CHAPTER 12

LOCAL ANGIOTENSIN GENERATION AND AT₂ RECEPTOR ACTIVATION

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1. INTRODUCTION

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and body fluid homeostasis. Traditionally, the RAS has been viewed as a circulating system (“circulating” RAS). However, it is now well-established that angiotensin (Ang) generation also occurs at tissue sites (“tissue” RAS). The complexity of the system has increased even further now that we know that Ang II activates more than one receptor, that Ang II has metabolites which activate their own receptors, and that there may even be receptors for renin and prorenin. This review summarizes the latest insights on tissue angiotensin generation and focuses in particular on the activation of the Ang II type 2 (AT₂) receptor by locally generated Ang II.

2. THE RENIN-ANGIOTENSIN SYSTEM

2.1. Renin, Prorenin and (Pro)Renin Receptors

Renin belongs to the family of aspartyl proteases and has only one known substrate, angiotensinogen, the precursor of all angiotensin peptides. Structure analysis revealed that renin consists of 2 homologous lobes which form a cleft containing the active site. Renin has an inactive precursor, prorenin, in which the active site is covered by the prosegment.

The renin gene was cloned in the 1980s in human, rat and mouse. Most species have one renin gene (ren-1a), although some mouse strains have two renin genes, ren-1b and a submandibular variant, designated as ren-2. The ren-2 gene is encoding for a nonglycosylated prorenin, as opposed to the ren-1 gene which can be glycosylated at three asparagine residues. The renin gene is located on chromosome 1 in human and mouse, whereas it is localized on chromosome 13 in rat.
The renin gene encodes for pre-prorenin consisting of a presegment of 23 amino acids, a prosegment of 43 amino acids and the actual renin protein of 340 amino acids (Morris 1992). The presegment functions as a signal peptide directing prorenin to the secretory pathway. Recently, a splice-variant of the renin gene was discovered which lacks the signal peptide and part of the prosegment. This truncated prorenin displays enzymatic activity because the truncated prosegment only partially covers the enzymatic cleft. It is thought to remain intracellular (Clausmeyer et al 2000), although truncated prorenin has also been demonstrated extracellularly (Shinagawa et al 1992).

Mice lacking the ren-1d gene are characterized by sexually dimorphic hypotension (leading to a significant reduction of blood pressure in female mice), absence of dense secretory/storage granule formation in juxta-glomerular cells, altered morphology of the kidney, and a significant increase of plasma prorenin levels (Clark et al 1997). Deletion of the ren-2 gene resulted in increased renin and decreased prorenin levels (Sharp et al 1996), but no changes in blood pressure, nor morphological changes occurred.

Transgenic mice overexpressing human renin did not develop hypertension whereas transgenic mice expressing both human renin and human angiotensinogen showed a significantly increased blood pressure (Fukamizu et al 1993). The plasma concentrations of Ang I and Ang II were 3-5-fold increased in double transgenic mice as compared to either control mice or transgenic mice overexpressing human renin. These results demonstrate that human renin does not crossreact with mouse angiotensinogen, thereby illustrating the unique species specificity of the RAS.

Prorenin can be activated through cleavage of the prosegment (proteolytic activation) or via a conformational change induced by low pH or low temperature (non-proteolytic activation) (Danser and Deinum 2005) (Fig. 1). Proteolytic activation is an irreversible process in which the prosegment is cleaved, e.g., by kallikrein, trypsin or plasmin. In vivo, proteolytic activation is probably mediated by a proconvertase in the renin-producing cells of the juxta-glomerular apparatus of the kidney. Non-proteolytic activation of prorenin is a reversible process in which prorenin is converted from the ‘closed’ (inactive) to the ‘open’ (active) conformation by unfolding of the prosegment from the enzymatic cleft (Suzuki et al 2003). Acid activation leads to complete activation of prorenin whereas exposure to cold (‘cryoactivation’) only leads to partial activation (~15%). Kinetic studies have shown that an equilibrium exists between the closed and open conformations of prorenin, and that under physiological conditions (pH 7.4, 37°C) <2% of prorenin is in the open conformation (Danser and Deinum 2005).

The kidneys are the main source of circulating (pro)renin. However, following a bilateral nephrectomy, prorenin, in contrast with renin, remains detectable. This suggests that prorenin is also produced outside the kidney. Potential extrarenal prorenin-producing tissues are the eye, adrenal, ovary and testis (Sealey et al 1988; Danser et al 1989; Itskovitz et al 1992; Clausmeyer et al 2000). Normally, the concentration of prorenin in human plasma is 10 times higher than that of renin. The reasons for this excess are unknown, as prorenin does not seem to be activated
Figure 1. Proteolytic and non-proteolytic activation of prorenin. Aog, angiotensinogen; Ang, angiotensin. See text for explanation

outside the kidney (Lenz et al 1990). One possibility is that prorenin has functions unrelated to angiotensin generation. In this regard, it is of interest to note that it has recently been suggested that prorenin binds to a ‘(pro)renin receptor’, thereby activating second messenger pathways in a manner that is independent of Ang II (Nguyen et al 2002; Saris et al 2006). (Pro)renin receptors may also mediate the uptake of renin and/or prorenin into tissues that do not synthesize renin and prorenin themselves, like the heart and the vessel wall.

To date, two (pro)renin-binding receptors have been identified: the mannose-6-phosphate (M6P) receptor (Saris et al 2001) and the above-mentioned (pro)renin receptor. The M6P receptor is identical to the insulin-like growth factor II (IGFII) receptor and binds IGFII, M6P-containing proteins such as prorenin and renin, and retinoic acid at distinct sites (Kornfeld 1992; Kang et al 1997). Prorenin and renin are both rapidly internalized after binding to this receptor, and internalized prorenin is proteolytically converted to renin. However, binding to this receptor did not result in angiotensin generation, either intra- or extracellularly. This, in combination with the fact that intracellularly generated renin was found to be degraded within a few hours, suggests that M6P/IGFII receptors function as clearance receptors for (pro)renin. Alternatively, since binding of M6P-containing proteins to M6P/IGFII receptors results in the activation of second messenger pathways involving G-proteins (Di Bacco and Gill 2003), (pro)renin may act as an M6P/IGFII receptor agonist.

The (pro)renin receptor was cloned by Nguyen and co-workers (Nguyen et al 2002). Prorenin and renin bind equally well to this receptor, without being internalized or degraded. Interestingly, the catalytic activity of bound renin was increased
5-fold, and receptor-bound prorenin became fully active in a non-proteolytic manner. Thus, apparently, this receptor allows prorenin to generate angiotensins at tissue sites. Importantly, binding of (pro)renin to the (pro)renin receptor in human mesangial cells also induced Ang II-independent effects, such as an increase in DNA synthesis, activation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK)1 (p44)/ERK2 (p42), and plasminogen-activator inhibitor-1 release. Furthermore, in cardiomyocytes, prorenin activated the p38 MAPK/heat shock protein 27 pathway, resulting in changes of actin filament dynamics (Saris et al 2006). These non-angiotensin-mediated effects may underlie the blood pressure-independent cardiac hypertrophy in rats with a hepatic prorenin overexpression (Véniant et al 1996).

Finally, Peters and co-workers demonstrated ren-2 prorenin internalization in cardiomyocytes of transgenic rats expressing the mouse ren-2 gene in the liver (Peters et al 2002). Since ren-2 prorenin is nonglycosylated, this phenomenon cannot be mediated by M6P/IGFII receptors. The internalization contrasts with the observations on the recently cloned (pro)renin receptor. Thus, there may be a third (pro)renin receptor, the identity of which is currently unclear.

2.2. Angiotensinogen

Angiotensinogen, the precursor of all angiotensin metabolites, is the only known substrate for renin. The angiotensinogen gene encodes for a glycoprotein of 453 amino acids with a molecular weight of ~60 kDa. The gene is located as a single copy on, respectively, chromosome 19 in rats, chromosome 8 in mice and chromosome 1 in humans. In 1983, Doolittle reported a significant sequence homology of angiotensinogen to α₁-antitrypsin (23%), ovalbumin (21%) and antithrombin III (18%) (Doolittle 1983). These proteins are members of the serine proteinase inhibitor family and are closely associated with acute inflammation reactions. Acute inflammation induces gene expression via acute-response which increases the angiotensinogen concentration in plasma (Kageyama et al 1985). The similarity between the structural organization of the angiotensinogen and α₁-antitrypsin genes suggests that both genes have evolved from a common ancestor (Kitamura et al 1987).

Although angiotensinogen mRNA has been detected in brain, adipocytes, heart and the reproductive system, its main source is the liver (Paul et al 2006). Hepatocytes constitutively secrete angiotensinogen into the extracellular fluid, without intracellular storage. Blood plasma/extracellular fluid functions as the major reservoir for angiotensinogen. Angiotensinogen plasma concentrations (~1 μM) approximate the Michaelis-Menten constant of the renin reaction, which makes RAS activity sensitive to small changes in angiotensinogen concentration. Deletion of the angiotensinogen gene in mice leads to hypotension, low body weight gain after birth, and an abnormal morphology of kidney and heart (Niimura et al 1995). In turn, overexpression of angiotensinogen led to the development of hypertension (Kimura et al 1992).
2.3. ACE and ACE2

Two isoforms of ACE exist: somatic ACE and testis (germinal) ACE. Somatic ACE is abundantly expressed throughout the body, whereas testis ACE is exclusively expressed in the testis. Cloning of the ACE gene provided a better understanding of the relationship between somatic and testis ACE. Both forms are transcribed from the same gene by using different promoters (Hubert et al 1991). In humans the ACE gene is located on chromosome 17. Somatic ACE has 2 homologous domains which share 60% sequence homology. Both domains contain a catalytically active site (Wei et al 1991a) and are situated at the N- and C-terminal side of ACE. According to their position they are designated as N- and C-domain. The majority of somatic ACE is membrane-bound on endothelial cells. Circulating ACE is derived from ACE-expressing cells by proteolytic cleavage at the juxta-membrane stalk region (Wei et al 1991b). Testis ACE possesses only one catalytic domain which is identical to the C-domain of somatic ACE. Studies selectively blocking the C- and N-domain of somatic ACE revealed that conversion of Ang I to Ang II by membrane-bound ACE depends on the C-domain, whereas both domains contribute to this conversion in soluble ACE (van Esch et al 2005). Degradation of bradykinin at tissue sites also required both domains (Tom et al 2001). Deletion of both somatic and testis ACE (ACE−/−) in mice led to hypotension, male infertility and changes in kidney morphology (Esther et al 1996). Vascular expression of germinal ACE in Ace null mice restored renal morphology but did not normalize blood pressure, thus demonstrating that germinal ACE cannot functionally substitute for somatic ACE (Kessler et al 2007).

Recently, a homologue of somatic ACE called ACE2 was discovered (Donoghue et al 2000). ACE2 shares 42% homology with the C- and N-terminal domains of somatic ACE. The gene encoding ACE2 is located on the X chromosome and ACE2 is mainly expressed in endothelial cells of heart, kidney and testis. Like somatic ACE, ACE2 can be released into the circulation after proteolytic cleavage (Turner and Hooper 2002). Unlike somatic ACE, ACE2 has only one catalytically active site which can convert Ang I and Ang II to Ang (1-9) and Ang (1-7), respectively (Donoghue et al 2000; Vickers et al 2002). These data suggest a potential role of ACE2 in the counterregulation of high blood pressure by inactivation of Ang II. Indeed, in a model of Ang II-dependent hypertension, blood pressures were substantially higher in ACE2-deficient mice than in wildtype controls (Gurley et al 2006). Mice lacking the ACE2 gene were originally described to develop an abnormal heart function with severely impaired contractility (Crackower et al 2002), but this was not confirmed in a follow-up study (Gurley et al 2006). Remarkably, ACE2 also functions as a receptor for the virus causing severe acute respiratory syndrome, thereby stressing the importance of ACE2 in a manner unrelated to the RAS (Li et al 2003).

2.4. Angiotensin II Receptors

Initially, it was thought that the responses to Ang II were mediated by a single Ang II receptor. At the end of the 1980s, the discovery of specific Ang II receptor
ligands such as losartan, PD12377, PD123319 and CGP42112 made it possible to identify several Ang II receptor subtypes. We now know that the biological actions of Ang II in man are mediated by at least two types of Ang II receptors: Ang II type 1 (AT₁) and AT₂ receptors (Fig. 3).

2.4.1. AT₁ receptor

AT₁ receptors mediate virtually all the known physiological actions of Ang II, such as vasoconstriction, inotropy, chronotropy, aldosterone release, noradrenaline release and growth stimulation. The AT₁ receptor gene encodes for a protein of 359 amino acids with a molecular weight of 41 kDa. The gene was first cloned in 1991 from rat vascular smooth muscle cells (Murphy et al. 1991) and bovine adrenal gland (Sasaki et al. 1991). Cloning and genetic analysis of the human AT₁ receptor gene revealed that the human AT₁ receptor gene is located on chromosome 3 and can produce two isoforms by alternative splicing. Both isoforms have similar binding - and functional properties.

In rodents two subtypes of the AT₁ receptor have been identified: AT₁A and AT₁B (Elton et al. 1992). The origin of these subtypes lies in a gene duplication which occurred after the divergence of rodents from the human/artiodactyls group about 24 million years ago. AT₁A and AT₁B share 94% sequence homology and are located on chromosome 17 and 2 in rat and chromosome 13 and 3 in mice, respectively. Not surprisingly, both subtypes have similar ligand binding affinities and signal transduction properties but varying expression levels in different tissues. The AT₁A receptor predominates in heart, kidney, lung, liver and vascular smooth muscle, whereas the AT₁B receptor is mainly expressed in the adrenal and pituitary gland (Burson et al. 1994). To date, there are no pharmacological antagonists which clearly discriminate AT₁A and AT₁B receptors.

Studies in mice using targeted gene manipulation provided more insight in the functional role of both subtypes in vivo. Deletion of the AT₁A receptor gene significantly decreased resting blood pressure in both heterozygous AT₁A⁺⁻ and homozygous AT₁A⁻⁻ receptor mice (Ito et al. 1995). Ang II infusions resulted in a diminished pressor response in AT₁A⁺⁻ receptor mutants whereas this response was virtually abolished in AT₁A⁻⁻ mutants. Additionally, both the expression levels of renin mRNA and plasma renin activity were markedly increased in AT₁A receptor knockout mice (Sugaya et al. 1995). Deletion of the AT₁B receptor gene did not affect resting blood pressure, nor altered the pressure response to Ang II (Chen et al. 1997). Taken together, these findings indicate the important role of the AT₁A receptor in mediating the pressure response in mice. AT₁A or AT₁B receptor deficiency is not associated with an impaired development or survival, but double knockout mice lacking both receptors display a phenotype similar to that observed in angiotensinogen knockout mice (Tsuchida et al. 1998). These observations, together with the fact that Ang II does cause a pressor response in AT₁A knockout mice after enalapril pretreatment (Oliverio et al. 1997), suggest a compensatory role for the AT₁B receptor. Additionally, in vitro studies demonstrated that the AT₁B receptor
is the most important regulator of Ang II contractile responses in the mouse aorta
and femoral artery (Zhou et al 2003).

The AT$_1$ receptor belongs to the seven-transmembrane G-protein-coupled
receptor superfamily, and couples to a wide variety of second messenger systems,
including the phospholipase C/inositol-1,4,5-triphosphate/diacylglycerol/protein
kinase C pathway, the phospholipase A$_2$/arachidonic acid pathway, the phospho-
lipase D/phosphatidylcholine/phosphatidic acid pathway, and tyrosine kinases such
as the MAP kinases ERK1/2, p38 and c-jun N-terminal kinase (Mehta and
Griendling 2007).

AT$_1$ receptor stimulation results in a rapid internalization of the Ang II-AT$_1$
receptor complex, followed by either receptor degradation in lysosomes or receptor
recycling to the cell surface (Mehta and Griendling 2007). Internalized Ang II has
been proposed to activate cytoplasmic or nuclear receptors prior to its intracel-
lular degradation (Thomas et al 1996). Furthermore, Zou and co-workers recently
demonstrated that mechanical stretch resulted in AT$_1$ receptor activation in a ligand-
-independent manner. Interestingly, the consequences of such activation could be
prevented by an AT$_1$ receptor blocker (Zou et al 2004).

Several reports have described crosstalk between AT$_1$ receptor and other
receptors, e.g. the bradykinin type 2 (B$_2$) receptor, the AT$_2$ receptor, and the $\alpha_1$–
adrenoceptor. AT$_1$ and B$_2$ receptors form stable heterodimers with an enhanced
G-protein activation and altered receptor sequestration (AbdAlla et al 2000).
AT$_1$ receptor-$\alpha_1$-adrenoceptor crosstalk enhances the response to $\alpha_1$-adrenoceptor
agonists (Purdy and Weber 1988). Interestingly, although the postjunctional AT$_1$
receptor interacting with the $\alpha_1$-adrenoceptor is of the AT$_{1A}$ subtype, the prejunc-
tional AT$_1$ receptor which facilitates noradrenaline release from sympathetic nerve
endings is of the AT$_{1B}$ subtype (Guimaraes and Pinheiro 2005).

2.4.2. AT$_2$ receptor

In contrast to the well-characterized AT$_1$ receptor, the function of the AT$_2$ receptor
is much less understood. In general, it is assumed that AT$_2$ receptors counteract
the responses mediated by the AT$_1$ receptor (Hein et al 1995; Ichiki et al 1995;
AbdAlla et al 2001; Schuijt et al 2001; Batenburg et al 2004). AT$_2$ receptors are
involved in physiological processes like development and tissue remodeling (by
inhibiting cell growth and by stimulating apoptosis), regulation of blood pressure
(vasodilatation), natriuresis and neuronal activity.

Evidence for AT$_2$ receptor mediated vasodilatation is largely based on two
approaches: an indirect approach, showing an enhanced response to Ang II in the
presence of AT$_2$ receptor blockade or gene disruption (Hein et al 1995; Ichiki et al
1995; Batenburg et al 2004; van Esch et al 2006), and a direct approach showing
AT$_2$ receptor-induced responses by applying either the (partial) AT$_2$ receptor agonist
CGP42112A or Ang II in the presence of an AT$_1$ receptor blocker (Widdop et al
2002; Li and Widdop 2004).

The AT$_2$ receptor gene was first cloned in 1993 (Mukoyama et al 1993). The AT$_2$
receptor gene shares 34% sequence homology with its AT$_1$ receptor counterpart
and encodes for a protein of 363 amino acids with a molecular mass of 41 kDa. It is located on the X chromosome in both humans and rodents. In fetal tissues the AT$_2$ receptor is the predominant subtype. This situation changes rapidly after birth, resulting in the AT$_1$ receptor becoming the dominant subtype in most adult tissues (Widdop et al. 2003). Yet, in adults, AT$_2$ receptors can still be detected in a variety of tissues, including uterus, ovary, adrenal medulla, heart, blood vessels and brain (Bottari et al. 1993). Here it is important to consider that the distribution of the AT$_2$ receptor depends on age and species, but is also subject to changes in expression during pregnancy and pathological conditions such as hypertension, heart failure and vascular injury (see below) (Bottari et al. 1993; de Gasparo et al. 2000).

In 1995, two groups reported that deletion of the AT$_2$ receptor in mice led to an increased pressor response to Ang II (Hein et al. 1995; Ichiki et al. 1995). Additionally, Ichiki et al. reported a significant increased blood pressure in hemizygous AT$_2^{-/Y}$ receptor mice whereas blood pressure was not significantly increased in a similar model described by Hein and co-workers. Mutants lacking the AT$_2$ receptor gene showed a lower body temperature and impaired exploratory behavior. Remarkably, despite its wide expression in the fetus, the AT$_2$ receptor does not seem to be required for embryonic development, as no morphological and developmental differences were found between homozygous AT$_2^{-/-}$ or hemizygous AT$_2^{-/Y}$ receptor mice and their wildtype controls. Possibly, AT$_2$ receptor knockout mice display a delayed expression of calponin and h-caldesmon after birth (Yamada et al. 1999). During pregnancy, Ang II levels are elevated. Because the fetus is also exposed to these high Ang II levels, it has been postulated that the AT$_2$ receptor plays a role in the regulation of Ang II responsiveness in order to prevent fetal hypertension (Perlegas et al. 2005).

Like AT$_1$ receptors, AT$_2$ receptors belong to the G protein-coupled receptor superfamily. However, in contrast to the AT$_1$ receptor, the AT$_2$ receptor is not internalized upon binding of Ang II (Widdop et al. 2003). Two major pathways have been described for AT$_2$ receptor signaling (Nouet and Nahmias 2000): (a) activation of protein phosphatases causing protein dephosphorylation and (b) activation of the nitric oxide (NO)/guanosine cyclic 3', 5'-monophosphate (cGMP) pathway. Up to now, three specific phosphatases have been linked to AT$_2$ receptor activation: MAPK phosphatase 1, SH2-domain-containing phosphatase 1 and protein phosphatase 2A. Growth factors, including Ang II via the AT$_1$ receptor, mediate their growth promoting actions through tyrosine kinase receptors and several kinase-driven phosphorylation steps. Activation of the AT$_2$ receptor counteracts these growth-promoting actions by dephosphorylation through subsequent activation of phosphatases. In addition to the inhibitory effect on growth, dephosphorylation (e.g., of ERK1/2) also seems to play an important role in the stimulation of apoptosis (Horiuchi et al. 1998).

Several studies have shown that AT$_2$ receptor-mediated vasodilation is an endothelium-dependent phenomenon involving B$_2$ receptors, NO and cGMP (Wiemer et al. 1993; Siragy and Carey 1997) (Fig. 2). Initially, in vitro studies using endothelial cells showed that the stimulatory effect of Ang II on cGMP production,
Figure 2. $AT_2$ receptor-mediated relaxation involves either intracellular activation of kininogenase and subsequent bradykinin type 2 ($B_2$) receptor activation, or a direct activation of NO synthase (NOS).

As both $AT_2$ and $B_2$ receptors are co-expressed in various tissues, the hypothesis was raised that both receptors form heterodimers which can interact through receptor crosstalk. Recent studies in rat pheochromocytoma cells, applying fluorescence resonance energy transfer, confirmed this hypothesis (Abadir et al 2006). Heterodimer formation appeared to be dependent on the receptor number that was expressed, but also required $AT_2$ receptor stimulation. As a consequence of heterodimer formation, it is possible that $AT_2$ receptor activation results in $B_2$ receptor activation without intermediate bradykinin synthesis (Batenburg et al 2004).

In addition to its interaction with the $B_2$ receptor, $AT_2$ receptors are also known to interact with their $AT_1$ counterpart. Transfection studies in fetal fibroblasts showed...
that AT\textsubscript{1} and AT\textsubscript{2} receptors form heterodimers in which the AT\textsubscript{2} receptor functions as a specific AT\textsubscript{1} receptor antagonist (AbdAlla \textit{et al.} 2001). Possibly, AT\textsubscript{2} receptor-induced vasodilation depends on simultaneous AT\textsubscript{1} receptor activation, as no AT\textsubscript{2} receptor-mediated responses were noted in the absence of AT\textsubscript{1} receptors (van Esch \textit{et al.} 2006).

Furthermore, it is important to consider that data obtained in absence of the AT\textsubscript{2} receptor are complex because AT\textsubscript{2} receptors downregulate AT\textsubscript{1} receptors in a ligand-independent manner (Jin \textit{et al.} 2002) and AT\textsubscript{2} receptor knockout mice display an increased AT\textsubscript{1} receptor expression (Tanaka \textit{et al.} 1999). In addition to its interaction with AT\textsubscript{1} receptors, the AT\textsubscript{2} receptor also downregulates renin biosynthesis, thereby inhibiting the formation of Ang II (Siragy \textit{et al.} 2005).

2.5. Angiotensin-Derived Metabolites and Their Receptors

Ang I and II are metabolized by a whole range of peptidases (‘angiotensinases’). Although initially it was thought that all metabolites other than Ang II were inactive, it is now clear that at least several of these metabolites have functions of their own, which are sometimes mediated via non-AT\textsubscript{1}/AT\textsubscript{2} receptors. The most important of these peptides are Ang (1-7), Ang (2-8) (Ang III) and Ang (3-8) (Ang IV) (Fig. 3).

Ang (1-7) can be formed from Ang I by the action of neutral endopeptidase or prolyl endopeptidase but also from the Ang I degradation products Ang (1-9) and Ang II (Vickers \textit{et al.} 2002). Ang (1-7) is generally believed to counteract the response of Ang II although there are reports of similar or distinct actions from Ang II (Santos \textit{et al.} 2000). Ang (1-7) induces relaxation in several vascular beds. The fact that this relaxation could be blocked by the selective Ang (1-7) antagonist A-779 [D-Ala\textsubscript{7}-Ang (1-7)] suggested the involvement of a specific Ang (1-7) receptor (Santos \textit{et al.} 2000). Indeed, in 2003 the Mas proto-oncogene, a G protein-coupled receptor, was proposed to be the receptor for Ang (1-7) (Santos \textit{et al.} 2003). Ang (1-7) potentiates bradykinin-induced responses (Tom \textit{et al.} 2001) and releases NO (Brosnihan \textit{et al.} 1996) via Mas receptor stimulation. Mas receptor mRNA expression has been detected in heart, testis, kidney and brain (Metzger \textit{et al.} 1995). Mice deficient for the Mas-receptor lack the antidiuretic action of Ang (1-7) after an acute water load, and their aortas no longer relax in response to Ang (1-7) (Santos \textit{et al.} 2003). Mas\textsuperscript{−/−} mice are also characterized by an impaired heart function, indicating an important role of the Mas receptor in the maintenance of the structure and function of the heart (Santos \textit{et al.} 2006).

Although the Mas-receptor is now held responsible for most of the responses to Ang (1-7), there are several other pharmacological mechanisms and receptors that are affected by Ang (1-7). As a slow substrate for ACE, Ang (1-7) may also function as an ACE inhibitor, resulting in decreased Ang II formation and potentiation of bradykinin-induced vasodilatation (Tom \textit{et al.} 2001). Furthermore, Ang (1-7) acts as an AT\textsubscript{1} receptor antagonist at low concentrations (Stegbauer \textit{et al.} 2003), and exerts AT\textsubscript{1} receptor agonistic effects at high concentrations (van Rodijnen \textit{et al.} 2002). A link between Ang (1-7) and the AT\textsubscript{2} receptor has recently been
Figure 3. Schematical overview of the generation of angiotensin I and its metabolites. NEP, neutral endopeptidase; PEP, prolyl endopeptidase; PCP, prolyl carboxypeptidase; APA, aminopeptidase A; APN, aminopeptidase N

proposed, because infusion of Ang (1-7) during AT\textsubscript{1} receptor blockade unmasked a vasodepressor response in conscious SHR rats that could be attenuated by blockade of AT\textsubscript{2} receptors, B\textsubscript{2} receptors and NOS (Walters et al 2005). Possibly, Mas-AT\textsubscript{1} and/or Mas-AT\textsubscript{2} receptor heterodimers exist (Castro et al 2005; Lemos et al 2005).

Through the action of aminopeptidase A, Ang II is converted to Ang III, which in turn can be converted to Ang IV by aminopeptidase N (Ardaillou and Chansel 1997). Ang III mediates some of the classical responses of Ang II (such as stimulation of aldosterone secretion and vasoconstriction) and this most likely involves binding to AT\textsubscript{1} and AT\textsubscript{2} receptors. The affinity of Ang III for these receptors is somewhat lower than that of Ang II (Wright and Harding 1995). The responses to Ang III are less efficacious than those of Ang II, possibly due to its accelerated metabolism in the circulation. The latter relates to the wide distribution of aminopeptidase N that initiates the hydrolysis of Ang III but not Ang II. It is thought that Ang III might be the final mediator of some of the actions of Ang II. For example, the central action of Ang II on vasopressin secretion in rats is dependent on Ang III, as this effect was absent after specific blockade of aminopeptidase A (Zini et al 1996).
Additionally, Ang III, and not Ang II, mediates the excretion of Na\(^+\) excretion through AT\(_2\) receptors in the presence of AT\(_1\) receptor blockade (Padia et al 2006).

Ang IV was initially believed to have no biological activity. This was based on two important findings: both AT\(_1\) and AT\(_2\) receptors display a poor affinity for Ang IV, and Ang IV does not elicit the characteristic Ang II responses like Ang III. The discovery of a specific Ang IV binding site, designated as the AT\(_4\) receptor, changed this view (Swanson et al 1992). After purification, the receptor was identified as insulin-regulated aminopeptidase (Albiston et al 2001), a protein which is abundantly found in vesicles containing the insulin-sensitive glucose transporter (GLUT4) (Keller et al 1995). AT\(_4\) receptor expression occurs in brain, spinal cord, heart, kidney, colon, prostate, adrenal gland, bladder and vascular smooth muscle cells (Wright and Harding 1995; de Gasparo et al 2000). Ang IV and the AT\(_4\) receptor appear to be involved in the facilitation of memory and learning (Wright et al 1999). Ang IV infusions cause vasorelaxation in cerebral and renal vascular beds, possibly by increasing NOS activity (Patel et al 1998). On the other hand, there are also studies showing that Ang IV, because of its weak agonistic activity towards the AT\(_1\) receptor, induces vasoconstriction (van Rodijnen et al 2002). The close association of the AT\(_4\) receptor with GLUT4 suggests that Ang IV might modulate glucose uptake.

3. TISSUE ANGIOTENSIN GENERATION

As soon as it was realized that angiotensin production at tissue sites is of greater importance than angiotensin generation in the circulation, many investigators started to unravel how and where such local angiotensin production might occur. Initially, it was thought that all components required for local Ang II production (i.e., renin, angiotensinogen and ACE) would be produced at tissue sites. Infusions of radiolabeled angiotensins, allowing the quantification of uptake of blood-derived angiotensin in tissues, confirmed that the majority of tissue Ang I and II is produced at tissue sites, and not derived from blood (Schuijt and Danser 2002). ACE is well-known to be abundantly expressed in virtually every tissue of the body, its main site being the surface of endothelial cells. Thus, its local synthesis is beyond doubt. Although angiotensinogen mRNA has been detected outside the liver, direct proof for actual angiotensinogen synthesis at important sites of local angiotensin production (e.g., heart and vessel wall) is lacking. For instance, the isolated perfused heart does not release angiotensinogen (de Lannoy et al 1997). Therefore, the majority of tissue angiotensinogen is probably of hepatic origin. The fact that angiotensinogen is neither internalized, nor binds to membranes, combined with the observation that angiotensinogen-synthesizing cells release angiotensinogen to the extracellular space (Klett et al 1993), rather than storing it intracellularly, indicates that angiotensin generation must occurs extracellularly. Thus, tissue angiotensin generation is restricted to the interstitial space and/or the cell surface (Fig. 4).
Figure 4. Model of angiotensin generation at cardiac tissue sites

Following a bilateral nephrectomy, tissue renin and angiotensin levels drop to levels at or below the detection limit (Campbell et al. 1993; Danser et al. 1994; Katz et al. 1997). This suggests that the majority of tissue renin is not locally produced, but kidney-derived, and that without renin, there is no angiotensin production. The presence of renin in cardiac membrane fractions (Danser et al. 1994) suggested that circulating renin, in addition to its diffusion into the interstitial space (Katz et al. 1997; van den Eijnden et al. 2002), may bind to renin-binding proteins or receptors at tissue sites. The recent discovery of several of such receptors, as discussed above, supports this concept. An interesting additional observation is that these receptors also bind prorenin, and that prorenin, upon binding, becomes catalytically active. In view of the much higher prorenin than renin levels, an attractive concept is that prorenin rather than renin contributes to tissue angiotensin generation. Studies with (pro)renin receptor blockers in diabetic rats confirmed this concept (Ichihara et al. 2004). Unexpectedly however, these blockers did not affect tissue angiotensin levels in control rats, although the prorenin levels of the latter rats were only ≈2-fold lower than those of the diabetic rats. Moreover, despite the fact that prorenin is still present in circulating blood after a nephrectomy (Danser et al. 1994), tissue angiotensin levels are close to zero. This suggests that, if prorenin contributes to tissue angiotensin production, this involves prorenin of renal rather than extrarenal origin. Currently, the only known difference between renal and extrarenal prorenin relates to their degree of glycosylation.

In vitro studies using the isolated perfused rat Langendorff heart fully confirmed the idea of renin and angiotensinogen uptake underlying tissue angiotensin production. During buffer perfusion, no release of RAS components could be
demonstrated in the coronary effluent or interstitial fluid (de Lannoy et al 1997). After adding renin to the perfusion fluid, renin started to accumulate in the interstitial fluid, reaching steady-state levels in this compartment that were identical to its levels in the coronary circulation. Findings on angiotensinogen were similar. Stopping the exposure to renin revealed a biphasic washout curve, in agreement with the concept that renin is not only present in extracellular fluid but also binds to receptors. Angiotensinogen washout was mono-phasic. Angiotensin synthesis only occurred during simultaneous perfusion with renin and angiotensinogen. Interestingly, in hearts of transgenic rats overexpressing angiotensinogen, angiotensin release continued after stopping the renin perfusion, i.e., when renin was no longer present in the coronary circulation (Müller et al 1998). This was due to the fact that receptor-bound renin continued to generate Ang I.

At steady state, the cardiac tissue levels of Ang I were as high as expected assuming that Ang I is restricted to the extracellular fluid (de Lannoy et al 1998; Schuijt et al 1999). In contrast, the tissue Ang II levels were much higher. Pretreatment with an AT$_1$ receptor antagonist greatly reduced the cardiac tissue Ang II levels during renin + angiotensinogen perfusion. This suggests that locally generated Ang II accumulates at tissue sites through binding to AT$_1$ receptors. Subsequent subcellular fractionation studies confirmed that tissue Ang II, but not Ang I, is located intracellularly (Schuijt et al 1999; van Kats et al 2001). This is due to the fact that AT$_1$ receptor-bound Ang II is rapidly internalized, after which intracellular degradation occurs. Based on these observations, it is not surprising that the tissue Ang II content correlates directly with tissue AT$_1$ receptor density (van Kats et al 1997).

A wide range of in vitro studies has provided evidence for the existence of enzymes other than renin and ACE generating Ang I and II, including cathepsin D, kallikrein, tonin and chymase (Hackenthal et al 1978; Urata et al 1990). The in vivo importance of these alternative pathways is questionable. The fact that Ang I and II are virtually absent in plasma and tissue of nephrectomized animals (including humans) argue against a role of non-renin angiotensinogen-converting enzymes in vivo. A similar situation exists for chymase which is present in the cardiac interstitium, mast cells and endothelial cells. In vitro studies have provided evidence for an important role of chymase in the conversion of Ang I to Ang II (Urata et al 1990; Tom et al 2003), but in vivo evidence for chymase-dependent Ang II generation could not be obtained (Saris et al 2000). Moreover, angiotensinogen and ACE knockout mice have similar phenotypes (Tanimoto et al 1994; Krege et al 1995), and ACE deletion reduced the Ang II levels in both tissue and circulation by up to 99% (Campbell et al 2004). Thus, at least in mice, ACE is the main, if not only Ang II-generating enzyme in vivo.

4. AT$_2$ RECEPTORS AND PATHOPHYSIOLOGY

As discussed above, AT$_2$ receptor expression is low or undetectable in adult tissues, in contrast with its high expression in fetal tissues. However, AT$_2$ receptors reappear under pathophysiological conditions.
For instance, in the kidney, AT$_2$ receptor expression increases when inflammation, apoptosis, and proteinuria occur (Ruiz-Ortega et al 2003). Interestingly, transgenic AT$_2$ receptor-overexpressing mice displayed less glomerular injury, proteinuria and transforming growth factor β expression in a subtotal nephrectomy model (Hashimoto et al 2004). This suggests that the re-appearance of AT$_2$ receptors under pathological conditions is part of a protective mechanism, for instance related to enhanced NO production (Hiyoshi et al 2005). However, not all studies confirm the counterregulatory, protective actions of AT$_2$ receptors in the kidney. Duke and co-workers report that AT$_2$ receptors mediate vasoconstriction in the renal medulla of 2-kidney, 1-clip rats, as opposed to the vasodilator effects mediated by AT$_1$ receptors in this model (Duke et al 2005).

In the heart, a wide range of animal studies revealed increased AT$_2$ receptor expression under pathological conditions, e.g. during pressure overload, hypertension and ischemia, and post-myocardial infarction (Wiener et al 1993; Wu et al 1994; Schuijt et al 2001; Yayama et al 2004). Studies in failing human hearts confirmed the animal data, and simultaneously showed a downregulation of AT$_1$ receptors (Asano et al 1997; Wharton et al 1998). From studies with AT$_1$ receptor antagonists it is widely accepted that AT$_1$ receptors play a major role in the post-myocardial remodeling process, mediating both fibrosis and hypertrophy (Schieffer et al 1994). Since the beneficial effects of AT$_1$ receptor blockade following myocardial infarction were diminished in AT$^{-/-}$Y receptor mice (Xu et al 2002), it is reasonable to assume that the increased Ang II levels that will occur during AT$_1$ receptor blockade (see below) exert beneficial effects via AT$_2$ receptor stimulation. Indeed, transgenic mice overexpressing AT$_2$ receptors in the heart displayed improved cardiac hemodynamics post-myocardial infarction in an NO-dependent manner (Yang et al 2002; Bove et al 2004). Furthermore, treatment with either an AT$_2$ receptor antagonist or a B$_2$ receptor antagonist reduced the beneficial effects of AT$_1$ receptor blockade in wildtype mice following myocardial infarction (Liu et al 2002). Therefore, the beneficial effects of AT$_2$ receptors in the heart involve the B$_2$ receptor/NO/cGMP pathway.

In contrast with these observations, a few studies have shown that AT$_2$ receptors, like AT$_1$ receptors, induce cardiac hypertrophy and fibrosis (Senbonmatsu et al 2000; Ichihara et al 2001). To explain these discrepant data, it has been hypothesized that AT$_2$ receptor upregulation is beneficial in the early pathological phase, by counteracting hypertrophy and fibrosis, but that chronic stimulation of the AT$_2$ receptor (for instance by the high Ang II levels that will occur during AT$_1$ receptor blockade) has deleterious effects on cardiac recovery (Schneider and Lorell 2001).

Knowledge on the effects of AT$_2$ receptors in the human heart comes from polymorphism studies, although the data are often contradictory. AT$_2$ receptor gene variants have been linked to both cardiac hypertrophy and coronary ischemia (Schmieder et al 2001; Herrmann et al 2002; Alfakih et al 2005), without knowing however whether this is based on increased or decreased AT$_2$ receptor density. AT$_2$ receptor-mediated vasodilation in isolated human coronary microarteries increases with age (Batenburg et al 2004). Since endothelial function decreases with age, this could point to increased AT$_2$ receptor expression in the face of decreased
endothelial function, again in agreement with the concept that AT$_2$ receptor density increases under pathological conditions. AT$_2$ receptor expression also increased in peripheral resistance arteries of hypertensive diabetic patients during treatment with an AT$_1$ receptor blocker, and this resulted in enhanced Ang II-induced vasodilation (Savoia et al 2007).

Recent studies have shown that AT$_2$ receptors are also expressed in various carcinomas (Deshayes and Nahmias 2005). Assuming that AT$_1$ receptors contribute to tumor growth and vascularization (Fujita et al 2002), one may predict that, here too, AT$_2$ receptors will counteract the effects of the AT$_1$ receptor stimulation, thus inhibiting growth and vascularization (Silvestre et al 2002). However, proangiogenic effects of AT$_2$ receptors have also been described, occurring in conjunction with AT$_1$ receptor activation (Walther et al 2003).

5. RAS BLOCKADE AND AT$_2$ RECEPTOR STIMULATION

Blocking the RAS is possible at three levels: renin, ACE and the AT receptors. Beta-adrenoceptor blockers, by antagonizing the renin-releasing $\beta_1$-adrenoceptors in the juxta-glomerular cells, were the first drugs to suppress the RAS. These drugs will lower renin (Campbell et al 2001), Ang I and Ang II, thereby reducing the degree of AT$_1$ and AT$_2$ receptor stimulation (Table 1).

Subsequently, the ACE inhibitors were introduced. These drugs will lower Ang II. Given the wide variety of available angiotensinases, this will not lead to substantial Ang I accumulation, but rather result in metabolism of Ang I through different (compensatory) pathways, e.g. by neutral endopeptidase. As a consequence, Ang-(1-7) levels will rise during ACE inhibition, thereby allowing Ang-(1-7) to contribute to the beneficial effects of ACE inhibitors (Tom et al 2001). Simultaneously, due to the interference with Ang II generation, the negative feedback loop system regulating renin release is affected, and thus, the kidneys will release more renin. Therefore, depending on the degree of ACE inhibition, Ang II levels may rise again, sometimes to levels above baseline (Campbell et al 1993; van Kats et al 2000). For instance, at 90% ACE inhibition, a 10-fold rise in renin is sufficient to fully restore Ang II levels, whereas a 20-fold rise in renin would increase Ang II twofold above its baseline levels. In addition, prolonged ACE inhibition is known to upregulate ACE. Given these compensatory mechanisms, it

| Renin blockers | Ang formation | Receptor stimulation |
|---------------|--------------|----------------------|
| Beta blocker  | ↓            | ↓                    | ↓        |
| Renin inhibitor | ↑          | ↓                    | ↓        |
| ACE inhibitor  | ↑            | ↑                    | ↓        |
| AT$_1$ receptor blocker | ↑ | ↑ | ↓ | ↓ |
is not surprising that it has proven difficult to show that blood plasma and tissue Ang II levels remain suppressed during continuous ACE inhibition (van Kats et al 2000).

Indeed, in pigs treated with captopril for 3 weeks post-myocardial infarction, cardiac Ang II levels were increased as compared to untreated control pigs (Fig. 5). Although this Ang II may theoretically stimulate AT$_1$ and AT$_2$ receptors, it must be kept in mind that such receptor stimulation may occur less efficiently than normal. Without ACE inhibitor treatment, ACE generates Ang II in a highly efficient manner, in close proximity of AT receptors. During chronic ACE inhibition, the increase in Ang I generation will still allow Ang II generation, either by non-inhibited ACE or by non-ACE converting enzymes like chymase (van Kats et al 2005). However, this type of Ang II generation is less efficient, because it does not result in a high level of regional AT receptor stimulation. In particular, Ang II generated by chymase (which is localized in the adventitia) will be subject to rapid metabolism in the interstitial space on its way to AT receptors (Schuijt et al 1999; de Lannoy et al 2001) and thus is less likely to result in a high regional AT receptor occupancy. Therefore, a low overall AT receptor occupancy will occur, below the minimum per cell required to induce an effect.

AT$_1$ receptor blockers, available since the early 1990s, will also cause a rise in renin. Ang I and II in blood and tissues (as well as their metabolites) will increase in parallel with renin, and although this will not result in AT$_1$ receptor stimulation, non-AT$_1$ receptors (including AT$_2$ receptors and Mas) may now be stimulated excessively. As discussed above, it is feasible that, at least part of the beneficial effect of AT$_1$ receptor blockers is due to such AT$_2$ receptor stimulation (Widdop et al 2002).

Finally, renin inhibitors will soon be clinically available. These drugs lower both Ang I and II, and evidence for this, at least in blood plasma, is already available (Nussberger et al 2002; Azizi et al 2004). Whether renin inhibitors also decrease tissue Ang I and II levels is not yet known. This relates to the fact

![Figure 5. Plasma and cardiac tissue angiotensin levels in pigs that were either untreated or treated with the ACE inhibitor captopril or the AT$_1$ receptor antagonist eprosartan for 3 weeks after a myocardial infarction. *P<0.05 vs. untreated. Data are derived from (van Kats et al 2000)](image-url)
that renin inhibitors primarily block human renin, and not (or to a much lesser degree) rat, mouse or porcine renin. Thus, renin inhibitors cannot be tested easily in well-established animal models. Theoretically, the decreased Ang I and II levels during renin inhibition will prevent AT\(_1\) and AT\(_2\) receptor stimulation, as well as the stimulation of any other receptor by angiotensin metabolites. Although renin will rise during renin inhibitor treatment (like it does during any RAS blocker treatment), this renin cannot be enzymatically active due to the presence of the renin inhibitor. Thus, renin inhibitors may offer a more complete suppression of the RAS, although this also implies that the putative beneficial effects mediated by AT\(_2\) or Mas receptors will now no longer occur. So far, this does not appear to diminish the effects of renin inhibitors, at least on blood pressure (Gradman et al 2005).

6. CONCLUSIONS

Ang II generated at tissue sites stimulates both AT\(_1\) and AT\(_2\) receptors. This local generation depends largely on angiotensinogen and renin and/or prorenin taken up from blood, the latter uptake possibly involving the recently discovered (pro)renin receptor. ACE is generated locally, and appears to be the main, if not the only, Ang II-generating enzyme. Ang II has a whole range of metabolites, the most important of which are Ang (1-7), Ang III and Ang IV. The enzymes generating these metabolites, including ACE2, have recently been characterized, as well as their putative (non-AT\(_1\)/AT\(_2\)) receptors, like the Mas and AT\(_4\) receptor. Stimulation of AT\(_2\) receptors most likely contributes to the beneficial effect of RAS blockers, in particular during AT\(_1\) receptor antagonism. These receptors are upregulated under pathophysiological conditions, and are generally believed to counteract the effects of AT\(_1\) receptor stimulation. However, not all studies agree on this aspect, and thus it remains to be seen how the effect of drugs that completely suppress the RAS, i.e., renin inhibitors, compare to those that allow/require AT\(_2\) receptor stimulation, like ACE inhibitors and AT\(_1\) receptor antagonists.

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