ventricles and atria of neonatal and young rat hearts. The AT₂R expression was significantly more in the neonatal rat heart as compared to young rat heart (Wang et al. 1998).

5

AT₁/AT₂ Interactions

The interaction between receptor subtypes may be important to the physiological response to the agonist. Integrated responses to Ang II are the result of combined AT₁R- and AT₂R-mediated actions (Berry et al. 2001). It has been postulated that Ang II effects at the AT₁R are opposed by actions at the AT₂R (Carey et al. 2000b). Furthermore, it has been viewed that infusion of Ang II with an AT₂R antagonist represents an alternative model of AT₁R stimulation (Diep et al. 1999). Thus the concept of “crosstalk” was proposed where one Ang II subtype receptor will affect the other.

AT₁R activation stimulates vasoconstriction, vascular cell hypertrophy and hyperplasia, sodium retention (Berry et al. 2001), stimulation of reactive oxygen species (ROS) production (Berry et al. 2000), and induction of inflammatory (Muller et al. 2000), thrombotic (Vaughan et al. 1995) and fibrotic processes (Boffa et al. 1999), and under some conditions can lead to apoptosis in some cell types (Li et al. 1999). In contrast, AT₂R has vasodilatory, antigrowth, and apoptotic actions that oppose AT₁R actions (Berry et al. 2001).

Several organs and tissues showed manifestations of the crosstalk between AT₁R and AT₂R. In the heart, these receptors seem to exert opposite effects in terms of cardiovascular hemodynamics and cell growth (Xoriuchi et al. 1999). In neuronal cells, Laflamme et al. illustrated negative crosstalk interaction between AT₁R and AT₂R receptors (Laflamme et al. 1996). Tsutsumi et al. demonstrated that AT₁R and AT₂R play opposite roles in aortic vascular smooth muscles (VSM). In transgenic mice overexpressing AT₂R, chronic infusion of Ang II completely abolished the AT₁R-mediated pressor effects. (Tsutsumi et al. 1999) In the mesenteric artery of SD rats, Bonnet et al. demonstrated that AT₁R plays a role in the up-regulation of the AT₂R following Ang II infusion (Bonnet et al. 2001).

In the kidneys, the AT₁R is shown to induce vasoconstriction and sodium retention, whereas the AT₂R is postulated to promote vasodilation and natriuresis (Haithcock et al. 1999; Siragy and Carey 1999; Siragy et al. 1999). AT₂R inhibition prevents the hypotensive effects of AT₁R blockade. Thus the AT₂R seems to

mediate the depressor responses to Ang II (Inagami et al. 1999; Siragy and Carey 1999; Tanaka et al. 1999). AT₂R has been shown to induce vasodilation through bradykinin (BK)-/nitric oxide (NO)-dependent pathways (Siragy and Carey 1999; Siragy et al. 2000). It has been suggested that AT₂R may play a role in the BP-lowering effect of AT₁R blockers.

Several studies examined the underlying mechanism(s) of the crosstalk occurring between AT₁ and AT₂ receptors. Hunley et al. showed that AT₂ decreases angiotensin converting enzyme (ACE) activity tonically, which by decreasing the formation of Ang II, attenuates AT₁R-mediated actions (Hunley et al. 2000). Moreover, the AT₂R appears to counterbalance the AT₁R by increasing the production of BK, NO, and cGMP, thus mediating vasodilation and BP-lowering actions.

6

AT₂R/NO Interaction

NO is synthesized from the amino acid L-arginine in endothelial as well as other cell types by the action of NO synthase (NOS) (Moncada et al. 1991). In the kidney, the NOS isoenzymes are distributed in close proximity to the sites of the RAS components, which may explain the interaction between these two systems (Millatt et al. 1999). Intrarenal NO helps to maintain the normally low renal vascular resistance, being responsible for up to one-third of the renal blood flow (Navar et al. 1996). It participates in the maintenance of renal perfusion and glomerular filtration in the normal kidney by its vasodilatory effect, mainly on the afferent arterioles of the superficial glomeruli (Kone 1997; Kone and Baylis 1997). NO also plays an important role in controlling tubular function. It acts as a natriuretic factor, by directly inhibiting Na⁺ reabsorption in the proximal and collecting tubules, by inhibiting Na⁺/H⁺ exchange and Na⁺-K⁺-ATPase (Majid and Navar 1997). Other actions that involve NO include controlling cell growth, apoptosis, and inflammation (Gross and Wolin 1995).

The stimulation of AT₂R is associated with increased generation of BK (Siragy et al. 1996, 1998), NO (Siragy et al. 1998; Carey et al. 2000a), and cGMP (Siragy and Carey 1996), all of which have vasodilatory properties (Carey et al. 2000b). A vasotonic role for BK production during AT₂R activation has been reported in several animal models such as in the stroke-prone spontaneously hypertensive rats (SHR-SP) aorta (Gohlke et al. 1998) and in rats with two-kidney, one figure-eight wrap hypertension (Siragy and Carey 1999). Seyedi et al. studied coronary microvessels and large coronary arteries obtained from normal dogs. Nitrite, a product of NO metabolism, was increased following Ang II infusion, an effect blocked by BK B₂R antagonist, AT₁R antagonist, and AT₂R antagonist (Seyedi et al. 1995). These effects suggested a role for local kinin formation in the coronary vessels, and that AT₁R and AT₂R may play a role in mediating the release of nitrite. Golke et al. demonstrated that the attenuation in Ang II-induced pressor response in SHR-SP rats by AT₁R blockade was associated with a rise in aortic cGMP concentration. BK-B₂R antagonist or L-nitroarginine

methyl ester (L-NAME) (Golke et al. 1998) inhibited this increase in cGMP concentration. This study suggests that AT₂R activation during AT₁R blockade is associated with an increase in BK production, which in turn stimulates NO generation and can cause a vasodilatory effect. Bucher et al. described another interaction between AT₂R and NO. In this study SD rats were injected with either lipopolysaccharide or lipoteichoic acid to stimulate gram-negative and gram-positive sepsis, respectively. In both models of sepsis, AT₂R expression in the adrenal gland was downregulated and NOS II expression was induced. This downregulation of AT₂R was prevented by blocking of NO synthesis (Bucher et al. 2001). This study points to another important aspect of the AT₂R–NO interaction, where AT₂R could play an important role in the pathogenesis of septic shock that is NO-dependent.

Siragy and Carey demonstrated that Ang II stimulates cGMP production in the kidney, which could be inhibited by AT₂R blockade or NOS inhibitor (Siragy and Carey 1996, 1997a). These data demonstrated that AT₂R activation leads to an increase in renal NO production. They further demonstrated that the physiological action of Ang II in the kidney, such as the release of cGMP by the AT₂R, is potentiated during stressful conditions such as sodium depletion in which the RAS is stimulated. Reports from the same laboratory also demonstrated that the increase in Ang II, in response to AT₁R blocker valsartan, stimulated AT₂R, which mediates a BK and NO cascade (Siragy et al. 2000). Furthermore, it was shown that the AT₂-null mouse has low basal levels of renal BK and renal cGMP, an index of NO production, with no change in these levels following Ang II infusion or dietary salt restriction (Siragy et al. 1999). Israel et al. showed that systemic administration of AT₁R blocker, losartan, not only inhibited the pressor response induced by mild footshocks but also resulted in vasodepression. When AT₂R was blocked by PD123319 in combination with losartan, the vasodepressor response was eliminated (Israel et al. 2000). These data suggested that the vasodepressor response to footshocks in the presence of AT₁R antagonist is triggered by activation of AT₂R. This vasodepressor response was also blunted by BK, NO, and prostaglandin (PG) inhibitors, suggesting the involvement of BK, NO, and PG in the depressor response triggered by AT₂R activation in that model. Taken together, these results demonstrate that AT₂R is necessary for normal physiological responses of BK and NO, and that the renal NO production by AT₂R activation can be a major contributing factor in the regulation and control of multiple renal functions.

7

AT₂R and the Kidney

The kidney serves as one of the major target tissues that mediate the physiological actions of Ang II. Ang II has multiple effects on renal function including modulation of renal blood flow, glomerular filtration rate (GFR), tubular epithelial transport, renin release, and cellular growth (Matsubara 1998). Ang II levels in renal interstitial fluid (RIF) were reported to be 1,000 times higher than sys-

temic plasma levels, suggesting the importance of Ang II in the local regulation of renal function (Siragy et al. 1995). Furthermore, Nishiyama et al. confirmed that RIF concentrations of Ang I and Ang II are substantially higher than the corresponding plasma concentrations (Nishiyama et al. 2002). The high RIF concentrations of Ang II were not responsive to acute ACE inhibition or volume expansion, suggesting the compartmentalization and independent regulation of RIF Ang II.

AT₂R has specific effects on renal function. The detection of increased incidence of congenital anomalies of the kidney and the urinary tract in AT₂R-deficient mice, suggests a role for AT₂R in genitourinary development (Guron and Friberg 2000). Among the renal effects of the AT₂R stimulation are: mediation of renal interstitial production of NO, BK, and cGMP; counter-regulatory protective role against AT₁R-mediated anti natriuretic and pressor actions of Ang II; and enhancing the activity of 9-ketoreductase enzyme leading to conversion of prostaglandin E2 (PGE2) to prostaglandin F2 alpha (PGF2 alpha) (Siragy and Carey 1996, 1997b).

7.1

AT₂R Localization in the Kidney

A paucity of information exists about the localization of the AT₂R in the kidney. Autoradiography coupled with competitive binding studies have been used to characterize the distribution of Ang II receptors subtypes in renal tissues. By using these techniques, the distribution of AT₁R and AT₂R subtypes showed remarkable species-differences (Table 2).

Table 2 AT₂ localization in the kidney

Species	Age	Location
Mouse	Fetal	Mesenchymal cells of mesonephros surrounding the mesonephric tubules
Rat	Fetal	Mesenchymal cells surrounding the epithelial structure and ureteric buds, immature glomeruli, primitive cortical and medullary tubules, interstitial cells
	Newborn	Glomeruli, proximal and distal tubules, blood vessel
	Young adult	Glomeruli and tubules. Re-expressed with sodium depletion in glomerular mesangial cells, tubules and mesenchyma, interstitial cells of outer cortex
	Mature adult	Glomeruli and tubules. Enhanced by sodium depletion in glomeruli, interstitial cells, and adventitia of preglomerular arcuate and interlobular arteries.
Rabbit		Fibrous sheath around the kidney
Rhesus monkey		JGA, vasculature of renal cortex
Human	Fetal	Mesenchymal cells adjacent to the Stalk of ureter epithelium, superficial cortex, between collecting ducts
	Children	Interlobular arterial
	Adult	Glomeruli and tubules, large cortical preglomerular vessels, interlobular arteries, tubulo-interstitium

Kakuchi et al. reported that AT₂R is intensely expressed in the mesenchymal cells of the mesonephros surrounding the mesonephric tubules of fetal mouse kidney (Kakuchi et al. 1995). In the rat fetal kidney, AT₂R is heavily expressed in immature glomeruli, cortical and medullary tubules and interstitial cells, but within few days of birth, AT₂R expression diminishes rapidly, and is observed only at low levels in adult kidney (Ozono et al. 1997; Carey et al. 2000b).

Ozono et al. published an eloquent study using immunohistochemical staining to detect AT₂R gene in fetal (day 14 and 19 of fetal life), newborn (day 1 postpartum) and adult rat kidney (4 weeks and 3 months old). The study showed heavy immunohistochemical staining for the AT₂ receptor in the undifferentiated mesenchymal cells surrounding the epithelial structures and in the ureteric buds in the F14 fetus. In the F19 fetus, AT₂ receptor signal was detected in the S-shaped glomeruli, primitive tubules, and mesenchymal tissue. The study further showed positive staining in glomeruli, proximal and distal renal tubule cells, and blood vessels of the newborn rat kidney at D1. In young adult (4-week-old) rats on normal sodium intake, renal staining for the AT₂ receptor was markedly reduced but remained detectable in the glomeruli and tubules. Sodium depletion increased AT₂R expression in young adult rats, in the glomeruli, tubules, and mesenchyma. The glomerular staining was localized to mesangial cells and also was present in the interstitial cells of the outer cortex. Similar results were shown in the mature adult (3-month-old) rats. On normal sodium intake, there was some glomerular and tubule expression of AT₂R protein signal that was enhanced during sodium depletion predominantly in the glomeruli and in the interstitial cells of the outer cortex (Ozono et al. 1997).

The status of renal AT₂R in the adult rat kidney has been controversial. Initial studies (Gibson et al. 1991) suggested that there was no gene expression for AT₂R in adult kidney. However, using immunohistochemical techniques and autoradiography, it has been shown that AT₂R is present in low levels in the glomerular epithelial cells, cortical tubules, and interstitial cells of glomeruli and tubules of rat kidney. (Ozono et al. 1997; Wang et al. 1999). Miyata et al., using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) found the AT₂R mRNA and protein expression throughout the rat kidney, but little expression was found in the glomerulus and the medullary thick ascending limbs of Henle (Miyata et al. 1999). Sharma et al. demonstrated the presence of AT₁R and AT₂R in rat glomerular epithelial cells, and proposed that Ang II and its interaction with AT₁R and AT₂R on glomerular epithelial cells play an important part in the regulation of glomerular function (Sharma et al. 1998).

AT₂R and its mRNA are most prominent in the fetal and newborn mammalian kidney. In contrast, the expression of AT₂R in the adult mammalian kidney has been reported to be very low and localized mainly in the glomerular and mesangial cells (Goto et al. 1997; Ozono et al. 1997), or adventitia of the preglomerular arcuate and interlobular arteries (Zhou et al. 1996). Autoradiography revealed that in rabbit, the fibrous sheath around the kidney contains AT₂R binding sites (Herblin et al. 1991), and that in rhesus monkeys it is present

in juxta-glomerular apparatus (JGA) and in the vasculature of the renal cortex (Gibson et al. 1991)

In the human fetal kidney, the presence of AT₂Rm RNA was detected in undifferentiated mesenchymal cells adjacent to the stalk of the ureter epithelium, in the superficial cortex near nephrogenic areas, and in the area between collecting ducts (Grone et al. 1992). In adult human kidney, AT₂ mRNA was localized in the medial layers of interlobular arteries (Matsubara et al. 1998). AT₂R was also found in large cortical preglomerular vessels and the tubulo-interstitium (Grone et al. 1992; Goldfarb et al. 1994; Zhou et al. 1996). The presence of AT₂R in the interlobular arteries was also shown in children. Vinswanathan et al. examined kidneys obtained at autopsy from five children (aged 1 month to 2 years) during the first 24 h after death. Ang II receptor subtypes were localized by quantitative autoradiography. While renal glomeruli expressed exclusively AT₁R, 80% of the Ang II receptors in the interlobular artery belonged to AT₂R (Vinswanathan et al. 2000). Immunohistochemistry of the human adult kidney with the use of a polyclonal antibody to the AT₂R revealed a positive signal in the glomerular epithelial elements, tubules, and large-order renal blood vessel (Carey et al. 2000b).

7.2

AT₂R Actions and the Kidney

Ang II takes part in a number of regulatory events in the kidney mainly the control of renal vascular resistance, glomerular filtration, and tubular epithelial transport. These renal actions of Ang II are mediated by AT₁R, which predominates in glomeruli, JGA, and proximal tubules in adult human kidneys. However, the AT₂R also seems to have some specific effects on renal functions. AT₂R is postulated to play a role in pressure natriuresis, vascular and BP regulation, apoptosis and antiproliferation, glomerular action, and certain pathologic renal conditions.

7.2.1

Pressure Natriuresis and Diuresis

Controversial data exist regarding the role of AT₂R in pressure natriuresis and diuresis. While most studies proposed that AT₂R enhance both pressure natriuresis and diuresis (Siragy et al. 1999; Gross et al. 2000), other studies did not support this proposal (Lo et al. 1995; Madrid et al. 1997). Siragy et al. suggested that AT₂R stimulation can physiologically increase pressure natriuresis and proposed that the effect is mediated by BK/NO cascade. In AT₂-null mice, chronic infusion of sub-pressor doses of Ang II for 1 week increased the systolic BP (SBP) and reduced urinary sodium excretion (U_{NAV}), whereas neither SBP nor U_{NAV} changed in WT mice. Furthermore, in WT mice, dietary sodium restriction or Ang II infusion increased RIF BK and cGMP, with no changes noted in the AT₂-null mice. These results suggest that the absence of AT₂R leads to sodium retention and hypertension in the presence of physiologically increased

Ang II (Siragy et al. 1999). In agreement with Siragy et al. was the study by Gross and colleagues. Under the same renal perfusion pressure (RPP), Gross et al. noted that the control WT mice excreted threefold the quantity of sodium and water compared to the AT₂ null mice, suggesting that AT₂R plays a role in enhancing pressure natriuresis and diuresis (Gross et al. 2000). Haithcoch et al. showed that AT₂R is linked to inhibition of bicarbonate reabsorption in the proximal tubule, an effect that opposes AT₁R-mediated facilitation of sodium and bicarbonate reabsorption and suggests a role for AT₂R in natriuresis. These natriuretic and diuretic responses were not related to hemodynamic changes (Haithcoch et al. 1999)

7.2.2

Effects on Blood Vessels

Ang II directly influences blood vessels structure and function. The atrophic and proliferative effects of Ang II on the vasculature are modulated by both AT₁R and AT₂R. Tsutsumi et al. demonstrated that AT₁R and AT₂R play opposite roles in aortic VSM. In transgenic mice with overexpressed AT₂, chronic infusion of Ang II completely abolished the AT₁R-mediated pressor effects, suggesting an AT₂-mediated vasodilation (Tsutsumi et al. 1999). Sheuer et al. and Munzenmaier et al. showed data confirming the vasodilatory actions of AT₂R. Sheuer et al. demonstrated that AT₂ mediates the depressor phase of the biphasic BP response to Ang II in mature rats (Sheuer et al. 1993), while Munzenmaier and coworkers showed the pressor action of Ang II to be enhanced with chronic treatment with AT₂R antagonist PD123319 in SD rats (Munzenmaier et al. 1996). In agreement with these data is the observation that the pressor actions of Ang II are further enhanced in the AT₂-null mice model (Hein et al. 1995; Ichicki et al. 1995). Furthermore, Ichiki et al. and Siragy et al. demonstrated that AT₂R-null mice have a higher sensitivity to BP and exert enhanced pressor actions of Ang II (Ichicki et al. 1995; Siragy et al. 1999).

To delineate the mechanisms of the AT₂R vasodilatory effects, Tsutsumi et al. chronically infused Ang II into mice overexpressing the AT₂R. In these studies, Ang II completely abolished the AT₁-mediated pressor effect, which was blocked by inhibitors of bradykinin type 2 receptor (icatibant) and NO synthase (L-NAME). The vasoconstrictive response of Ang II was enhanced by AT₂R blockade, suggesting that AT₂ antagonizes the AT₁-mediated vasoconstrictive actions in aortic VSMC through the stimulation of bradykinin-NO-cGMP cascade (Tsutsumi et al. 1999). The proposed mechanism of stimulation of this cascade is that AT₂ causes intracellular acidic changes through inhibition of amiloride-sensitive Na⁺/H⁺ exchanger activity. The increase in cellular acidity stimulates the activity of kininogenases that in turn increase formation of bradykinin (Tsutsumi et al. 1999). Arima and Ito proposed another mechanism while studying rabbit afferent arteriole (Af-Art). They showed that AT₁R blockade abolished the Ang II-induced vasoconstriction. Further infusion of Ang II in the setting of AT₁R blockade caused dose-dependent dilation, which was abolished by

AT₂R blockade, suggesting an AT₂R-mediated vasodilation. While the dilation was not affected by inhibiting the synthesis of NO or prostaglandin (PG), it was completely abolished by either disrupting the endothelium or inhibiting the synthesis of epoxyeicosatrienoic acid (EET), a cytochrome P-450 (CYP) epoxygenase metabolite of arachidonic acid that is a potent vasodilator in the Af-Art. Thus, AT₂R induces endothelium-dependent and EET-mediated vasodilation, which is independent of NO release (Arima and Ito 2000). Using the same model of rabbit Af-Art, Kohagura et al. induced vasoconstriction by inhibiting NOS or EET using L-NAME or miconazole respectively. The addition of Ang II enhanced further this vasoconstriction. When the experiment was repeated with the addition of AT₂R blocker PD123319, only L-NAME augmented the vasoconstrictive activity of Ang II, while EET inhibitor, miconazole had no effect. These studies suggest an additional mechanism whereby AT₂R mediates vasodilation via coupling to EET (Kohagura et al. 2000).

Contrary to the proposed vasodilator effect of AT₂R, Croft et al. showed that Ang II increases CYP monooxygenase in preglomerular microvessels (PGMV) of SD rats, which in turn cause an increase in the vasoconstrictor 20-hydroxyeicosatrienoic acid (HETE) by two- to threefolds. This effect was blocked by AT₂R blockade (PD123319) and phospholipase C (PLC) inhibitor (U-73122). These data suggested that AT₂R-PLC effector unit is associated with synthesis of a vasoconstrictor product in PGMVs (Croft et al. 2000).

7.2.3

Blood Pressure Regulation

Several studies suggest that AT₂R plays a role in BP control, counterbalancing the AT₁R pressor action and causing a reduction in Ang II-induced hypertension. AT₂R has been suggested to have a direct vasodilatory action that can cause reduction in BP. Furthermore, AT₂R through its pressure natriuresis and diuresis actions can achieve similar BP reduction effects. The malfunction of pressure natriuresis and diuresis leads to arterial hypertension.

In a series of studies (Siragy and Carey 1996, 1997a, 1999; Siragy et al. 1996), Siragy et al. provided evidence for a vasodilatory cascade mediated by AT₂R. These studies showed that Ang II, via AT₂R, triggers a cascade of increase tissue BK, synthesis of NO, and cGMP, and ultimately leading to vasodilation and reduction of the increased BP associated with Ang II.

The protective role of the renal AT₂R was further investigated in a 2-kidney, 1 figure-8 wrap model (Siragy and Carey 1999). Siragy et al. observed that BK, NO, and cGMP were all higher in the intact kidney than in the wrapped kidney. While AT₁R blockade increased BK, NO, and cGMP further in the intact kidney, AT₂R blockade decreased these products in both kidneys. They concluded that Ang II triggers the production of BK in the kidney via the stimulation of AT₂R, which then releases NO and cGMP to produce counter-regulatory vasodilation in this model. The study also suggests that the hypotensive response associated with AT₁R blockade is partially mediated by the AT₂R. To further expand on the

role that AT₂R plays in BP regulation, Carey et al. infused the selective AT₂R agonist CGP42112A, and showed that it decreased BP. They further showed that this BP-lowering response was blocked by either AT₂R blockade PD123319 or the NOS inhibitor L-NAME, suggesting that the AT₂R induces a vasodilator response mediated by NO (Carey et al. 2001).

The role of AT₂R in BP regulation was studied further in AT₂-null mice (Hein et al. 1995; Ichiki et al. 1995; Siragy et al. 1999). At base line in the absence of AT₂R, AT₂-null mice have slightly elevated BP. This mouse model demonstrated pressor hypersensitivity to Ang II compared with WT controls. When the RAS was activated by dietary sodium restriction or Ang II infusion, the AT₂-null mice did not increase BK, NO, nor cGMP in contrast to the WT control mice (Siragy et al. 1999). These data support the hypothesis that AT₂R plays a role in lowering BP through the vasodilatory actions of BK/NO/cGMP.

Additional studies using AT₁R blockers (Matrougui et al. 1999) alone or in combination with AT₂R agonist (Barber et al. 1999) or in combination with salt restriction (Siragy et al. 2000) confirmed the vasodilator actions of AT₂R stimulation and its role in BP regulation.

7.2.4

Apoptosis

Apoptosis is characterized by a series of morphological events such as shrinkage of the cell, condensation of chromatin, fragmentation into apoptotic bodies, and rapid phagocytosis by neighboring cells. It plays a crucial role in the normal development as well as in the pathophysiology of a variety of tissues that have undergone injury or damage. Among many other factors, the balance between pro- and anti-apoptotic proteins determines whether a cell will undergo programmed cell death or survive.

AT₂R stimulation has been demonstrated to induce apoptosis in a number of cells in correlation with extensive cell death in the developing fetus. AT₂R induces apoptosis in cells of neuronal origin, such as PC12 W (Yamada et al. 1996) and cultured neurons from newborn rat (Shenoy et al. 1999), rat ovarian granulosa cells (Tanaka et al. 1995), fibroblast from mouse embryo (Li et al. 1998), and endothelial cells from human (Dimmeler et al. 1997). AT₂R was also shown to mediate vascular mass regression by stimulating smooth muscle cells apoptosis (Tea et al. 2000). In a study by Suzuki et al., vascular injury was induced by polyethylene cuff placement around the left femoral artery of AT₂-null mice. They found that the number of apoptotic cells decreased in this mouse model, pointing to a proapoptotic effect of AT₂R on VSMC in the process of neointimal formation after vascular injury (Suzuki et al. 2002).

In the kidney, it has been suggested that the balance between cell proliferation and apoptosis plays an important role in the pathophysiology of various renal diseases (Kovacs and Gomba 1998; Thomas et al. 1998; Truong et al. 1998). Ang II was shown to induce apoptosis in renal tubular cells. This was demonstrated by the immunohistochemical techniques and confirmed by electron mi-

croscopy. Both AT₁R and AT₂R antagonists attenuated this effect of Ang II on apoptosis. In a kidney model of rat with unilateral ureteral obstruction, AT₂R blockade suppressed tubular cell apoptosis (Morrissey and Klahr 1999). Using the same model of unilateral ureteral obstruction in male WT and AT₂R-deficient mice, Ma et al. investigated the role of Ang II in renal remodeling. They observed that the obstructed kidney from the AT₂R knock-out mice showed significantly fewer apoptotic cells compared to WT control mice, supporting a role for AT₂R in apoptotic cell death (Ma et al. 1998).

Ceramide, an intracellular lipid second messenger and one of the most hydrophobic molecules in mammalian cells, has been implicated as an important mediator of apoptosis (Pushkareva et al. 1995). Gallinat et al. demonstrated that Ang II increased ceramide levels during apoptosis in PC12 W cells, and that this action was completely abolished by co-incubation with an AT₂R antagonist, confirming that apoptosis is mediated by AT₂R (Gallinat et al. 1999). Miura and Karnik further showed that induction of apoptosis could be a constitutive function of the AT₂R, and proposed that overexpression of the AT₂R itself may act as a signal for apoptosis that did not require Ang II (Miura and Karnik 2000).

7.2.5

Antiproliferative Effects

As mentioned before, the balance between cell proliferation and apoptosis plays an important role in the pathophysiology of various diseases (Kovacs and Gomba 1998; Thomas et al. 1998; Truong et al. 1998). Ang II was noted to be able to induce both cellular proliferation (Bunkenburg et al. 1992; Kunert-Radek et al. 1994) and apoptosis (Anaka et al. 1995; Kajstura et al. 1997) in renal cystic diseases, renal fibrosis, and obstructive uropathy. While the proliferative properties of Ang II were considered to be mediated by AT₁R, the AT₂R was considered to promote apoptosis (Nakajima et al. 1995; Yamada et al. 1996). The opposite was also suggested where AT₁R mediates apoptosis (Croft et al. 2000) and AT₂R mediates proliferation (Levy et al. 1996). To establish a mechanistic pathway through which Ang II exerts its growth promoting effect, Li et al. infused Ang II to WKY rats with AT₁R blocker or AT₂R blocker for 21 days. They noted that growth of the heart, aorta, coronary, renal, mesenteric, and femoral arteries are mediated by the AT₁R, with little evidence of a role of AT₂R (Li et al. 1998). This result raised the question of whether AT₂R subtype has growth-inhibitory and proapoptotic properties or no role in growth at all. Stoll et al. demonstrated an antiproliferative effect of Ang II on coronary endothelial cells that could be blocked by the AT₂R blocker PD123319 (Stoll et al. 1995). These results suggest that the antiproliferative effect of Ang II was mediated via AT₂R. In agreement with this observation were Brogelli et al. and Suzuki et al. Using an *in vitro* organ culture model, Brogelli et al. demonstrated that the growth of VSMC of male Wistar rat aorta was inhibited by AT₁R blockade, yet increased by the AT₂R antagonist. This data demonstrated that AT₂R-mediated inhibition of VSMC proliferation in adult vessels (Brogelli et al. 2002). Suzuki et al. demon-

strated that neointimal formation as well as DNA synthesis in VSMC was exaggerated in AT_2 -null mice following vascular injury, suggesting an antiproliferative effects of AT_2R (Suzuki et al. 2002).

In the kidney, Goto et al. examined AT_2 expression using culture mesangial cells (MC) from normotensive WKY and from SHR-SP rats. MC from SHR-SP, whose proliferative activity was much higher than WKY, showed only AT_1R subtype. The lower expression of AT_2R in MC from SHR-SP may suggest its involvement in the MC higher proliferative activity and possibly in the development of renal disorders (Goto et al. 1997).

7.2.6

Glomerular Actions

AT_2R seems to play a role in regulation of glomerular blood flow (GBF). Selective activation of AT_2R using afferent arteriole microperfusion technique was shown to cause endothelium-dependent vasodilation via CYP, possibly by releasing vasodilatory EET (Kohagura et al. 2000). On the other hand, AT_2R was shown to be able to do the opposite by increasing synthesis of the vasoconstrictor product of arachidonic acid metabolite, 20 HETE (Croft et al. 2000).

In a human study, Mifune et al. examined kidney samples from 21 patients with or without glomerular lesions. The glomerular lesions included IgA nephropathy, minimal change disease, and membranous glomerulonephritis. While mild–moderate immunohistochemical staining of AT_2R was seen in the blood vessels, weaker staining was found in the glomeruli. These results were observed in both the normal and diseased kidney samples, suggesting a role for the AT_2R in both normal and diseased human kidneys (Mifune et al. 2001).

7.2.7

Diabetes Mellitus

Diabetes mellitus (DM) has grown into an epidemic. Several studies pointed toward a significant role of the RAS activation in DM. Clinical studies demonstrated a role for interrupting the RAS at the ACE level and/or at the AT_1R levels, using ACEI and AT_1R blockade, in delaying progression of diabetic nephropathy (Lewis et al. 1993, 2001; Parving et al. 2001). In spite of that, the source of intrarenal Ang II formation remains unclear. Several studies have shown that renal renin mRNA and activity, proximal tubule angiotensinogen expression, and proximal tubule cell mRNA expression are increased in the diabetic model (Anderson et al. 1993; Wang TT et al. 1998; Zimpelmann et al. 2000). Once Ang II is formed in the kidney, it exerts most of its effects as vasoconstriction, increased glomerular capillary pressure, and mechanical stretch-induced glomerular injury via AT_1R (Yuan et al. 1990; Akai et al. 1994). Recently, Wehbi et al. studied the role of intrarenal AT_2R in the progression of diabetic nephropathy in streptozotocin (STZ)-induced diabetic SD rats. The study demonstrated by RT-PCR that early diabetes had no significant effect on glomerular mRNA ex-

pression of renin, angiotensinogen, or ACE. While the non-glycosylated AT₁R expression was increased in the diabetic rats, there was a significant decrease in glomerular AT₂R protein expression. Furthermore, AT₂R expression was decreased in all kidney regions in early diabetes. These data suggest that alterations in the balance of kidney AT₁R and AT₂R expression may contribute to Ang II-mediated glomerular injury in progressive diabetic nephropathy (Wehbi et al. 2001).

Bonnet et al. further confirmed that AT₂R was reduced in long-term diabetic SHR. In a study where they assessed gene and protein expression of the AT₁R and the AT₂R using RT-PCR, immunohistochemistry, and autoradiography in STZ-induced diabetes in SHR and WKY, they showed that both AT₁R and AT₂R mRNA levels in the kidney, glomerular, and tubular-interstitial staining for both AT₁R and AT₂R, and their binding was reduced (Bonnet et al. 2002). These data suggest a role for the AT₂R in the pathogenesis of renal injury, including DM.

7.2.8

Congenital Anomalies of the Kidney and Urinary Tract

The RAS plays a role in cell proliferation and differentiation, and organogenesis, especially nephrogenesis (Wolf and Neilson 1996). AT₂R is more expressed during development and appears to affect tissue remodelling through apoptosis and plays a role in nephrogenesis.

Congenital anomalies of the kidney and urinary tract account for more than 50% of cases of abdominal mass found in neonates, and involve 0.5% of all pregnancies (Scott et al. 1988). Studies of the mice and human AT₂R gene showed an association between being AT₂R null and congenital anomalies of the kidney and urinary tract (Nishimura et al. 1999). The AT₂R is postulated to be involved in the development of the ureteric lumen through apoptotic resorption of cells. Thus, the occurrence of a mutation of the AT₂R was shown to occur more frequently in individuals with pelvi-ureteric junction obstruction as well as lower ureter malformation (Nishimura et al. 1997). Hohenfellner et al., while confirming a role for AT₂R for the normal development of the ureter, could not find a relationship between AT₂R and vesico-ureteral reflux (Hohenfellner et al. 1999).

8

Conclusion

While our knowledge of AT₁R actions in the kidney is better delineated, most of the data regarding the role of AT₂R in normal physiological and in pathological situations remain unclear. A vast number of basic and clinical studies over the past few decades have confirmed a major role for drugs that interrupt the RAS at the ACE or the AT₁R levels in the management of several diseases including hypertension, diabetic nephropathy, congestive heart failure, and some of the glomerular diseases.

The possible protective role that AT₂R may play in some of these diseases is intriguing. Further studies aiming at better understanding of the physiologic and pathophysiologic roles of AT₂R and evaluating the potential beneficial actions of drugs that work as agonists for this receptor is much needed.

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AT₂ Function and Target Genes

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Abstract Angiotensin II (ANG II), the biologically active component of renin-angiotensin system (RAS), acts through two receptor subtypes, the AT₁ and the AT₂ receptors. All classic physiological effects of ANG II, such as vasoconstriction, aldosterone and vasopressin release, sodium and water retention, as well as ANG II-mediated growth, are mediated by the AT₁ receptor. Recent investigations have established a role for the AT₂ receptor in cardiovascular, brain, and renal function as well as in the modulation of various biological processes involved in development, cell differentiation, and tissue repair. Furthermore, binding of ANG II to AT₂ receptors inhibits, in certain cells, proliferation, mediates differentiation in neural cell lines, and even induces apoptosis. Interestingly, one study suggests that AT₂ receptor-mediated apoptosis may be independent of the ligand ANG II and requires only AT₂-receptor expression on a specific target cell. As all these effects of AT₂ receptor activation are the opposite of those mediated through AT₁ receptors, a ying-yang hypothesis has been proposed in which AT₁ and AT₂ receptors have principally contrary functions. This review summarizes new insights in the function of the AT₂ receptor and its affected target genes.

Keywords Angiotensin II · AT₂ receptor · Function · Target gene · Angiotensin receptors

1 Introduction

The peptide hormone angiotensin II (ANG II) exerts a broad field of physiological and pathophysiological actions on the cardiovascular and nervous system, fetal development, and in response to injuries. In 1989, two independent groups of investigators provided pharmacological evidence for the existence of two major subtypes of ANG II receptor (Chiu et al. 1989; Whitebread et al. 1989). These subtypes were subsequently named the AT₁ and AT₂ receptors. There may be even more ANG II receptor types, but they are not yet cloned. The AT₁ receptor is responsible for mediating many of the well-known stimulatory physiological actions of ANG II, including vasoconstriction, secretion of aldosterone, and renal sodium reabsorption. The AT₁ receptor can activate G protein-coupled and -uncoupled signal transduction pathways, such as down-regulation of adenylate cyclase, activation of phospholipase A₂, and protein kinase C, stimulation of the Janus kinase/STAT (signal transducer and activator of transcription) cascade, and opening of calcium channels. The AT₁ receptor is the predominant subtype in the adult and is responsible for the most physiological effects of ANG II (de Gasparo et al. 2000).

The AT₂ receptor shares only about 33% amino acid sequence identity with its AT₁ counterpart (Nakajima et al. 1993). The lowest homology between these receptors exists between their third intracellular loops (ICL). The third ICL is regarded as one of the major regions of the G protein-coupled receptor that is responsible for their signal transduction. Delivery of synthetic third ICL peptides of the AT₂ receptor into vascular smooth muscle cells (VSMC) results in decreased DNA synthesis, diminished cell proliferation, and a reduction in mitogen-activated protein (MAP) kinase activity, similar to the effects of ANG II acting via the AT₂ receptor (Hayashida et al. 1996a). Moreover, in neurons cultured from newborn rat hypothalamus and brain stem, intracellular injection of a 22-amino acid peptide (PEP-22) corresponding to the putative third ICL of the AT₂ receptor elicited an increase in delayed rectifier K⁺ current (K_v) similar to that obtained with ANG II acting via AT₂ receptors in these cells (Kang et al. 1996). Using a chimeric receptor in which the third ICL of the AT₂ receptor was replaced with that of the AT₁ receptor, Dittus et al. observed a loss in affinity to [¹²⁵I-Sar¹-Ile⁸]ANG II and ¹²⁵I-CGP42112A (Dittus et al. 1999). Collectively these data support the hypothesis that the third ICL of the AT₂ receptor is as closely linked to intracellular signaling as to the determination of ligand-binding properties.

Regardless of the low homology of the AT₁ and AT₂ receptors, ANG II exhibits a similar affinity to both receptor subtypes. Discrimination of these subtypes became possible with the development of highly selective receptor antagonists (Timmermans et al. 1993). AT₁ receptors exhibit a low affinity to tetrahy-

droimidazolepyridines, such as PD123177 and PD123319, and a high affinity for a class of compounds called “sartans” which, from the chemical point of view, belong to the biphenylimidazoles. On the other hand, AT₂ receptors show a very low affinity for biphenylimidazoles and a high affinity for tetrahydroimidazolepyridines. Moreover, compared with AT₁ receptors, AT₂ receptors also display a high affinity for the peptide CGP42112A, which is a partial agonist (Brechler et al. 1993).

The exposure of G protein-coupled receptors (GPCRs) to agonists often results in a rapid attenuation of receptor responsiveness. This process, termed desensitization, is the consequence of a combination of different mechanisms including the uncoupling of the receptor from heterotrimeric G proteins, the internalization of cell surface receptors to intracellular membranous compartments, and the down-regulation of the total cellular receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Ferguson et al. 2001). In contrast to this concept, the AT₂ receptor neither undergoes internalization after agonist binding (Hein et al. 1997) nor down-regulation of mRNA or protein level. Contrariwise, the AT₂ receptor density on the cell surface is adjusted by self up-regulation mechanisms on transcriptional and translational levels by the receptor itself (Shibata et al. 1997; Li et al. 1999).

The AT₂ receptor gene (AGTR2) is located on the X chromosome. The gene comprises three exons with the entire coding region in the third exon (Ichiki et al. 1995a). There is no intron in the coding region, thus excluding multiple forms of the AT₂ receptor derived by alternative splicing.

The majority of studies performed on AT₂ receptor regulation have been in vitro cell culture experiments. In the course of these experiments, it became evident early on that the cell culture conditions can affect the expression of AT₂ receptors. For example, in the fibroblast cell line R3T3, which is known to express AT₂ but not AT₁ receptors, the number of AT₂ binding sites depends greatly on the cell density (Dudley et al. 1993). In actively growing R3T3 cells, the expression of AT₂ receptors is very low, but their expression is substantially increased when the cells become confluent. This regulation of AT₂ receptors seems to occur at multiple levels, involving translational and/or posttranslational as well as transcriptional control (Camp et al. 1995). The fact that serum dramatically influences the expression of AT₂ receptors in cultured cells led to the idea that growth factors may have a role in the regulation of these receptors, and this is the case. The promoter activity of the AT₂ receptor gene is indeed modulated by several factors. To be specific, the AT₂ receptor is consistently down-regulated by factors which are known to induce cell proliferation, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and ANG II via AT₁ receptor (Ichiki et al. 1995a; Kizima et al. 1996; Li et al. 1998, 1999). In contrast, AT₂ receptor expression is up-regulated during serum deprivation and cell contact inhibition as well as by factors like insulin, insulin-like growth factor, the inflammatory cytokine interleukin 1b, estrogen, and ANG II via the AT₂ receptor itself

(Ichiki et al. 1995b; Kambayashi et al. 1996; Shibata et al. 1997; Armando et al. 2002; Stoll et al. 2002). It should also be pointed out that the effects of growth factors or serum on AT₂ receptor expression in PC12W and R3T3 cells are dependent on the respective cell passage (Li et al. 1998), indicating another level of complexity in the regulation of AT₂ receptor expression in these cells. In primary neuronal cultures, short-term incubations with NGF resulted in increased AT₂ receptor mRNA levels, whereas long-term incubations with this factor generated the opposite effect (Huang et al. 1997). The finding that the effects of certain factors on the AT₂ receptor expression are not necessarily identical in different cell types emphasizes the importance of thoroughly discriminating data gathered in each situation.

In vivo, AT₂ receptors are abundantly expressed in fetal organs but are present in distinct tissues in the adult organism, including brain, adrenal glands, uterine myometrium, ovarian follicles, kidney, and heart (Matsubara et al. 1998). In the brain, the receptor is expressed in brain stem, several thalamic nuclei, lateral septum, and amygdala (Tsutsumi and Saavedra 1991). Interestingly, the AT₂ receptor is re-expressed under pathological conditions such as congestive heart failure, renal failure, or following incidents like skin lesions, vascular injury, myocardial infarction, and lesions of the nervous system like brain ischemia, brain lesions, sciatic, or optic nerve transection (Unger 1999).

The signal transduction pathways for the AT₂ receptor still remain enigmatic. Although the AT₂ receptor possesses all features of a G protein-coupled receptor, the coupling of the receptor to G proteins is still a matter of controversy. The insensitivity of the AT₂ receptor to guanosine triphosphate (GTP) γ S raised the assumption that this receptor is not a member of the G protein-coupled receptor family (Bottari et al. 1991). Evidence that linked the AT₂ receptor to G proteins came from studies in neuronal cells demonstrating that the receptor modulates K⁺ channel activity by the G protein G_i (Kang et al. 1994). It turned out that at least three phosphatases lead to the growth-inhibitory signals of the AT₂ receptor: mitogen-activated protein kinase phosphatase (MKP)-1, protein phosphatase 2A (PP2A) and SH2 domain-containing phosphatase (SHP)-1 (Nouet and Nahmias 2000). Moreover, stimulation of the AT₂ receptor in certain cell types leads to changes in cellular cGMP levels and nitric oxide (NO) production (Sumners et al. 1991; Siragy and Carey 1997; Gohlke et al. 1998). In renal tubular epithelial cells and in cultured neurons, the receptor is also linked to a membrane-associated phospholipase A2 (PP2A) pathway which involves the G protein p21ras. This pathway can be blocked by the inhibition of the PP2A (Zhu et al. 1998). Another fatty acid used by the AT₂ receptor as a second messenger is the sphingolipid ceramide. In PC12W cells, a rat pheochromocytoma cell line, long-term activation of the AT₂ receptor increases the synthesis of ceramide and may lead via this pathway to the AT₂ receptor-mediated apoptosis (Gallinat et al. 1999; Lehtonen et al. 1999).

Unexpectedly, studies using AT₂ receptor knockout mice show that the AT₂ receptor appears to have a physiological role in blood pressure control as well as behavior modulation (Hein et al. 1995; Ichiki et al. 1995c; Sakagawa et al.

2000). These knockout models are part of a separate section of this volume (i.e., see Brede and Hein) and discussed there in more detail.

As most of these effects of AT₂ receptor activation are opposing those mediated by AT₁ receptor activation, a yin–yang hypothesis has been proposed in which the AT₁ and AT₂ receptors have principally opposed functions (Unger 1999; Gallinat et al. 2000; Stoll and Unger 2001; Kaschina and Unger 2003).

2 Function

ANG II has a number of well-known physiological effects, including stimulation of drinking behavior, increases in blood pressure, modulation of baroreceptor function, and stimulation of vasopressin release. However, the majority of early studies have indicated that they are AT₁ receptor-mediated effects and have failed to recognize any role of AT₂ receptors (Hogarty et al. 1992; Qadri et al. 1993). One notable exception involves studies showing that administration of the AT₂ receptor antagonist PD123177 potentiates the stimulatory effects of ANG II on drinking behavior and vasopressin secretion (Höhle et al. 1996). This is one example of a situation in which AT₂ receptors appear to oppose AT₁ receptor-mediated effects.

The AT₂ receptor is expressed at a very high level in numerous fetal tissues (Shanmugam and Sandberg 1996). In contrast, its expression is restricted to certain organs and brain areas in the adult. The AT₂ receptor is re-expressed in the context of cardiac and vascular injury (Nakajima et al. 1995) and nerve crush (Gallinat et al. 1998; Lucius et al. 1998) as well as during wound healing (Kimura et al. 1992) or kidney obstruction (Morrissey and Klahr 1999). These observations led to the hypothesis that the AT₂ receptor exerts its main functions during the processes of cellular development and reorganization. Therefore, in this chapter, we will focus on the role of the AT₂ receptor during ontogenesis and in tissue regeneration.

2.1 Growth and Development

During the process of ontogenesis, the AT₂ receptor is expressed in an abundant and transient manner. In fetal tissues, the AT₂ receptor emerges on embryonic days 11–13 (E11–E13) and reaches its maximal level on E19. The AT₂ receptor is localized in tissues derived from embryonic mesoderm such as the striated and smooth muscle, undifferentiated mesenchymal tissues surrounding the developing cartilage, diaphragm, tongue, connective tissue, bronchi, blood vessels, ligaments, and submucosal layer of the stomach and intestine, and the choroid surrounding the retina and dermis. The AT₂ receptor expression then rapidly declines in after birth to lower or undetectable levels (Table 1; Shanmugam and Sandberg 1996). In comparison, the AT₁ receptor is expressed less in fetal organs such as the aorta, kidney, adrenal gland, liver, and lungs (Grady et al.

Table 1 Tissue distribution of the AT₂ receptor and ontogenic change

Tissue	Fetus	New-born	3 week	8 week	Reference(s)
Adrenal					
Cortex	+	+	+	+	Shanmugam et al. 1995
Medulla		+	+	+	Feuillan et al. 1993
Ovary					
Follicular granulosa	+	+		+	Pucell et al. 1991
Kidney					
Cortex	+++	+			Shanmugam et al. 1995
Medulla (outer strip)	+	++			Shanmugam et al. 1995; Kakuchi et al. 1995
Heart	±				Shanmugam et al. 1995
Uterus					
Myometrium					
Blood vessels				+	
Heart	±				Shanmugam et al. 1995
Aorta	+			+	Feuillan et al. 1997; Shanmugam et al. 1995
Pancreas	+				Chappell et al. 1992
Trachea	+				Shanmugam et al. 1995
Stomach	+				Shanmugam et al. 1995
Mesenchyme	+				
Skin	+++	±			Grady et al. 1991; Shanmugam et al. 1995; Feuillan et al. 1993
Tongue	+++	±			Grady et al. 1991; Feuillan et al. 1993
Skeletal muscle	+				Feuillan et al. 1993

1991) which are the tissues where ANG II is known to play a physiological role during adult life.

2.1.1

Development of the Nephron and Aorta

In the fetal kidney on the embryonic days E12–E16, the AT₂ receptor is expressed in mesenchymal cells of differentiating cortex and medulla, which surround the glomeruli in the cortex and the tubular tissues in the medulla (Kakuchi et al. 1995). These mesenchymal cells will finally be replaced by tubular tissue after their apoptosis. On E14, the AT₂ receptor emerges in the interstitial mesenchym of the kidney, but not in the glomeruli or the S-body, whereas the AT₁ receptor is richly expressed in the preglomeruli and S-bodies. On E16, AT₂ receptor mRNA is seen in the renal capsule and inner medulla where it is prominent along the papillary duct and between the collecting ducts. In this context, the apoptosis of the AT₂ receptor-expressing cells in fetal tissue development is an attractive concept. On the other hand, mice with targeted deletions

of genes for angiotensinogen or angiotensin-converting enzyme (ACE) revealed more severe renal abnormalities compared to AT₁ or AT₂ receptor knockout animals with milder abnormalities of the kidney (Hein 1998). These findings suggest that AT₁ and AT₂ receptors are both involved in the developmental process of the nephron and that ANG II growth-stimulatory effects, though AT₁ receptors may be adjusted by AT₂ receptor-mediated apoptosis and growth inhibition. Therefore, changes in AT₂ receptor signaling may lead to changes in the sensitive balance between growth stimulation and inhibition and to alterations in tubular formation.

In the fetal aorta, AT₂ receptor expression is undetectable in early stages (E15) but rises to very high levels during late embryonic development (E16–E21) whereupon it decreases 2 weeks after birth (D22) (Viswanathan et al. 1991). A similar pattern of AT₂ receptor expression during development was also observed in other cardio-pulmonary tissues. By in utero application of the AT₂ receptor antagonist PD123319, the role of the AT₂ receptor in the development of the fetal aorta could be examined. The drug was administered for 3 days prior to tissue harvesting and the rates of DNA synthesis in the fetal aortae in utero were measured. During early embryonic development (E15), when the AT₂ receptor expression is low and the aortic DNA synthesis rates are near maximum, PD123319 has no effect on DNA synthesis. Between E16 and E21, the growth rates in the fetal aorta decline as the AT₂ receptor is expressed. PD123319 treatment attenuates significantly this reduction in aortic DNA synthesis (Nakajima et al. 1995). These results suggest that the AT₂ receptor mediates an antigrowth effect on the aorta in vivo which may be causally related to the delayed vasculogenesis observed in AT₂ receptor knockout mice (Yamada et al. 1998).

2.1.2

Development of the Brain and Cognitive Function

Although circumventricular organs of the brain respond to circulating ANG II, an endogenous brain renin-angiotensin system can generate ANG II in tissues protected from circulating ANG II by the blood-brain barrier. ANG II generated by this system reacts with angiotensin receptors within the brain. The brain angiotensin receptors have been studied and reviewed extensively (Gehlert et al. 1991a,b; Rowe et al. 1992; Saavedra 1992; Song et al. 1992; Höhle et al. 1995; Lenkei et al. 1996). An expression of the AT₂ receptor in fetal (E18) brain tissues was reported in several areas: inferior olive, paratrigeminal nucleus, and hypoglossal nucleus (Table 2). Saavedra determined AT₁ and AT₂ receptor expression in various regions of the brain, and the changes of the expression depending on age (Saavedra 1992). Although AT₁ receptor expression did not show marked age dependence, the AT₂ receptor showed a significant decrease from 2 to 8 weeks of age. Using competitive radioligand binding the distribution of ANG II receptor types in adult rat brain nuclei were investigated (Obermüller et al. 1991). Whereas midbrain and brain stem contained AT₁ and AT₂ receptors in

Table 2 Distribution of the AT₂ receptor in the brain: effect of age (fmol/mg protein, mean±SEM)

	2-week-old	8-week-old
Regions containing only the AT ₂ receptor		
Persistent AT ₂ receptor with age		
Lateral septal nucleus	58±6	18±3
Ventral thalamic nuclei	101±8	24±3
Mediodorsal thalamic nucleus	165±11	38±13
Locus ceruleus	289±19	98±13
Principal sensory trigeminal nucleus	75±6	15±4
Parasolitary nucleus	220±15	57±14
Inferior olive	1328±61	181±32
Medial amygdaloid nucleus	159±8	94±9
Medial geniculate nucleus	338±24	71±6
Transient expression of the AT ₂ receptor		
Anterior pretectal nucleus	53±8	ND
Nucleus of the optic tract	101±13	ND
Ventral tegmental area	101±11	ND
Posteodorsal tegmental nucleus	110±21	ND
Hypoglossal nucleus	141±11	ND
Central medial and paracentral thalamic nucleus	202±14	ND
Laterodorsal thalamic nucleus	110±10	ND
Oculomotor nucleus	98±13	ND
Regions containing both AT ₁ and AT ₂ receptors		
Persistent AT ₂ receptor with age		
Superior colliculus	145±5	65±8
Cingulate cortex	19±4	8±4
Transient expression of the AT ₂ receptor		
Cerebellar cortex	59±6	ND

ND, not determined.

comparable concentrations, AT₁ receptors were by far predominant in several hypothalamic nuclei, although a limited amount of AT₂ receptors was detectable in most of them. Although minor differences may exist among these studies, they share the following observations: AT₂ receptor expression is consistently high in the cerebellar nuclei, inferior olive, and locus coeruleus in the brain stem, which is rich in noradrenergic neurons. In contrast to the AT₁ receptor, which participates in various central cardiovascular functions and is expressed in the hypothalamus and in brain stem nuclei (nucleus of the solitary tract, dorsal motor nucleus of vagus at a low level), the AT₂ receptor is much less present in distinct hypothalamic and brain stem nuclei associated with the regulation of cardiovascular functions. The presence of the AT₂ receptor in the dorsal motor nucleus of vagus is not completely settled.

The AT₂ receptor is exclusively or predominantly expressed in two limbic systems, the lateral septal nucleus, and the medial amygdaloid nucleus. This distribution of the AT₂ receptor remains unchanged during development and in the adult animal; however, the density of the AT₂ receptors shows a marked decline after birth (Millan et al. 1991; Tsutsumi and Saavedra 1991). The fact that the

AT₂ receptor is much more highly expressed in fetal brain tissues compared to adult suggests that the AT₂ receptor may modulate auditory, visual, motor, and limbic formation during the process of development. In vitro studies also support that assumption, e.g., the long-term treatment of the AT₂ receptor with ANG II induces two major morphological changes in 3-day-old rat cerebellar cells: increased elongation of neurites and cell migration from the edge of the microexplant toward the periphery. These are two important processes in the organization of the various layers of the cerebellum (Cote et al. 1999).

The role of the AT₂ receptor in brain development may explain the altered behavior of AT₂ receptor knockout mice. These mice have been reported to show an anxiety-like and markedly reduced exploratory behavior (Hein et al. 1995a; Ichiki et al. 1995c), and the pain threshold was significantly lower in AT₂ receptor knockout compared to findings in wild-type mice. Furthermore, AT₂ receptor knockout mice show a greater stimulation of dipsogenesis after water deprivation (Hein et al. 1995a). In the passive avoidance task and cold injury, no differences were found between wild-type and AT₂-deficient mice (Sakagawa et al. 2000).

Another genetic study points to a role for the AT₂ receptor in brain development and cognitive function in human. This study shows that AT₂ receptor expression was missing in a female patient with mental retardation (MR) who had a balanced X;7 chromosomal translocation. Additionally, 8 of 590 unrelated male patients with MR were found to have mutations in the AT₂ receptor (Vervoort et al. 2002). Thus it appears that the AT₂ receptor in human seems to be involved in the development of cognitive function and learning behavior.

Although the distribution of AT₂ receptors in the brain is well known, their effects are still not clear. As outlined above, brain AT₂ receptors may play a role in cognitive functions and certain types of behavior, such as exploration or drinking (Hein et al. 1995a). On the other hand, they may also antagonize the central effects of angiotensin peptides in osmoregulation mediated via AT₁ receptors (Höhle et al. 1995, 1996). Taken together, the data suggest the AT₂ receptor regulates the differentiation of various precursor cells and enables them to assume their final tasks. A possibly important role of AT₂ receptors in neuroregeneration and neuroprotection will be dealt with below.

2.2 Tissue Regeneration and Pathophysiological Aspects

AT₂ receptor expression is dramatically altered in a number of tissues after experimental- or disease-induced damage, and there is much evidence that AT₂ receptors contribute to the cellular processes that follow injury situations. The role of AT₂ receptors in pathological situations will be reviewed in the following paragraphs, with particular focus on their function in tissue regeneration.

2.2.1

Nerve Regeneration

In vitro treatment of undifferentiated, NGF pre-stimulated NG108-15 or PC12W cells, which are used as in vitro models of sympathetic neurons, with ANG II induces growth arrest and morphological differentiation to neuronal cells via a Rap1/B-Raf-pathway. This effect can be blocked by the selective AT₂ receptor antagonist PD123177 (Laflamme et al. 1996; Meffert et al. 1996; Stroth et al. 1998; Gendron et al. 2002). During this differentiation process the cytoskeleton of PC12W cells undergoes substantial morphological changes, pointing to an involvement of the AT₂ receptor in axonal regeneration (Fig. 1). These morphological changes are paralleled by alterations in the expression patterns of different cytoskeletal proteins, such as neurofilament-M (NF-M) subunit (Gallinat et al. 1997) and microtubule-associated proteins (Stroth et al. 1998), pointing to an involvement of the AT₂ receptor in axonal regeneration. As demonstrated by Gallinat et al., a lasting and pronounced increase in the AT₂ receptor gene expression after sciatic nerve transection occurs in both sciatic nerves as well as in dorsal root ganglia neurons (Gallinat et al. 1998). Together with the observation that Schwann cells express AT₂ receptors (Bleuel et al. 1995), this specific AT₂ receptor expression pattern after peripheral nerve injury points to a role of these cells in neuroregenerative events.

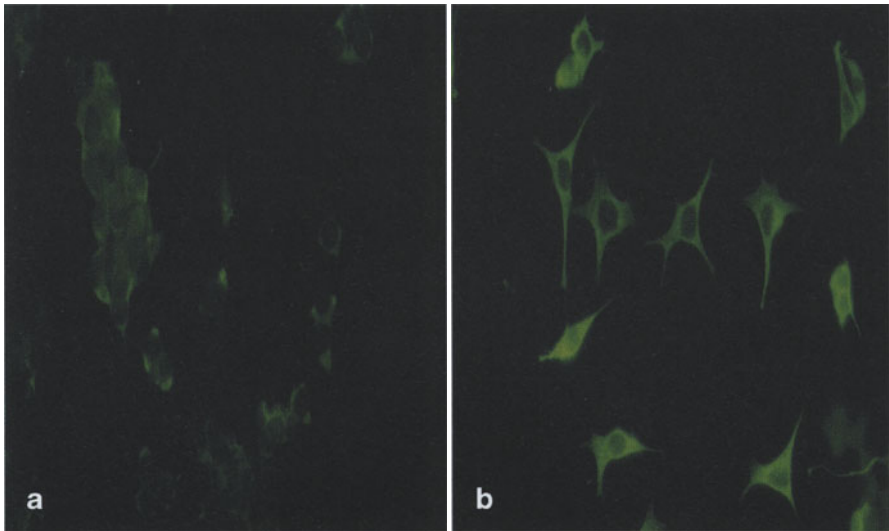
Both in vitro and in vivo, the AT₂ receptor promotes axonal elongation of postnatal retinal explants and dorsal root ganglia neurons and, in adult rats, axonal regeneration of retinal ganglion cells after optic nerve crush. It has been demonstrated that AT₂ receptor stimulation directly contributes to the process of axonal regeneration in the CNS of adult rats. Intravitreal application of ANG II-soaked collagen foams significantly improved the axonal regeneration of retinal ganglion cells after optic nerve crush. Co-treatment with the AT₂ receptor antagonist PD123177, but not the AT₁ receptor antagonist losartan, entirely abolished the ANG II-induced axonal regeneration, providing for the first time evidence for receptor-specific neurotrophic actions of ANG II via the AT₂ receptor (Figs. 2 and 3; Lucius et al. 1998).

Thus, the AT₂ receptor plays a role in neuronal cell differentiation and nerve regeneration via regulation of the neural cytoskeleton of affected neurons (for review, Rosenstiel et al. 2002).

2.2.2

Remodeling of the Heart After Injury

Pharmacological evidence indicates that most of the known effects of ANG II in adult cardiovascular tissues are due to the AT₁ receptor predominantly expressed in the adult heart. However, the relative proportion of the AT₁ and AT₂ receptor density changes during myocardial infarction (MI) (Fig. 4). AT₂ receptor density was shown to be increased in experimental MI 1 day after infarction in the infarcted area. Additionally, the AT₂ receptor was further upregulated



control

ANG II



**Ang II +
PD123177**

Fig. 1a–c Immunofluorescence microscopy analysis of the effect of ANG II on β -tubulin in PC12W cells. **a** Control; **b** ANG II; **c** ANG II+

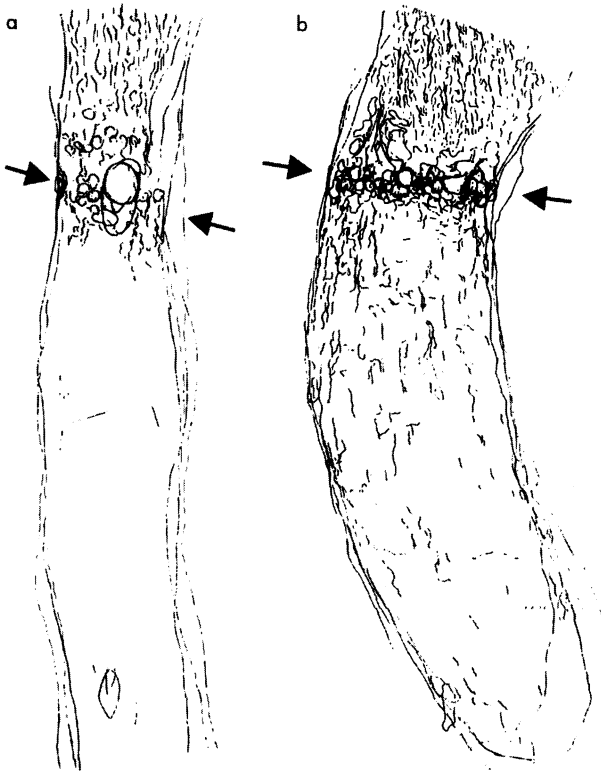


Fig. 2a, b Camera-lucida projections from serial sections of paraffin-embedded and GAP-43-stained optic nerves. The lesion site is demarcated by *two arrows*. In control animals (**a**), only few fibers grew over the lesion, whereas in animals receiving the ANG II gel foam (**b**), numerous axons crossed the lesion site and regenerated over a distance of several millimeters. ANG II increased the number of GAP-43-positive axons in the proximal optic nerve stump compared with controls

7 days post infarction in both the infarcted and the noninfarcted area (Nio et al. 1995; Busche et al. 2000). These findings resemble the hypertrophied heart, where the ratio of AT_2 to AT_1 receptor densities is also increased (Lopez et al. 1995).

Most studies using AT_2 receptor-deficient mice suggested that AT_2 receptors inhibit hypertrophy and vascular fibrosis (Akishita et al. 2000b; Brede et al. 2001; Sandmann et al. 2001a). A recent study demonstrated the role of the AT_2 receptor in wound healing after MI using an *in vivo* intervention study in AT_2 receptor knockout mice with MI. Mice lacking the AT_2 receptor revealed a decreased collagen deposition, followed by cardiac rupture. The impaired wound healing caused a significantly greater mortality rate of the AT_2 receptor knockout mice compared to wild-type mice (Ichihara et al. 2002). Experiments using cultured cell systems showed that experimental overexpression of the AT_2 receptor in VSMC (vascular smooth muscle cells) induced collagen expression (Mifune et al. 2000). Further *in vitro* studies demonstrated that the AT_2 receptor in-

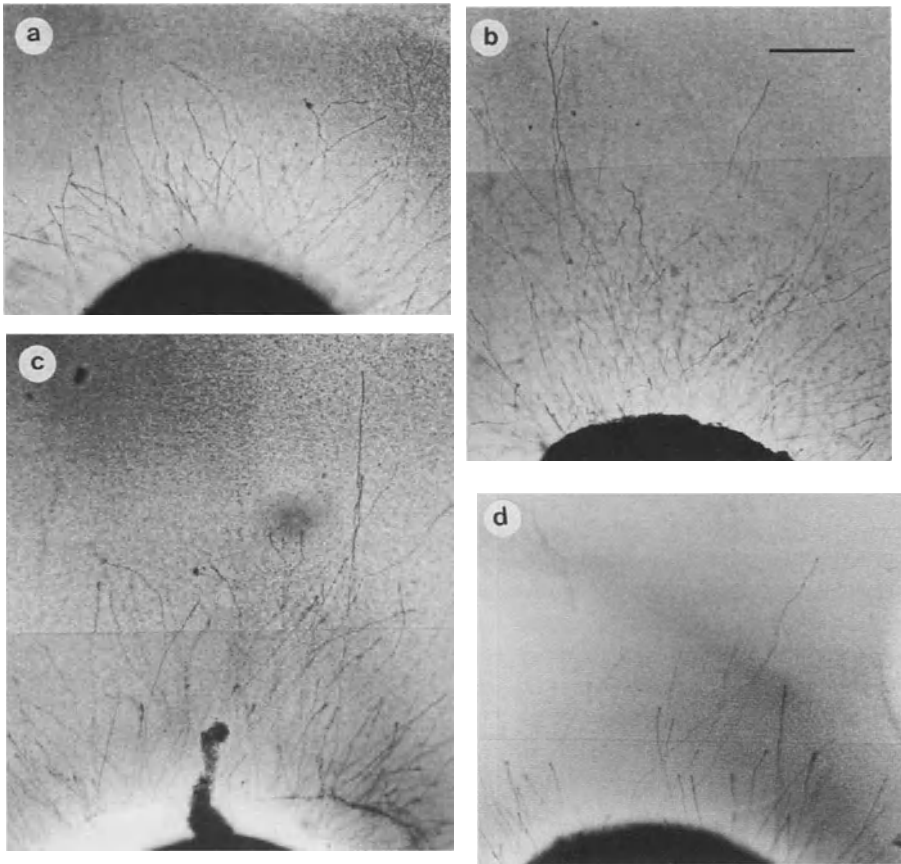


Fig. 3. **a** Retinal explant (postnatal day 11) in control cultures after 3 days in vitro. Many regenerating, but short neurites were detected. **b** Addition of BDNF (10 ng/ml) enhanced axonal growth up to $160\pm 3\%$. **c** In the presence of ANG II (10^6 M), the number and length of neurites was increased to comparable amounts ($162\pm 4\%$), whereas **d** the AT₂ receptor antagonist, PD123177 (10^5 M), blocked axonal regrowth ($102\pm 3\%$). On the other hand, the AT₁ receptor-antagonist, losartan (10^5 M) did not influence the ANG II-induced axonal elongation (data not shown). Bar=100 μ m

hibited the proliferation of rat coronary endothelial cells and transfected VSMCs (Stoll et al. 1995; Fischer et al. 2001) and prevented ANG II-induced growth of cultured neonatal rat myocytes (Booz and Baker 1996). The AT₂ receptor appears also to control the transcriptional regulation of the Na⁺-H⁺-exchanger NBC-1 in the ischemic myocardium and contributes to the control of pH regulation in the diseased cardiac tissue (Sandmann et al. 2001b). Therefore, the AT₂ receptor counteracts acidification in the affected areas through cytoplasmic alkalization and thereby reduces apoptosis and inflammation, both consequences of acidification.

Considering the ying-yang hypothesis, where AT₂ receptor functions are the opposite of those mediated through the AT₁ receptor, the up-regulation of the

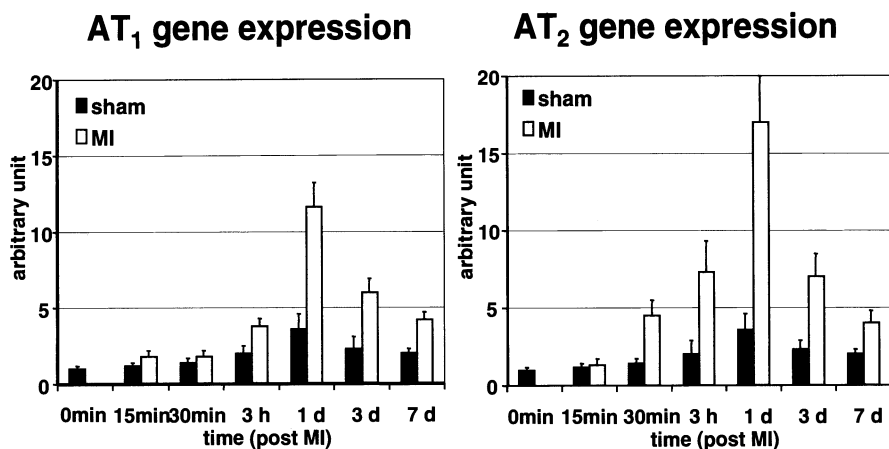


Fig. 4 Regulation of AT receptors following myocardial infarction

AT₂ receptor in the diseased heart leads to interesting pharmacological consequences of AT₁ receptor antagonist treatment. AT₁ receptor antagonists cause a reduction in blood pressure, cardiac and vascular hypertrophy, proteinuria, and glomerular sclerosis. By blocking the AT₁ receptor, the circulating ANG II preferentially binds to the cardiac AT₂ receptor. There is evidence that the AT₂ receptor is activated in failing hearts, especially in cardiac fibroblasts. Liu et al. reported that the cardioprotective function of AT₁ receptor antagonists is mainly exerted by the selective stimulation of AT₂ receptor, which is mediated partly by the kinin/NO system. ANG II-induced cardiac fibrosis is increased by chronic inhibition of NO synthase, and generation of coronary kinin mediates NO release after ANG II receptor stimulation (Liu et al. 1997). Moderate endothelial NO synthase (eNos) activity and NO generation is cardio protective because NO reversibly depresses mitochondrial respiration and hence decreases basal myocardial oxygen consumption (Xie et al. 1996). Interestingly, Ritter et al. showed that AT₂ receptor stimulation with ANG II increases eNOS protein expression 3.3-fold in rat cardiomyocytes (Ritter et al. 2003; see Sect. 11). AT₂ receptor-mediated activation of the kinin/NO system is also involved in pressure natriuresis and diuresis of the kidneys. Kijima et al. reported that the AT₂ receptor in hypertrophic myocytes at least partially exerts an inhibitory effect on AT₁ receptor-mediated positive chronotropic or hypertrophic actions by showing up-regulation of AT₂ receptors in stretch-induced myocyte hypertrophy (Kijima et al. 1996). Taken together, these results show that the AT₂ receptor plays an important role in cardiovascular recovery in various ways (Table 3; Matsubara 1998; Sandmann and Unger 2002).

Table 3 Pathophysiological roles of AT₂ receptors in cardiovascular systems

Effect	Cell/tissue	Reference(s)
Growth inhibition	VSMC	Nakajima et al. 1995
	Endothelial cell	Stoll et al. 1995
	Neonatal rat myocytes	Fischer et al. 2001
	Cardiomyocyte	Booz and Baker 1996
	Cardiac fibroblast	Van Kesteren et al. 1997
Apoptosis	Endothelial cell	Ohkubo et al. 1997
	VSMC	Dimmeler et al. 1997
	Cardiomyocyte	Yamada et al. 1998
		Leri et al. 1998
Differentiation	VSMC	Goldenberg et al. 2001
	Heart	Yamada et al. 1999
Decrease in cellular matrix	Heart	Ohkubo et al. 1997
		Fischer et al. 2001
		Shimizu-Hirota et al. 2001
		Chassagne et al. 2002
pH regulation	Heart	Kohout et al. 1995
Vasodilatation NO production	Glomerular afferent arteriole	Sandmann et al. 2001b
	Coronary artery and microvessel	Arima et al. 1997
	Kidney	Seyedi et al. 1995
	Aorta	Siragy et al. 1996
	Heart	Gohlke et al. 1998
		Ritter et al. 2002

2.2.3

Renal Physiological and Pathophysiological Function

The AT₁ receptor is clearly expressed in the glomeruli and in the inner stripe of the outer medulla. Glomerular AT₁ receptors may control the hemodynamic function of the kidney. The proportion of the AT₂ receptor in the kidney cortex is less than 10% of the AT₁ receptor in the rat and rabbit, and roughly 55% in monkey (Chang and Lotti 1991). Nevertheless, the AT₂ receptor influences renal function by controlling pressure natriuresis (Lo et al. 1995). In sodium-depleted rats it is involved in decreasing cGMP production by a bradykinin-dependent mechanism (Siragy and Carey 1996; Chung und Unger 1998).

Morrissey and Klahr determined the effects of the AT₂ receptor antagonist PD123319 on pathophysiological processes in the kidneys of rats undergoing unilateral ureteral obstruction. The authors showed that blockade of the AT₂ receptor flattens the increase in interstitial volume and the collagen IV matrix score of the ureteral-obstructed kidney. The AT₂ receptor antagonist inhibited apoptosis of tubular cells, α -smooth muscle actin expression within the interstitium, and p53 expression in the ureteral-obstructed kidney. These findings suggest that the AT₂ receptor exerts an antifibrotic effect under pathophysiological conditions, again an effect that is opposite to the profibrotic effects of the AT₁ receptor (Morrissey and Klahr 1999). In support of this hypothesis, an in vitro

study shows that ANG II induces growth inhibition and apoptosis in the cultured proximal tubule cell line LLC-PK1 overexpressing of AT₂ receptors (Zimpelmann and Burns 2001). Taken together, these reports demonstrate that the AT₂ receptor also plays a pivotal role in renal remodeling following injury.

3 Target Genes

As demonstrated above, the AT₂ receptor is mainly involved in cellular development and tissue rearrangement after injury. In order to elucidate the expression profile of AT₂ receptor target genes involved in these processes, various studies have been performed. Recently, two studies using differential display performed in cell-culture systems discovered some of these target genes. Wolf and co-workers investigated the alteration in gene expression in PC12W cells after AT₂ receptor stimulation. They were able to show that ANG II suppresses the mRNA expression of SM-20, a growth factor-responsive gene. The SM-20 transcription was also reduced by ANG II acting on AT₂ receptors in rat glomerular endothelial cells that express both AT₁ and AT₂ receptors (Wolf et al. 2002). These effects could be antagonized by PD123177, but not by losartan, a selective AT₁ antagonist. SM-20 is associated with mitochondria and is involved in neurotrophin withdrawal-mediated apoptosis through a caspase-dependent mechanism (Wax et al. 1994). Its down-regulation via AT₂ receptors may set the course from apoptosis to differentiation of the affected cells.

Stoll and co-workers identified two additional target genes of the AT₂ receptor. One of them was the transcription factor Zfh_{ep}, a zinc finger homeodomain enhancer-binding protein which is implicated in various developmental processes. The expression patterns of Zfh_{ep} *in vivo* and in a P19 cell model of neurogenesis suggest that Zfh_{ep} may play a role in the differentiation of neural cells (Yen et al. 2001). In quiescent PC12W cells, Zfh_{ep} mRNA was time-dependently induced by ANG II, an effect that could be blocked by the selective AT₂ antagonist PD123177. These observations were supported by data from a coronary endothelial cell model. In this model, Zfh_{ep} mRNA expression was also induced after AT₂ receptor stimulation. Interestingly, this Zfh_{ep} induction was only observed when the AT₁ receptor was blocked by losartan. This observation suggests that a negative regulatory influence of AT₁ receptors on AT₂ induced Zfh_{ep} mRNA expression. Zfh_{ep} is present in both hyperphosphorylated and hypophosphorylated forms. It is primarily phosphorylated on serine and threonine residues and dephosphorylation enables the translocation from the cytosol into the nucleus of the transcription factor. This dephosphorylation could be accomplished by PP2A (Costantino et al. 2002), a phosphatase which is activated via AT₂ receptors. In this way the AT₂ receptor may modulate the differentiation process in neuronal cells.

The second gene identified by Stoll et al. was the AT₂ receptor itself (Stoll et al. 2002), an observation that is consistent with an older study using cultured rat cortical cells (Shibata et al. 1997). This self up-regulation is exceptional be-

cause the majority of receptors are down-regulated after stimulation, preventing an "overload" of the receptor signal by a positive feedback loop. But this observation can be reconciled with the general unique nature of the AT₂ receptor signaling, including the lack of ligand-stimulated receptor internalization and desensitization of the receptor (Mukoyama et al. 1993; Mukoyama et al. 1995). These findings led to the assumption of the existence of an unknown endogenous factor which, like the AT₂ receptor ligands, seems to prevent AT₂ receptor degradation (Csikos et al. 1998). Miura and Karnik were first to report that the stimulation of the AT₂ receptor via ANG II is not obligatory for its signaling and also that the receptor expression level is crucial. They proposed that an overexpression of the AT₂ receptor itself is a receptor-intrinsic signal for apoptosis that does not require ANG II (Miura and Karnik 2000).

Since then, several publications have confirmed this concept of an intrinsic activity of the AT₂ receptor. Another interesting finding is that the AT₂ receptor binds directly to the AT₁ receptor and thereby antagonizes the function of the AT₁ receptor. This inhibitory effect of the AT₂ receptor is independent of the stimulation status. This report is notable since it is the first identified interaction between two G protein-coupled receptors, where one receptor acts like an antagonist (AbdAlla et al. 2001).

All these data fit to two recently published studies which show that overexpression of the AT₂ receptor significantly alters the expression profile of cells, given the receptor density crosses the critical threshold. In VSMC, overexpressed AT₂ receptor leads to decreased expression of AT₁ and transforming growth factor (TGF)- β type I receptors (Su et al. 2002). In the same cell line, over-expressed AT₂ receptor upregulates bradykinin and inducible NO in the absence of ANG II. The bradykinin B2 receptor antagonist HOE-140 and the NO synthase inhibitor *N*^o-nitro-L-arginine methylester (L-NAME) inhibit the decrease in AT₁ receptor expression via the overexpression of AT₂ receptor in VSMCs, whereas L-arginine enhances a decrease of AT₁ receptor expression (Jin et al. 2002). By this, the AT₂ receptor antagonizes the AT₁ receptor via a bradykinin B2 receptor/NO pathway without the requirement of ANG II (Fig. 5). These observations are consistent with the presumed AT₂ receptor-mediated vasodilator signaling cascade that includes bradykinin, nitric oxide, and cyclic guanosine 5-monophosphate (see Sect. 9).

Another gene that is negatively regulated by AT₂ receptor overexpression is the TGF- β I receptor. Conversely, AT₁ receptor stimulation is accompanied by an up-regulated TGF- β 1 gene expression. TGF- β 1 is a potent regulator of VSMC proliferation, migration, and extracellular matrix synthesis, components which partially contribute to the pathogenesis of cardiovascular end-organ damage. Furthermore, the AT₁ receptor stimulates collagen production in VSMCs via a paracrine/autocrine loop of TGF- β (Ford et al. 1999). The suppression of the AT₁ receptor as well as the TGF- β I receptor appear to have a potential benefit in preventing pathological myocardial fibrosis.

To investigate the molecular events that follow the initiation of neurite outgrowth in PC12W cells via AT₂ receptor stimulation, Gallinat and co-workers

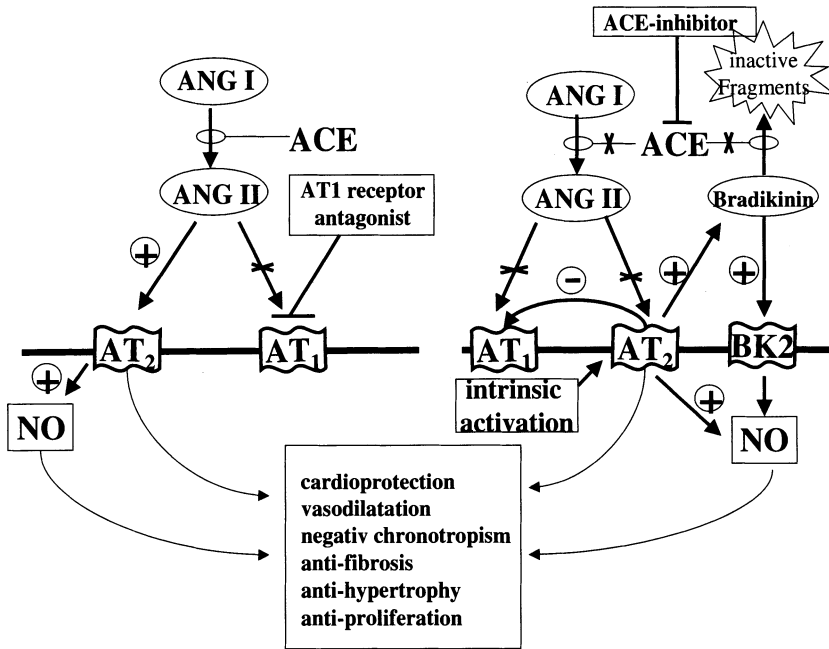


Fig. 5 Influence of AT₁ receptor antagonist and ACE-inhibitor on the AT₂ receptor effects

determined the expression pattern of the middle-sized neurofilament subunit (NF-M). They showed that the protein and mRNA levels of NF-M was not only down-regulated by ANG II via AT₂ receptor directly, but also by counteracting the NGF-mediated NF-M up-regulation. In view of previous findings of decreased NF levels in regenerating neurons and in neuronal cultures undergoing apoptosis, this observation suggests a role of AT₂ receptors in either of these processes (Gallinat et al. 1997).

As outlined above, the AT₂ receptor is re-expressed and plays a role in tissue remodeling. Change in the expression profile of the concerned areas is one possible option of the cells to implement this reconstruction. The AT₂ receptor appears to take a direct and active part in the remodeling of the extracellular matrix of the injured regions. Fischer et al. showed that stimulation of the AT₂ receptor in VSMCs, retrovirally transfected with the AT₂ receptor, resulted in a significant increase in thrombospondin 1 synthesis, which was abolished by an AT₂ receptor antagonist and attenuated by pretreatment with pertussis toxin, suggesting a G protein-dependent pathway (Fischer et al 2001). Using the same cell model, Mifune and co-workers were able to show that the AT₂ receptor stimulation increases collagen synthesis also via a G protein-mediated mechanism (Mifune et al. 2000).

In the myocardium, acidosis depresses the contractility of cardiac myocytes by affecting virtually every step in excitation-contraction coupling. Hence, in order to preserve a proper function of the heart, cardiac myocytes possess pH-

regulating transporters that maintain the pH within very narrow limits. Ion exchange in myocardial cells represents a major mechanism for pH regulation during normal physiological processes, but particularly during ischaemia and early reperfusion. The AT₂ receptor, being upregulated itself within the infarcted area, upregulates and activates the Na⁺-HCO₃ symporter NBC-1 in the ischemic myocardium and thereby contributes to the control of pH in cardiac tissue (Kohout et al. 1995; Sandmann et al. 2001b). Since acidification is also a feature of apoptosis and inflammation, the AT₂ receptor acts to obviate this process by preventing acidification through cytoplasmic alkalization.

A recent study using *in vivo* and *in vitro* experiments determined the influence of the AT₂ receptor on myocardial eNOS (NOS-III) protein expression. AT₂ receptor stimulation with ANG II increases the eNOS protein expression 3.3-fold in rat cardiomyocytes. This augmentation could be blocked by PD123319, whereas inhibition of the AT₁ receptor did not reduce ANG II-mediated eNOS protein expression. The modulatory effects of the AT₂ receptor on eNOS expression was confirmed *in vivo* using an AT₂ receptor knockout mouse. Mice lacking AT₂ receptors express significantly less eNOS protein in the myocardium than wild-type mice (Ritter et al. 2003). As outlined above, AT₂ receptor stimulation releases NO (see Sect. 9). This has been shown for coronary arteries and the aorta (Seyedi et al. 1995; Golhke et al. 1998). Moderate eNOS activity and NO generation is considered to be cardioprotective, because NO reversibly depresses mitochondrial respiration and hence decreases basal myocardial oxygen consumption (Xie et al. 1996; Liu et al. 1997).

Many inflammatory renal diseases are connected with glomerular influx of monocytes/macrophages (M/M). The signals guiding the M/M into the glomerulus are cytokines and chemokines. *In vitro* ANG II stimulation of the AT₂ receptor induces mRNA and protein expression of the chemokine RANTES in cultured rat glomerular endothelial cells, a chemokine that is chemotactic for monocytes. In an *in vivo* study it was shown that intraperitoneal infusion of ANG II into naive rats for 4 days significantly stimulated glomerular RANTES mRNA and protein expression compared to control. Immunohistochemistry revealed induction of RANTES protein mainly in glomerular endothelial cells and small capillaries. Under medical conditions the AT₂ receptor may have a proinflammatory implication mediated through its target gene RANTES (Wolf et al. 1997).

In summary, the functions of the AT₂ receptor in development and regeneration imply a wide range of target genes (Table 4) that are controlled via ANG II stimulation and AT₂ receptor density. The suppression of proliferation signals by the AT₂ receptor via activation of various phosphatases is mediated via down-regulation of growth-mediating receptors as well as up-regulation of the receptor itself, thereby producing a positive feedback loop. During its function in cell differentiation, the AT₂ receptor regulates miscellaneous genes in order to reorganize precursor cells during maturation and enable them to assume their finale tasks.

Table 4 Target genes modulated by the AT₂ receptor

Target gene	Regulation	Reference
AT ₁ receptor	Down	Su et al. 2002
AT ₂ receptor	Up	Stoll et al. 2002
Bradykinin	Up	Jin et al. 2002
Collagen	Up	Mifune et al. 2000
eNOS	Up	Ritter et al. 2002
NBC-1 Na ⁺ -HCO ₃ symporter	Up	Sandmann et al. 2001b
Thrombospondin 1	Up	Fischer et al. 2001
RANTES	Up	Wolf et al. 1997
SM-20	Down	Wolf et al. 2002
TGF- β type I receptor	Down	Su et al. 2002
Zfhsp	Up	Stoll et al. 2002

4

Summary and Conclusion

The AT₂ receptor signals and functions in unexpected ways compared to the “classical” AT₁ receptor. Moreover, some of the actions of the AT₂ receptor are even directly opposing those of the AT₁ receptor, especially concerning the growth- and differentiation-modulating actions after ANG II stimulation. The modulatory or suppressive function of the AT₂ receptor often counteracts stimulatory signals. This requires measuring reduction in activities, which is technically more difficult than measurement of increased activity from a low level. This may have been the reason the AT₂ receptor has sometimes escaped the attention of investigators.

The signaling mechanisms of the AT₂ receptor are diverse, and only a few of them have yet been characterized reasonably well. The unique nature of the AT₂ receptor includes the lack of desensitization and internalization after ligand binding and the intrinsic activity of the receptor in itself. AT₂ receptor expression in fetal tissues is widespread. However, in several tissues the receptor disappears after birth or—under in vitro conditions—when cells are transferred in the presence of serum and growth factors. On the other hand, a sometimes dramatic up-regulation of the AT₂ receptor does occur after tissue injuries. Especially during heart failure its expression is significantly increased. Pharmacologically relevant is the fact that AT₂ receptor stimulation inhibits cardiac fibroblast growth and extracellular matrix formation and exerts a negative chronotropic effect, indicating that AT₂ receptor stimulation has a cardioprotective effect (Fig. 5). It appears that the AT₂ receptor accepts the role as a modulator of biological programs in embryonic development and cellular processes following injuries, particularly by altering the expression profile of its target genes.

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Angiotensin-(1-7). Its Contribution to Arterial Pressure Control Mechanisms

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Abstract A formidable body of evidence accumulated to date reveals the pleiotropic actions of the renin-angiotensin system and its role in cardiovascular medicine. The characterization of the biological actions of angiotensin-(1-7) expanded knowledge of the endogenous control mechanisms regulating the actions of angiotensin II and revealed alternate enzymatic pathways for the processing of angiotensin I and angiotensin II. The following chapter reviews the tissue-specific pathways for formation and metabolism of this member of the renin-angiotensin system as well as its localization within brain, renal, and cardiac tissue. The unique actions of angiotensin-(1-7) in general oppose those of angiotensin II, and the preponderance of data identifies the regulation of the balance between the two peptides as important to overall cardiovascular health. We emphasize the

physiological actions of the peptide in vascular, neural, and renal systems and provide a comprehensive review of its trophic and signaling mechanisms. The current status of the receptors mediating the actions of the peptide and the potential for non-receptor mechanisms are also presented. Finally, we discuss the significance of angiotensin-(1-7) in human disease, based on evidence emerging from recent studies suggesting that deficits in angiotensin-(1-7) are associated with hypertension in specific subpopulations of patients.

Keywords Blood pressure · Angiotensin-(1-7) · Hypertension · Kidney · Heart · Vasculature · Brain

1

Introduction

With the publication of the first edition of *Angiotensin* 30 years ago (Ryan 1974), it appeared that the role of the renin–angiotensin system in the regulation of arterial pressure was cast in stone. In retrospect, this was not the case. The formidable body of evidence accumulated to date has provided a more complete understanding of the pleiotropic actions of the system, its role in cardiovascular medicine, and the critical role of the angiotensin peptide system in heart failure (Pitt 2002), hypertension (Dahlof et al. 2002), diabetes (Brenner et al. 2001; Dahlof et al. 2002), atherosclerosis (Strawn et al. 2000), and end-stage renal disease (Brenner et al. 2001; Lewis et al. 2001).

The advent of new technologies and the synthesis of selective antagonists to subtype 1 and 2 angiotensin receptors provided the opportunity to explore the molecular mechanisms at a more complex level. A re-examination of the putative role of angiotensin fragments other than angiotensin II (Ang II) was one of the outcomes of these endeavors. The characterization of the biological actions of angiotensin-(1-7) [Ang-(1-7)] expanded knowledge of the endogenous control mechanisms regulating the actions of Ang II and uncovered alternate enzymatic pathways for the processing of angiotensin I (Ang I) and Ang II. Until recently, the dogma was that the carboxyl-terminus of the Ang II molecule was required for binding to its receptor. This idea, advanced by Bumpus and associates (1961), required revision with the discovery that Ang-(1-7), a fragment where the C-terminal end of the Ang II molecule was cleaved at the eighth position, had pharmacological activity. A comprehensive review of the lessons learned from studying the biological role of Ang-(1-7) in both animals and humans is provided below.

2

Production and Metabolism of Angiotensin-(1-7)

The newer concept, that the biochemical mechanisms for the processing of Ang I into smaller biologically active forms, departed significantly from the original concept, which implicated angiotensin converting enzyme (ACE) and

aminopeptidases as the two critical enzymes for synthesis and degradation of angiotensin peptides.

2.1

Pathways of Angiotensin-(1-7) Formation and Degradation

Illustrated in Fig. 1 are the major bioactive components of the renin-angiotensin system following conversion of angiotensinogen to the decapeptide Ang I. The peptide cascade diverges with the processing of Ang I to Ang II and Ang-(1-7) yielding products with different carboxy termini and contrasting biological actions. Ang II generation occurs primarily by the hydrolysis of the Phe⁸-His⁹ bond of Ang I by ACE (EC 3.4.15.1) (Corvol et al. 1995; Deddish et al. 1998). Numerous studies demonstrated that the mast cell-derived serine protease chymase also generates Ang II from Ang I or the ACE-resistant analog [Pro¹¹,Ala¹²]-Ang I (Urata et al. 1990; Balcells et al. 1996). However, the lack of direct evidence that chymase contributes to endogenous production of Ang II questions the relevance of this pathway. Ang II is metabolized further either at its amino terminus by aminopeptidases, internally at the Tyr⁴-Ile⁵ bond by endopeptidases, or at the carboxy terminus by carboxypeptidases. Several enzymes expressing carboxypeptidase-like activity, including prolyl oligopeptidase and prolyl carboxypeptidase, hydrolyze the Pro⁷-Phe⁸ bond to generate Ang-(1-7) (Welches et al. 1991; Welches et al. 1993). Both enzymes are primarily cytosolic with prolyl carboxypeptidase localized to lysosomes. Their peptide substrates generally require a carboxy terminal proline residue. Shariat-Madel et al. (2002) demonstrated a novel activity of prolyl carboxypeptidase that processes prokallikrein to the active form of the enzyme in human endothelial cells. The ability of prolyl carboxypeptidase to activate kallikrein as well as to form Ang-(1-7) is of particular interest in lieu of the positive interaction between Ang-(1-7) and bradykinin in vascular function (see below).

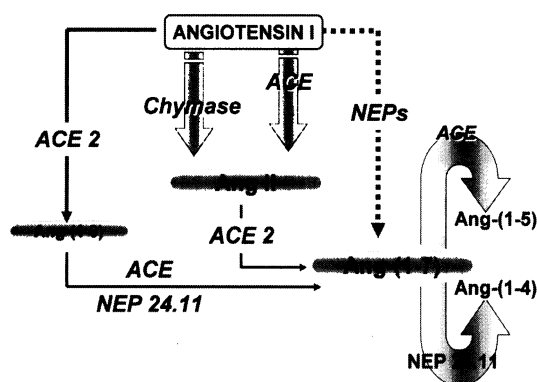


Fig. 1 Biochemical pathways involved in the synthesis and degradation of angiotensin-(1-7). ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2 (Crackower et al. 2002); NEPs, neutral endopeptidases 24.11, 24.26, and 24.15

Recent studies identified a third enzymatic candidate that processes Ang II to Ang-(1-7). Although originally termed ACE2 due to its sequence homology with ACE, this enzyme does not cleave Ang I to Ang II nor is it sensitive to ACE inhibitors (Donoghue et al. 2000). ACE2 is a carboxypeptidase that exhibits a high catalytic efficiency for the generation of Ang-(1-7) from Ang II—almost 500-fold greater than that for the conversion of Ang I to Ang-(1-9) and 10- to 600-fold higher than that of prolyl oligopeptidase and prolyl carboxypeptidase, respectively, to form Ang-(1-7). From an array of over 120 peptides, only dynorphin A and apelin 13 were hydrolyzed by ACE2 with comparable kinetics to the conversion of Ang II to Ang-(1-7) (Vickers et al. 2002). ACE2 is a type I membrane protein that is primarily localized to the heart (myocytes), kidney (endothelium and tubular elements), and testes (Donoghue et al. 2000). Although the physiological role of ACE2 in the formation of Ang-(1-7) is unknown, ACE2 knockout mice have altered heart function that may be associated with changes in the cardiac renin-angiotensin system (Crackower et al. 2002). Moreover, ACE2 shows a parallel distribution with Ang-(1-7) in kidney tubules and is increased in the spontaneously hypertensive rat (SHR) following combined ACE-nepriylisin blockade with omapatrilat (Chappell et al. 2002).

Ang-(1-7) is formed directly from Ang I or Ang-(1-9) by multiple endopeptidases that cleave the Pro⁷-Phe⁸ bond, completely bypassing the formation of Ang II (Gafford et al. 1983; Stephenson and Kenny 1987; Welches et al. 1993). In the circulation, this pathway is mediated principally by the metalloendopeptidase neprilysin (EC 3.4.24.11) (Campbell et al. 1998; Iyer et al. 1998b; Chappell et al. 2000). Similar to ACE and ACE2, neprilysin is a membrane-bound enzyme located on the luminal side of the endothelium with access to circulating peptides. Although the kidney, particularly the brush border of the proximal tubules, exhibits the highest concentration of neprilysin, significant activity is found in extra-renal sites including the lung, aorta, mesenteric artery, endothelial cells, and neutrophils (Erdos et al. 1989; Tamburini et al. 1989; Llorens-Cortes et al. 1992; Soleilhac et al. 1992; Graf et al. 1995; Tharaux et al. 1997). Neprilysin inhibitors significantly reduce circulating levels of Ang-(1-7), particularly in the presence of ACE inhibition; however, they do not completely abolish its production, suggesting the contribution of other enzymatic pathways (Anastasopoulos et al. 1998; Iyer et al. 1998b; Chappell et al. 2000; Ferrario et al. 2002a). The extent to which other endopeptidases form endogenous Ang-(1-7) from Ang I is not adequately defined at the present time nor have complete kinetic analyses been obtained. Endopeptidases that process Ang I to Ang-(1-7) *in vitro* include endothelin-converting enzyme in human endothelial cells (Pirro et al. 2001), thimet oligopeptidase (EC 3.4.24.15) in rat vascular smooth muscle cells (Chappell et al. 1994, 2000) as well as prolyl oligopeptidase in neuronal cells, brain tissue, and the perfused hindlimb (Santos et al. 1988; Welches et al. 1991).

As the pathways for the formation of Ang-(1-7) were defined, the route for the metabolism of the peptide was investigated. We and others showed that Ang-(1-7) is hydrolyzed at the Ile⁵-His⁶ bond to form angiotensin-(1-5) and the

dipeptide His-Pro by ACE (Chappell et al. 1998; Deddish et al. 1998; Allred et al. 2000). Although somatic ACE contains two active sites (N and C domains), Ang-(1-7) and the hematopoietic fragment Acetyl-Ser-Asp-Lys-Pro are the only two known substrates hydrolyzed exclusively by the N-domain of human ACE (Deddish et al. 1998). The favorable kinetic constants ($K_m=0.8 \mu\text{M}$, k_{cat}/K_m of $2,200 \text{ mM}^{-1} \text{ sec}^{-1}$ for canine ACE) suggest an *in vivo* role for ACE in the metabolism of Ang-(1-7). The K_m of ACE for Ang-(1-7) is surpassed only by that of the potent vasodilatory peptide bradykinin. Similar to kinins, the ACE-dependent metabolism may account for the remarkably short half-life of Ang-(1-7) in the circulation ($t_{1/2}$ of 9 s versus 60 s for Ang II) (Yamada et al. 1998). Moreover, chronic treatment with the ACE inhibitor lisinopril prolonged the half-life of infused Ang-(1-7) approximately six-fold in the SHR while the AT_1 antagonist losartan had no effect (Yamada et al. 1998). The marked increase in circulating levels of Ang-(1-7) following ACE inhibition most likely reflects increased synthesis (due to higher Ang I levels) as well as reduced metabolism. In the circulation, ACE constitutes a nexus in this pathway as it may balance the magnitude of the pressor and proliferative properties of Ang II and the depressor and anti-proliferative actions of Ang-(1-7) and bradykinin. However, in the kidney, both ACE and neprilysin are involved in the metabolism of Ang-(1-7). In salt-sensitive hypertensives and the SHR, chronic administration of the combined ACE/neprilysin inhibitor omapatrilat resulted in a significant elevation in the urinary excretion of Ang-(1-7) (Ferrario et al. 2002a,b). In isolated brush border membranes from rat, we demonstrated that neprilysin hydrolyzed the Tyr⁴-Ile⁵ bond of Ang-(1-7) to form Ang-(1-4) and the tripeptide Ile-His-Pro (Allred et al. 2000). Moreover, addition of omapatrilat increased the $t_{1/2}$ of Ang-(1-7) in urine approximately eight-fold (5.5 min to 34 min) (Chappell et al. 2001; Ferrario et al. 2002a). Thus, in tissues such as the kidney with high concentrations of neprilysin, this endopeptidase may primarily contribute to Ang-(1-7) metabolism.

2.2 Immunocytochemical Findings

Selective antibodies to Ang-(1-7) reveal some of the sites at which the heptapeptide may act in a true paracrine or autocrine form. The earliest observations were obtained in rat brain where it was first shown that Ang-(1-7) modulated the secretion of vasopressin (Schiavone et al. 1988, 1990; Moriguchi et al. 1994) and the central control of the baroreflex system (Campagnole-Santos et al. 1989). Localization of immunoreactive (ir-) Ang-(1-7) within the rat hypothalamus (Block et al. 1988) gave impetus to more detailed studies of Ang-(1-7) distribution in rat brain. Ang-(1-7) immunolabeling was detected in neurons in the nucleus circularis and in the suprachiasmatic, supraoptic, and paraventricular nuclei (PVN) of [mRen-2]27 transgenic hypertensive rats (Krob et al. 1998). Interestingly, robust immunolabeling was present in the posterior magnocellular division of the PVN (Fig. 2, top panel), while double labeling experiments provided direct evidence that vasopressin and Ang-(1-7) co-localized within in-

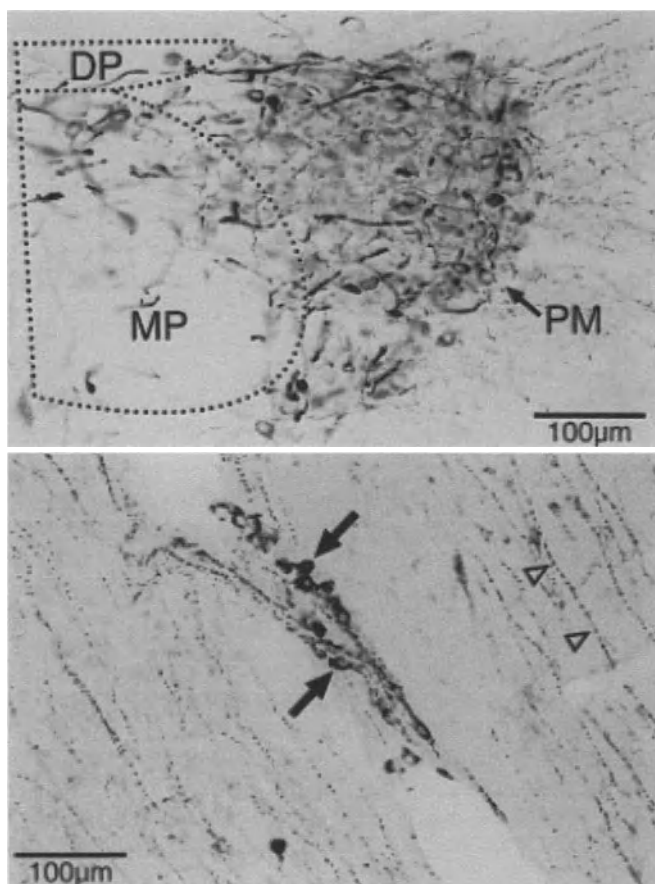


Fig. 2 *Top panel:* Ang-(1-7) immunoreactive labeling in the posterior paraventricular nucleus in a [mRen-2]27 transgenic hypertensive rat. Labeled neurons are primarily found in the dorsal and lateral parts of the posterior magnocellular division. *DP*, dorsal component of the paraventricular nuclei; *MP* and *PM*, medial and posterior components of the paraventricular nuclei. *Bottom panel:* Ang-(1-7) immunoreactive neurons associated with a blood vessel in the hypothalamus immediately anterior to nucleus circularis. The vessel lumen, which is visible on *top* and *bottom*, is obscured by labeled neurons (*black arrows*) and processes. *Arrowheads at the right* point to a beaded axon that is typical of the axons showing ir-Ang-(1-7). (Reprinted by permission from Krob et al. 1998)

dividual neurons projecting to the median eminence, neurohypophysis, and both PVN and supraoptic nuclei (Krob et al. 1998). The anatomical distribution of Ang-(1-7) neurons is in keeping with the seminal observation of Schiavone et al. (1988) showing Ang-(1-7)-mediated release of vasopressin from isolated rat hypothalamic-hypophysial explants and the finding that central Ang-(1-7) infusions stimulated vasopressin secretion in normal rats (Moriguchi et al. 1994). Furthermore, the presence of ir-Ang-(1-7) in neuron-like cells surrounding ce-

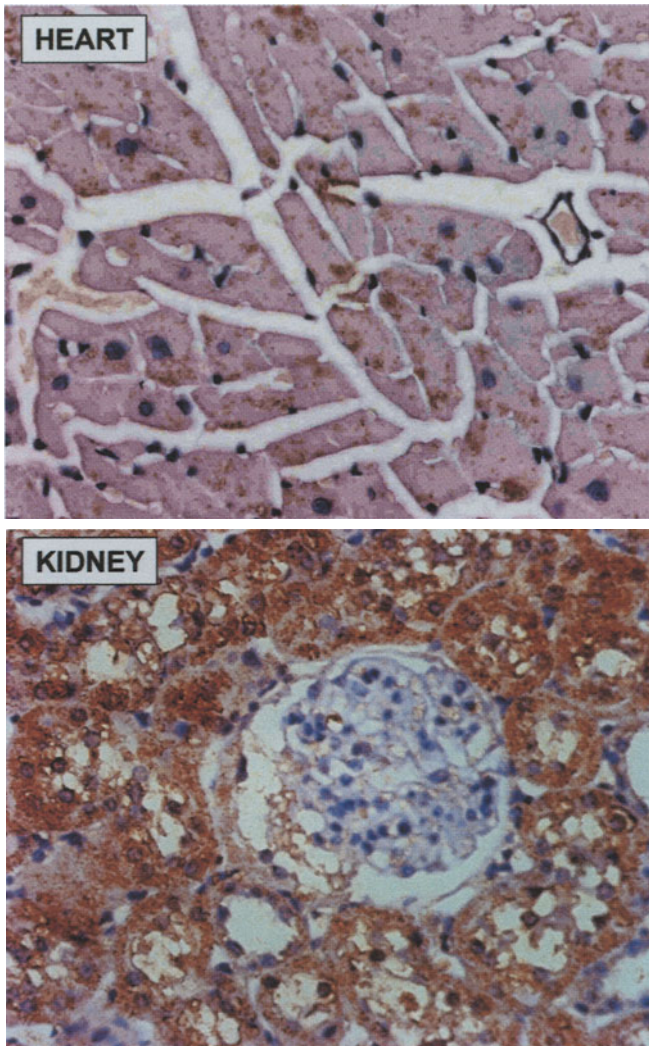


Fig. 3 Localization of immunoreactive Ang-(1-7) products in cardiac myocytes (*top*) and kidneys (*bottom*) provide additional evidence for paracrine functions of the peptide. Dense immunoreactive products positive for Ang-(1-7) are present in cardiac myocytes of the left ventricle from a normotensive Lewis rat. In the kidney, immunoreactive Ang-(1-7) is localized in proximal and distal tubules but completely absent in the glomeruli. (From studies reported by Ferrario; Ferrario 2002; Ferrario et al. 2002a)

rebral arterioles (Fig. 2, lower panel) is consistent with the observation that Ang-(1-7) dilates cerebral vessels (Meng and Busija 1993).

Recent studies demonstrated intense ir-Ang-(1-7) in rat cardiac (Ferrario 2002) and renal (Ferrario et al. 2002a) tissues. In the heart, ir-Ang-(1-7) was found only in cardiac myocytes (Fig. 3, upper panel) (Ferrario 2002). Moreover, we also found that Ang-(1-7) staining was more intense in myocytes found with-

in the region of the penumbra surrounding the infarct zone (Ferrario 2002). These findings correlate with previous observations showing the presence of Ang-(1-7) in the venous effluent from the canine coronary sinus (Santos et al. 1990) and within the interstitial fluid collected from microdialysis probes placed in canine left ventricle (Wei et al. 2002).

As discussed below, Ang-(1-7) has important actions in the control of glomerulotubular function. That these actions may be mediated, at least in part, by local production of Ang-(1-7) in renal tubules gained credence with the demonstration of intense ir-Ang-(1-7) in renal proximal tubules and the ascending loop of Henle in the rat (Ferrario et al. 2002a). The lower panel of Fig. 3 shows that Ang-(1-7) immunostaining is absent in renal glomeruli but present in the cytoplasm of renal proximal convoluted tubules and the thick ascending limbs of loops of Henle (Ferrario et al. 2002a).

3 Physiological Actions of Angiotensin-(1-7)

The pithed rat served as the first biological model to characterize the effects of Ang-(1-7) on blood pressure and vascular tone (Benter et al. 1993). In this animal model, the absence of autonomic nervous system function achieved through spinal cord destruction provided a tool to determine the vasoactive properties of Ang-(1-7) in the systemic circulation. Injection of the peptide into a peripheral vein in the pithed rat produced a biphasic response in blood pressure that was blocked by the non-selective Ang II antagonist [Sar¹Thr⁸]-Ang II. The initial short-lasting pressor component was blocked by losartan which also attenuated the duration, but not the magnitude of the depressor component. The entire hypotensive response was blocked by indomethacin. These early studies, confirmed by others, showed that the depressor component of the response may be variably influenced by the species studied, whether animals are anesthetized or non-anesthetized, and the dose of the peptide employed (Santos et al. 2000). With one exception (Kono et al. 1986), a vasodilator response was recorded in pigs (Porsti et al. 1994), dogs (Brosnihan et al. 1996), and rabbits (Ren et al. 2002). In general, the vasodilator effect was largely intact after administration of either AT₁ or AT₂ receptor blockers, a finding that supports the possibility that the vascular effects of Ang-(1-7) are mediated by a receptor subtype that is not sensitive to either AT₁ or AT₂ receptor blockade.

Vasodilators such as bradykinin, prostaglandins, and nitric oxide (NO) constitute a system of autacoid regulators acting to oppose the pressor molecules such as Ang II, catecholamines, and endothelin. With the possible exceptions of bradykinin and atrial natriuretic peptide, none of these factors shares biochemical pathways for either formation or degradation of the biologically active products. In contrast, enzymes which form and degrade angiotensin peptides characteristically share common substrates and biochemical pathways. The intertwining of these pathways suggested that the vasodilator properties of Ang-(1-7) could represent a mechanism within the renin-angiotensin system to op-

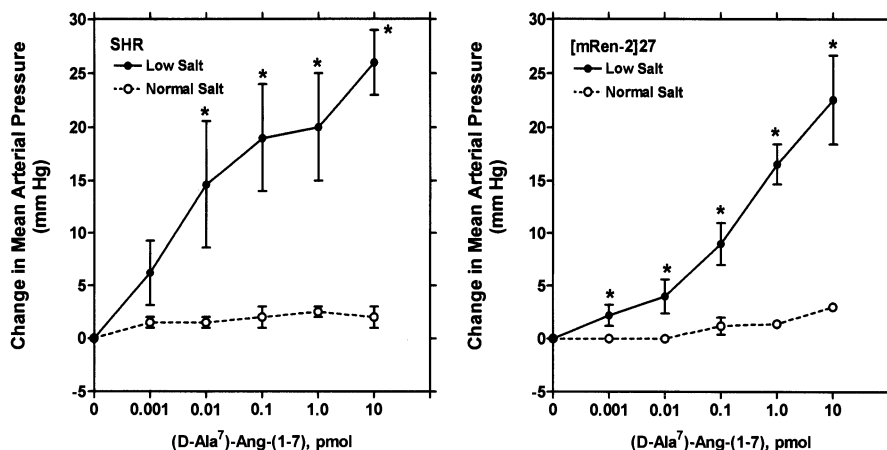


Fig. 4 Comparative effect of intravenous infusions of $[d\text{-Ala}^7]\text{Ang-(1-7)}$ on the blood pressure of conscious SHR (*left panel*) and $[m\text{Ren-2}]27$ hypertensive transgenic rats maintained in either a normal (*open symbols, dashed lines*) or reduced intake of sodium (*filled symbols, solid lines*) 12 days after initiation of the dietary regimen. (Reprinted by kind permission from Iyer et al. 2000a)

pose the pressor and trophic actions of Ang II. This concept was first evaluated in two-kidney one-clip hypertensive dogs, as the elevated blood pressure in this model of experimental hypertension is clearly dependent on increased circulating levels of Ang II. Nakamoto et al. (1995) showed that the vasodilator component of the blood pressure response due to intravenous injections of Ang-(1-7) was augmented in both untreated and L-arginine-treated two kidney, one-clip hypertensive dogs but significantly attenuated in hypertensive dogs fed the NO synthase inhibitor.

In these studies (Nakamoto et al. 1995), the magnitude of the vasodilator effects of Ang-(1-7) was consistently dependent on whether the renin-angiotensin system was activated. Ferrario et al. (1997) thus proposed that Ang-(1-7) may serve as an intrinsic negative regulator of the pressor actions of Ang II. The concept was explored further in studies in which dietary salt depletion was used to assess the contribution of the renin-angiotensin system to the regulation of arterial pressure (Iyer et al. 2000a). During salt depletion, renin-angiotensin system activation fails to elevate blood pressure, presumably due to blood volume contraction and downregulation of AT_1 receptors. However, recent experiments implicate a role for Ang-(1-7) in opposing the actions of Ang II. Fig. 4 shows the dose-dependent effects of administering a selective antagonist of Ang-(1-7) (Santos et al. 1994)— $[D\text{-Ala}^7]\text{Ang-(1-7)}$ —to SHR and $[m\text{Ren-2}]27$ transgenic hypertensive rats maintained on a low-salt diet for 11 days (Iyer et al. 2000a). Blockade of endogenous Ang-(1-7) activity caused dose-dependent increases in the arterial pressure of salt-restricted rats, suggesting that the heptapeptide prevented the increased levels of Ang II from raising blood pressure. The pharmacological effects of the selective Ang-(1-7) receptor antagonist were observed in the presence of increased plasma levels of renin and Ang II and duplicated by

administering an Ang-(1-7) antibody (Iyer et al. 1998b). These data provide a mechanistic understanding for the finding that stimulation of the renin-angiotensin system by salt depletion is not accompanied by increases in arterial pressure.

A tonic role of Ang-(1-7) in contributing to blood pressure regulation was also demonstrated in hypertensive rats in which the elevated blood pressure was normalized by chronic oral administration of an ACE inhibitor or an Ang II receptor blocker. In both situations, control of arterial blood pressure by either agent was reversed in part by acute administration of a selective Ang-(1-7) antibody (Iyer et al. 1998a) or inhibition of neprilysin (Iyer et al. 1998b). In these treated hypertensive rats, the pressor effects of Ang-(1-7) blockade could not be ascribed to activation of AT₂ receptors or a direct effect of bradykinin (Iyer et al. 1998a,b).

3.1

Vascular Mechanisms

3.1.1

Species and Regional Bed Differences in Angiotensin-(1-7) Vasodilator Responses

The vascular-related actions of Ang-(1-7) are species-, region-, and mediator-specific. In porcine and canine coronary arteries, Porsti et al. (1994) and Brosnihan et al. (1996) reported that Ang-(1-7) produced a dose-dependent dilation that was dependent upon an intact endothelium. The vasodilator response was mediated by a non-AT₁/non-AT₂ angiotensin receptor that stimulated the endothelium-dependent release of NO and bradykinin, but not prostaglandins (Porsti et al. 1994; Brosnihan et al. 1996). The confirmatory nature of these two studies strongly supports the characterization of Ang-(1-7) receptors in canine coronary endothelium (Ferrario et al. 1997). In contrast, Gorelik et al. (1998) reported no vasodilator response to Ang-(1-7) in porcine coronary arteries. It is difficult to explain the results of Gorelik et al. (1998) since the preparation used (species, pre-constricting agent, and regional bed) was similar to those reported by Porsti et al. (1994). In piglet pial arterioles (Meng and Busija 1993), Ang-(1-7) and Ang II produced mild vasodilation at concentrations of 10⁻⁵ and 10⁻⁴ M, as assessed with intravital microscopy. Intravenous administration of indomethacin blocked the dilator responses. The results of these studies may reflect a lack of pre-constriction of the pial preparation as well as differences in regional beds. Furthermore, in canine coronary vessels, Ang II and Ang-(1-7) at equivalent concentration ranges produced diametrically opposite changes in the contractile state of coronary artery rings (Fig. 5) (Brosnihan et al. 1996). Weaker agonistic vasoconstrictor and vasodilator actions were elicited by Ang-(3-8) and Ang-(3-7), respectively.

Intraarterial injections of Ang-(1-7) in feline superior mesenteric vascular beds produced vasodilation at 1-, 3-, and 10- μ g doses and vasoconstriction at 30 and 100 μ g (Osei et al. 1993). In the hindquarter vascular bed, intraarterial in-

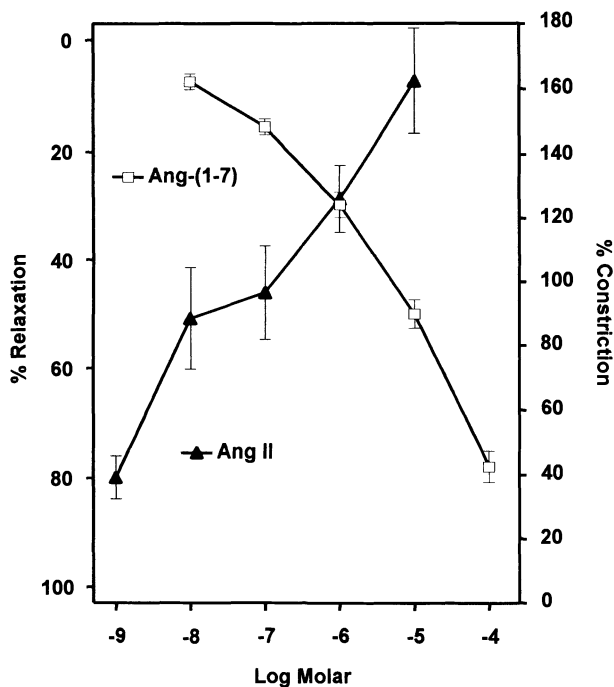


Fig. 5 Contrasting effects of Ang II and Ang-(1-7) on isolated coronary artery rings obtained from dogs illustrates the comparative effects of the peptides on vascular tension. (Adapted from studies reported in Brosnihan et al. 1998)

jection of 1 μg produced an increase in perfusion pressure, whereas a decrease in perfusion pressure was found at 10- and 30- μg doses. While the pressor effects of Ang-(1-7) were prevented by blockade of AT_1 receptors with losartan, the depressor responses to lower doses of the peptide were mediated by release of NO (Osei et al. 1993).

In Wistar rat mesenteric vessels, topical applications of Ang-(1-7) elicited 4% and 10% vasodilation at 100 and 1000 pmol, respectively (Oliveira et al. 1999). The vasodilator response was reduced but not abolished by pretreatment with either N^G -nitro-L-arginine methylester (L-NAME) or indomethacin but was unaffected by the bradykinin B_2 receptor antagonist HOE 140. [D-Ala⁷]Ang-(1-7) completely blocked the Ang-(1-7) response, whereas losartan was without effect. Le Tran and Forster (Forster and le Tran 1997) showed that Ang-(1-7) dilated pre-contracted rat aorta (ED_{50} of 2.3 nM) in an endothelial-dependent manner. Pre-contraction was obtained with 20 mM KCl, which produced a submaximal contraction. The degree of pre-contraction and the agent used appear to be critical factors in assessing the vasodilator actions of Ang-(1-7). Ang-(1-7) caused the vasodilation of norepinephrine-precontracted rabbit renal afferent arterial vessels with an ED_{50} of 2 nM (Ren et al. 2002). The response was mediated by NO and was selectively blocked by [D-Ala⁷]Ang-(1-7). Ang-(1-7) caused the re-

lease of prostanoids including prostacyclin from cultured rabbit aortic vascular smooth muscles cells (Muthalif et al. 1998), rat and porcine aortic vascular smooth muscle cells (Jaiswal et al. 1993a,b), and endothelial cells (Jaiswal et al. 1992). It is thus unclear why Ang-(1-7)-stimulated release of prostacyclin does not cause vasodilation in *in vitro* vascular preparations that are endothelium-denuded. In humans, Ueda et al. (2000) showed that Ang-(1-7) alone had no significant effect on forearm blood flow at concentrations from 1 to 2,000 pmol/min; at greater concentrations (0.1–40 nmol/min) they observed a reduction in forearm blood flow. However, the antagonism of Ang II-mediated vasoconstriction of human forearm resistance vessels by nanomolar concentrations of Ang-(1-7) observed by Ueda et al. (2000) may result from activation of an AT₍₁₋₇₎ receptor and the release of compensatory vasodilators. Wilsdorf et al. (Wilsdorf et al. 2001) also found no vasodilator response to Ang-(1-7) in human forearm. Finally, Davie and McMurray (1999) reported no vasodilator but a slight vasoconstrictor response in patients with heart failure and treated with ACE inhibitors.

A recent innovative study demonstrated the vasodilator effect of Ang-(1-7) in mature skin and sponge-induced neovasculature in mice (Machado et al. 2002). Blood flow increased in the presence of Ang-(1-7) and was blocked by [D-Ala⁷] Ang-(1-7), NO synthase inhibitors, or indomethacin. Treatment with an AT₂ receptor antagonist failed to alter the growth of newly formed vessels but prevented the vasodilation in the pre-existing vessels. These studies suggest that Ang-(1-7) may contribute to newly formed vascular bed formation during wound healing, chronic inflammatory processes, and tumor growth and development.

3.1.2

Interactions of Angiotensin-(1-7) and Bradykinin in the Vasculature

The interactions of Ang-(1-7) and bradykinin were first demonstrated by Paula et al. (1995, 1999), who showed that Ang-(1-7) potentiates the hypotensive effect of bradykinin in conscious normotensive and hypertensive rats. The response was attenuated by pretreatment with indomethacin and exaggerated with ACE inhibition. The potentiation of bradykinin by Ang-(1-7) was also shown in isolated dog and pig coronary arteries (Li et al. 1997; Gorelik et al. 1998), rat mesenteric microvessels (Oliveira et al. 1999; Fernandes et al. 2001), isolated rat heart, and human forearm brachial arteries (Ueda et al. 2001). The specificity of the response was demonstrated by showing that Ang-(1-7) did not affect the vasodilator responses of acetylcholine or sodium nitroprusside (Li et al. 1997; Oliveira et al. 1999) and neither Ang I nor Ang II potentiated the bradykinin response (Li et al. 1997). The potentiating effect of Ang-(1-7) was blocked by a NO synthase inhibitor in canine and porcine coronary arteries (Schappert 1996; Gorelik et al. 1998), rat mesenteric vessels (Oliveira et al. 1999), and human forearm brachial arteries (Ueda et al. 2001) while indomethacin inhibited the response in the isolated heart preparation (Almeida et al. 2000), and in mesenteric microvessels of Wistar (Oliveira et al. 1999) and SHR rats (Fernandes et al.

2001). In contrast, endothelium-derived hyperpolarizing factor participated in the response in SHR microvessels (Fernandes et al. 2001). In addition, [D-Ala⁷]Ang-(1-7), which is not a substrate or inhibitor of ACE, blocks the Ang-(1-7) potentiation of the responses to bradykinin in both Wistar rats (Almeida et al. 2000) and SHRs (Fernandes et al. 2001). These results suggest that Ang-(1-7) potentiates bradykinin through the release of prostaglandins, NO or endothelium-derived hyperpolarizing factor (EDHF), depending upon the species and the vascular bed, and is mediated by an AT₍₁₋₇₎ receptor.

Alternatively, Ang-(1-7) may potentiate bradykinin responses through its ability to inhibit ACE activity. Micromolar concentrations of Ang-(1-7) inhibit ACE activity, through interactions at the C-terminal domain (Chappell et al. 1998; Deddish et al. 1998). However, the K_m for Ang-(1-7) is similar to that of bradykinin, suggesting that competition for the enzyme would occur even at modestly elevated levels of either peptide. Indeed, ACE inhibitors increase levels of both peptides. ACE inhibitors potentiate responses to bradykinin, by inhibiting bradykinin B₂ receptor desensitization (Minshall et al. 1997; Benzing et al. 1999). This resensitization is thought to occur through protein-protein interactions between the bradykinin receptor and ACE. In porcine coronary arteries, bradykinin-induced vasodilation was potentiated by captopril, quinaprilat, or Ang-(1-7) (Tom et al. 2001). The mechanisms for the interaction between Ang-(1-7) and bradykinin in the dilation of blood vessels and the regulation of blood pressure are complex and require further investigation.

3.2

Brain/Neural Mechanisms

The first report of a biological function for Ang-(1-7) indicated that it was as potent as Ang II in releasing vasopressin from isolated hypothalamic-pituitary explants (Schivavone et al. 1988). Since that observation, a wide variety of actions were reported for the peptide in the central nervous system (CNS). Unlike the counter-regulatory actions described thus far, many actions of Ang-(1-7) in the CNS mimic those of Ang II at a cellular level. However, when Ang-(1-7) is evaluated by effects exerted through integrative regulatory systems, the heptapeptide generally opposes the actions of Ang II. Ang-(1-7) is present in brain tissues such as the hypothalamus and medulla oblongata at concentrations equivalent to or greater than those of Ang II (Chappell et al. 1989). The distribution of the peptide in neurons and pathways of the medulla oblongata, PVN (Fig. 2), median eminence and pituitary-hypophysial portal system (Block et al. 1988; Lawrence et al. 1992; Krob et al. 1998) and its co-distribution with nicotinamide adenine dinucleotide phosphate, reduced diaphorase (NADPH) (Calka and Block 1993) and vasopressin (Block et al. 1988) underscore the importance of this peptide as a critical component of the mechanisms that participate in fluid balance and osmoregulation.

Hypothalamus. Ang-(1-7) stimulated release of vasopressin from isolated explants (Schiavone et al. 1988) and from hypothalamic sites in the whole animal (Moriguchi et al. 1994; Qadri et al. 1998). This is consistent with excitatory actions on paraventricular neurons (Felix et al. 1991). However, plasma vasopressin levels were not different in these studies, suggesting that the peptide acted as a local modulator of tissue vasopressin release. Although the release of vasopressin from isolated organ explants by Ang-(1-7) was comparable to Ang II, plasma release of vasopressin in response to paraventricular injections of the peptide was less than that of Ang II following injection (Qadri et al. 1998). Moreover, the effects in whole animals were not accompanied by the increase in blood pressure or drinking normally seen with Ang II (Moriguchi et al. 1994; Qadri et al. 1998). In the hypothalamus of [mRen-2]²⁷ transgenic hypertensive rat, both peptides were elevated to levels 10- to 20-fold higher than in normotensive Hannover Sprague Dawley (SD) rats (Senanayake et al. 1994). Although no direct effects of the peptide on blood pressure were observed, Moriguchi et al. (1995b) clearly showed that in [mRen-2]²⁷ transgenic hypertensive rats, Ang II and Ang-(1-7) produced opposing actions on blood pressure through central mechanisms. Intracerebroventricular administration of an Ang II antibody lowered pressure, while an antibody that selectively bound to Ang-(1-7) raised pressure.

The overall effect of exogenously administered Ang-(1-7) on plasma vasopressin may depend upon the fluid balance state of the animal and the balance between endogenous levels of Ang II and Ang-(1-7). Long-term infusion of Ang-(1-7) in Wistar-Kyoto (WKY) or SD rats tended to increase plasma vasopressin levels (Benter et al. 1995b). In contrast, Ang-(1-7) treatment of SHR rats with elevated levels of plasma vasopressin lowered plasma vasopressin (Benter et al. 1995b). These data, which interestingly mimicked the effects of ACE inhibition (Thibonnier et al. 1981), showed that effects of Ang-(1-7) were dependent upon the state of the animal and that Ang-(1-7) acts as a modulator rather than a direct secretagogue. Similarly, in [mRen-2]²⁷ transgenic hypertensive rats with elevated hypothalamic tissue levels of both Ang II and Ang-(1-7) (Senanayake et al. 1994), the release of vasopressin (Moriguchi et al. 1994) or substance P (Diz et al. 1997) in response to Ang-(1-7) was diminished.

The failure of Ang-(1-7) to stimulate thirst or increase blood pressure while modulating vasopressin release provided some of the first indications that Ang-(1-7) and Ang II must be acting through separate receptors and transmitter systems or pathways. Indeed, patterns of release for norepinephrine, dopamine, and substance P were distinctly different for the two peptides. Ang II released norepinephrine, dopamine, and substance P from hypothalamic slices, whereas Ang-(1-7) primarily released substance P, although delayed effects on dopamine and norepinephrine were observed (Diz and Pirro 1992). Ang-(1-7) may inhibit potassium-stimulated release of norepinephrine from hypothalamic slices (Diz and Pirro 1992; Gironacci et al. 2000) through a mechanism mediated by NO (Gironacci et al. 2000). Ang-(1-7) shows a remarkable co-distribution with NADPH diaphorase, suggesting that NO may play a role in the effects of Ang-

(1-7) in the brain (Calka and Block 1993). However, studies in glial cell cultures support a role for the peptide in release of other neuroactive substances, such as prostaglandins (Jaiswal et al. 1991b; Tallant et al. 1991b).

The failure of Ang-(1-7) to evoke release of the monoamines in a similar time frame as Ang II may be the mechanism for the absence of drinking and pressor responses to the heptapeptide (Kawabe et al. 1986). However, since tachykinins participate in Ang II-mediated release of vasopressin (Perfumi et al. 1988; Massi et al. 1991a,b), the similar effects of the two angiotensin peptides on substance P may explain their concerted ability to evoke release of vasopressin. Intracerebroventricular injection of Ang-(1-7) induced *c-fos* expression primarily in the organum vasculosum of the lamina terminalis and median preoptic nucleus, whereas enhanced *c-fos* expression by Ang II was observed in the PVN and subfornical organ as well as the organum vasculosum of the lamina terminalis and median preoptic nucleus, providing additional evidence that the two peptides may act through different pathways (Mahon et al. 1995).

Rowe et al. (1995) showed that Ang-(1-7) has moderate affinity for the AT₁ receptor (~100 nM) at several brain sites including the PVN. The affinity of neuronal AT₁ receptors for Ang-(1-7) is greater than that reported for the peptide at peripheral AT₁ sites (>1 μM) although lower than Ang II. Qadri et al. (1998) showed that actions of Ang-(1-7) in the PVN required higher doses of Ang-(1-7) than Ang II, and actions of both peptides were blocked by AT₁ receptor antagonists. The AT₂ antagonist PD123177 also blocked the response to Ang-(1-7) (Qadri et al. 1998). In fact, Ang-(1-7) stimulated dopamine release only after AT₁ receptor blockade (Pawlak et al. 2001). Electrophysiological studies of Ang-(1-7) in the PVN showed that neuronal responses to Ang-(1-7) were blocked by [D-Ala⁷]Ang-(1-7) as well as by AT₁ and AT₂ antagonists (Felix et al. 1991; Ambuhl et al. 1992) but that blockade by AT₂ antagonists required higher doses, suggesting an interaction with AT₁ receptors. Substance P release (Diz and Pirro 1992) and dopamine release (Pawlak et al. 2001) in response to Ang-(1-7) were attenuated in hypothalamic slices by [D-Ala⁷]Ang-(1-7) and AT₂ antagonists as well as [D-Ala⁷]Ang-(1-7) blocked the release of substance P or catecholamines in response to Ang II (Diz and Pirro 1992; Pawlak et al. 2001). Critical evaluation of these findings clearly support the interpretation that the receptor activated by Ang-(1-7) does not show the same pharmacologic selectivity as the classic AT₁ or AT₂ receptors or the non-AT₁, non-AT₂ receptor present in the peripheral vasculature, as discussed elsewhere in the chapter.

Medulla Oblongata. Ang-(1-7) elicited excitatory neuronal actions in the nucleus of the solitary tract (nTS) and the dorsal motor nucleus of the vagus (dmnX) (Barnes et al. 1990). In both nuclei, the percentage of cells excited was comparable to that of Ang II. However, some cells responded to both peptides or only one peptide, suggesting that separate receptors mediated the observed actions. Microinjection of low doses of Ang-(1-7) into the dorsomedial medulla evoked depressor and bradycardic responses, whereas higher doses of the peptide produced a biphasic response consisting of an initial increase in blood pressure fol-

lowed by a decrease in blood pressure (Campagnole-Santos et al. 1989). These responses appeared similar to those evoked by injection of Ang II into the nTS (Diz et al. 1984). However, subsequent studies showed that unilateral sino-aortic denervation potentiated the cardiovascular responses to ipsilateral injection of Ang-(1-7), whereas responses to Ang II injection were not affected (Campagnole-Santos et al. 1990). Studies also demonstrated that Ang II may act at nerve terminals in the nTS as well as at intrinsic neurons of the nTS (Qu et al. 1996; Diz et al. 2001). Thus, Ang II has both pre- and post-synaptic sites of actions in the dorsomedial medulla and Ang II has excitatory actions on cells of the nodose ganglion (Widdop et al. 1992). In contrast, Ang-(1-7) is devoid of actions on cells in the nodose ganglion (Widdop et al. 1992). The site of action of Ang-(1-7) (pre- or post-synaptic) awaits further studies.

The response patterns to Ang-(1-7) in the medulla oblongata are dependent on the cardiovascular state of the animal, similar to the actions at forebrain sites. For example, the depressor/bradycardic responses to Ang-(1-7) injected into the nTS were greater in the SHR. In contrast, microinjection of Ang-(1-7) in the nTS of ASrAogen rats (angiotensinogen antisense linked to a glial fibrillary acidic protein) caused smaller depressor responses than those produced by injection of the peptide in the nTS of Hannover SD rats (Couto et al. 2002). Thus, the decrease in tissue levels of angiotensin peptides in the brain stem of ASrAogen rats (Schinke et al. 1999; Monti et al. 2001) and/or the relatively lower level of arterial pressure in these rats altered the cardiovascular response to Ang-(1-7). The opposite effects observed in the SHR may reflect the importance of resting levels of pressure on the response.

In terms of the baroreceptor reflex, the effects of Ang II and Ang-(1-7) are in direct opposition. Ang II attenuated, whereas Ang-(1-7) augmented, baroreceptor reflex control of heart rate. Ang-(1-7) given by either intracerebroventricular administration (Campagnole-Santos et al. 1992; Oliveira et al. 1996) or long-term peripheral infusion (Benter et al. 1995a) also increased the gain of the baroreceptor reflex control of heart rate in SHR and normotensive rats. This effect was mediated at sites in the nTS (Chaves et al. 2000; Diz and Westwood 2000), where a tonic action of the peptide contributed to the resting level of reflex function in normotensive rats (Diz and Westwood 2000). Actions of Ang-(1-7) in the dorsal medulla were apparently influenced by hypertension and by anti-hypertensive treatments. In SHRs with an impaired baroreceptor reflex, the role of endogenous Ang-(1-7) was enhanced, since the injection of [D-Ala⁷]Ang-(1-7) inhibited the reflex to a greater extent than that produced by injection of the Ang-(1-7) antagonist in the nTS of Wistar rats (Chaves et al. 2000). In [mRen-2]²⁷ transgenic hypertensive rats with lower tissue levels of Ang-(1-7), [D-Ala⁷]Ang-(1-7) had no effect on the reduced baroreflex sensitivity (Senanayake et al. 1994; Diz and Westwood 2000). In ASrAogen animals (Couto et al. 2002), Ang-(1-7) no longer facilitated the reflex and, surprisingly, inhibited baroreflex sensitivity. When ACE inhibitors were given to SHRs (Heringer-Walther et al. 2001) or rats with renal hypertension (Britto et al. 1997), the ability of ACE inhibition to improve the baroreceptor reflex was mediated, in part, by endogenous

Ang-(1-7). The role of Ang-(1-7) in this beneficial effect of ACE inhibition was substantiated by the finding that [D-Ala⁷]Ang-(1-7) reversed the enhanced gain in the reflex seen with this type of treatment. These findings underscore that Ang II and Ang-(1-7) exist in a balance to set the sensitivity or gain of the baroreceptor reflex at the level of the nTS.

In the ventrolateral medulla (VLM), Ang-(1-7) like Ang II has actions that mimic excitatory neurotransmitters in the caudal and rostral cell groups involved in blood pressure control. In the caudal VLM, Ang-(1-7) lowered arterial pressure by activation of pathways providing tonic inhibition to the pressor rostral VLM (Potts et al. 2000a,b). In the rostral VLM, the peptide excited pathways leading to activation of the sympathetic nervous system (Fontes et al. 1994; Lima et al. 1996; Fontes et al. 1997). These actions mimic those observed when Ang II or glutamate was injected into the caudal or rostral VLM (Averill and Diz 1999; Diz et al. 2002). The effects of Ang-(1-7) at the rostral VLM are mediated by both vasopressin and the sympathetic nervous system (Oliveira et al. 1998). However, the increases in blood pressure in response to Ang-(1-7) injections into the rostral VLM were enhanced after hemorrhage, while those to Ang II were not (Lima et al. 1999). In animals with reduced levels of Ang II (Schinke et al. 1999; Monti et al. 2001) and presumably Ang-(1-7), the ASrAogen animals, there was no role of endogenous Ang-(1-7), since [D-Ala⁷]Ang-(1-7) did not decrease pressure observed in a SD control animal (Baltatu et al. 2001). In [mRen-2]27 transgenic animals with higher levels of Ang II and lower levels of Ang-(1-7) than in SD rats (Senanayake et al. 1994), [D-Ala⁷]Ang-(1-7) caused a greater fall in pressure than in SD rats, suggesting a greater contribution of the Ang-(1-7) to the elevated pressure in the rostral VLM (Fontes et al. 2000). Taken together with the effects of Ang-(1-7) on the baroreceptor reflex in the dorsal medulla, the data suggest that, much like Ang II (Weinstock and Gorodetsky 1995), the overall influence of Ang-(1-7) depends upon its site of action, the relative balance of Ang II and Ang-(1-7) endogenously present, the prevailing level of arterial pressure and heart rate, and the sensitivity of the reflex.

We observed no effect of Ang-(1-7) on basal release of serotonin and substance P in slices prepared from the medulla oblongata (Diz and Pirro 1992). However, Ang-(1-7) caused a significant release in the presence of a potassium stimulus (Diz and Pirro 1992). Since Ang-(1-7) facilitated, whereas Ang II attenuated, the gain of the baroreceptor reflex control of heart rate, this pattern of transmitter release is particularly relevant given that serotonin and substance P are known to enhance reflex function. The relationship between these changes in transmitters and the actions of Ang II or Ang-(1-7) in the ventral medulla are not known. However, both peptides may act directly upon neurons of the caudal and rostral VLM and not through release of additional transmitters at those sites similar to other excitatory neurotransmitters (Li and Guyenet 1995, 1996). Ang II acts in the rostral VLM to enhance the release of glutamate (Moriguchi et al. 1995a; Yamada et al. 1995; Zhu et al. 1998), suggesting a presynaptic effect for Ang II. We do not know if Ang-(1-7) shares a similar mechanism of action.

As indicated above, Ang-(1-7) has moderate affinity for the AT₁ receptor in the nTS (~100 nM) (Rowe et al. 1995). We reported that losartan competed for Ang II binding in the nTS and dmX of the dog, suggesting AT₁ sites predominated in the dorsal medulla (Diz and Ferrario 1996). However, in the more rostral aspects of these nuclei, these losartan-sensitive binding sites were 10- to 100-fold more sensitive to Ang-(1-7) and the AT₂ antagonist PD123177. Couto et al. (1998) reported a smaller response to Ang II in the rostral nTS of the rat, whereas Ang-(1-7) acted similarly at both sites. Importantly, the actions of the Ang-(1-7) were blocked by [D-Ala⁷]Ang-(1-7) (Santos et al. 1994). In contrast, Potts et al. (2000b) found that the actions of Ang-(1-7) in the ventrolateral medulla required higher doses than Ang II and actions of both peptides were blocked by AT₁ receptor antagonists (either losartan or candesartan). [D-Ala⁷]Ang-(1-7) also blocked the actions of both Ang II and Ang-(1-7) in this study (Potts et al. 2000b). Thus, receptors responding to Ang-(1-7) in the medulla oblongata are also sensitive to AT₁ and AT₂ antagonists; however, in contrast to the hypothalamus, there is less of an effect of the AT₂ antagonists in this brain region. There remains a great deal of controversy about the nature of the receptors mediating responses to angiotensin peptides in the ventrolateral medulla. The sarcosine-substituted peptide antagonists have actions that do not appear related to either Ang II, Ang-(1-7), or other angiotensin peptides (Potts et al. 2000a) and the specificity of the actions of losartan are questionable (Averill et al. 1994).

3.3

Angiotensin-(1-7) in the Kidneys

The kidney is a critical target site for the actions of the renin-angiotensin system. The ability of Ang II to promote the retention of sodium and water through its potent vasoconstrictor effects, reduction in the filtration coefficient, and stimulation of various transporters in the proximal epithelium are well-characterized (Ichikawa and Harris 1991). In contrast, infusion of Ang-(1-7) into the renal artery stimulates a marked diuresis and natriuresis in both the rat and dog, as well as an increase in glomerular filtration rate (GFR) (DelliPizzi et al. 1994; Heyne et al. 1995; Hilchey and Bell-Quilley 1995; Handa et al. 1996; Vallon et al. 1998; Heller et al. 2000). The diuretic and natriuretic actions of Ang-(1-7) are independent of either AT₁ or AT₂ receptors, but are attenuated by the [D-Ala⁷]Ang-(1-7) antagonist. At concentrations 1,000-fold higher than that of Ang II, Ang-(1-7) did not antagonize Ang II-dependent vasoconstriction in the rat kidney (Handa et al. 1996). Moreover, in the rabbit, low nanomolar doses of Ang-(1-7) induced vasodilation of pre-constricted afferent arterioles that was attenuated solely by [D-Ala⁷]Ang-(1-7) (Ren et al. 2002). These studies suggest that the renal actions of Ang-(1-7) encompass both tubular and vascular sites that are sensitive to blockade of a non-AT₁/AT₂ receptor. Ang-(1-7) or its metabolite Ang-(3-7) are potent inhibitors of Na⁺,K⁺-ATPase activity in isolated convoluted proximal tubules and the renal cortex (Handa et al. 1996; Lopez et al.

1998; Handa 1999). In cultured rabbit renal tubular epithelial cells, Ang-(1-7) stimulated transcellular flux of sodium which was associated with activation of phospholipase A₂ (Andreatta-Van Leyen et al. 1993). Furthermore, inhibition of sodium transport by Ang I was markedly potentiated by captopril—strong evidence for the bio-activation to Ang-(1-7) in the proximal tubules of the kidney. Indeed, the natriuretic and diuretic actions of Ang-(1-7) in the perfused kidney were associated with increased levels of prostacyclin that were attenuated by the cyclooxygenase inhibitor indomethacin (Hilchey and Bell-Quilley 1995). Furthermore, low picomolar doses of Ang-(1-7) stimulated phosphatidylcholine incorporation in the renal cortex, a mechanism that may provide a substrate for arachidonic acid synthesis (Gironacci et al. 2002). Recently, we showed that the Ang-(1-7)-dependent inhibition of ouabain-sensitive rubidium (Rb⁸⁶) influx was blocked by both [D-Ala⁷]Ang-(1-7) and inhibitors of the cytochrome P450 (CYP450) system in isolated tubules (Chappell 2002). These data suggest that Ang-(1-7) may selectively activate the proximal epithelial CYP450 system to augment sodium excretion, in contrast to the Ang II-dependent stimulation of the vascular CYP450 system to produce potent vasoconstrictors (McGiff et al. 1993; Harder et al. 1995; Roman and Alonso-Galicia 1999). Our studies with the mixed vasopeptidase inhibitor omapatrilat in the SHR model also support the renal actions of Ang-(1-7) described above. The pronounced diuresis accompanying the chronic administration of omapatrilat was associated with a large increase in urinary excretion of Ang-(1-7) and enhanced immunocytochemical staining of the peptide in the kidney (Ferrario et al. 2002a).

Other reports suggest that the renal actions of Ang-(1-7) are somewhat comparable to those of Ang II. For example, in a perfused preparation of straight proximal tubules, peritubular application of Ang-(1-7) displayed biphasic effects on bicarbonate transport that were essentially identical to Ang II (Garcia and Garvin 1994). A low concentration (10^{-12} M) of Ang-(1-7) stimulated transport, while higher levels (10^{-8} M) inhibited absorption, probably by altering the Na⁺/H⁺ exchanger. At this site, the biphasic actions of Ang-(1-7) were completely blocked by losartan, and partially attenuated by the AT₂ antagonist PD123319 (Garcia and Garvin 1994). Intratubular application of Ang-(1-7) stimulated transport in the loop of Henle, but did not affect reabsorption in either the proximal or distal tubule (Vallon et al. 1997). In this case, the lack of an effect may result from the high luminal concentrations of Ang-(1-7) in the proximal or distal segments of the tubule. Indeed, luminal application of an AT₁ antagonist or ACE inhibitor attenuates basal reabsorption in the proximal tubule, suggesting that endogenous levels of Ang II are sufficient to facilitate water and sodium transport (Quan and Baum 1996). In contrast to these actions, Santos and colleagues (Santos and Baracho 1992) found that subcutaneous administration of Ang-(1-7) promoted an anti-diuretic action in water-loaded Wistar rats, an effect blocked by both losartan and [D-Ala⁷]Ang-(1-7), but unaffected by an AT₂ antagonist or a vasopressin V₂ antagonist (Santos et al. 1996; Baracho et al. 1998). Studies to date have not determined whether other AT₁ antagonists also inhibit this response, nor have the cellular mechanisms been defined. Ang-(1-7)

stimulates water transport in collecting duct tubules, suggesting that these terminal epithelial cells may be the site of action for Ang-(1-7) (Santos et al. 1996). Borges et al. (2002) also investigated the influence of Ang-(1-7) on water absorption in the jejunum and reported similar antidiuretic actions that were blocked by [D-Ala⁷]Ang-(1-7). However, in the presence of losartan, the same dose of Ang-(1-7) potentially inhibited water reabsorption. Finally, a series of studies reported that Ang-(1-7) alone stimulated ouabain-insensitive Na-ATPase activity in the ovine renal cortex, but inhibited Ang II-dependent stimulation of this ATPase through a [D-Ala⁷]Ang-(1-7)-sensitive site (Caruso-Neves et al. 2000; Lara et al. 2002). Although Burgelova et al. (2002) showed that intrarenal administration of Ang-(1-7) produced natriuresis, the peptide also blocked the anti-natriuretic actions of Ang II. In this case, Ang-(1-7) did not attenuate the reduction in GFR or increased renal perfusion pressure associated with Ang II. Furthermore, infusion of the [D-Ala⁷]Ang-(1-7) alone produced a comparable response to that of Ang II (Burgelova et al. 2002). Importantly, these latter studies emphasize the complex array of actions of Ang-(1-7) and suggest that the overall state of the renin-angiotensin system as well as the dose, route of administration, and site(s) of the nephron exposed to Ang-(1-7) clearly influence the physiological actions of the peptide in the kidney.

3.4

Angiotensin-(1-7) and Trophic Mechanisms

Early observations of responses to Ang-(1-7) included its ability to regulate cellular growth by either preventing mitogen-stimulated proliferation or stimulating the growth of quiescent cells. We and others showed that nanomolar concentrations of Ang-(1-7) inhibited mitogen-stimulated growth of cultured rat thoracic aortic vascular smooth muscle cells (VSMCs) through activation of a [D-Ala⁷]Ang-(1-7)-sensitive receptor (Freeman et al. 1996; Zeng et al. 2001). Ang-(1-7) also has antiproliferative properties *in vivo*, reducing neointimal formation following balloon catheter injury to the rat carotid artery (Strawn et al. 1999). These results demonstrate that Ang-(1-7) inhibits smooth muscle cell growth following vascular injury and could be used to reduce restenosis following angioplasty and stent placement. In similar studies in neonatal rat cardiomyocytes, Ang-(1-7) reduced Ang II-mediated hypertrophy by preventing the increase in protein synthesis and cell surface area which was insensitive to either AT₁ or AT₂ receptor antagonists, suggesting that Ang-(1-7) inhibits myocyte hypertrophy through activation of a novel receptor (Zeng et al. 2000). These studies are in agreement with a recent report by Loot et al. (2002) demonstrating that infusion of Ang-(1-7) reduced myocyte surface area in infarcted rat hearts following coronary artery ligation. Cardiac tissue contains an endogenous renin-angiotensin system (Dzau and Re 1994), and infusion of Ang I into the canine left ventricle results in an 800-fold elevation in interstitial Ang-(1-7) (Wei et al. 2002). In addition, we recently showed significant amounts of immunoreactive Ang-(1-7) in rat myocardium (Ferrario 2002). These results suggest a

potential role for Ang-(1-7) in the regulation of myocyte growth and an impact on cardiac dynamics.

Ang-(1-7) also regulates the formation of new capillary blood vessels. Machado et al. (2000) demonstrated an antiangiogenic effect of Ang-(1-7) using a mouse sponge model. These results suggest a potential therapeutic use for Ang-(1-7) in angiogenesis-dependent diseases such as tumor growth. We recently showed that Ang-(1-7) inhibited DNA synthesis in human lung and breast cancer cells, effects mediated by a non-AT₁, non-AT₂ angiotensin peptide receptor sensitive to [D-Ala⁷]Ang-(1-7) (Tallant et al. 2001). Collectively, these results suggest a potential role for Ang-(1-7) as a chemopreventive and chemotherapeutic agent in human cancers through a reduction in cancer cell growth and angiogenesis.

In contrast to the antiproliferative responses to Ang-(1-7), the heptapeptide stimulates the growth of human skin and cardiac fibroblasts. Nickenig et al. (1997) reported that nanomolar concentrations of both Ang II and Ang-(1-7) stimulated DNA synthesis in cultured human skin fibroblasts. Although the proliferative response to Ang II was partially blocked by an AT₁ receptor antagonist, the stimulatory effect of Ang-(1-7) was insensitive to either an AT₁ or an AT₂ receptor antagonist. Fleck and colleagues (Neuss et al. 1994, 1996) also showed that Ang II and Ang-(1-7) stimulated the growth of cardiac fibroblasts isolated from the left ventricle of explanted end-stage failing hearts. The proliferative responses to Ang II and Ang-(1-7) had EC₅₀s in the nanomolar range and were not prevented by AT₁ or AT₂ receptor antagonists. These results suggest that human fibroblasts contain a novel angiotensin peptide receptor that binds both Ang II and Ang-(1-7) and is coupled to the stimulation of cell growth. Ang II and Ang-(1-7) were also equipotent in increasing DNA synthesis in neonatal rat cortical astrocytes and human astrocytoma cells, responses insensitive to either AT₁ or AT₂ receptor antagonists (Fogarty et al. 2002). However, micromolar concentrations of Ang II and Ang-(1-7) were required to stimulate astrocyte proliferation, leading to questions about the physiological role of these responses. The ability of Ang-(1-7) to either inhibit or stimulate cell growth is dependent upon the type of cell, suggesting that the AT₍₁₋₇₎ receptor may activate unique signaling pathways in different types of cells.

Finally, roles for Ang-(1-7) in hematopoietic recovery following radiation injury and in dermal repair and wound healing were recently suggested by Rodgers et al. (2001, 2002). Treatment of irradiated mice with either Ang II or large doses of Ang-(1-7) accelerated hematopoietic recovery by increasing the number of hematopoietic progenitors in the bone marrow and in the blood. Although losartan tended to block the response to Ang II and not to Ang-(1-7), experiments with additional angiotensin receptor antagonists are required to identify the receptor mediating these responses (Rodgers et al. 2002). Ang II and Ang-(1-7) also accelerated dermal repair in four different models of wound healing, including excision wounds and thermal injury and increased keratinocyte proliferation (Rodgers et al. 2001). Since Ang II and Ang-(1-7) were equipotent in stimulating hematopoietic progenitor formation and accelerating dermal repair but Ang-(1-7) does not increase blood pressure, Ang-(1-7) may

have important therapeutic effects following myelosuppressive irradiation and dermal injury.

4

Mechanisms of Signal Transduction by Angiotensin-(1-7)

Prostanoid Production by Ang-(1-7). We showed that Ang-(1-7) stimulates the release of prostaglandin E₂ (PGE₂) and prostacyclin from C6 glioma cells, human astrocytes, porcine aortic endothelial cells, both porcine and rat aortic VSMCs and rabbit vas deferens (Trachte et al. 1990; Jaiswal et al. 1991a,b, 1992, 1993a,b; Tallant et al. 1991b). In cultured rabbit renal tubular epithelial cells, Ang-(1-7) activated phospholipase A₂ to release arachidonic acid (Andreatta Van Leyen et al. 1993). An antisense oligonucleotide to the cytosolic phospholipase A₂ blocked arachidonic acid release by Ang-(1-7) in rabbit VSMCs, and Ang-(1-7) stimulated translocation of the cytosolic phospholipase A₂ to the nuclear membrane, demonstrating that Ang-(1-7) activates phospholipase A₂ to release arachidonic acid for prostanoid production (Muthalif et al. 1998). In addition, Ang-(1-7) stimulation of arachidonic acid release in rabbit VSMCs was dependent upon the sequential activation of calmodulin-dependent protein kinase II and mitogen-activated protein kinase prior to activation of the cytosolic phospholipase A₂. Thus, Ang-(1-7) stimulates phospholipase A₂ activity to release arachidonic acid for prostanoid production. In contrast to Ang II, Ang-(1-7) does not activate phospholipase C in vascular smooth muscle or endothelial cells, astrocytes, or mesangial cells (Tallant et al. 1991b; Jaiswal et al. 1993a; Chansel et al. 2001; Heitsch et al. 2001; Tallant and Higson 1997) or phospholipase D in VSMCs (Freeman and Tallant 1994).

Many physiological responses to Ang-(1-7) are dependent upon prostanoid production, based upon studies using the cyclooxygenase inhibitor indomethacin. Indomethacin blocked the Ang-(1-7)-mediated inhibition of VSMC growth by Ang-(1-7) (Tallant and Clark 2003), the reduced incidence and duration of arrhythmias in rat hearts (Ferreira et al. 2001), the vasodilation of piglet pial arteries (Meng and Busija 1993), and the depressor component in the pithed rat (Benter et al. 1993). In SHR treated with lisinopril, losartan, or both, an antibody against Ang-(1-7), an inhibitor of Ang-(1-7) formation, or [D-Ala⁷]Ang-(1-7) caused an increase in blood pressure (Iyer et al. 2000b). Indomethacin caused a similar increase in blood pressure, and the increase in blood pressure by [D-Ala⁷]Ang-(1-7) was prevented by pretreatment with the cyclooxygenase inhibitor. These results suggest that vasodilatory prostaglandins mediate many of the vascular effects of Ang-(1-7).

In Dahl salt-sensitive rats, the Ang-(1-7)-mediated reduction in mean arterial pressure was associated with an increase in prostacyclin and a reduction in thromboxane A₂ levels (Bayorh et al. 2002). Since thromboxanes are vasoconstrictive, the decrease in thromboxane production by Ang-(1-7) may contribute to its hypotensive response. Treatment of two-kidney, one-clip (2K-1C) hypertensive rats with Ang-(1-7) reduced thrombus weight following vena cava occlu-

sion (Kucharewicz et al. 2000). Ang-(1-7) also reduced collagen adhesion to platelets isolated from 2K-1C rats. These antithrombotic effects could be due to increased production of prostaglandins or reduced thromboxane synthesis by Ang-(1-7), since prostaglandins are known anti-thrombotic mediators and thromboxanes induce platelet aggregation. Since Ang-(1-7) also increased plasminogen activator inhibitor (PAI)-1 and tissue plasminogen activator (TPA) release from human endothelial cells (HUVECs) (Yoshida et al. 2002), the potential role for Ang-(1-7) as an antithrombotic agent requires further investigation.

Prostaglandins also participate in many of the renal effect of Ang-(1-7). Ang-(1-7) stimulated arachidonic acid release from renal tubular epithelial cells (Andreata-Van Leyen et al. 1993). Infusion of Ang-(1-7) into isolated rat kidneys caused an increase in prostacyclin in both the urine and the perfusate; pretreatment with indomethacin blocked the increase in prostacyclin as well as the diuretic effects (Hilchey and Bell-Quilley 1995). Ang-(1-7) infusion into SHR's caused diuresis and natriuresis during the first 3 days of infusion, concomitant with increased urinary prostaglandins measured on day 2 of the infusion (Benter et al. 1995b). In agreement with these studies, patients chronically treated with the ACE inhibitor captopril had increased plasma concentrations of Ang-(1-7) and prostacyclin with no significant effect on plasma concentrations of Ang II (Luque et al. 1996). These results suggest that Ang-(1-7) and prostacyclin may contribute to the antihypertensive effects of chronic therapy with captopril.

Nitric Oxide Production by Ang-(1-7). The Ang-(1-7)-mediated vasodilation of canine and porcine coronary arteries, isolated feline mesenteric and hindquarter vascular beds, canine middle cerebral arteries, and rabbit afferent arterioles were attenuated by NO synthase inhibitors (Osei et al. 1993; Porsti et al. 1994; Brosnihan et al. 1996; Feterik et al. 2001; Ren et al. 2002). Ang-(1-7) reduced norepinephrine release from the rat hypothalamus, an additional response which was blocked by NO synthase inhibitors (Gironacci et al. 2000). In addition, the antiangiogenic effects of Ang-(1-7) in the mouse sponge model was mediated by NO (Machado et al. 2001). Since the effects of Ang-(1-7) on the canine, porcine, and rabbit arteries as well as in the rat hypothalamus were also blocked by the bradykinin B₂ receptor antagonist HOE 140, Ang-(1-7) may stimulate the production of vasoactive kinins to release NO.

The reduction in mean arterial pressure of Dahl salt-sensitive rats by Ang-(1-7) was associated with an enhanced release of prostacyclin, as described above, as well as enhanced NO production (Bayorh et al. 2002). Ang-(1-7) also caused the release of NO from aortic rings isolated from Dahl salt-sensitive rats on a low- or high-salt diet. In addition, Ang-(1-7) stimulated the production of aortic cyclic guanosine monophosphate (cGMP). These results suggest that the release of NO by Ang-(1-7) stimulates the production of cGMP to induce vascular relaxation.

Stimulation of NO synthase by Ang-(1-7) was measured in isolated canine vessels and bovine aortic endothelial cells. In isolated canine microvessels or coronary arteries, Ang-(1-7) caused nitrite release but only at a concentration of 10 μ M (Seyedi et al. 1995). The effect of Ang-(1-7) was blocked by HOE 140 or

losartan and may result from activation of AT₁ receptors at this concentration. Ang-(1-7)-mediated NO release from bovine aortic endothelial cells was only observed at micromolar concentrations of the peptide, was blocked by HOE 140, and was partially attenuated by [D-Ala⁷]Ang-(1-7), losartan, or PD123177 (Heitsch et al. 2001). The requirement of micromolar concentrations of Ang-(1-7) and blockade of NO production by various receptor antagonists may result from activation of AT₍₁₋₇₎, AT₁, AT₂, or B₂ receptors by the peptide or through inhibition of ACE activity.

Effects of Ang-(1-7) at the AT₁ Receptor. Pharmacological concentrations of Ang-(1-7) bind to the AT₁ receptor and either stimulate AT₁-like responses or antagonize Ang II responses at this receptor. Chansel et al. (2001) showed that Ang-(1-7) prevented the Ang II-mediated increase in Ca²⁺ in rat mesangial cells. However, the effect of Ang-(1-7) occurred at micromolar concentrations of the heptapeptide which competed at the AT₁ receptor in these cells. Ang-(1-7) also constricted rat renal microvessels at micromolar concentrations (van Rodijnen et al. 2002). Consistent with these findings, we showed that micromolar concentrations of Ang-(1-7) reduced Ang II binding to the AT₁ receptor and Ang II-mediated phospholipase C activity in rat aortic VSMCs and CHO cells transfected with the AT_{1A} receptor (Clark et al. 2001a,b). Micromolar concentrations of Ang-(1-7) compete for binding to the AT₁ receptor in VSMCs and CHO-AT_{1A} cells and AT₁ receptor antagonists blocked downregulation of the AT₁ receptor by Ang-(1-7), demonstrating that Ang-(1-7) interacts with the AT₁ receptor to reduce Ang II-mediated responses. We also showed that Ang-(1-7) downregulates the AT₁ receptor in kidney slices (Clark et al. 2003). This effect was blocked by [D-Ala⁷]Ang-(1-7) and indomethacin, suggesting that it occurred through activation of an AT₍₁₋₇₎ receptor. Since ACE inhibitors increase Ang-(1-7) levels (Kohara et al. 1993), the decreases in AT₁ receptor density seen in brain areas such as the nTS or PVN of SHR treated with ACE inhibitors (Diz et al. 1992, 1994) and hypothalamic or medullary neuronal cells in culture (Wilson et al. 1988) may reflect actions of Ang-(1-7) at the AT₁ receptor.

Competition for the AT₁ receptor by pharmacological concentrations of Ang-(1-7) may be responsible for blockade of contractile responses to Ang II in rabbit aortic rings by Ang-(1-7), which required 1–30 μM Ang-(1-7) (Mahon et al. 1994) and human internal mammary arteries, in response to 10 μM Ang-(1-7) (Roks et al. 1999). However, this latter study also addressed the role of Ang-(1-7) as an ACE inhibitor to reduce Ang I contractions in human atrial tissue. They demonstrated that Ang-(1-7) had an IC₅₀ value of 3 μM as an ACE inhibitor and acted in a noncompetitive/mixed way. In functional studies, Ang-(1-7) inhibited the contraction to Ang I and [Pro¹⁰]Ang I, a substrate solely converted by ACE, but not to [Pro¹¹-D-Ala¹²] Ang I, an ACE-resistant substrate. Thus, Ang-(1-7) could also modulate responses at the AT₁ receptor through inhibition of ACE; however, the concentrations required for ACE inhibition by Ang-(1-7) do not occur under physiological conditions. In contrast to inhibition of Ang II responses at micromolar concentrations, the antagonism of Ang II-mediated vaso-

constriction of human forearm resistance vessels by nanomolar concentrations of Ang-(1-7) observed by Ueda et al. (2000) may result from activation of an $AT_{(1-7)}$ receptor and the release of compensatory vasodilators.

Although Ang-(1-7) acutely downregulates the AT_1 receptor, Neves et al. (2000) showed that a 24-h treatment with Ang-(1-7) upregulated AT_1 mRNA in VSMCs isolated from the Akron strain of WKY and SHR rats. This suggests that chronic exposure to Ang-(1-7) may alter the expression of the AT_1 receptor in a strain-specific manner.

4.1 Angiotensin-(1-7) Receptor(s)

Recognition of the physiological responses to Ang-(1-7) occurred concurrently with the identification of subtype-selective ligands for different molecular forms of Ang II receptors. AT_1 angiotensin receptors are pharmacologically defined by their selectivity for the prototypical ligand losartan and similar antagonists (valsartan, L-158,809, etc.), while AT_2 receptors show selectivity for the antagonist PD123177 and PD123319 as well as the agonist CGP42112 (de Gasparo et al. 1995). Characterization of physiological or cellular responses to Ang-(1-7) was therefore accompanied by attempts to define the receptor mediating these responses. Ang-(1-7) is a poor competitor (IC_{50} s in the micromolar range) at pharmacologically defined AT_1 receptors in VSMC (Jaiswal et al. 1993a,b) or AT_2 receptors in differentiated NG108-15 cells, pancreatic cells, and human myometrium (Tallant et al. 1991a; Chappell et al. 1995; Bouley et al. 1998). [D-Ala⁷]Ang-(1-7), a modified form of Ang-(1-7) in which proline at position 7 is replaced with D-alanine, was designed as a selective antagonist for the Ang-(1-7) receptor (Santos et al. 1994). In initial studies, [D-Ala⁷]Ang-(1-7) blocked hemodynamic and renal responses to Ang-(1-7), did not compete for binding of ¹²⁵I-Ang II to rat adrenal AT_1 or AT_2 receptors, and did not block pressor or contractile responses to Ang II, demonstrating selectivity for Ang-(1-7) (Santos et al. 1994).

We identified an Ang-(1-7) binding site on bovine aortic endothelial cells (BAEC) which was competed by [D-Ala⁷]Ang-(1-7) and [Sar¹-Ile⁸]Ang II, but not by losartan or PD123319 (Tallant et al. 1997). In agreement with this identification, treatment of BAEC with Ang-(1-7) stimulated NO release, an effect blocked by [D-Ala⁷]Ang-(1-7) (Heitsch et al. 2001). A similar ¹²⁵I-Ang-(1-7) binding site, sensitive to Ang-(1-7) and [D-Ala⁷]Ang-(1-7), was visualized in the endothelium of canine coronary artery rings (Ferrario et al. 1997), consistent with functional effects of Ang-(1-7) in endothelium-intact canine and porcine coronary arteries (Xiang et al. 1985; Porsti et al. 1994; Brosnihan et al. 1996). We recently identified a similar binding site for Ang-(1-7) in the rat vasculature that is sensitive to both [D-Ala⁷]Ang-(1-7) and [Sar¹-Thr⁸]Ang II, but not to losartan or PD123319 (Iyer et al. 2000b). Ang-(1-7) inhibited the mitogen-stimulated growth of rat VSMCs which was blocked by [D-Ala⁷]Ang-(1-7) and sarcosine analogs of Ang II but not by losartan and PD123177 (Freeman et al. 1996; Tallant et al. 1999; Min et al.

2000). Additionally, the specific receptor antagonist [D-Ala⁷]Ang-(1-7) blocked the Ang-(1-7)-induced inhibition of angiogenesis in the mouse sponge model (Machado et al. 2001). The identification of high-affinity binding sites for Ang-(1-7) that are selectively competed by [D-Ala⁷]Ang-(1-7) agrees with the ability of this ligand to block functional responses at these sites.

A multitude of physiological responses to Ang-(1-7) are mediated by a specific AT₍₁₋₇₎ receptor that is selectively blocked by [D-Ala⁷]Ang-(1-7) or the sarcosine analogs of Ang II, but not by pharmacologically-defined AT₁ or AT₂ receptor antagonists. Studies in the pithed rat (Benter et al. 1993) provided the first evidence for a hypotensive response to Ang-(1-7) that was mediated by a non-AT₁, non-AT₂ receptor. Moreover, treatment with an Ang-(1-7) antibody, the non-selective angiotensin peptide antagonist [Sar¹-Thr⁸]Ang II, or a neprilysin inhibitor to prevent Ang-(1-7) formation caused a rapid increase in blood pressure in SHR chronically treated with an ACE inhibitor and an AT₁ receptor antagonist (Iyer et al. 1998a,b). These effects were not blocked by the AT₂ receptor antagonist PD123319. Similar effects of the Ang-(1-7) antibody, a neprilysin inhibitor, or [D-Ala⁷]Ang-(1-7) were observed in salt-depleted SHR or [mRen-2]27 hypertensive rats (Iyer et al. 2000a). In agreement, Ang-(1-7) infusion decreased blood pressure in SHR on day 4 and 5 which was blocked by [D-Ala⁷]Ang-(1-7) (Widdop et al. 1999). Bayorh et al. (2002) recently showed that Ang-(1-7) also caused a depressor response in salt-sensitive Dahl rats which was specifically blocked by [D-Ala⁷]Ang-(1-7); Ang-(1-7) infusion caused an increased in the release of prostacyclin and NO and reduced thromboxane A₂ levels, all of which were prevented by pretreatment with the selective AT₍₁₋₇₎ receptor antagonist. Collectively, these results demonstrate that the hypotensive response to Ang-(1-7) was mediated by a non-AT₁, non-AT₂ [D-Ala⁷]-Ang-(1-7)-sensitive receptor. Other studies reviewed above are also consistent with the existence of an AT₍₁₋₇₎ receptor in brain (Ambuhl et al. 1994; Fontes et al. 1997; Chaves et al. 2000; Fontes et al. 2000) and renal tissue (Vallon et al. 1998; Gironacci et al. 2002; Lara et al. 2002). Taken together, these data provide overwhelming evidence that the actions of Ang-(1-7) are mediated by a non-AT₁, non-AT₂ receptor. We refer to this receptor as the AT₍₁₋₇₎ receptor, in accordance with the guidelines established by the International Union of Pharmacology Nomenclature Subcommittee for Angiotensin Receptors (Bumpus et al. 1991; de Gasparo et al. 1995). The unique AT₍₁₋₇₎ receptor is defined by its sensitivity to Ang-(1-7), its antagonism by [Sar¹-Thr⁸]Ang II and [D-Ala⁷]Ang-(1-7), and its lack of response to losartan or PD123319, either functionally or in competition for binding.

Recently, Santos et al. (2002) reported the G protein-coupled receptor *mas* as a functional AT₍₁₋₇₎ receptor. Ang-(1-7) bound with high affinity to either CHO or HEK cells transfected with the *mas* gene. [D-Ala⁷]Ang-(1-7) blocked the heptapeptide binding to the transfected CHO cells; however, the AT₁ or AT₂ receptor antagonists had no effect. The results suggest that *mas* serves as a selective Ang-(1-7) binding site. *Mas* is predominately expressed in the testis and the hippocampus and amygdala of the mammalian forebrain with minimal but detectable levels in the rodent heart and kidney (Metzger et al. 1995). This tissue distribu-

tion differs from many previous reports of Ang-(1-7) binding and functional responses, suggesting the possibility of additional receptor subtypes.

Conversely, some of the renal effects of Ang-(1-7) implicate a losartan-sensitive receptor (Garcia and Garvin 1994). Ang-(1-7) inhibited sodium transport in the rat proximal tubule, an effect that was partially blocked by losartan but completely inhibited by [Sar¹-Thr⁸]Ang II (Handa et al. 1996). In the basolateral membranes of porcine renal proximal tubules, Ang-(1-7)-stimulated Na⁺-ATPase activity was reversed by losartan, but not by PD123319 or [D-Ala⁷]Ang-(1-7) (Caruso-Neves et al. 2000). In contrast to the diuretic and natriuretic effects of Ang-(1-7), the heptapeptide has anti-diuretic effects in water-loaded rats that were also specifically blocked by both [D-Ala⁷]Ang-(1-7) and losartan, but not by AT₂ receptor antagonists (Baracho et al. 1998). In support of losartan-sensitive responses to Ang-(1-7) in the kidney, Ang-(1-7) competed with high affinity ($K_i=8$ nM) for an ¹²⁵I-Ang II binding site in rat renal cortex that was totally displaced by losartan (Gironacci et al. 1999). Likewise, microinjection of [D-Ala⁷]Ang-(1-7) or losartan into the ventrolateral medulla inhibited both the pressor and depressor responses to Ang-(1-7) (Potts et al. 2000b). Although Ang-(1-7)-stimulated prostaglandin release from C6 glioma cells was also blocked by the AT₁ antagonist, micromolar concentrations of the antagonist were required, suggesting that this may be a non-selective effect of the antagonist (Jaiswal et al. 1991a). Collectively, these results suggest that some of the Ang-(1-7)-stimulated responses in the kidney and brain are partially mediated by a receptor that is sensitive to losartan and may be sensitive to other AT₁ receptor antagonists, but retains sensitivity to [D-Ala⁷]Ang-(1-7).

There are also several reports of responses to Ang-(1-7) that are sensitive to AT₂ receptor antagonists. Ang-(1-7) stimulated arachidonic acid release from rabbit VSMCs which was partially blocked by [D-Ala⁷]Ang-(1-7) or PD123319. However, the combination of [D-Ala⁷]Ang-(1-7) and PD123319 completely blocked the response to Ang-(1-7), suggesting that the heptapeptide activates both a [D-Ala⁷]Ang-(1-7)-sensitive and a PD123319-sensitive receptor on rabbit VSMCs (Muthalif et al. 1998). Ang-(1-7) inhibition of norepinephrine release from the rat hypothalamus was blocked by PD123319 and by [D-Ala⁷]Ang-(1-7), but not by losartan. These results suggest that the Ang-(1-7) inhibition of noradrenergic neurotransmission is mediated by a receptor that is sensitive to both [D-Ala⁷]Ang-(1-7) and PD123319 (Gironacci et al. 2000) or that the two receptors act in series. In cultured human astrocytes, the Ang-(1-7)-mediated release of both PGE₂ and prostacyclin was blocked by CGP42112A; however, other AT₂ receptor antagonists were not available at the time of these studies. Since CGP42112A is now recognized as an agonist for the AT₂ receptor, further studies are required to determine whether this response is mediated by an AT₂ receptor (Jaiswal et al. 1991b).

Identification of an AT₍₁₋₇₎ receptor is further confounded by reports of responses to Ang-(1-7) that are sensitive to both AT₁ and AT₂ receptor antagonists. Iontophoresis of Ang-(1-7) excited neurons in the paraventricular nucleus. Although both AT₁ and AT₂ receptor antagonists blocked the responses to Ang-

(1-7), AT₁ receptor antagonists were more potent than AT₂ receptor antagonists (Ambuhl et al. 1992). Ang-(1-7) caused the release of norepinephrine from the rat atria, which was inhibited by both losartan and PD123319 (Gironacci et al. 1994). Additionally, Ang-(1-7), at concentrations from 1 to 10 μ M, stimulated NO and superoxide release from bovine aortic endothelial cells. These responses were partially blocked by [D-Ala⁷]Ang-(1-7), losartan, or PD123177 (Heitsch et al. 2001). However, since the K_d for binding of Ang-(1-7) to the AT₍₁₋₇₎ receptor in bovine aortic endothelial cells was in the nanomolar range (Tallant et al. 1997), the observed responses may be due to interactions with ACE or resensitization of the B₂ receptor on endothelial cells. In porcine vascular smooth muscle cells, Ang-(1-7)-mediated prostanoid release was not inhibited by losartan but was partially inhibited by L-158,809, EXP3174, or PD123319 (Jaiswal et al. 1993b).

Finally, Handa (1999) reported that Ang-(1-7) recognizes the AT₄ receptor in renal proximal tubules and Madin-Darby canine kidney (MDCK) collecting duct cells. This interaction requires the metabolism of Ang-(1-7) to Ang-(3-7), a high-affinity agonist that subsequently binds to the AT₄ receptor (Handa 2000a,b). However, the AT₄ receptor was identified as the insulin-regulated aminopeptidase, suggesting a substrate-enzyme interaction rather than an agonist-receptor pathway (Albiston et al. 2001).

[D-Ala⁷]Ang-(1-7) exhibited agonistic properties in two recent reports. Gironacci et al. (2002) showed that [D-Ala⁷]Ang-(1-7) blocked Ang-(1-7)-stimulated phosphatidylcholine synthesis in rat renal cortical slices. However, [D-Ala⁷]Ang-(1-7) itself stimulated phosphatidylcholine synthesis. Additionally, Ang-(1-7) as well as [D-Ala⁷]Ang-(1-7) decreased the release of PAI-1 and TPA in human umbilical vein endothelial cells (Yoshida et al. 2002). These results suggest that [D-Ala⁷]Ang-(1-7) may also act as a partial agonist in some circumstances.

5

Role of Angiotensin-(1-7) in Human Hypertension

A limited number of studies in humans provides a foundation for Ang-(1-7) as another mechanism implicated in cardiovascular function and blood pressure dysregulation. The vasodilator effects of Ang-(1-7) contribute to the antihypertensive effects of ACE inhibitors (Ferrario et al. 1998) and the vaso-peptidase inhibitor omapatrilat (Ferrario et al. 2002b). These two studies provided evidence that salt-sensitive hypertensive patients may have lower excretion rates of Ang-(1-7), a finding that we interpreted as a deficit in Ang-(1-7) synthesis or activity as a contributor to the syndrome of low-renin hypertension. The bulk of the physiological, pharmacological, and molecular evidence gathered to date makes this field a fruitful point for further investigation.

Recent studies demonstrated that circulating and urinary levels of Ang-(1-7) are increased in normal human pregnancy. Valdes et al. (2001) showed that urinary Ang II and Ang-(1-7) increased throughout gestation. By the 35th week of

gestation, both urinary Ang II and Ang-(1-7) reached their highest levels, which were 16- and 20-fold higher than that obtained during the menstrual cycle. In addition, plasma levels of Ang I, Ang II, and Ang-(1-7) were increased in normotensive pregnant women during the third trimester of pregnancy (Merrill et al. 2002). These changes were accompanied by increased levels of other components of the renin-angiotensin system, including plasma renin activity and aldosterone. In age—and race—matched third-trimester preeclampsia subjects, plasma levels of Ang-(1-7) were significantly decreased compared with normal pregnant subjects. Other components of the renin-angiotensin system, with the exception of ACE, were reduced in preeclamptic subjects; only plasma Ang II remained elevated in preeclamptic subjects as compared to non-pregnant subjects. There was an inverse association between circulating Ang-(1-7) and both systolic ($r=-0.51$, $p<0.004$) and diastolic ($r=-0.44$, $p<0.02$) blood pressure, supporting a role of Ang-(1-7) in the control of blood pressure. This study suggests that Ang-(1-7) may contribute to the elevated blood pressure in preeclamptic women. Furthermore, these data are consistent with the negative correlation between urinary Ang-(1-7) and blood pressure previously reported in normotensive and hypertensive subjects (Ferrario et al. 1998).

6 Summary

Hypertension research has provided fundamental insights into the regulation of cardiovascular function and expanded knowledge of the biochemical mechanisms by which hormones and locally generated autacoids regulate tissue perfusion and blood distribution to maintain homeostasis. Research on Ang-(1-7) has expanded knowledge of the biochemistry of the renin-angiotensin system and its interaction with molecules participating in the processes of inflammation, growth, and vascular tone. The unique actions of Ang-(1-7) in generally opposing the pleiotropic effects of Ang II in cardiovascular function led us to propose that the heptapeptide represents a negative feedback mechanism controlling the agonistic actions of Ang II in tissue function and body fluid control mechanisms. The demonstration that ACE is the primary enzyme responsible for Ang-(1-7) inactivation provides a mechanistic point of balance between synthesis of Ang II and Ang-(1-7) degradation. The half-life of Ang-(1-7) in blood and tissue, similar to that of bradykinin and far shorter than the half-life of circulating Ang II, supports the role of this hormone as a vasodilator factor. Modulatory effects of Ang-(1-7) described in neural and central neuroendocrine mechanisms regulating blood pressure and fluid volumes are strong, as is the physiological and pharmacological evidence that Ang-(1-7) contributes to the glomerulotubular regulation of sodium and, perhaps, water excretion.

While there is overwhelming evidence that Ang-(1-7) binds to a unique, non-AT₁, non-AT₂ receptor, additional studies suggest that the molecular interactions of Ang-(1-7) with its cellular receptor are complex. Data accumulated thus far indicate that the heptapeptide also may bind to additional receptors that are

sensitive to AT₁ and/or AT₂ receptor antagonists as well as blocked by [D-Ala⁷] Ang-(1-7). Alternatively, Ang-(1-7) may bind to a unique receptor that physically interacts with the pharmacologically defined AT₁ and AT₂ receptors, resulting in a protein complex with combinatorial characteristics. Such a heterodimer is formed between the AT₁ and bradykinin B₂ receptors, resulting in a complex with altered G protein signaling and receptor endocytosis (AbdAlla et al. 2000). Cloning of the unique non-AT₁, non-AT₂ receptor as well as further studies directed toward protein-protein interactions between the AT₍₁₋₇₎ receptor and other membrane receptors are needed to elucidate the precise molecular mechanisms involved in Ang-(1-7)-mediated responses.

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Angiotensin AT₄ Receptor

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Abstract Although angiotensin IV (Ang IV) was thought initially to be an inactive product of angiotensin II (Ang II) degradation, the hexapeptide was subsequently shown to markedly enhance learning and memory in normal rodents and reverse memory deficits observed in animal models of amnesia. These central nervous system effects of Ang IV are mediated by binding to a specific site known as the AT₄ receptor which is found in appreciable levels throughout the brain and concentrated particularly in regions involved in cognition. The AT₄ receptor has a broad distribution and is found in a range of tissues including

the adrenal gland, kidney, lung and heart. In addition to Ang IV, the peptide LVV-haemorphin-7 binds with high affinity to the AT₄ receptor and has been demonstrated to mediate the same effects as Ang IV. In the kidney Ang IV has been demonstrated to mediate a number of effects including increasing renal cortical blood flow and decreasing Na⁺ transport in isolated renal proximal tubules. Biochemical studies define the AT₄ receptor as a single transmembrane glycoprotein, 165 kDa in size. This field of research was redefined by the identification the AT₄ receptor as the transmembrane enzyme insulin-regulated membrane aminopeptidase (IRAP). Insulin-regulated aminopeptidase is a type II integral membrane-spanning protein belonging to the M1 family of aminopeptidases. Ang IV has been demonstrated to be a potent inhibitor of IRAP enzymatic activity.

Keywords Aminopeptidase · Angiotensin · AT₄ receptor · Memory · Haemorphin · Insulin-regulated aminopeptidase

1

Definition of the AT₄ Receptor

Angiotensin IV (Ang IV), the 3–8 fragment of angiotensin II (Ang II), is formed by consecutive actions of aminopeptidase A and N on Ang II (Zini et al. 1996). This hexapeptide was initially thought to be inactive because it does not elicit the classical effects of Ang II on blood pressure and fluid balance. However, in 1992 a specific high-affinity binding site was discovered for Ang IV in bovine adrenal membranes (Swanson et al. 1992). This binding site is pharmacologically distinct from angiotensin AT₁ and AT₂ receptors and binds Ang II with only micromolar affinity. This binding site was named the angiotensin AT₄ receptor by an International Union of Pharmacology (IUPHAR) nomenclature committee (de Gasparo et al. 1995).

Ang IV exhibits a high affinity for the AT₄ receptor in bovine adrenal cortical membranes with a K_D of between 0.2 and 0.6 nM and a B_{max} of between 0.5 and 2.9 pmol/mg protein (Swanson et al. 1992; Bernier et al. 1994). The binding of Ang IV to the AT₄ receptor in bovine adrenal cortical membranes is saturable and reversible.

2

Ligands of the AT₄ Receptor

The amino acids of Ang IV critical for binding to the AT₄ receptor have been identified (Sardinia et al. 1993, 1994; Krishnan et al. 1999). The presence of an amino-terminal valine or, more precisely, a primary α -amine in the L-amino acid conformation in position 1, is important for binding to the AT₄ receptor. Glycine substitutions at positions 1, 2 or 3 of Ang IV greatly reduce affinity for the AT₄ receptor, whereas substitutions at positions 4, 5 or 6 of Ang IV have little effect (Sardinia et al. 1993). N-terminal elongation of Ang IV to Ang III and

Ang II results in a marked reduction in affinity, whilst the C-terminal extensions (Ang I 3–9, Ang I 3–10) do not alter binding affinity (Sardinia et al. 1993). Thus, the N-terminal amino acids of the Ang IV peptide are critical for receptor binding, whilst the C-terminal portion is less critical.

Two N-terminally modified analogues of the Ang IV, namely Nle¹-Ang IV and norleucinal Ang IV, exhibit higher affinities for the AT₄ receptor site from bovine adrenal membranes, with K_i of 3.6×10^{-12} and 1.8×10^{-10} M respectively (Sardinia et al. 1994). These peptides were thought to be more resistant to degradation by aminopeptidases and were found to be “agonists” at the AT₄ receptor site, eliciting vasodilatation and facilitation of memory as discussed in the following sections. Replacement of the Ile³ with Val³ and the amide bonds connecting Val¹ and Tyr², Val³ and His⁴ with methylene peptide bond isosteres [Vψ(CH₂-NH₂)YVψ(Ch₂-NH₂)HPF] results in a non-peptide partial antagonist of the AT₄ receptor named divalinal Ang IV (Krebs et al. 1996). Divalinal Ang IV bound to the AT₄ receptor from bovine adrenal membranes with a K_D of 0.4 nM (Krebs et al. 1996).

In addition to Ang IV and its analogues, we isolated a decapeptide, LVVYPWTQRF, from sheep cerebral cortex that binds with high affinity to the AT₄ receptor (Moeller et al. 1997). This peptide is identical to an internal sequence of β,δ,γ and ε globin and has been called LVV-haemorphin-7 because it was derived from haemoglobin and exhibited weak opioid activity in the guinea-pig ileum (Garreau et al. 1995). In bovine adrenal membranes, LVV-haemorphin-7 competes for ¹²⁵I-Ang IV binding with an IC₅₀ of 35 nM, while Ang IV competes for the same binding site with an IC₅₀ of 2.3 nM (Moeller et al. 1997). The binding of ¹²⁵I-LVV-haemorphin-7 and ¹²⁵I-Ang IV on adjacent sections of the sheep hindbrain exhibit identical patterns (Moeller et al. 1997), suggesting that both of the peptides bind to the same site, the AT₄ receptor. In contrast to Ang IV, LVV-haemorphin-7 is more resistant to degradation in vitro (Moeller et al. 1999a).

Deletion of the last four amino acids from the carboxy terminus or N-terminal truncation of Leu¹ does not alter binding of LVV-haemorphin-7 to the AT₄ receptor. In fact, removal of Val² from the N-terminus results in a tenfold increase in its affinity for the receptor site. By contrast, substitution of Tyr⁴ and Trp⁶ residues with alanine results in a tenfold decrease in affinity with respect to LVV-haemorphin-7. However, the replacement of Pro⁵ and Thr⁷ with alanine has little effect on the affinity of the decapeptide for the AT₄ receptor.

3 Distribution of the AT₄ Receptor

Although the AT₄ receptor was first identified and characterised in bovine adrenal membranes, where it occurs in high levels in the zona glomerulosa (Krebs et al. 1996), the receptor has since been detected in many tissues. These include heart, spleen, colon, kidney and brain of the guinea-pig, human prostate and bladder, rhesus monkey bladder and rat kidney (reviewed in Wright et al. 1995).

3.1

Cardiovascular

In the guinea-pig heart, the AT₄ receptors were found in cardiomyocytes, blood vessels, epicardium and endocardium (Hanesworth et al. 1993) and in the rabbit in cardiac fibroblasts (Wang et al. 1995b). The AT₄ receptor has also been detected in bovine coronary (Hall et al. 1995), bovine aortic (Bernier et al. 1995) and porcine aortic (Riva and Galzin 1996) endothelial cells, and in bovine aortic vascular smooth muscle cells (Hall et al. 1993). In rabbit carotid arteries, we found the AT₄ receptor distributed most abundantly in the smooth muscle layer and in vaso vasorum of the adventitia. Much lower levels are detected in the endothelium (Moeller et al. 1999b). Moreover, we found that the AT₄ receptor was upregulated in the media and the thickened neointima following endothelial denudation (Moeller et al. 1999b).

3.2

Renal

In the rat kidney, the AT₄ receptors have been reported in outer stripe of the medulla (Harding et al. 1994; Coleman et al. 1998; Handa et al. 1998) and at lower levels in the cortex, including the proximal and distal tubules. However, we find a different pattern of distribution of the AT₄ receptors in sheep, guinea-pig, rabbit, mouse and human kidney (A.L. Albiston, unpublished results). In our studies, high levels of the AT₄ receptors occur in the medullary rays and also in the inner medulla. Moderate levels of the receptor are also detected in the proximal and distal tubules, over the glomeruli and in the renal vasculature (Fig. 1). In WKY rats fed a high salt diet (8%), the AT₄ receptors in the kidney were reported to increase by 28% (Grove and Deschepper 1999).

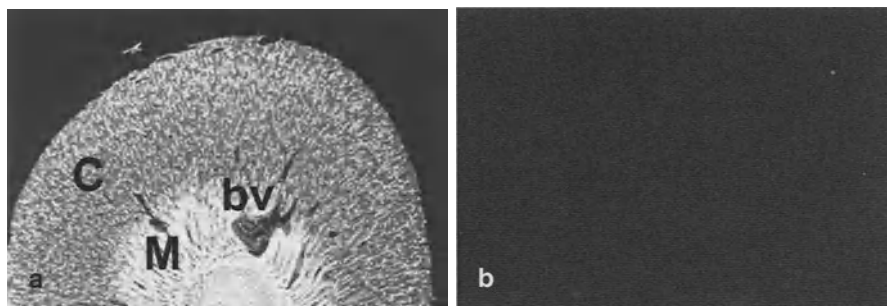


Fig. 1a, b Receptor autoradiography using [¹²⁵I]Nle¹-Ang IV on 10- μ m adjacent ovine kidney sections. Incubations were carried out as described previously. (Chai et al. 2000). The binding sites are displayed as white regions against the black background. Total: [¹²⁵I]Nle¹-Ang IV binding, (a) non-specific : [¹²⁵I]Nle¹-Ang IV binding in the presence of 1 μ M Ang IV (b). C, cortex; M, medulla; bv, blood vessel

3.3

Brain

Angiotensin AT₄ receptors, as detected by [¹²⁵I]Ang IV or [¹²⁵I]Nle¹-Ang IV binding, are distributed widely in the brains of the guinea-pig (Miller-Wing et al. 1993), rat (Roberts et al. 1995), sheep (Moeller et al. 1995), macaque monkey (Moeller et al. 1996a) and human (Chai et al. 2000). The distribution of the AT₄ receptors is highly conserved throughout these species. The AT₄ receptors are found in high concentrations in the basal nucleus of Meynert, in the pyramidal cell layer of CA1 to CA3 of Ammon's horn of the hippocampus, and throughout the neocortex in layers IV/V. This pattern of AT₄ receptor distribution closely resembles that of cholinergic neurons and their projections and its abundance in brain regions involved in cognitive processing is consistent with the memory-enhancing properties of the AT₄ receptor agonists. High levels of binding also occur in most brain regions involved in motor control including the motor cortex, ventral lateral thalamic nucleus, cerebellum and the cranial nerve motor nuclei and spinal cord ventral horn motor neurons. Many sensory regions, and some hypothalamic regions, also contained moderate levels of the AT₄ receptor, suggesting a more widespread role for the receptor in the brain.

The AT₄ receptor has also been demonstrated in primary cultures of embryonic rat neurons and neonatal rat astrocytes where the receptor was found to be abundant with B_{\max} s of 1.7 and 1.4 pmol/mg protein respectively (Greenland et al. 1996).

4

Effects of Ang IV and Other AT₄ Receptor Ligands in the CNS

The first report of central effects of Ang IV was by Braszko and colleagues in 1988 who infused Ang IV (1 nmol) into the lateral cerebral ventricles of the rat and observed improved acquisition of conditioned avoidance responses and improved recall in the passive avoidance paradigm (Braszko et al. 1988). They also reported stimulation of exploratory locomotor activity and enhanced stereotypy with apomorphine or amphetamine (Braszko et al. 1988).

Following the discovery of the AT₄ receptor in discrete regions of the guinea-pig brain, particularly in the cerebral cortex and hippocampus, the effect of intracerebroventricular infusion of Ang IV on the passive avoidance paradigm was reinvestigated (Wright et al. 1993). A single dose of Ang IV (100 and 1,000 pmol) given before the final conditioning trial improved performance thereby enhancing memory retention (Wright et al. 1993). Ang IV (10, 100 and 1,000 pmol), given prior to retesting on day 2, was found to enhance retrieval of memory (Wright et al. 1993). In contrast to the earlier study by Braszko, no significant increase was observed in exploratory behaviour in the open field test (Wright et al. 1995).

In a spatial learning paradigm, the Morris water maze, chronic infusion of Nle¹-Ang IV (0.1–0.5 nmol/h for 6 days) facilitated the rate of acquisition of this

task for the first 3 days (Wright et al. 1999). In the same study, chronic infusion of the "antagonist" divalinal Ang IV (0.5–5 nmol/h for 6 days) interfered with the acquisition process on days 3–6 (Wright et al. 1999). In our study using the Barnes circular maze, another spatial learning paradigm, a single dose of Nle¹-Ang IV (100 or 1,000 pmol) or the structurally unrelated AT₄ ligand, LVV-haemorphin-7 (1,000 pmol), given intracerebroventricularly dramatically accelerated spatial learning by up to 3 days compared to control rats. The concurrent delivery of 10 nmol of the antagonist divalinal Ang IV with 100 pmol of Nle¹-Ang IV prevented the increased rate of acquisition mediated by the AT₄ receptor.

The role of AT₄ receptors in memory was reported even in invertebrates, since both Ang IV and Ang II facilitated long-term memory of an escape response in the crab *Chasmagnathus* (Delorenzi et al. 1997). However, they later concluded that Ang II, but not Ang IV, is the endogenous mediator of long-term memory in this species (Delorenzi and Maldonado 1999).

In addition to its memory-enhancing effects in normal animals, AT₄ receptor ligands are effective in various models of amnesia. Bolus i.c.v. injection of 1 nmol Nle¹-Ang IV prior to the testing reverses scopolamine-induced amnesia in rats, demonstrated in the Morris water maze paradigm (Pederson et al. 1998). Subsequently it was demonstrated that i.c.v. injection of Nle¹-Ang IV dose-dependently (0.1–1 nmol) reversed scopolamine-induced amnesia in the same paradigm, an effect blocked by the AT₄ receptor antagonist, Nle¹,Leu⁵-Ang IV [1 nmol (Pederson et al. 2001)]. Similarly, we find that i.c.v. injection of the AT₄ receptor ligand, LVV-haemorphin-7 (100 pmol), reverses scopolamine-induced amnesia in a passive-avoidance paradigm (S.Y. Chai et al., unpublished results). In a second rat model of amnesia, a single i.c.v. injection of the AT₄ receptor agonist, norleucinal (1 nmol), 5 min before the training trial each day, reversed the deficit in spatial learning induced by bilateral perforant pathway knife-cuts in the rat (Wright et al. 1999).

Recent studies demonstrate a role for AT₄ receptors in long-term potentiation (LTP) (Kramar et al. 2001; Wayner et al. 2001), a cellular basis for learning and memory. Application of Nle¹-Ang IV (1 μ M) enhances synaptic transmission during low-frequency test pulses (0.1 Hz), and increases tetanus-induced LTP by 63% with respect to control in CA1 region of the rat hippocampus in vitro. Paired stimulation before and during infusion of Nle¹-Ang IV had no effect on paired-pulse facilitation, suggesting that the Nle¹-Ang IV-induced increase in synaptic transmission and LTP is likely to be a post-synaptic event. Similarly, administration of Ang IV (0.8 μ M) enhanced LTP in the rat dentate gyrus in vivo (Wayner et al. 2001). A complex time-related effect was observed with a maximum at 5 min, a return to normal LTP at 30 min, a minimum below normal at 90 min, and a return to normal at 120 min. The Ang IV-induced enhancement of LTP was attenuated in the presence of divalinal-Ang IV (5 μ M).

In cultured embryonic chicken sympathetic neurons Ang IV inhibited neurite outgrowth in a dose-dependent manner (Moeller et al. 1996b; Reed et al. 1996). This effect was blocked by the Ang IV analogues WSU 4042, Nle¹-Y-I-amide or Nle¹-Ang IV, but not by the Ang II antagonists, [Sar¹, Ile⁸]Ang II or CGP 42112,

indicating an AT₄ receptor-mediated effect (Moeller et al. 1996b). Other compounds, such as acetylcholine, have been shown to reduce neurite outgrowth of embryonic chicken ciliary neurons (Pugh and Berg 1994).

Based on the distribution of the AT₄ receptor in the brain, and involvement of the cholinergic system in hippocampal-dependent memory acquisition, retention and retrieval, we investigated the effect of AT₄ receptor ligands on acetylcholine release from hippocampal slices. Ang IV and LVV-haemorphin-7 both potentiated depolarisation-induced [³H]-acetylcholine release from the rat hippocampus in a concentration-dependent manner with the maximal effective dose (0.1 μM) of each inducing increases of 45% and 96% above control, respectively (Lee et al. 2001). Potentiation of release by both agonists was attenuated by the AT₄ receptor antagonist, 1 μM divalinal-Ang IV. Ang IV-induced potentiation was not affected by AT₁ and AT₂ receptor antagonists (Lee et al. 2001). AT₄ receptors therefore can potentiate depolarisation-induced release of acetylcholine from hippocampal neurons and may be one mechanism by which AT₄ receptor ligands enhance cognition.

There is considerable evidence to support a role for AT₄ receptors in mitogenesis (reviewed in Mustafa 2002). Work from this laboratory demonstrated that the AT₄ receptor ligand, LVV-haemorphin-7, stimulates DNA synthesis, in the neuronal/glial hybrid cell line, NG108-15 (Moeller et al. 1999a). However, treatment with Ang IV alone had no effect on DNA synthesis, and co-incubation of the cells with excess Ang IV (1 μM) inhibited LVV-haemorphin-7-stimulated DNA synthesis. In contrast, we also demonstrated that both Ang IV and LVV-haemorphin-7 (100–1.0 nM) stimulated DNA synthesis in the human neuroblastoma cell line SK-N-MC by up to 72% and 81% above control levels (Mustafa et al. 2001). These trophic effects may play a role in the AT₄ receptor-mediated reversal of memory deficits resulting from bilateral perforant pathway lesion (see above).

5

Effects of Ang IV and Other AT₄ Receptor Ligands in Peripheral Tissues

5.1

Vasculature

Intra-arterial infusions of either Ang IV or Nle¹-Ang IV at 100 pmol/min into the carotid artery increased cerebral blood flow in anaesthetised rats, an effect blocked by pretreatment with N^ω-nitro-L-arginine methylester (L-NAME), suggesting the involvement of nitric oxide (Kramar et al. 1998). Intravenous infusions of Ang IV (1 μg/kg/min) also reversed acute cerebral blood flow reduction after subarachnoid haemorrhage (Naveri et al. 1994).

Ang IV dose-dependently stimulated plasminogen activator inhibitor-1 (PAI-1) expression in cultured bovine aortic endothelial cells (Kerins et al. 1995) and Ang IV at 1 μM increased PAI-1 mRNA and protein levels in human coronary artery endothelial cells (Mehta et al. 2002). The actions of Ang IV on the plasm-

nogen activator system were inhibited by divalinal Ang IV, indicating an AT₄ receptor-mediated effect. In porcine pulmonary aortic endothelial cells, Ang IV stimulated nitric oxide release (Hill-Kapturczak et al. 1999) and increased activity of endothelial cell nitric oxide synthase (Patel et al. 1998). Ang IV also mobilised intracellular calcium and increased release from intracellular stores in porcine pulmonary endothelial cells (Patel et al. 1999; Chen et al. 2000). These findings suggest that the Ang IV-mediated vasorelaxation results from mobilisation of calcium and release of nitric oxide from the endothelium.

5.2

Heart

The AT₄ receptor has been characterised in guinea-pig and rabbit heart membranes (Hanesworth et al. 1993) and in rabbit cardiac fibroblasts (Wang et al. 1995b) where Ang IV stimulates DNA and RNA synthesis (Wang et al. 1995a). This contrasts with an earlier report that Ang IV (at 1 μ M) inhibited the effect of Ang II to increase protein synthesis in chick myocytes whilst having no effect alone (Baker and Aceto 1990). In the isolated rabbit heart preparation, Nle¹-Ang IV (100 pM) was reported to modulate left ventricular systolic function by reducing pressure-generating capability and increasing the sensitivity of pressure development to volume change. The AT₄ receptor agonist also reduced left ventricular ejection capability and accelerated relaxation (Slinker et al. 1999). Treatment with 100 pM Nle¹-Ang IV also decreased *c-fos* and *egr-1* mRNA expression in the mechanically loaded, isolated rabbit heart (Yang et al. 1997).

5.3

Renal

In the anaesthetised rat, intra-renal arterial infusion of Ang IV or Nle¹-Ang IV (0.1–100 pmol/min) produced a dose-dependent increase in renal cortical blood flow without altering systemic blood pressure (Coleman et al. 1998). Increased renal cortical blood flow and increased sodium excretion following intra-renal arterial infusion of Ang IV occurred with no change in mean arterial pressure, glomerular filtration rate or urinary volume (Hamilton et al. 2001). These effects were blocked by pretreatment with divalinal Ang IV (Coleman et al. 1998; Hamilton et al. 2001). In contrast, another study in the anaesthetised rats reported no vasodilation after intra-renal infusion of Ang IV (10, 100 and 1,000 pmol/min) or LVV-haemorphin-7 (10, 100) (Fitzgerald et al. 1999).

In view of the abundance of AT₄ receptors in renal proximal tubules (Handa et al. 1998), the effects of Ang IV have been investigated in a number of proximal tubular epithelial cell lines. In isolated rat proximal tubules, Ang IV dose-dependently (0.1 pM–1 μ M) decreased transcellular Na⁺ transport as measured by proximal tubule oxygen consumption rates (Handa et al. 1998). The effect of 1 pM Ang IV on Na⁺ transport was mimicked by 1 pM Nle¹-Ang IV and blocked by 1 μ M divalinal Ang IV (Handa et al. 1998). In the HK2 human immortalised

proximal tubular epithelial cells, Ang IV stimulated PAI-1 expression (Gesualdo et al. 1999). In the same cell line, Ang IV induced a concentration-dependent increase in intracellular sodium and activation of extracellular signal regulated kinase (ERK)-2 and p38 (Handa 2001). The intracellular calcium response to Ang IV was biphasic, increasing from 10^{-10} to 10^{-8} then decreasing at 10^{-7} and 10^{-6} M (Handa 2001). Surprisingly, divalinal Ang IV also increased intracellular sodium and calcium levels and ERK-2 and p38 phosphorylation (Handa 2001). Ang IV also dose-dependently stimulated tyrosine phosphorylation of p125-focal adhesion kinase and p68-paxillin in the LLC-PK1/Cl4 porcine proximal tubule epithelial cells (Chen et al. 2001).

In the distal tubular-like epithelial cells, the Mardin-Darby bovine kidney (MDBK) epithelial cell line Ang IV (0.1 and 10 nM) increased intracellular calcium concentration (Handa et al. 1999), a response which was no longer evident at 1 μ M Ang IV. Interestingly, in this cell line, as in the HK-2 cells, divalinal Ang IV also produced the same effect as Ang IV (Handa et al. 1999).

Angiotensin AT₄ receptors have also been detected in a human collecting duct cell line, where 0.1 μ M Ang IV stimulated adenosine monophosphate (cAMP) production in the presence of forskolin (Czekalski et al. 1996).

6 Biochemical Properties of the AT₄ Receptor

The biochemical properties of the AT₄ receptor have been characterised in bovine adrenal and aortic endothelial cells using photosensitive analogues of Ang IV (Bernier et al. 1998; Zhang et al. 1998). In bovine aortic endothelial cells, the AT₄ receptor was found to be a glycoprotein of 186 kDa, with N-linked carbohydrate moieties which accounted for 30% of the mass of the receptor (Bernier et al. 1998). Mild trypsin treatment of intact endothelial cell membranes produced the release of a high molecular weight fragment of the AT₄ receptor of approximately 177 kDa into the supernatant, suggesting that the receptor comprises a large extracellular domain (Bernier et al. 1998). A smaller protein was reported in the bovine adrenal membranes with a molecular weight of 165 kDa containing approximately 20% N-linked carbohydrates (Zhang et al. 1998). The receptor occurred as a dimer, with the ligand-binding subunit of 165 kDa and a smaller subunit of approximately 70 kDa (Zhang et al. 1998).

Structural analysis of the AT₄ receptor in different bovine tissues suggests the presence of a brain-specific isoform of the receptor. In contrast to the AT₄ receptor in other peripheral tissues, the receptor in the hippocampus was smaller (150 kDa) and did not occur as a dimer (Zhang et al. 1999). The difference in size between AT₄ receptor from the hippocampal and the other sources could be attributed to differential glycosylation of the proteins. There were also minor differences between the hippocampal AT₄ receptor and the receptor from peripheral sources that were uncovered by endopeptidase C fingerprinting, although these were poorly characterised (Zhang et al. 1999).

In both the bovine aortic endothelial and rat neuronal NG108-15 cells, the cell surface AT₄ receptor is rapidly internalised upon binding of [¹²⁵I]Ang IV (Briand et al. 1999; Moeller et al. 1999a) whereas binding of [¹²⁵I]divalinal Ang IV did not result in internalisation of the receptor (Briand et al. 1999).

7

Identification of the AT₄ Receptor

7.1

Purification

In order to identify the AT₄ receptor, our group purified the receptor from bovine adrenal membranes, an abundant source ($B_{\max}=3$ nmol/mg protein) of this receptor (Wright et al. 1995; Zhang et al. 1998). The AT₄ receptor was cross-linked to a photoactivatable analogue of Ang IV (Mustafa et al. 2001) and solubilised. After ion exchange chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), a silver-stained protein band co-migrating with the radioactive band was excised and digested with trypsin. Tryptic peptides were subjected to capillary-column reversed phase-high performance liquid chromatography, coupled to an electrospray ionisation ion trap mass spectrometer for peptide sequencing, as described (Simpson 2000).

Of three peptides identified, one was 95% identical to amino acid sequence of human oxytocinase, the homologue of rat IRAP. IRAP was the most likely candidate since its size (Keller et al. 1995) and tissue distribution (Keller et al. 1995) closely resemble those of the AT₄ receptor, and the enzyme had been demonstrated to bind Ang IV (Herbst et al. 1997).

7.2

Proof of Concept: The AT₄ Receptor Is IRAP

In order to demonstrate that the "AT₄ receptor" is in fact IRAP, HEK 293T cells were transfected with the full-length cDNA for human IRAP (pCI-IRAP), and analysed for the biochemical and pharmacological properties of "AT₄ receptor" (Albiston et al. 2001).

First, in cells transfected with IRAP, the specific binding of [¹²⁵I]-Nle¹-Ang IV was 30- to 40-fold higher than binding to membranes from cells transfected with empty vector. Unlabelled Ang IV and LVV-haemorphin-7 competed for the binding of [¹²⁵I]-Nle¹-Ang IV with IC₅₀ values of 32 nM and 140 nM, respectively, which are in close agreement with the IC₅₀ values for the endogenous "AT₄ receptor" (Mustafa et al. 2001). This indicates that the pharmacological profile of IRAP was identical to that of the AT₄ receptor (Albiston et al. 2001).

Second, membranes from transfected cells were cross-linked with [¹²⁵I]Nle¹-BzPhe⁶-Gly⁷-Ang IV and resolved by SDS-PAGE. In cells transfected with pCI-IRAP, but not with empty vector, a major radiolabelled band of 165 kDa and a minor band of greater than 250 kDa were observed under non-reducing condi-

tions. Thus, the molecular weight of IRAP was identical to that of the AT₄ receptor observed in previous studies (Bernier et al. 1998; Briand et al. 1998; Mustafa et al. 2001). Both bands were absent in the presence of 10 μ M unlabelled Ang IV, confirming the specific interaction of the photoactivatable Ang IV analogue with the "AT₄ receptor" expressed from the IRAP cDNA (Albiston et al. 2001).

Third, we compared the distribution of "AT₄ receptors", detected by ¹²⁵I-Nle¹-Ang IV binding, with the distribution of IRAP mRNA and IRAP immunoreactivity in the mouse brain (Albiston et al. 2001). In line with previous studies (Miller-Wing et al. 1993; Moeller et al. 1996a; Chai et al. 2000), ¹²⁵I-Nle¹-Ang IV binding sites were widely distributed and occurred in high abundance in the medial septum, in the pyramidal cell layer of CA1 to CA3 region of the hippocampus, and throughout the neocortex in the mouse brain. High levels of binding were also found in brain regions involved in motor control, such as the cerebellum. Using in situ hybridisation histochemistry and immunohistochemistry, the distribution of IRAP mRNA and immunoreactivity closely paralleled

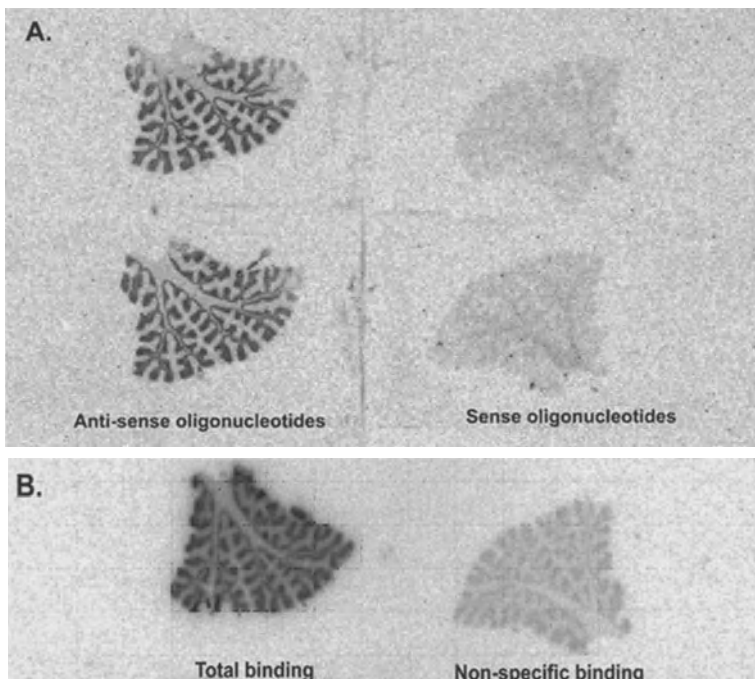


Fig. 2A, B Parallel distribution of [¹²⁵I]Nle-Ang IV binding and IRAP gene expression in human cerebellar cortex. **A** Distribution of IRAP mRNA in human cerebellar sections determined by in situ hybridisation histochemistry, using antisense and sense oligonucleotide sequences derived from the human IRAP cDNA sequence. The antisense sequences used include nucleotides 1136–1109, nucleotides 1407–1377 and nucleotides 2587–2560 of the human IRAP sequence (Human Genbank Acc.U62768). **B** The adjacent sections of the cerebellum were labelled with [¹²⁵I]Nle-Ang IV as previously described. (Chai et al. 2000)

$^{125}\text{I-Nle}^1\text{-Ang IV}$ binding in the mouse brain (Albiston et al. 2001). Specifically, IRAP mRNA and protein was localised in regions such as the medial septum, hippocampus, neocortex and cerebellum. In the CA1–CA3 region of the hippocampus, IRAP-positive immunoreactive cells co-localised with NeuN staining in pyramidal neurons, but not in glial cells, as revealed by glial fibrillary acidic protein (GFAP) staining (Albiston et al. 2001). We also find a parallel distribution of IRAP mRNA and $^{125}\text{I-Nle}^1\text{-Ang IV}$ binding in the human brain (Fig. 2).

8 Insulin-Regulated Aminopeptidase

8.1 Biochemical Properties of IRAP

IRAP is a 916 amino acid protein with a calculated molecular mass of 104,575 Da. The predicted 105-kDa molecular mass for IRAP is less than the apparent 165-kDa size of the protein on SDS-PAGE, which is due to glycosylation of the protein. IRAP is a type II integral membrane-spanning protein such that the C-terminal domain containing the catalytic site is extracellular followed by a single 22 amino acid potential transmembrane domain, and a short 109 amino acid hydrophilic N-terminal segment, which projects into the cytoplasm (Keller et al. 1995). The N-terminal cytoplasmic domain contains two dileucine motifs, which are preceded by highly acidic regions; these motifs are involved in vesicular trafficking (Fig. 3) (Johnson et al. 2001).

The large extracellular region of IRAP contains the catalytic domain consisting of zinc binding [HEXXH-(18–64X)-E] and exopeptidase (GAMEN) motifs, making it a member of the M1 family of zinc metallopeptidases (Fig. 3) (Laustsen et al. 1997, 2001; Rasmussen et al. 2000). IRAP degrades a range of peptides *in vitro* including oxytocin, arginine vasopressin, Ang II, Ang III, somatostatin, met-enkephalin, dynorphin A (1–8), neurokinin A, neuromedin B and CCK-8 (26–33; Tsujimoto et al. 1992; Herbst et al. 1997; Matsumoto et al. 2000). In hydrolysing fully active hormones into inactive peptides or alternatively processing precursors into active peptides, IRAP may regulate *in vivo* peptide levels and their specific actions.

IRAP was initially cloned from rat adipocytes and skeletal muscle (Keller et al. 1995), where it is found predominantly in vesicles containing the insulin-regulated glucose transporter (GLUT4) (Kandror et al. 1994; Keller et al. 1995). This enzyme is also known as placental leucine amino peptidase (P-LAP), or oxytocinase (OTase), because it was also cloned from a human placental cDNA library as the peptidase involved in the degradation of oxytocin (Rogi et al. 1996; Laustsen et al. 1997). Analysis of the amino acid sequence revealed that human P-LAP/oxytocinase shares 85% identity with the rat IRAP sequence (Keller et al. 1995), indicating that IRAP is the rat homologue of human P-LAP.

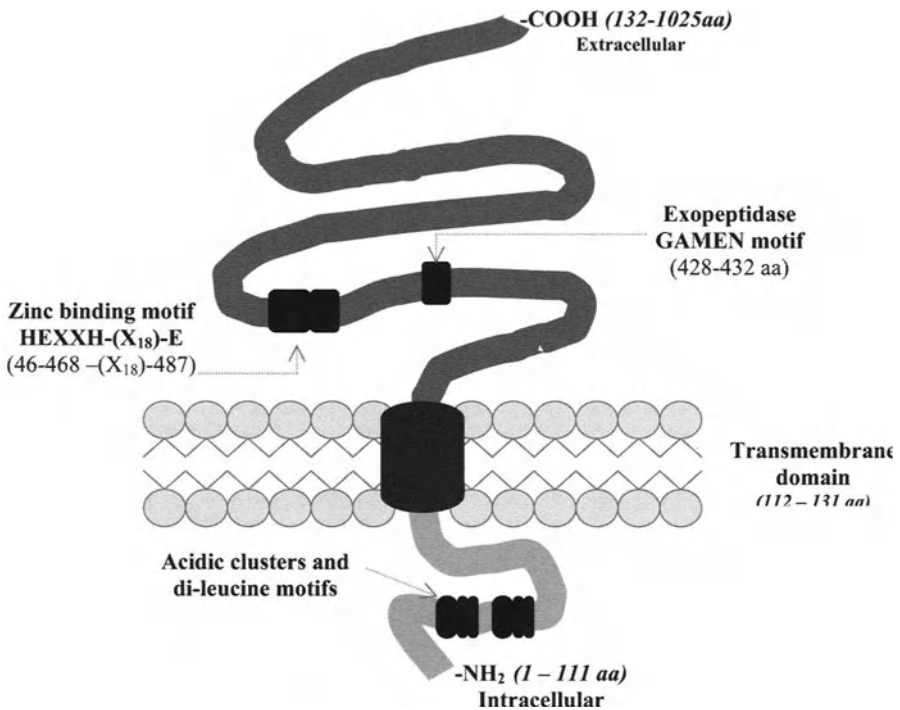


Fig. 3 Schematic diagram representing the structure and plasma membrane topology of IRAP. The large extracellular catalytic domain of IRAP (131–1025 aa) contains the Zn²⁺ binding (HEXXH-E) and exopeptidase motif (GAMEN) that is conserved in all M1 zinc metallopeptidases family members. The short intracellular domain (1–112 aa) contains di-leucine motifs in an acidic region

8.2

Tissue Distribution of IRAP

In addition to its characterisation in classic insulin responsive tissues (adipocytes, skeletal muscle and cardiomyocytes), IRAP has been identified in a range of other tissues. IRAP was first described in syncytiotrophoblasts of the placenta and was predicted to be the primary site of synthesis and release of the enzyme into the maternal circulation during pregnancy (Small and Watkins 1975). Following cloning of IRAP, the enzyme was also detected by both Western and Northern blot analysis in the heart, skeletal muscle, placenta, brain and pancreas (Keller et al. 1995; Rogi et al. 1996; Laustsen et al. 1997). Immunohistochemical localisation of IRAP protein has subsequently been demonstrated not only present in the placenta (Nagasaka et al. 1997) but also in other reproductive tissues such as human umbilical vascular endothelial cells (Nakamura et al. 2000) and endometrial epithelial cells (Toda et al. 2002).

Earlier studies reported immunohistochemical localisation of IRAP in neuronal cells but did not describe the brain region from which the sections were tak-

en (Nagasaka et al. 1997). Later studies extended these findings, localising the enzyme in neurons of the rat cerebral cortex, basal ganglion, and cerebral cortex (Matsumoto et al. 2001). Localisation of IRAP at a subcellular level in neurons revealed granular and punctate binding throughout the cell body and dendritic spines, suggesting localisation of IRAP in cytoplasmic vesicles (Nagasaka et al. 1997; Matsumoto et al. 2001). The rat pheochromocytoma cell line PC12 endogenously expresses a high level of IRAP in the absence of GLUT4.

8.3

Trafficking of GLUT4/IRAP Vesicles

In the basal state, the majority of IRAP and GLUT4 are co-localised intracellularly in specialised post-endosomal vesicles, and also in the trans-golgi network in adipocytes (Kandror 1999; Pessin et al. 1999; Holman and Sandoval 2001; Simpson et al. 2001). During insulin stimulation, both GLUT4 and IRAP rapidly translocate to the cell surface (Kandror and Pilch 1994; Ross et al. 1996) and are inserted into the plasma membrane. This process, in part, resembles synaptic vesicle trafficking and docking in the regulation of neurotransmitter release in the brain. IRAP and GLUT4 are both trafficked by the same specialised insulin-regulated pathway in adipocytes, and a similar trafficking mechanism has also been reported for skeletal muscle (Sumitani et al. 1997; Zhou et al. 2000). Other than insulin, treatment with peptides such as endothelin and oxytocin has also been shown to stimulate the translocation of IRAP in adipocytes and endothelial cells, respectively (Wu-Wong et al. 1999; Nakamura et al. 2000). The sub-cellular localisation of IRAP in non-insulin responsive tissues is poorly defined, as are the factors that may mediate its translocation to the plasma membrane in these cells. The physiological relevance of the translocation of IRAP to the cell surface remains to be elucidated.

8.4

AT₄ Ligands

The aminopeptidase activity of IRAP can be measured by the hydrolysis of a synthetic fluorescent substrate, L-leucine- β -naphthylamide ($K_m=31 \mu\text{M}$). We have used this system to investigate the effect of AT₄ ligands on IRAP catalytic activity. As stated in Sect. 16, transient transfection of HEK293T cells with pCI-IRAP results in a roughly 30- to 40-fold increase of IRAP expression compared to cells transfected with the vector alone, as assessed by [¹²⁵I]Nle¹-Ang IV binding to cell membranes. For evaluation of effects on IRAP catalytic activity, HEK293T-solubilised membranes were incubated with 10 μM of the substrate, L-leucine- β -naphthylamide in the absence and presence of AT₄ ligands (Albiston et al. 2001). Addition of increasing concentrations (0.1–10 μM) of the AT₄ receptor ligands Ang IV and LVV-H7 resulted in a marked dose-dependent decrease in catalytic activity. These findings indicate that AT₄ receptor ligands bind with high affinity to IRAP and dose-dependently inhibit catalytic activity.

To determine whether the AT₄ ligands are potential substrates of IRAP, Ang IV was incubated with solubilised, crude membranes from HEK293T-transfected cells and peptide degradation determined by HPLC separation with on-line mass spectrometric detector analysis. There was no difference in the degradation profile between membranes from IRAP-transfected and vector-only-transfected cells, indicating that Ang IV is not a substrate for IRAP (R.A. Lew et al., unpublished results).

We therefore propose that AT₄ ligands may, at least in part, mediate their effects by acting as inhibitors of IRAP. The AT₄ ligands are structurally similar to a number of substrates for IRAP, being small peptides of less than 10 amino acids in length and with at least two aromatic residues. Therefore, it is likely that the AT₄ ligands are substrate analogue inhibitors of IRAP, binding at the catalytic site. If this is the case, we would predict that all AT₄ ligands are competitive inhibitors of IRAP.

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