CHAPTER 12

LOCAL ANGIOTENSIN GENERATION AND AT_2 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 (AT_2) 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-1*^{c}), although some mouse strains have two renin genes, *ren-1*^{d} 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.

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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-1*^{d} 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 specifity 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^{o}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

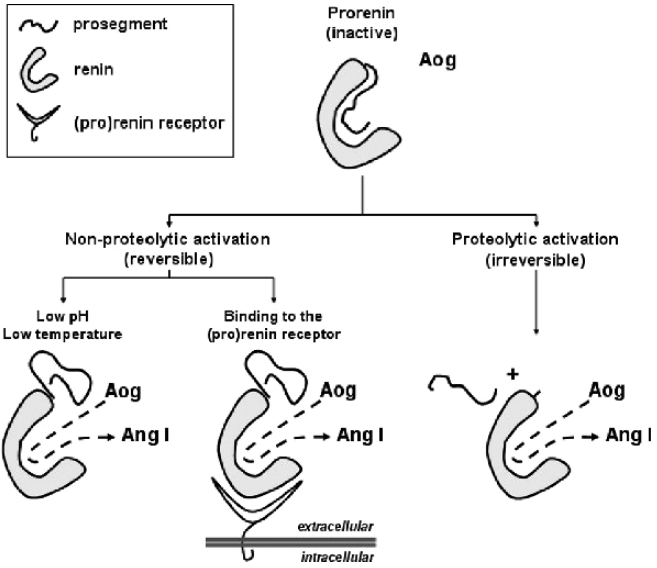

Figure 1. Proteolytic and non-proteolytic activation of prorenin. Aog, angiotensinogen; Ang, angiotensin. See text for explanation

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 α_1-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 α_1-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 (AT_1) and AT_2 receptors (Fig. 3).

2.4.1. AT_1 receptor

AT_1 receptors mediate virtually all the known physiological actions of Ang II, such as vasoconstriction, inotropy, chronotropy, aldosterone release, noradrenaline release and growth stimulation. The AT_1 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 AT_1 receptor gene revealed that the human AT_1 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 AT_1 receptor have been identified: AT_{1A} and AT_{1B} (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. AT_{1A} and AT_{1B} 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 AT_{1A} receptor predominates in heart, kidney, lung, liver and vascular smooth muscle, whereas the AT_{1B} receptor is mainly expressed in the adrenal and pituitary gland (Burson *et al* 1994). To date, there are no pharmacological antagonists which clearly discriminate AT_{1A} and AT_{1B} receptors.

Studies in mice using targeted gene manipulation provided more insight in the functional role of both subtypes *in vivo*. Deletion of the AT_{1A} receptor gene significantly decreased resting blood pressure in both heterozygous ${AT_{1A}}^{+/-}$ and homozygous ${AT_{1A}}^{-/-}$ receptor mice (Ito *et al* 1995). Ang II infusions resulted in a diminished pressor response in ${AT_{1A}}^{+/-}$ receptor mutants whereas this response was virtually abolished in ${AT_{1A}}^{-/-}$ mutants. Additionally, both the expression levels of renin mRNA and plasma renin activity were markedly increased in AT_{1A} receptor knockout mice (Sugaya *et al* 1995). Deletion of the AT_{1B} 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 AT_{1A} receptor in mediating the pressure response in mice. AT_{1A} or AT_{1B} 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 AT_{1A} knockout mice after enalapril pretreatment (Oliverio *et al* 1997), suggest a compensatory role for the AT_{1B} receptor. Additionally, *in vitro* studies demonstrated that the AT_{1B} 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 phospholipase 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 intracellular 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 α_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-α_1-adrenoceptor crosstalk enhances the response to α_1-adrenoceptor agonists (Purdy and Weber 1988). Interestingly, although the postjunctional AT_1 receptor interacting with the α_1–adrenoceptor is of the AT_{1A} subtype, the prejunctional 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,

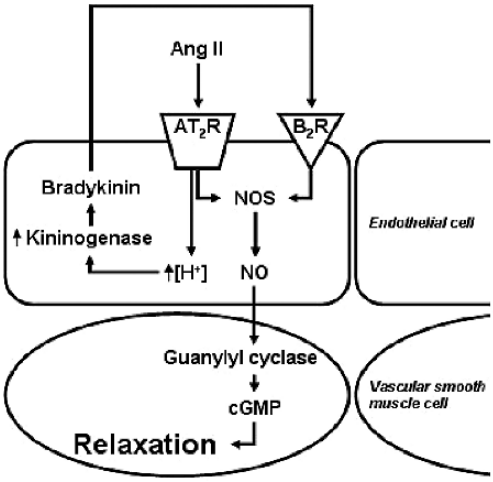

Figure 2. AT_2 receptor-mediated relaxation involves either intracellular activation of kininogenase and subsequent bradykinin type 2 (B_2) receptor activation, or a direct activation of NO synthase (NOS)

a downstream signaling product of NO production, was abolished by blocking both B_2 receptors and nitric oxide synthase (NOS) (Wiemer *et al* 1993). Subsequent *in vivo* studies confirmed that the AT_2 receptor-induced rise in cGMP involves bradykinin and NO (Siragy and Carey 1997). *In vitro* studies in endothelial cells reported that intracellular acidosis, as a result of AT_2 receptor activation, stimulates bradykinin formation by activating kininogenases (Tsutsumi *et al* 1999). Katada and Majima were able to show production of bradykinin after AT_2 activation in rat mesenteric arteries, suggesting that the B_2 receptor mediates vasodilatation by endogenous bradykinin released upon AT_2 receptor activation (Katada and Majima 2002). In agreement with this concept, deletion of the B_2 receptor enhanced the Ang II-induced hypertensive response *in vivo* (Cervenka *et al* 2001). Additional studies concluded that NO production following AT_2 receptor stimulation may also occur independently of B_2 receptors, through direct NOS activation (Abadir *et al* 2003), possibly involving the calcineurin/nuclear factor of activated T cells pathway (Ritter *et al* 2003).

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_1 and AT_2 receptors form heterodimers in which the AT_2 receptor functions as a specific AT_1 receptor antagonist (AbdAlla *et al* 2001). Possibly, AT_2 receptor-induced vasodilatation depends on simultaneous AT_1 receptor activation, as no AT_2 receptor-mediated responses were noted in the absence of AT_1 receptors (van Esch *et al* 2006).

Furthermore, it is important to consider that data obtained in absence of the AT_2 receptor are complex because AT_2 receptors downregulate AT_1 receptors in a ligand-independent manner (Jin *et al* 2002) and AT_2 receptor knockout mice display an increased AT_1 receptor expression (Tanaka *et al* 1999). In addition to its interaction with AT_1 receptors, the AT_2 receptor also downregulates renin biosynthesis, thereby inhibiting the formation of Ang II (Siragy *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_1/AT_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 *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 *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^7-Ang (1-7)] suggested the involvement of a specific Ang (1-7) receptor (Santos *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 *et al* 2003). Ang (1-7) potentiates bradykinin-induced responses (Tom *et al* 2001) and releases NO (Brosnihan *et al* 1996) via Mas receptor stimulation. Mas receptor mRNA expression has been detected in heart, testis, kidney and brain (Metzger *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 *et al* 2003). $Mas^{-/-}$ 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 *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 *et al* 2001). Furthermore, Ang (1-7) acts as an AT_1 receptor antagonist at low concentrations (Stegbauer *et al* 2003), and exerts AT_1 receptor agonistic effects at high concentrations (van Rodijnen *et al* 2002). A link between Ang (1-7) and the AT_2 receptor has recently been

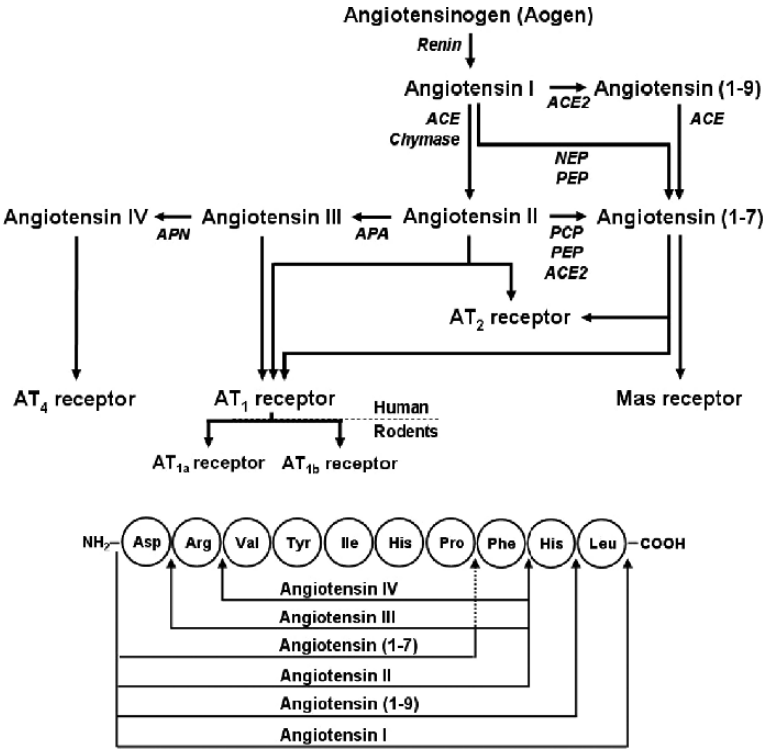

Figure 3. Schematical overview of the generation of angiotensin I and its metabolites. NEP, neutral endopeptidase; PEP, prolyl endopeptidase; PCP, prolyl carboxypeptidase; APA, aminopeptidase A; APN, aminopeptidase N

proposed, because infusion of Ang (1-7) during AT_1 receptor blockade unmasked a vasodepressor response in conscious SHR rats that could be attenuated by blockade of AT_2 receptors, B_2 receptors and NOS (Walters *et al* 2005). Possibly, Mas-AT_1 and/or Mas-AT_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_1 and AT_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).

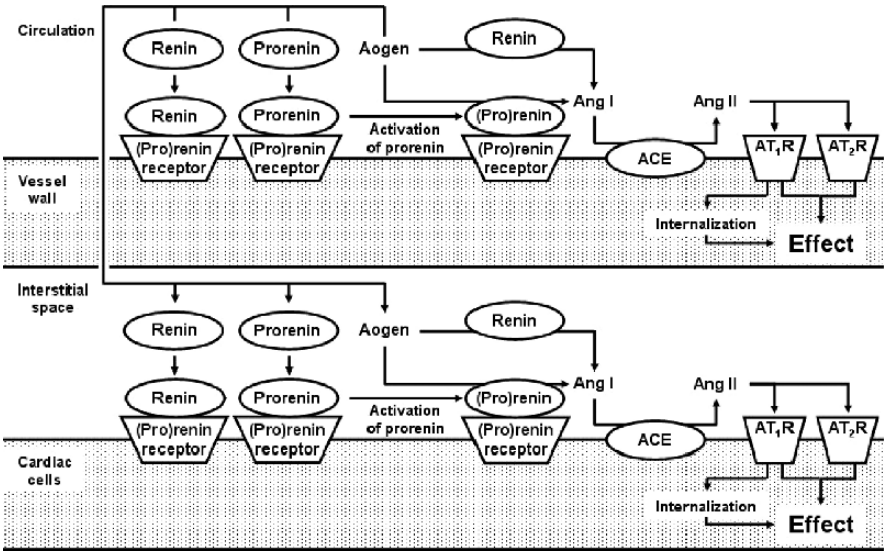

Figure 4. Model of angiotensin generation at cardiac tissue sites

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 (Wiemer *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 inceased 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 β_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

Table 1. Effects of various RAS blockers on renin, angiotensins and AT receptor stimulation

	Renin		Ang formation		Receptor stimulation	
	[Protein]	Activity	[Ang I]	[Ang II]	AT_1	AT_2
β 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

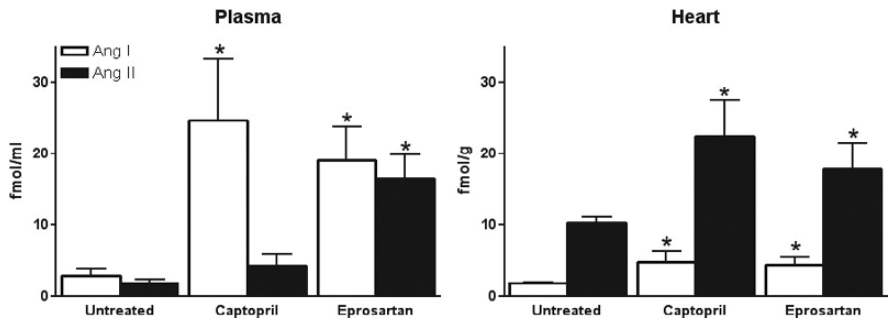

*Figure 5. Plasma and cardiac tissue angiotensin levels in pigs that were either untreated or treated with the ACE inhibitor captopril or the AT_1 receptor antagonist eprosartan for 3 weeks after a myocardial infarction. *P<0.05 vs. untreated. Data are derived from (van Kats et al 2000)*

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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