Unraveling the Complexities of the Renin-Angiotensin System:

From ACE to renin inhibition

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Unraveling the Complexities of the Renin-Angiotensin System:
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Thesis, Erasmus University, Rotterdam. With summary in Dutch
ISBN: 978-90-8559-378-2

Printed by [Optima] Grafische Communicatie, Rotterdam

Unraveling the Complexities of the Renin-Angiotensin System:

From ACE to renin inhibition

Ontrafeling van de complexiteit van het Renine-angiotensine systeem:

Van ACE tot renine remming

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaats vinden op
woensdag 4 juni 2008 om 11.45 uur

door

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geboren te Tilburg

ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

Promotor	:	Prof.dr. A.H.J. Danser
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Part of the research desribed in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-2007B019).

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Financial support by the following companies and foundations is gratefully acknowledged:
J.E. Jurriaanse Stichting
Data Sciences International
Uno Roestvaststaal B.V.
Harlan Netherlands B.V.
Sanofi-Aventis Netherlands B.V.

Voor ons pap en ons mam

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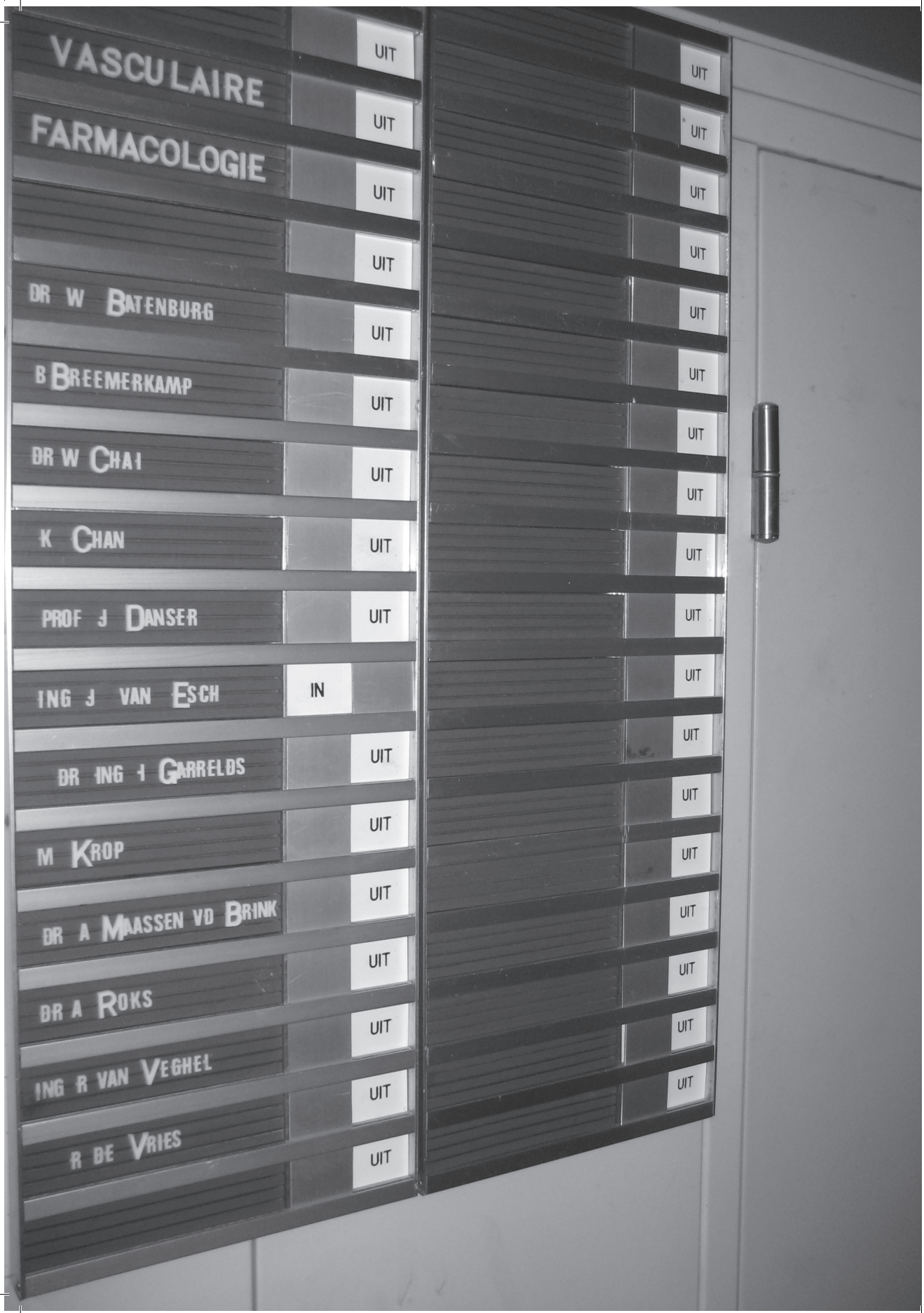
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Chapter 1

General introduction

Based on:
Joep H.M. van Esch, A.H. Jan Danser. Local angiotensin generation and AT_2 receptor activation.

Proteases in biology and disease - Frontiers in research of the RAS on human disease, Leung, P.S. (Ed): 2007, Vol 7, Chapter 12: 246-271.

Abstract

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.

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.

The renin angiotension system

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.

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[2]. 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[3], although truncated prorenin has also been demonstrated extracellularly.[4]

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[5]. Deletion of the ren-2 gene resulted in increased renin and decreased prorenin levels[6], 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.[7] 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.

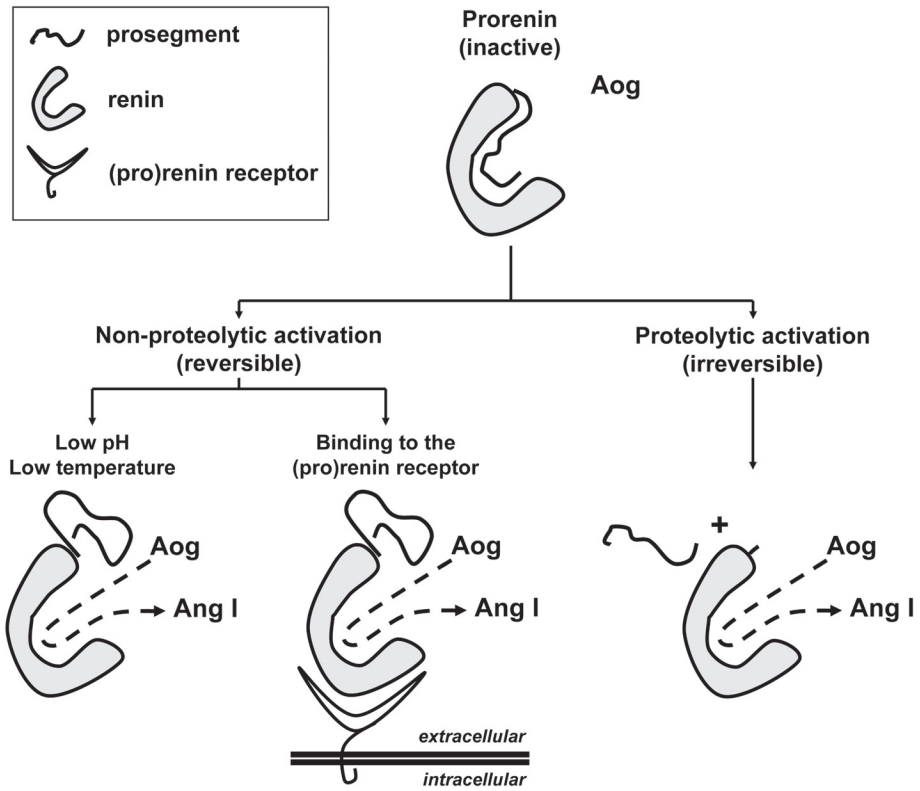


Figure 1. *Proteolytic and non-proteolytic activation of prorenin. Aog, angiotensinogen; Ang, angiotensin. See text for explanation.*

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)[8] (Figure 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.[9] 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.[8]

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.[3,10-12] 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 outside the kidney.[13] 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.[14,15] (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[16] 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.[17,18] 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[19], (pro)renin may act as an M6P/IGFII receptor agonist.

The (pro)renin receptor was cloned by Nguyen and co-workers.[15] 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.[14] These non-angiotensin-mediated effects may underlie the blood pressure-independent cardiac hypertrophy in rats with a hepatic prorenin overexpression.[20]

Finally, Peters and co-workers demonstrated *ren-2* prorenin internalization in cardiomyocytes of transgenic rats expressing the mouse *ren-2* gene in the liver.[21]

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.

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%).[22] 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[23]. The similarity between the structural organization of the angiotensinogen and α_1-antitrypsin genes suggests that both genes have evolved from a common ancestor.[24]

Although angiotensinogen mRNA has been detected in brain, adipocytes, heart and the reproductive system, its main source is the liver.[25] 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.[26] In turn, overexpression of angiotensinogen led to the development of hypertension.[27]

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.[28] 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[29] 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.[30] 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.[31] Degradation of bradykinin at tissue sites also required both domains.[32] Deletion of both somatic and testis ACE ($ACE^{-/-}$) in mice led to hypotension, male infertility and changes in kidney morphology.[33] 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.[34]
Recently, a homologue of somatic ACE called ACE2 was discovered.[35] 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.[36] 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.[35,37] 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.[38] Mice lacking the ACE2 gene were originally described to develop an abnormal heart function with severely impaired contractility[39], but this was not confirmed in a follow-up study.[38] 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.[40]

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 (Figure 3).

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[41] and bovine adrenal gland.[42] 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}.[43] 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.[44] 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.[45] 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.[46] Deletion of the AT_{1B} receptor gene did not affect resting blood pressure, nor altered the pressure response to Ang II.[47] 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.[48] These observations, together with the fact that Ang II does cause a pressor response in AT_{1A} knockout mice after enalapril pretreatment[49], 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.[50]

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 A2/arachidonic acid pathway, the phospholipase D/phosphatidyl-choline/phosphatidic acid pathway, and tyrosine kinases such as the MAP kinases ERK1/2, p38 and c-jun N-terminal kinase.[51]

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.[51] Internalized Ang II has been proposed to activate cytoplasmic or nuclear receptors prior to its intracellular degradation.[52] 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.[53]

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.[54] AT_1 receptor-α_1-adrenoceptor crosstalk enhances the response to α_1-adrenoceptor agonists.[55] 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.[56]

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.[57-61] 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[57,58,60,62], 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.[63,64]

The AT_2 receptor gene was first cloned in 1993.[65] 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.[66] 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.[67] 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).[67,68]

In 1995, two groups reported that deletion of the AT_2 receptor in mice led to an increased pressor response to Ang II.[57,60] 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.[69] 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.[70]

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.[66] Two major pathways have been described for AT_2 receptor signalling[71]: (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.[72]

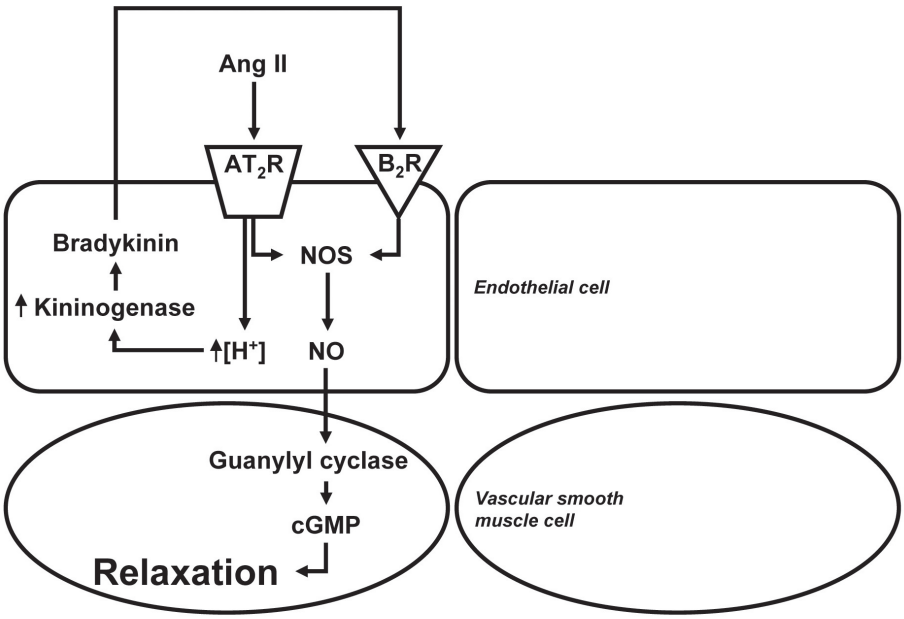


Figure 2. *AT_2 receptor-mediated relaxation involves either intracellular activation of kininogenase and subsequent bradykinin type 2 (B_2) receptor activation, or a direct activation of NO synthase (NOS).*

Several studies have shown that AT_2 receptor-mediated vasodilation is an endothelium-dependent phenomenon involving B2 receptors, NO and cGMP[73,74] (Figure 2). Initially, in vitro studies using endothelial cells showed that the stimulatory effect of Ang II on cGMP production, a downstream signaling product of NO production, was abolished by blocking both B_2 receptors and nitric oxide synthase (NOS).[73] Subsequent in vivo studies confirmed that the AT_2 receptor-induced rise in cGMP involves bradykinin and NO.[74] In vitro studies in endothelial cells reported that intracellular acidosis, as a result of AT_2 receptor activation, stimulates bradykinin formation by activating kininogenases.[75] 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.[76] In agreement with this concept, deletion of the B_2 receptor enhanced the Ang II-induced hypertensive response in vivo.[77] Additional

studies concluded that NO production following AT_2 receptor stimulation may also occur independently of B_2 receptors, through direct NOS activation[78], possibly involving the calcineurin/nuclear factor of activated T cells pathway.[79]
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.[80] 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.[58]
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.[61] 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.[62]
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[81] and AT_2 receptor knockout mice display an increased AT_1 receptor expression.[82] In addition to its interaction with AT_1 receptors, the AT_2 receptor also downregulates renin biosynthesis, thereby inhibiting the formation of Ang II.[83]

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) (Figure 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.[37] 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.[84] 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-Ala7-Ang-(1-7)] suggested the involvement of a specific Ang-(1-7) receptor.[84] Indeed, in 2003 the Mas proto-oncogene, a G protein-coupled receptor, was proposed to be the receptor for Ang-(1-7).[85] Ang-(1-7) potentiates bradykinin-induced responses[32] and releases NO[86] via Mas receptor stimulation. Mas receptor mRNA expression has been detected in heart, testis, kidney and brain.[87] 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).[85] 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.[88] 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[32]. Furthermore, Ang-(1-7) acts as an AT_1 receptor antagonist at low concentrations[89], and exerts AT_1 receptor agonistic effects at high concentrations[90]. A link between Ang-(1-7) and the AT_2 receptor has recently been 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[91]. Possibly, Mas-AT_1 and/or Mas-AT_2 receptor heterodimers exist.[92,93] 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.[94] 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.[95] 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.[96] 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.[97]

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.[98] After purification, the receptor was identified as insulin-regulated aminopeptidase[99], a protein which is abundantly found in vesicles containing the insulin-sensitive glucose transporter (GLUT4).[100] AT_4 receptor expression occurs in brain, spinal cord, heart, kidney, colon, prostate, adrenal gland, bladder and vascular smooth muscle cells.[68,95] Ang IV and the AT_4 receptor appear to be involved in the facilitation of memory and learning.[101] Ang IV infusions cause vasorelaxation in cerebral and renal vascular beds, possibly by increasing NOS activity.[102] 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.[90] The close association of the AT_4 receptor with GLUT4 suggests that Ang IV might modulate glucose uptake.

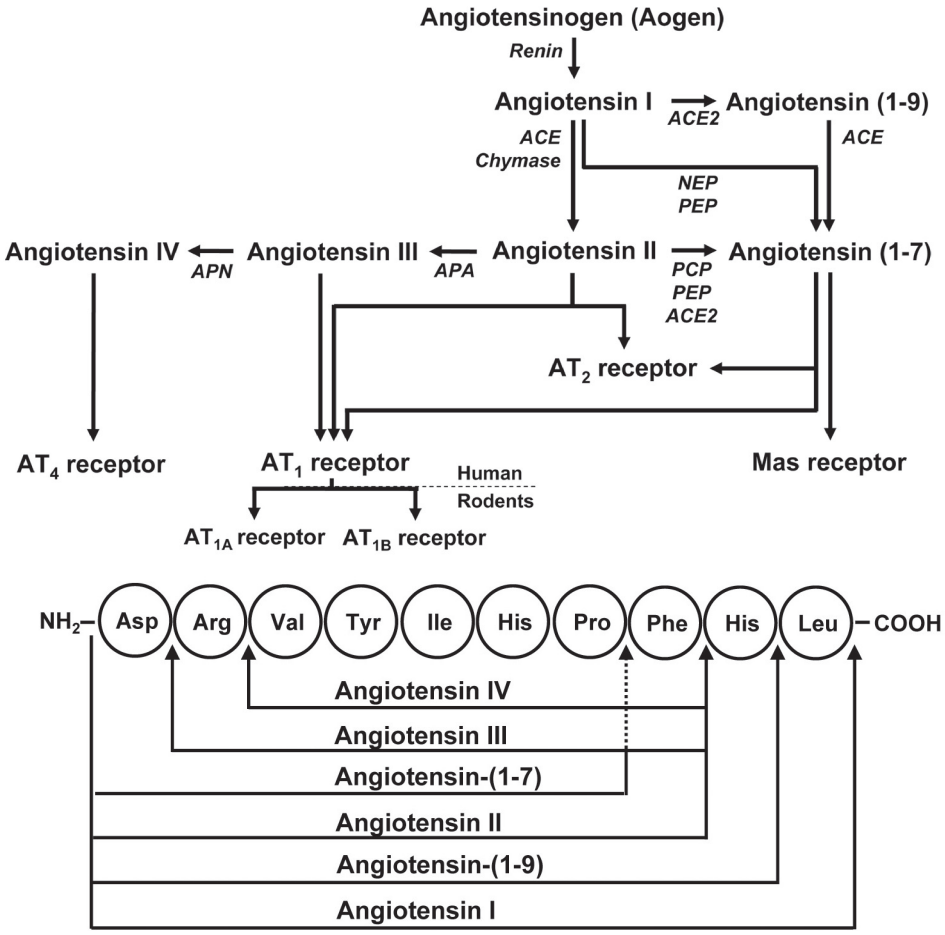


Figure 3. *Schematical overview of the generation of angiotensin I and its metabolites. NEP, neutral endopeptidase; PEP, prolyl endopeptidase; PCP, prolyl carboxypeptidase; APA, aminopeptidase A; APN, aminopeptidase N.*

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.[103]

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.[104] 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[105], 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 (Figure 4).

Following a bilateral nephrectomy, tissue renin and angiotensin levels drop to levels at or below the detection limit.[106-108] 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[106] suggested that circulating renin, in addition to its diffusion into the interstitial space[108,109], 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.[110]

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[106], 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.[104] 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,

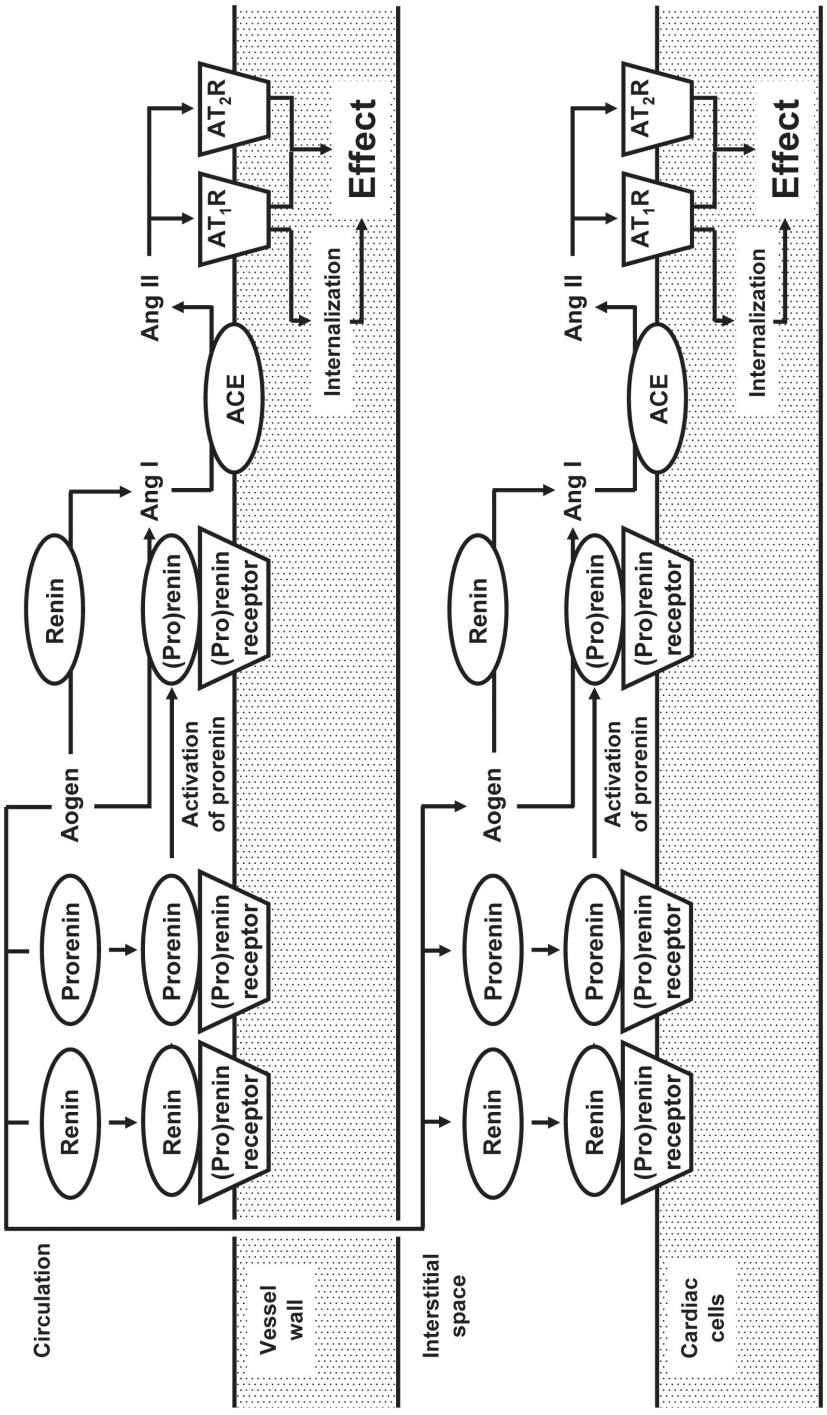


Figure 4. *Model of angiotensin generation at cardiac tissue sites.*

i.e., when renin was no longer present in the coronary circulation.[111] 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.[112,113] 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.[113,114] 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.[115]
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.[116,117] 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[116,118], but in vivo evidence for chymase-dependent Ang II generation could not be obtained.[119] Moreover, angiotensinogen and ACE knockout mice have similar phenotypes[120,121], and ACE deletion reduced the Ang II levels in both tissue and circulation by up to 99%.[122] Thus, at least in mice, ACE is the main, if not only Ang II-generating enzyme in vivo.

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 re-appear under pathophysiological conditions.
For instance, in the kidney, AT_2 receptor expression increases when inflammation, apoptosis, and proteinuria occur.[123] Interestingly, transgenic AT_2 receptor-overexpressing mice displayed less glomerular injury, proteinuria and transforming growth factor β expression in a subtotal nephrectomy model.[124] 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.[125] 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 AT1 receptors in this model.[126]
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.[59,73,127,128] Studies in failing human hearts confirmed the animal data, and simultaneously showed a downregulation of AT_1 receptors.[129,130] 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.[131] Since the beneficial effects of AT_1 receptor blockade following myocardial infarction were diminished in $AT_2^{-/Y}$ receptor mice[132], 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.[133,134] 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.[135] 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.[136,137] 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.[138]
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[139-141], 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.[58] 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.[142]
Recent studies have shown that AT_2 receptors are also expressed in various carcinomas.[143] Assuming that AT_1 receptors contribute to tumor growth and vascularization[144], one may predict that, here too, AT_2 receptors will counteract the effects of the AT_1 receptor stimulation, thus inhibiting growth and vascularisation.[145] However, proangiogenic effects of AT_2 receptors have also been described, occurring in conjunction with AT_1 receptor activation.[146]

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[147], Ang I and Ang II, thereby reducing the degree of AT_1 and AT_2 receptor stimulation (Table 1).

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	↑	↑	↑	↑	↓	↑

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.[32] 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.[1,107] 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 is not surprising that it has proven difficult to show that blood plasma and tissue Ang II levels remain suppressed during continuous ACE inhibition.[1]

Indeed, in pigs treated with captopril for 3 weeks post-myocardial infarction, cardiac Ang II levels were increased as compared to untreated control pigs (Figure 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.[148] 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[113,149] 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.[64]

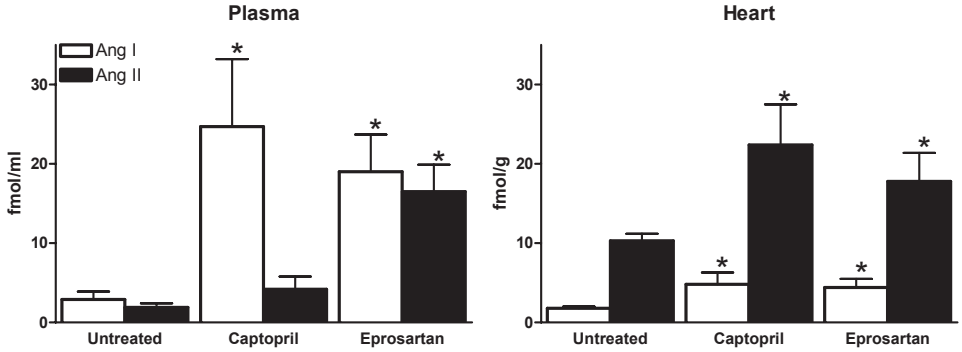


Figure 5. *Plasma and cardiac tissue angiotensin levels in pigs that were either untreated or treated with the ACE inhibitor captopril or the AT_1 receptor antagonist eprosartan for 3 weeks after a myocardial infarction. *P<0.05 vs. untreated. Data are derived from[1].*

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.[150,151] Whether renin inhibitors also decrease tissue Ang I and II levels is not yet known. This relates to the fact 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.[152]

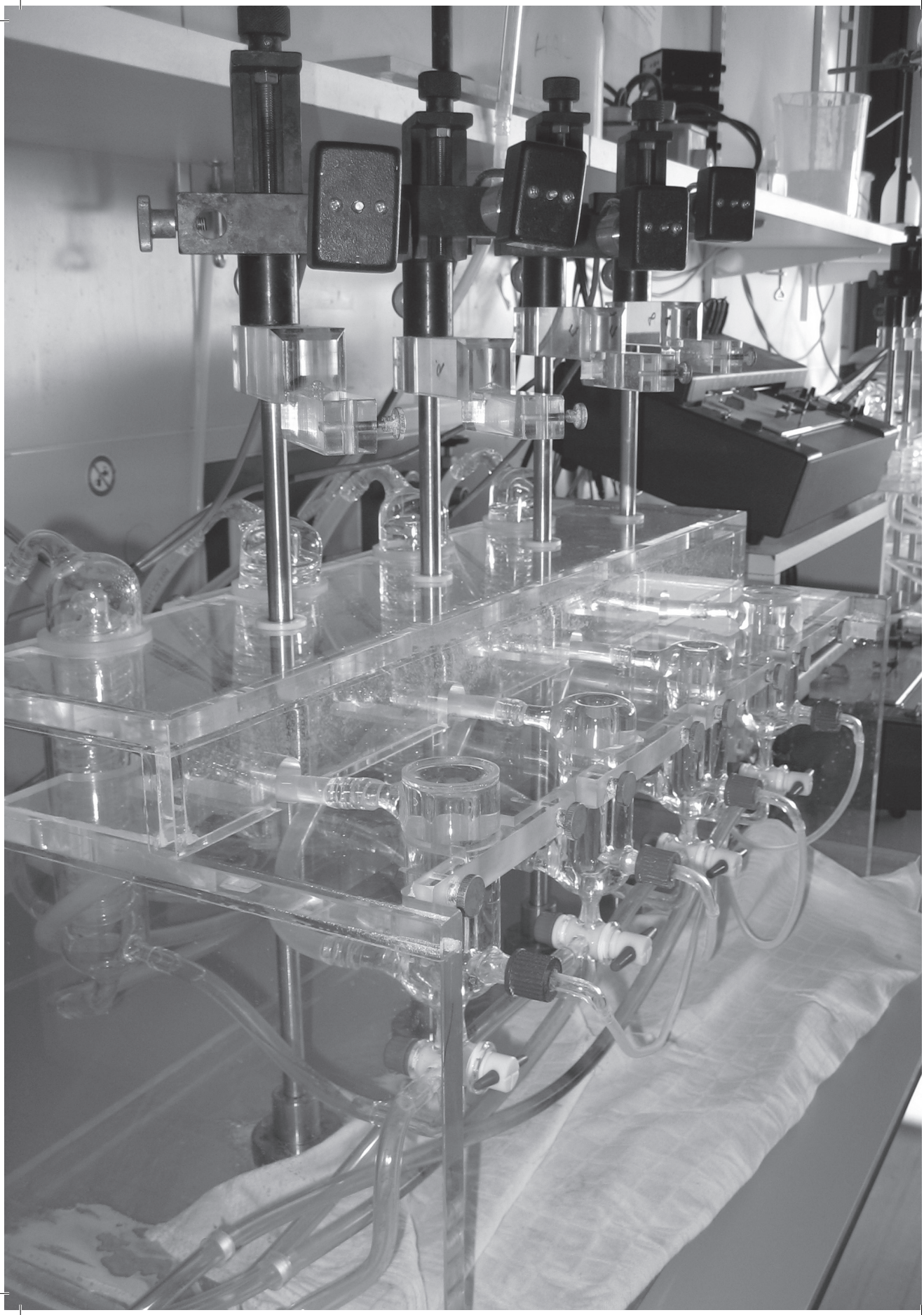
Aim of this thesis

Although the beneficial cardiovascular effects of ACE inhibitors and AT_1 receptor blockers are beyond doubt, it is clear that the renin-angiotensin system (RAS) still holds many secrets. A full understanding of this system might lead to the discovery of novel therapeutic targets and improved treatment strategies. To further unravel the complexities of the RAS, this thesis therefore has addressed the following three issues:

1. Somatic ACE contains two catalytic domains, the C- and N-domain. Their exact contribution to the generation of angiotensin II and the degradation of bradykinin in blood and tissues is unknown. In addition, although the so-called ACE insertion/deletion (I/D) polymorphism has received wide attention in the past decade, it is still uncertain to what degree the D allele (which results in higher ACE levels) truly associates with cardiovascular disease(s) and/or the effectiveness of RAS blockade. Of particular importance is the finding that DD subjects do not have higher angiotensin II levels than II subjects. Using selective ACE C- and N-domain inhibitors, we first quantified the contribution of both domains to angiotensin I and bradykinin hydrolysis. Next, we evaluated the possibility that the absence of an association between the ACE I/D polymorphism and angiotensin levels relates to differences in the contribution of the ACE C- and N-domain (Chapters 2 and 3).

2. Angiotensin II mediates its effects through 2 receptors, AT_1 and AT_2. In rodents, but not in humans, two AT_1 receptor subtypes have been identified: AT_{1A} and AT_{1B}. In addition, angiotensin I and II have several metabolites (angiotensin III, angiotensin IV and angiotensin-(1-7)) which may exert effects of their own, possibly via non-classical AT receptors. To get a better understanding of the contribution of each of these (AT/non-AT) receptors, and to determine their physiological agonist(s), we made use of classical pharmacological in vitro and in vivo models, selective AT receptor (ant)agonists and mice deficient in one, two or all three AT receptors (Chapter 4-6).

3. Renin inhibitors have now entered the clinical arena. Animal and clinical data suggest that the (short-term) blood pressure-lowering effect of renin inhibition equals that of ACE inhibition and AT_1 receptor blockade. Whether blocking the initial, rate-limiting step of the RAS also offers long-term (end-organ) protection still needs to be investigated. We therefore compared the cardiovascular efficacy of the renin inhibitor aliskiren, the AT_1 receptor blocker irbesartan and the ACE inhibitor captopril in a well-established physiological model, the spontaneously hypertensive rat, focusing in particular on coronary function and cardiac hypertrophy (Chapter 7).



Chapter 2

Selective ACE C-domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction

Based on:
Joep H.M. van Esch, Beril Tom, Vincent Dive, Wendy W. Batenburg, Dimitris Georgiadis, Athanasios Yiotakis, Jeanette M.G. van Gool, René J.A. de Bruijn, René de Vries, A. H. Jan Danser. Selective ACE C-domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction.

Hypertension. 2005; 45: 120-125.

Abstract

Somatic ACE contains two domains (the C- and N-domain) capable of catalyzing angiotensin I and bradykinin hydrolysis. Here we investigated the effect of increasing concentrations of the selective ACE C- and N-domain inhibitors RXPA380 and RXP407 on angiotensin I-induced vasoconstriction of porcine femoral arteries (PFAs) and bradykinin-induced vasodilation of preconstricted porcine coronary microarteries (PCMAs). Ang I concentration-dependently constricted PFAs. RXPA380, at concentrations >1 µmol/L, shifted the Ang I concentration-response curve (CRC) 10-fold to the right, and this was comparable to the maximal shift observed with the ACE inhibitors (ACEi) quinaprilat and captopril. RXP407 did not affect the Ang I CRC at concentrations up to 0.1 mmol/L. Bradykinin concentration-dependently relaxed PCMAs. 10 µmol/L RXPA380 and 0.1 mmol/L RXP407 potentiated bradykinin, both inducing a leftward shift of the bradykinin CRC that equalled ≈50% of the maximal shift observed with quinaprilat. Angiotensin I added to blood plasma, obtained from healthy volunteers, disappeared with a half life of 42±3 minutes. Quinaprilat increased the angiotensin I half life ≈4-fold, indicating that 71±6% of angiotensin I metabolism was due to ACE. RXPA380 (10 µmol/L) and RXP407 (0.1 mmol/L) increased the angiotensin I half life ≈2-fold, thereby suggesting that both domains contribute to conversion in plasma. In conclusion, tissue angiotensin I-II conversion depends exclusively on the ACE C-domain, whereas both domains contribute to conversion by soluble ACE and to bradykinin degradation at tissue sites. Since tissue ACE (and not plasma ACE) determines the hypertensive effects of angiotensin I, these data not only explain why N-domain inhibition does not affect angiotensin I-induced vasoconstriction in vivo, but also why ACEi exert blood pressure-independent effects at low (C-domain blocking) doses.

Introduction

Angiotensin (Ang) I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that generates Ang II and inactivates bradykinin, two peptides that play a key role in the regulation of blood pressure. Somatic ACE has two homologous domains, each containing an active center. According to their position (N- or C-terminal), these domains are designated as the N- and C-domain, respectively. Bradykinin potentiation by ACE inhibitors correlates directly with ACE C- and N-domain blockade[32,153] i.e., half maximal potentiation is observed during blockade of one domain, and full potentiation occurs during blockade of both domains. In contrast, selective inhibition of the N-domain with the phosphinic peptide RXP407 did not affect the blood pressure responses to Ang I bolus injections in mice[154], nor did targeted inactivation of the N-domain in mice affect blood pressure or plasma Ang II levels[155]. Although this suggests that the C-domain is the dominant Ang I-converting site, a recent study[153] demonstrated that selective inhibition of either the N-domain or the C-domain fully prevents conversion of systemically administered Ang I in mice. Moreover, in the latter study it was simultaneously demonstrated that, when using soluble ACE, full inhibition of Ang I cleavage is obtained only during blockade of both ACE active sites. Thus, Ang I-II conversion by membrane-bound and soluble ACE appears to obey to different mechanisms, and C-domain-selective ACE inhibitors like quinaprilat[156], Ang (1-7)[157] and the new phosphinic peptide RXPA380[153] may fully suppress tissue Ang I-II conversion at concentrations that neither fully block Ang I-II conversion in plasma nor fully potentiate bradykinin.

To study this possibility, we compared the inhibitory effects of the the C-domain-selective inhibitors RXPA380,[153] quinaprilat,[156] and Ang (1-7)[157] (selectivity for C-domain vs. N-domain resp. ≈1000-, 200- and 10-fold) and the N-domain-selective inhibitors captopril[158] and RXP407[154] (selectivity for N-domain vs. C-domain resp. ≈20- and 300-fold) towards membrane-bound and soluble ACE. Ang I-II conversion by membrane-bound ACE was quantified by investigating Ang I-induced contractions of porcine femoral arteries (PFAs). These contractions do not involve enzymes other than ACE[159,160] Ang I-II conversion by soluble ACE was studied by quantifying Ang II generation following the addition of Ang I to human blood plasma. Bradykinin potentiation was studied in porcine coronary (micro)arteries (PCAs, PCMAs). We excluded the possibility that the Ang (1-7)-induced potentiation of bradykinin[32,161] is mediated via the recently cloned Ang (1-7) receptors.[85] Finally, we studied the consequences of C-domain inhibition towards bradykinin in human coronary microarteries (HCMAs). The consequences of such inhibition towards Ang I-II conversion could not be studied in HCMAs, because in isolated human coronary vessels, Ang I conversion depends on chymase rather than ACE.[118,162]

Material and methods

Tissue and blood collection

HCMAs were obtained from 6 heart-beating organ donors (3 men, 3 women; age 13-61 years) who died of non-cardiac causes (3 subarachnoidal bleeding, 2 head trauma, 1 post-anoxic encephalopathy) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. Immediately after circulatory arrest, the hearts were stored in an ice-cooled sterile organ-protecting solution.[118,162] Upon arrival in the laboratory, tertiary branches of the left anterior descending coronary artery (diameter 280-600 µm, mean 420 µm) were removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. PCAs, PCMAs and PFAs were obtained from 32 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg) that had been used in in-vivo experiments studying the effects of calcitonin-gene related peptide receptor (ant)agonists under pentobarbital (600 mg i.v.) anaesthesia, and from 14 slaughterhouse pigs. The Ethics Committee of the Erasmus MC dealing with the use of animals for scientific experiments approved the protocol for this investigation. Arteries were either removed at the end of the experiment or after the heart had been brought to the laboratory in cold Krebs bicarbonate solution. Vessels were stored overnight in cold, oxygenated Krebs bicarbonate solution. Blood (50 mL) was collected from 6 healthy volunteers (4 men, 2 women; age 25-41 years) as described before.[163] Plasma was stored at -70°C.

Functional Studies

Following overnight storage, PCAs and PFAs were cut into segments of ≈4 mm length and mounted in 15-mL organ baths. HCMAs and PCMAs were cut into segments of ≈2 mm length and mounted in Mulvany myographs (J.P. Trading) with separated 6-mL organ baths.[164] HCMAs rather than large human coronary arteries were used, because only the former relax to bradykinin.[165] PCMAs were used in the bradykinin studies involving RXPA380 and RXP407 because of the limited availability of these drugs. All baths contained Krebs bicarbonate solution at 37°C, and were aerated with 95% O_2 and 5% CO_2. Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with the thromboxane A_2 analogue U46619. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes in the presence or absence of RXPA380, RXP407, quinaprilat, captopril, Ang (1-7) and/or D-Ala-Ang (1-7). Thereafter, HCMAs, PCMAs and PCAs were preconstricted with U46619 (10-100 nmol/L) and concentration-response curves (CRCs) were constructed to bradykinin. In PFAs, Ang I and II CRCs were constructed. The cyclo-oxygenase inhibitor indomethacin (5 µmol/L) was present during all experiments in HCMAs to suppress spontaneously occurring contractions and relaxations.

Metabolism Studies

To study Ang I-II conversion in plasma, 2.5 pmol Ang I was added to blood plasma diluted 1:2 or 1:20 in phosphate buffer (pH 7.4) in the presence or absence of increasing concentrations of RXPA380, RXP407, quinaprilat, captopril or Ang (1-7). The mixture was incubated at 37°C, and 200 µL samples were taken at 0, 5, 10 and 30 minutes (1:2 diluted plasma) or at 0, 60, 120 and 240 minutes (1:20 diluted plasma). The samples were immediately mixed with inhibitor solution,[118,162] and stored at -80°C until analysis. Ang I and II were measured with sensitive radioimmunoassays.[162]

Data analysis

Data are given as mean±SEM. CRCs were analysed as described before[162] to obtain pEC_{50} ($-^{10}\log[EC_{50}]$) values. Statistical analysis was by ANOVA, followed by post hoc evaluation according to Dunnett. $P<0.05$ was considered significant.

Results

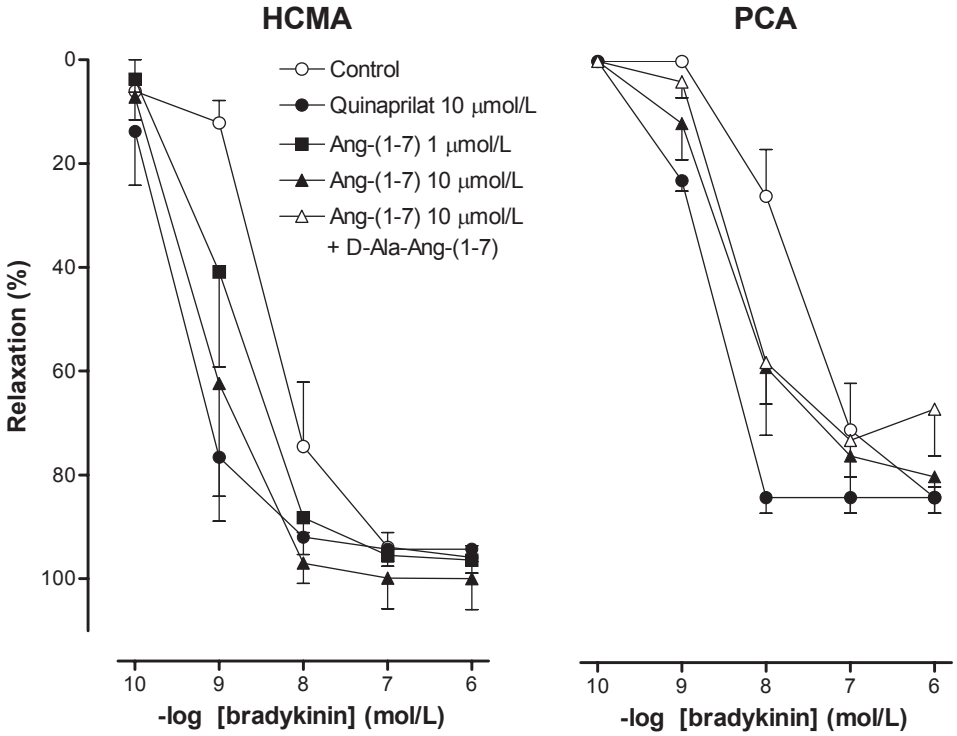


Figure 1. *Relaxations of U46619-preconstricted HCMAs (left) and PCAs (right) to bradykinin in the absence (control, open circles) or presence of 10 µmol/L quinaprilat (closed circles), 1 µmol/L Ang (1-7) (closed squares), 10 µmol/L Ang (1-7) (closed triangles), and 10 µmol/L Ang (1-7) + 10 µmol/L D-Ala-Ang (1-7) (open triangles). Data (mean±SEM of 4-6 experiments) are expressed as a percentage of the contraction induced by U44169.*

Bradykinin-Induced Relaxations

Bradykinin relaxed U46619-preconstricted HCMAs (n=6), PCAs (n=4) and PCMAs (n=14) (pEC_{50} 8.3±0.2, 7.4±0.3, and 8.5±0.2, respectively; Figures 1 and 2). Quinaprilat (10 μmol/L) shifted the bradykinin CRC in all three vessel types ≈10-fold to the left (pEC_{50} 9.5±0.2, 8.5±0.1, and 9.7±0.2, respectively; P<0.01 vs. control for all). Ang (1-7) also shifted the bradykinin CRC in HCMAs to the left, and, at a concentration of 10 μmol/L, its effect (pEC_{50} 9.1±0.3; P<0.05 vs. control) was

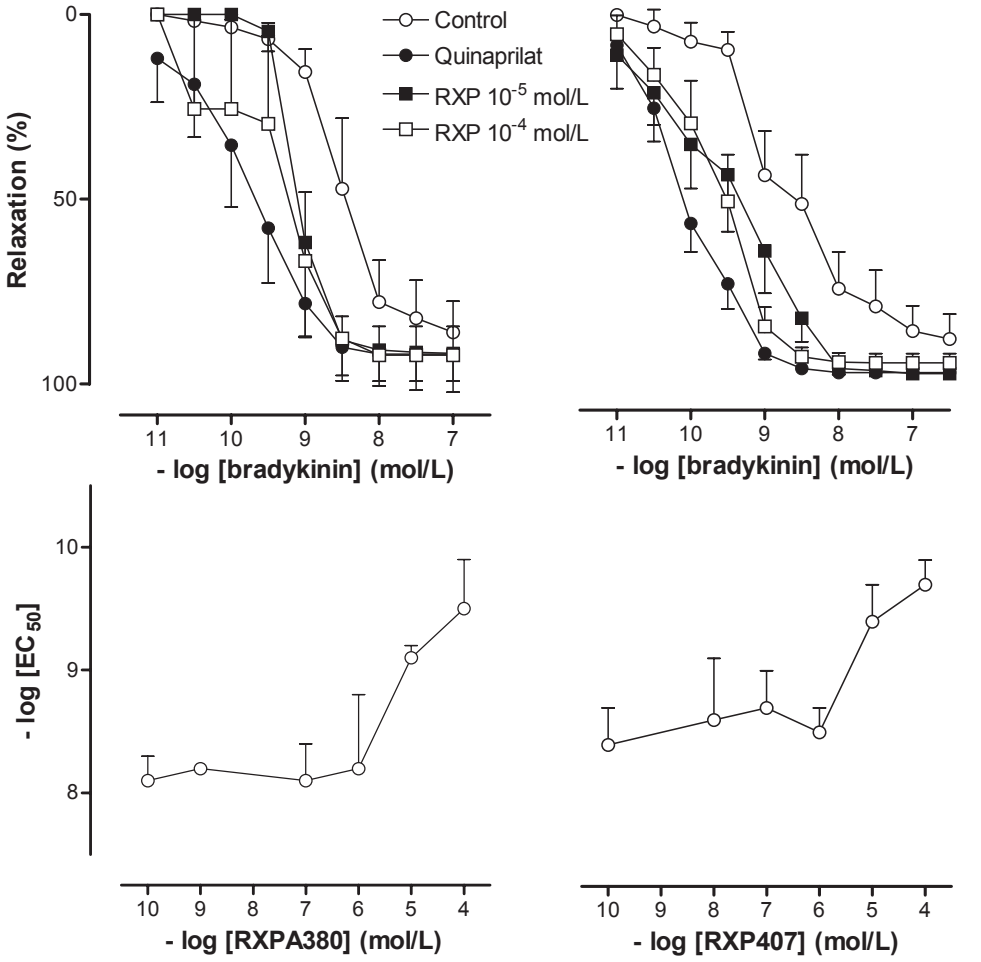


Figure 2. *Top panels: Relaxations of U46619-preconstricted PCMAs to bradykinin in the absence (control) or presence of the inhibitors RXPA380 (left) and RXP407 (right). For comparison, the effect of 10 μmol/L quinaprilat is also shown. Data (mean±SEM of 4-7 experiments) are expressed as a percentage of the contraction induced by U44169. Bottom panels: Change in -log[EC_{50}] of the bradykinin CRC in the presence of increasing concentrations of RXPA380 or RXP407. An increase in –log[EC_{50}] represents a leftward shift of the bradykinin CRC. Con, control. Significant differences (P<0.05) vs. control were obtained at the highest two concentrations only.*

comparable to that of 10 μmol/L quinaprilat (Figure 1). The leftward shift of 10 μmol/L Ang (1-7) in PCAs (pEC_{50} 7.9±0.5, P<0.05 vs. control) was not affected by the Ang (1-7) receptor antagonist D-Ala-Ang (1-7) (10 μmol/L) (Figure 1), nor did this drug exert additional effects on top of quinaprilat (n=4, data not shown).
RXPA380 and RXP407, at concentrations of 10 μmol/L and higher, induced a leftward shift of the bradykinin CRC in PCMAs that equalled 50% or more of the maximal shift observed with quinaprilat (Figure 2).

Angiotensin I-Induced Constrictions

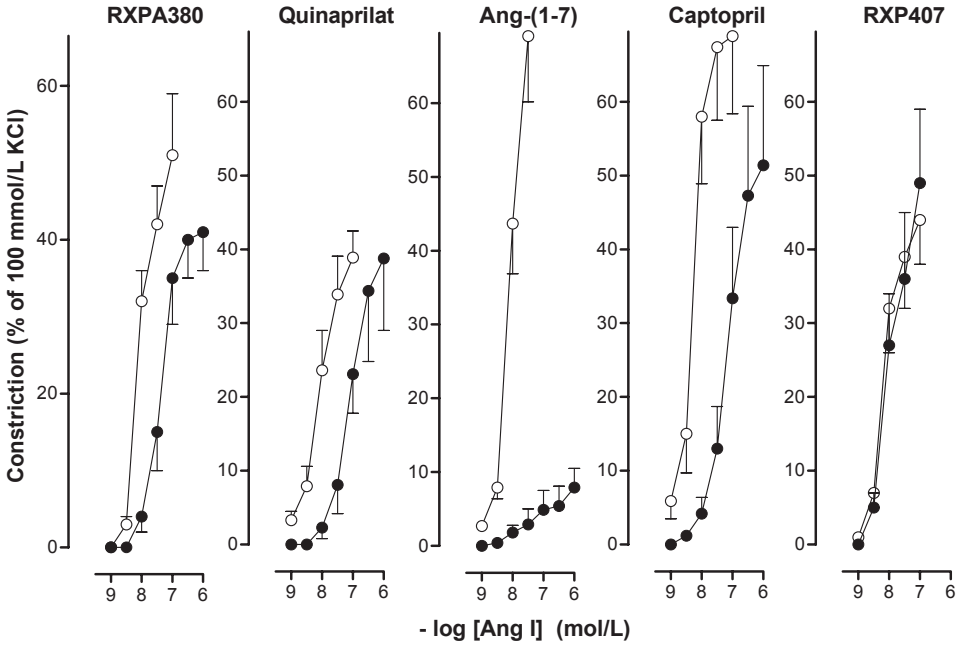


Figure 3. *Constrictions of PFAs to Ang I in the absence (control, open circles) or presence (closed circles) of 100 μmol/L RXPA380, 10 μmol/L quinaprilat, 10 μmol/L Ang (1-7), 100 μmol/L captopril (middle), or 100 μmol/L RXP407. Although a wide range of inhibitor concentrations was tested (see also Figure 4), for the sake of clarity only the CRC obtained in the presence of the highest inhibitor concentration is shown in each panel. Data (mean±SEM of 5-8 experiments) are expressed as a percentage of the contraction to 100 mmol/L K+.*

Ang I constricted PFAs (pEC_{50}=8.1±0.1, n=32; Figure 3) to maximally 40-60% of the contraction to 100 mmol/L K^+. RXPA380 (n=6), quinaprilat (n=8) and captopril (n=5) shifted the Ang I CRC to the right in a concentration-dependent manner, and a maximum (≈10-fold) shift occurred at concentrations of 1 μmol/L, 10 nmol/L and 100 μmol/L, respectively (Figures 3 and 4). Ang (1-7), up to a concentration of 1 μmol/L, did not shift the Ang I CRC to the right (n=6; Figure 4), although it did reduce the maximum constrictor effect of Ang I by >60% at the latter concentration. At a tenfold higher concentration, Ang (1-7) virtually abolished all Ang I-induced effects (Figure

3). This high Ang (1-7) concentration also reduced the maximum effect of Ang II (from 96±23% to 14±4% of the response to 100 mmol/L K^+, n=3), thereby indicating that its blocking effects towards Ang I are due to Ang II type 1 (AT_1) receptor antagonism rather than ACE inhibition. RXP407, up to a concentration of 100 µmol/L, did not significantly affect the Ang I CRC (Figures 3 and 4).

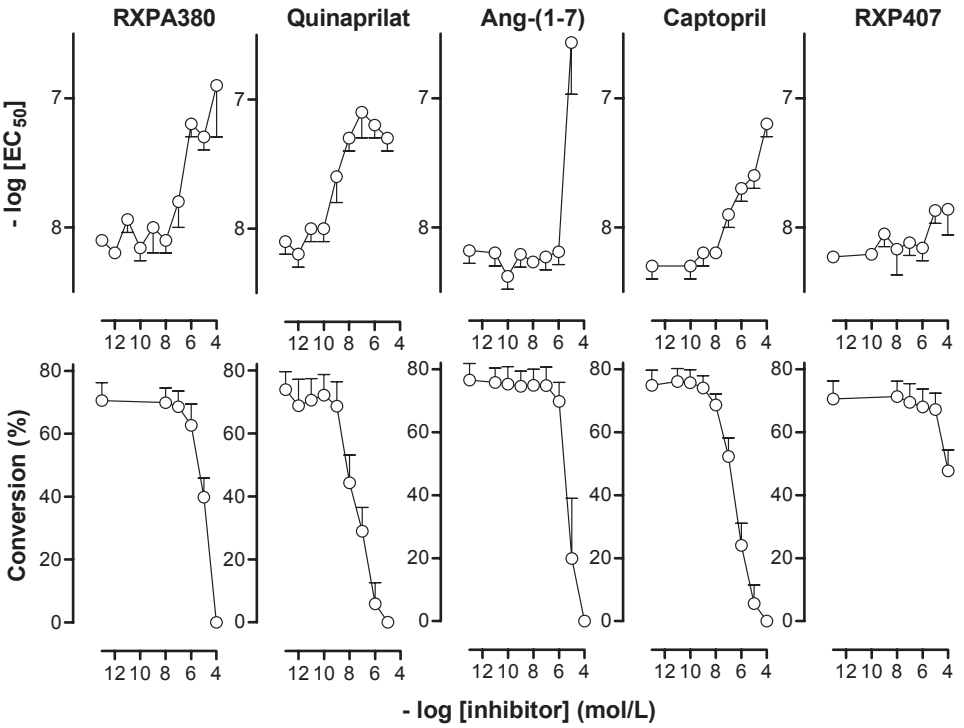


Figure 4. *Top panels: Change in –log[EC_{50}] of the Ang I CRC in the presence of increasing concentrations of RXPA380, quinaprilat, Ang (1-7), captopril, or RXP407. A decrease in –log[EC_{50}] represents a rightward shift of the Ang I CRC. Data (mean±SEM of 5-8 experiments) were obtained in PFAs (see also Figure 3). The first data point represents –log[EC_{50}] in the absence of inhibitor (i.e., control). Significant differences (P<0.05) vs. control were obtained for RXPA380, quinaprilat, Ang (1-7) and captopril at concentrations ≥ 1 µmol/L, 1 nmol/L, 10 µmol/L, and 0.1 µmol/L, respectively. Bottom panels: Inhibition of ACE-dependent Ang I-II conversion in the presence of increasing concentrations of RXPA380, quinaprilat, Ang (1-7), captopril, or RXP407. Data (mean±SEM of 4 experiments) were obtained in human blood plasma (see also Figure 5). The first data point represents conversion in the absence of inhibitor (i.e., control). Significant differences (P<0.05) vs. control were obtained for RXPA380, quinaprilat, Ang (1-7), captopril, and RXP407 at concentrations ≥ 10 µmol/L, 10 nmol/L, 10 µmol/L, 0.1 µmol/L, and 100 µmol/L, respectively. Note the difference in inhibition profile between the top and bottom panels.*

Angiotensin I Metabolism in Human Blood Plasma

The half life of Ang I added to human plasma (diluted 1:2 in phosphate buffer) was 7±0.2 minutes (n=4). Quinaprilat, captopril and Ang (1-7) increased the Ang I half life in 1:2 diluted plasma in a concentration-dependent manner to maximally 35±9,

29±4 and 32±6 minutes, respectively (n=4 for each; Figure 5). From these data it can be calculated[166] that, in the absence of inhibitors, 75±3% of the Ang I metabolism in human plasma is due to Ang I-II conversion by ACE (Figure 4). The highest quinaprilat and captopril concentrations that were tested in this study fully prevented the appearance of Ang II in the incubation mixture (data not shown). Ang (1-7), at concentrations up to 1 μmol/L, did not affect the generation of Ang II or the half life of Ang I. Higher concentrations of Ang (1-7) interfered with the Ang II (but not the Ang I) assay,[163] thus not allowing us to demonstrate that these concentrations also suppressed the generation of Ang II. However, the latter is highly likely in view of the similar increase in Ang I half life in the presence of the highest Ang (1-7) concentration as in the presence of the highest quinaprilat and captopril concentrations.

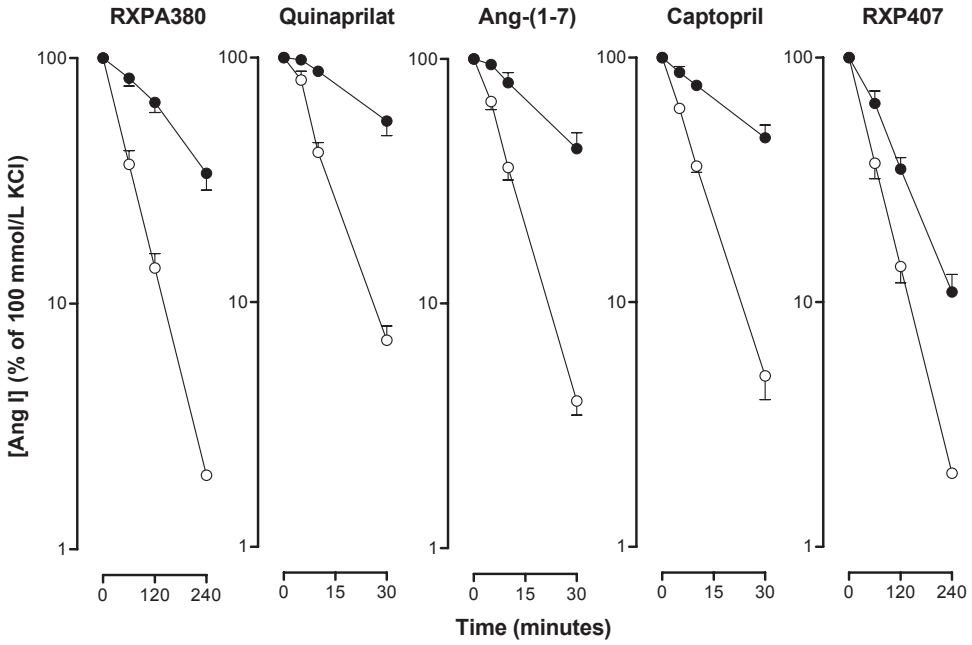


Figure 5. *Metabolism of Ang I in human plasma incubated at 37°C in the absence (control, open circles) or presence (closed symbols) of 100 μmol/L RXPA380, 10 μmol/L quinaprilat, 100 μmol/L Ang (1-7), 100 μmol/L captopril, or 100 μmol/L RXP407. Although a wide range of inhibitor concentrations was tested (see also Figure 4), for the sake of clarity only the results obtained in the presence of the highest inhibitor concentration are shown in each panel. Data (mean±SEM of 4 experiments) are expressed as a percentage of the Ang I levels at t=0.*

Importantly, the quinaprilat concentration that maximally shifted the Ang I CRC to the right (10 nmol/L; Figure 4) reduced Ang I conversion by plasma ACE by only 50%, and a 100-fold higher ($P<0.01$) quinaprilat concentration (1 μmol/L) was needed to fully suppress conversion (Figure 4). Conversely, the captopril concentration required to block plasma ACE by 50% (IC_{50}) was ≈5-fold lower (P=NS) than the captopril concentration required to cause a half-maximal shift of the Ang I CRC (pIC_{50} 6.5±0.2

vs. 6.0±0.3). No such comparisons could be made for Ang (1-7), because of its AT_1 receptor-blocking capacities in the organ bath experiments.
RXPA380 and RXP407, up to concentrations of 100 μmol/L, did not affect Ang I metabolism in 1:2 diluted plasma (n=4, data not shown). Subsequent measurement of these inhibitors in plasma revealed strong plasma protein binding, which reduced their free concentrations by >100-fold (V. Dive, unpublished observations). Therefore, to minimize the problems arising from protein binding, we studied the effects of these inhibitors in 1:20 diluted plasma. Under these conditions the half life of Ang I was 42±3 minutes (n=4; Figure 5). Quinaprilat (10 μmol/L) increased the Ang I half life to 166±22 minutes (P<0.01), thereby demonstrating that 71±6% of the Ang I metabolism in these samples is due to ACE (P=NS vs. 1:2 diluted samples). RXPA380 and RXP407 increased the Ang I half life in 1:20 diluted plasma in a concentration-dependent manner to maximally 156±19 (P=NS vs. quinaprilat) and 80±9 (P<0.01 vs. quinaprilat) minutes, respectively (Figure 5). Only the highest RXPA380 concentration (but not the highest RXP407 concentration) fully prevented the appearance of Ang II in the incubation mixture (data not shown). Importantly, the RXPA380 concentration that was required to fully block Ang I-II conversion in plasma (100 μmol/L) was 100 times higher (P<0.01) than the concentration required to fully shift the Ang I CRC to the right (Figure 4).

Discussion

The present study shows that low (C-domain selective) concentrations of RXPA380 and quinaprilat are sufficient to fully prevent Ang I-induced contractions of PFAs (i.e., to cause a maximum rightward shift of the Ang I CRC), whereas high concentrations (capable of blocking both the C- and N-domain) are required to fully block Ang I-II conversion in human blood plasma. Selective N-domain inhibition with RXP407 did not affect Ang I-induced constrictions, and reduced Ang I-II conversion in plasma by ≈50%. Taken together, these data suggest that only the C-domain contributes to Ang I-II conversion by membrane-bound ACE, and that both domains contribute to Ang II generation by soluble ACE. In contrast, bradykinin degradation by membrane-bound ACE depends on both domains, because, in agreement with the biphasic quinaprilat-induced leftward shift observed previously in PCAs,[32] RXPA380 as well as RXP407 shifted the bradykinin CRC to the left, and a maximum leftward shift was observed only when both domains were blocked. Using the same selective inhibitors, it has already been shown that both domains contribute to bradykinin degradation by soluble ACE.[153]
Our data on captopril, a modestly selective N-domain inhibitor,[158] are in full agreement with the above concept of C-domain-dependent Ang I-II conversion by membrane-bound ACE. When using this inhibitor, the concentrations required to shift the Ang I CRC to the right, if anything, were higher than the concentrations required to block Ang I-II conversion by circulating ACE. This directly opposes our findings with quinaprilat and RXPA380. The lack of a significant difference in the

present study most likely relates to the modest (≈20-fold) selectivity of captopril towards the N-domain.[158]

We also evaluated the effects of Ang (1-7), an angiotensin metabolite that selectively blocks the ACE C-domain.[157] Studies investigating the metabolism of Ang I in isolated human and porcine vessels have already shown that, under the present experimental conditions, Ang (1-7) will not be generated in sufficient amounts to exert effects.[118,159,160,162] First we investigated the potentiating capacity of Ang (1-7) towards membrane-bound ACE in HCMAs. To this end we constructed bradykinin CRCs rather than Ang I CRCs, because, due to the presence of chymase in human coronary arteries, ACE inhibition will not result in a significant rightward shift of the Ang I CRC in human vessels.[118,162] As expected, Ang (1-7) shifted the bradykinin to the left in a concentration-dependent manner, reaching the same maximal leftward shift as quinaprilat at a concentration of 10 μmol/L. The inhibitory capacities of Ang (1-7) towards human ACE were further supported by the fact that this inhibitor increased the Ang I half life in human blood plasma to exactly the same degree as the ACE inhibitors quinaprilat and captopril. Second, we excluded the possibility that the Ang (1-7)-induced leftward shift of the bradykinin CRC depends on the activation of Ang (1-7) receptors rather than ACE inhibition, using the selective Ang (1-7) receptor antagonist D-Ala-Ang (1-7).[85] As shown in Figure 1, this antagonist did indeed not affect the Ang (1-7)-induced leftward shift of the bradykinin CRC in PCAs, although it does block the direct vasodilator effects of Ang (1-7) in the isolated rabbit afferent arteriole.[167] Finally, we studied the effects of Ang (1-7) towards Ang I-induced constrictions in PFAs. PCAs are not suitable for such experiments, because of their limited reactivity to Ang II.[162] In agreement with our findings on quinaprilat, Ang (1-7) markedly shifted the Ang I CRC to the right, at concentrations that in PCAs selectively blocked the ACE C-domain.[118] However, it simultaneously reduced the maximum effect of Ang I, and identical observations were made towards Ang II.[168,169] This suggests that the Ang (1-7) concentrations that selectively block the C-domain are also capable of blocking AT_1 receptors, thereby not allowing us to demonstrate the functional consequence of selective C-domain inhibition by Ang (1-7) towards Ang I.

Our observation that N-domain inhibition does not block Ang I-II conversion by membrane-bound ACE is in full agreement with a previous study demonstrating no effect of the N-domain selective inhibitor RXP407 on the blood pressure responses to Ang I in mice.[154] In apparent contrast with our current data, as well as with the data on Ang I pressor responses in mice, Georgiadis et al.[153] observed that RXP407, at N-domain selective doses, did block the conversion of systemically administered Ang I in mice. This discrepancy may be explained in several ways. First, it could relate to in vitro Ang II generation at the time of blood sampling.[170] To avoid such in vitro generation, blood samples need to be collected with a syringe containing an angiotensinase inhibitor cocktail rather than mixing the samples with the inhibitor cocktail after they have been collected. This approach was not followed by Georgiadis et al..[153] Consequently, in their experimental setup, Ang II generation by plasma ACE may have continued ex vivo in the absence of inhibitors, but not (or to

a lesser degree) in the presence of RXP407 and RXPA380, thereby leading to the conclusion that both types of inhibitors are capable of blocking Ang II generation from systemically applied Ang I. Second, based on a recent study showing enhanced ACE-mediated outside-in signaling in the presence of ACE inhibitors,[171] one may speculate that ACE-induced signaling is determined by the N-domain. Consequently, RXP407, but not RXPA380, might exert effects through ACE that need to be taken into consideration when investigating Ang I-induced vasoconstriction.

Future perspective

This study is the first to show that low concentrations of (C-domain selective) ACE inhibitors are sufficient to fully inhibit Ang II generation by membrane-bound ACE. Although the molecular mechanism of this effect is currently not known, the fact that such low concentrations did not fully block plasma ACE offers an explanation for earlier studies showing that low doses of ACE inhibitors exert beneficial effects in the absence of blood pressure reduction.[172,173] Selective inhibition of tissue Ang II generation (in addition to AT_1 receptor blockade) might also explain why the beneficial effects of Ang (1-7) differ from those of ACE inhibitors in heart failure.[174]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Chapter 3

Different Contributions of the ACE C- and N-domain in Subjects with the ACE II and DD Genotype

Based on:
Joep H. M. van Esch, Jeanette M.G. van Gool, René J.A. de Bruin, John R. Payne, Hugh E. Montgomery, Magda Hectors, Jaap Deinum, Vincent Dive, A. H. Jan Danser. Different Contributions of the ACE C- and N-domain in Subjects with the ACE II and DD Genotype.

Journal of Hypertension. 2008; 26: 706-713.

Abstract

ACE I/D polymorphism-related differences in ACE concentration do not result in differences in angiotensin levels. In this study we investigated whether this relates to differences in the contribution of the ACE C- and N-domain, we quantified, using the C-domain-selective inhibitors quinaprilat and RXPA380, and the N-domain-selective inhibitor RXP407, the contribution of both domains to the metabolism of angiotensin I, bradykinin, the C-domain-selective substrate Mca-BK(1-8), and the N-domain-selective substrate Mca-Ala, in serum of II's, DD's, and 'hyperACE' subjects (i.e., subjects with increased ACE due to enhanced shedding). During incubation with angiotensin I, the highest angiotensin II levels were observed in sera with the highest ACE activity. This confirms that ACE is rate-limiting with regard to angiotensin II generation. C-domain-selective concentrations of quinaprilat fully blocked angiotensin I-II conversion in DD's, whereas additional N-domain blockade was required to fully block conversion in II's. Both domains contributed to bradykinin hydrolysis in all subjects, and the inhibition profile of RXP407 when using Mca-Ala was identical in II's and DD's. In contrast, the RXPA380 concentrations required to block C-domain activity when using Mca-BK(1-8) were three-fold higher in II's than DD's. The contributions of the C- and N-domain differ between DD's and II's, and RXPA380 is the first inhibitor capable of distinguishing D-allele ACE from I-allele ACE. The lack of angiotensin II accumulation in DD's *in vivo* is not due to the often quoted concept that ACE is a non-rate limiting enzyme. It may relate to the fact that in II's both the N- and C- domain generate angiotensin II, whereas in DD's only the C-domain converts angiotensin I.

Introduction

Membrane-bound ('tissue') ACE is the main, if not the only, contributor to angiotensin (Ang) I-II conversion in vivo.[163,175] A fraction of membrane-bound ACE is released into the extracellular fluid (shedding), but this 'soluble' ACE contributes little to Ang II generation.[176] Somatic ACE has two domains, each containing an active center. According to their position (N- or C-terminal), these domains are designated as the N- and C-domain, respectively. Selective C-domain inhibition fully blocks Ang I-induced vasoconstriction.[31] Thus, the Ang II that reaches vasoconstrictor Ang II type 1 (AT_1) receptors following its local generation from Ang I[118] requires the ACE C-domain. This implies that either the N-domain of tissue ACE does not contribute to Ang II generation or that it is responsible for the generation of Ang II that is released from tissues sites to act elsewhere.[153] The N-domain negatively affects shedding and the catalytic activity of the C-domain.[177,178]

The ACE I/D polymorphism determines part of the interindividual variability in tissue and soluble ACE, carriers of the D allele displaying higher tissue and serum ACE levels.[179-181] This is most likely due to the fact that the D allele leads to higher ACE mRNA expression.[182]. Remarkably, the ≈60% higher ACE levels in subjects with the DD genotype (as compared to II's) did not result in a 60% higher regional conversion rate nor in an alteration in the levels of circulating Ang II in DD's.[183-186]. Ang II levels also did not differ in mice with different numbers of Ace gene copies[187,188], and compensatory renin regulation, as well as changes in the levels of Ang I-degrading enzymes, have been shown to explain the lack of effect of ACE titration on Ang II in mice. D allele-related changes in renin or differences in Ang I degradation did not occur in humans[184,189] and, thus, alternative explanations must be put forward for the absence of a D allele-related change in Ang II in man.

In the present study, we set out to investigate the possibility that the contributions of the ACE C- and N-domain are different in D vs. I allele carriers. We made use of serum obtained from the II and DD subjects that displayed the lowest and highest serum ACE activity in a cohort of several hundred British army recruits. For comparison, we also used serum of 'hyperACE' subjects, i.e., subjects who, due to a point mutation in the stalk region of ACE (which enhances shedding), display serum ACE levels that are several fold above the levels in the general population.[190] Importantly, hyperACE subjects have normal plasma levels of Ang II and exhibit no clinical abnormalities. Finally, using porcine coronary arteries (PCAs), we studied whether Ang II release from tissue sites depends on the ACE N-domain. To distinguish the contribution of the two domains, we used the C-domain-selective inhibitors quinaprilat and RXPA380 and the N-domain-selective inhibitor RXP407.[31,32,153]

Material and methods

Tissue and blood collection

PCAs were obtained from slaughterhouse pigs. Arteries were removed after the heart had been brought to the laboratory in cold Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. Vessels were stored overnight in cold, oxygenated Krebs bicarbonate solution.

Serum was obtained from the 24 II and 25 DD subjects displaying the lowest and the highest serum ACE activity among a cohort of several hundred healthy British Army recruits (age 20 years) who were recruited to a study examining the role of genetic variation and environmental factors on cardiac growth in response to exercise. Genotyping occurred as described before.[191] Serum was also obtained from 15 hyperACE subjects (10 ID, 5 DD; 10 men and 5 women, age 25-69 years). Serum was stored at -80°C. The studies were approved by the UK Defence Medical Services Clinical Research (Ethics) Committee and the Ethics Committee of the UMCN St. Radboud.

Vascular studies

Following overnight storage, PCAs were cut into segments of ~4 mm length. The segments were incubated in 1 mL oxygenated Krebs bicarbonate solution at 37°C, aerated with 95% O_2 and 5% CO_2 in the absence or presence of increasing concentrations of RXPA380, RXP407 or quinaprilat for 30 minutes. Next, 100 pmol Ang I was added to the incubation fluid and the incubation was continued for 1 hour. Immediately thereafter, the incubation fluid was collected, mixed with inhibitor solution, and stored at -80°C. Ang II was measured with a sensitive radioimmunoassay.[118]

Serum studies

ACE activity was measured with a commercial kit (ACE Color, Fujirebio) using Hip-His-Leu (HHL) as substrate. Ang I-II conversion was studied by adding 10 pmol Ang I to serum diluted 1:20 (II and DD subjects) or 1:50 (hyperACE subjects) in phosphate buffer (pH 7.4; final volume 1 mL). The mixture was incubated at 37°C, and 200 µL samples were taken over a period of 4 hours. Samples were mixed with inhibitor solution,[118] and stored at -80°C. Ang I and II were measured with sensitive radioimmunoassay.[118] Bradykinin hydrolysis was studied by adding 250 pmol bradykinin to serum diluted 1:100 (II and DD subjects) or 1:300 (hyperACE subjects) in phosphate buffer (final volume 2 mL). The mixture was incubated at 37°C. Samples (200 µL) were taken over a period of 2 hours, mixed with ethanol, and bradykinin was quantified by UV detection (210 nm) after reversed-phase high-performance liquid chromatography according to Kuoppala et al.,[192] using bradykinin-(1-8) as internal standard. All studies with HHL, Ang I and bradykinin were repeated in the presence of increasing concentrations of quinaprilat. Finally, the inhibitory effects of RXP407 and RXPA380 were evaluated in serum diluted 1:20 (RXP407) or 1:100

(RXPA380) in 50 mmol/L HEPES buffer as described by Jullien et al.,[193] using the N- and C-domain-selective substrates Mca-Ala-Ser-Asp-Lys-DpaOH (Mca-Ala) and Mca-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DpaOH (Mca-BK(1-8)) at a concentration of 15 and 10 µmol/L, respectively.

Data analysis

Data are given as mean±SEM, and n-values represent the number of subjects. First order rate constants of Ang I and bradykinin metabolism by non-ACE enzymes ('degradation'; k_1) and ACE (conversion; k_2) in serum were calculated as described before,[160] and corrected for the dilution factor. In short, the Ang I and bradykinin elimination in the absence of quinaprilat represents both degradation and conversion ($k_{elimination} = k_1 + k_2$), whereas elimination in the presence of the highest quinaprilat dose (which fully blocks ACE) represents degradation only ($k_{elimination} = k_1$). By subtracting k_1 from the kelimination at each quinaprilat concentration, k_2 could be determined at each condition. Fractional conversion (i.e., the percentage of metabolism due to ACE) at each condition was subsequently calculated as $k_2 * 100/(k_1 + k_2)$. Statistical analysis was by t-test. $P<0.05$ was considered significant.

Results

Vascular studies

The Ang II levels in the incubation fluid after 1 hour of incubation at 37°C corresponded with ~5% of the Ang I levels that were added at t=0. RXPA380, but not RXP407, decreased Ang II in a concentration-dependent manner (Figure 1), reaching the same degree of inhibition as 10 µmol/L quinaprilat at a concentration of 0.1 mmol/L. Thus, Ang II release from porcine coronary arteries depends exclusively on the ACE C-domain.

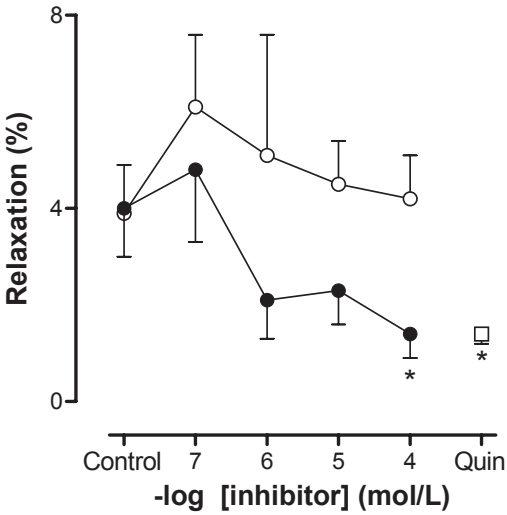


Figure 1. *Angiotensin II levels in incubation fluid following incubation of PCA segments in 1 mL fluid with 100 pmol Ang I in the absence or presence of increasing concentrations of RXP407 (open symbols) or RXPA380 (closed symbols). Results obtained with 10 µmol/L quinaprilat (quin) are shown for comparison. Data are mean±SEM of 4 experiments. *P<0.05 vs. control.*

Serum studies

HHL. Serum ACE activity in II, DD and hyperACE subjects was 19+1, 44+2 ($P<0.001$ vs. II), and 95+7 U/L ($P<0.001$ vs. DD), respectively. The inhibition profile of quinaprilat, tested in duplicate in serum pools containing serum of 10 subjects, was identical

in all groups (Figure 2). Quinaprilat concentrations of 1 μmol/L and higher were required to fully block HHL conversion.

Angiotensin I. The half life of Ang I added to serum of II (n=5) and DD (n=5) subjects (diluted 1:20) was 73±10 and 25±2 min (P<0.002), respectively (Figure 3). Peak Ang II levels were higher in subjects with the DD genotype (P<0.05). Quinaprilat increased the Ang I half life to maximally 246±60 and 170±37 min (P=NS for difference), respectively, and fully suppressed Ang II generation at concentrations >100 nmol/L. From these data it can be calculated that the first order rate constant of conversion in serum is ~4 times lower in II's than DD's (0.13±0.01 vs. 0.47±0.02 1/min; P<0.001; Figure 4), and that, in the absence of quinaprilat, 65±6% and 83±3% of Ang I metabolism is due to Ang I-II conversion by ACE (P<0.05). The first order rate constant of degradation (representing Ang I metabolism by non-ACE pathways) was similar in both groups (0.08±0.02 vs. 0.10±0.02 1/min; Figure 4). The inhibitory profile of quinaprilat was monophasic in DD's (fully blocking conversion at 10 nmol/L), and biphasic in II's (blocking conversion by 64±6% at 10 nmol/L (P<0.02 vs. DD), and fully at 1 μmol/L) (Figure 5). Since 10 nmol/L quinaprilat selectively blocks the C-domain, and 1 μmol/L quinaprilat blocks both domains,[31,32] these data indicate that the C-domain is the only Ang I-II-converting domain in DD subjects, and that both domains contribute to Ang I-II conversion in II subjects. The half life of Ang I added to serum of hyperACE (n=6) subjects (diluted 1:50) was 25±2 minutes (Figure 3). Peak Ang II levels were higher than in DD subjects (P<0.05), despite the 2.5-fold larger dilution of serum. Quinaprilat increased the Ang I half life to maximally 592±184 minutes, and fully suppressed Ang II generation at concentrations >100 nmol/L. From these data it can be calculated that the first order rate constant of conversion in serum of hyperACE subjects is 1.36±0.12 1/min (P<0.001 vs. DD's; Figure 4), and that, in the absence of quinaprilat, 94±2% (P<0.02 vs. DD's) of Ang I metabolism is due to Ang I-II conversion by ACE. The first order rate constant of degradation (0.09±0.03 1/min) was similar to that in II and DD subjects. The inhibitory profile of quinaprilat was biphasic in hyperACE subjects, blocking conversion by 43±3% at 10 nmol/L (P<0.05 vs. II, P<0.001 vs. DD), and fully at 1 μmol/L) (Figure 5). These data indicate that both ACE domains contribute to Ang I-II conversion in hyperACE subjects.

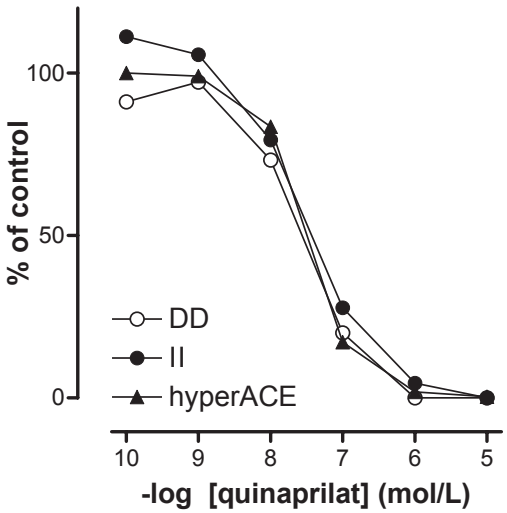


Figure 2. *Inhibitory effect of quinaprilat on ACE-mediated HHL conversion in serum of II, DD and hyperACE subjects. Data are means of duplicate measurements in pools containing serum of 10 subjects.*

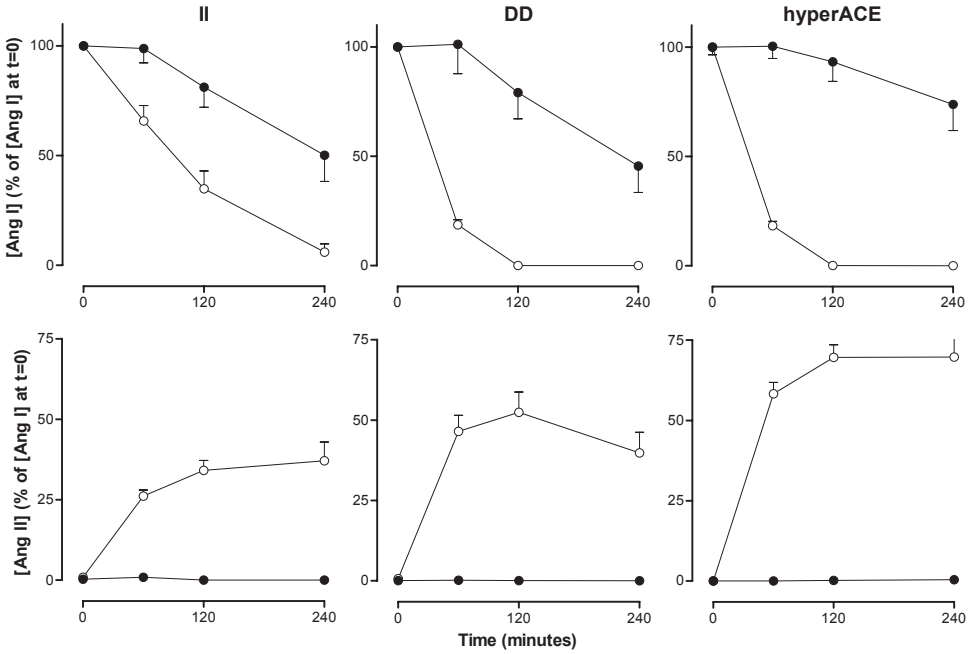


Figure 3. *Metabolism of angiotensin (Ang) I in serum obtained from II, DD and hyperACE subjects incubated at 37°C in the absence (open symbols) or presence (closed symbols) of quinaprilat. For the sake of clarity only data obtained with the highest quinaprilat concentration (10 μmol/L) are shown. Top, Ang I levels, bottom, Ang II levels. Data (mean±SEM of 5-6 experiments) are expressed as a percentage of the Ang I levels at t=0.*

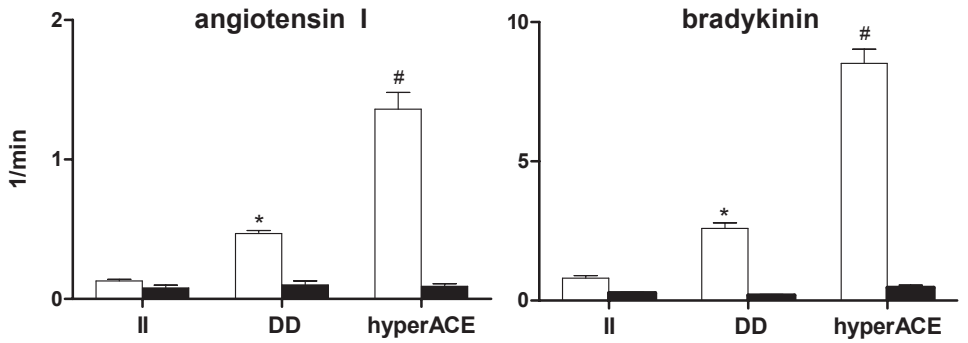


Figure 4. *First order rate constants of conversion (open bars) and degradation (closed bars) of angiotensin I and bradykinin in serum of II, DD and hyperACE subjects. Data are mean±SEM of 5-6 experiments. *P<0.001 vs. II, # P<0.001 vs. DD.*

Bradykinin. The half life of bradykinin added to serum of II (n=5) and DD (n=5) subjects (diluted 1:100) was 66±4 and 26±2 min (P<0.005), respectively (Figure 6). Quinaprilat increased the bradykinin half life to maximally 270±24 and 433±99 min

(P=NS for difference). From these data it can be calculated that the first order rate constant of conversion in serum is ~4 times lower in II's than DD's (0.81±0.09 vs. 2.59±0.20 1/min; P<0.001; Figure 4), and that, in the absence of quinaprilat, 75±4% and 93±1% of bradykinin metabolism is due to conversion by ACE (P<0.002). The first order rate constant of degradation (representing bradykinin metabolism by non-ACE pathways) was similar in both groups (0.19±0.03 vs. 0.27±0.03 1/min; Figure 4). The inhibitory profile of quinaprilat was identical in II's and DD's (Figure 5), requiring quinaprilat concentrations that blocks both domains to fully prevent bradykinin conversion. Thus, both ACE domains contribute to bradykinin hydrolysis in II and DD subjects. The half life of bradykinin added to serum of hyperACE (n=5) subjects (diluted 1:300) was 24±1 minutes (Figure 6). Quinaprilat increased the bradykinin half life to maximally 562±142 minutes. From these data it can be calculated that the first order rate constant of conversion in serum of hyperACE subjects is 8.52±0.51 1/min (P<0.001 vs. DD's; Figure 4), and that, in the absence of quinaprilat, 95±1% of bradykinin metabolism is due to conversion by ACE. The first order rate constant of degradation (0.46±0.10 1/min) was similar to that in II and DD subjects. The inhibitory profile of quinaprilat in hyperACE subjects was identical to that in II and DD subjects (Figure 5).

RXPA380 and RXP407. The inhibition profile of RXP407 was identical in II and DD subjects, and closely resembled that obtained when using

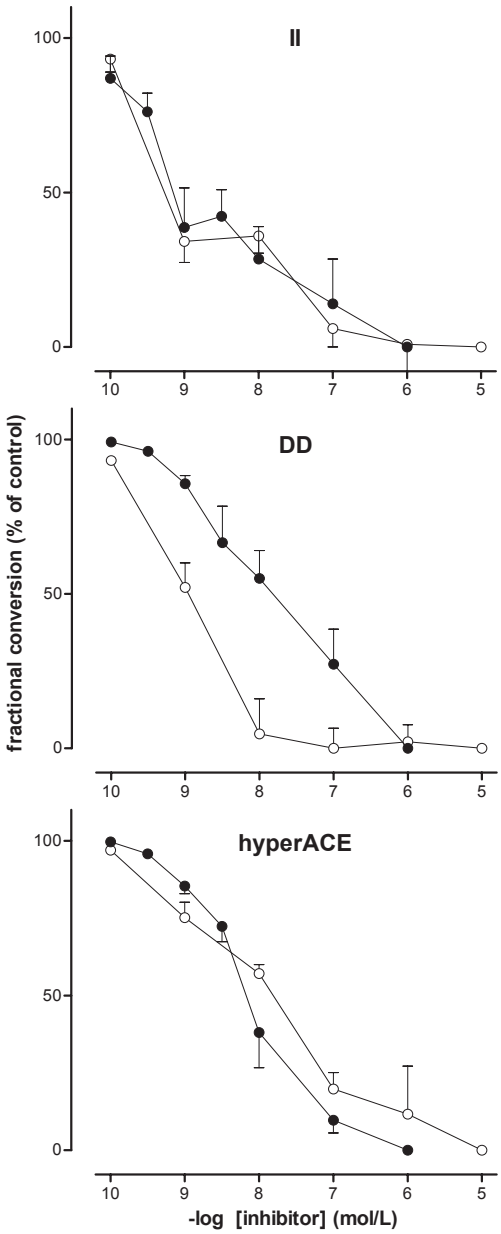


Figure 5. *Fractional conversion of Ang I (open symbols) and bradykinin (closed symbols) in serum obtained from II, DD and hyperACE subjects incubated at 37°C in the presence of increasing concentrations of quinaprilat. Data (mean±SEM of 5-6 experiments) are expressed as a percentage of the conversion in the absence of quinaprilat.*

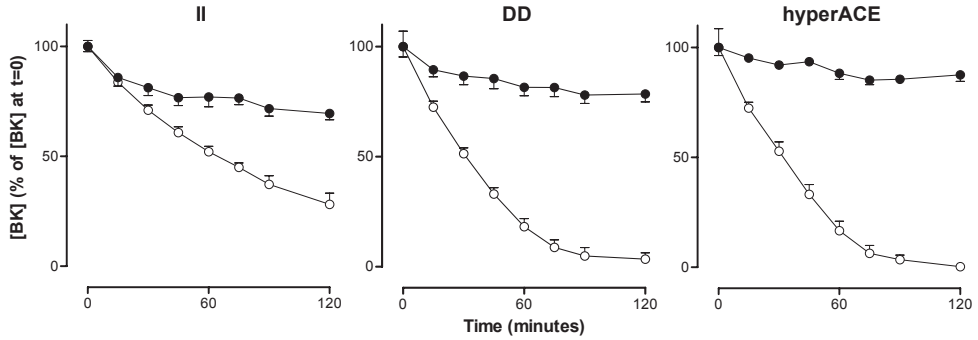


Figure 6. *Metabolism of bradykinin in serum obtained from II, DD and hyperACE subjects incubated at 37°C in the absence (open symbols) or presence (closed symbols) of quinaprilat. For the sake of clarity only data obtained with the highest quinaprilat concentration (10 μmol/L) are shown. Data (mean±SEM of 5 experiments) are expressed as a percentage of the bradykinin levels at t=0.*

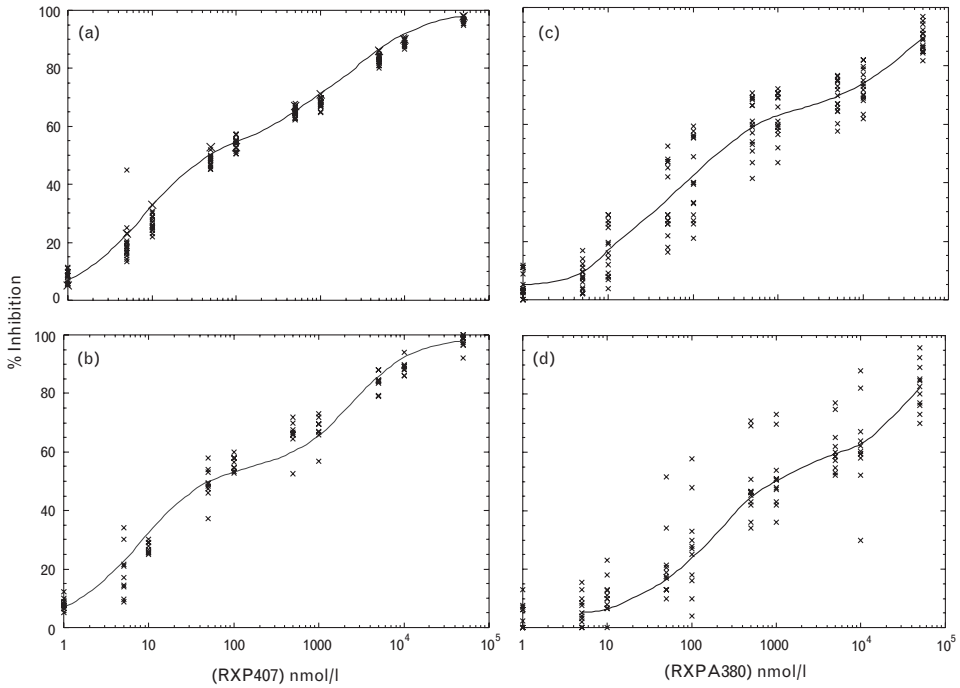


Figure 7. *Inhibition profiles (% inhibition) of human ACE with the N-domain-selective inhibitor RXP407 (A, B) or the C-domain-selective inhibitor RXPA380 (C, D) in serum obtained from 13 DD (top panels) and 10 II (bottom panels) subjects. Each symbol represents an individual measurement. Profiles were simulated using the following K_i values: panel A, 10 nmol/L for the N-domain, and 3 μmol/L for the C-domain; panel B, 10 nmol/L for the N-domain, and 5 μmol/L for the C-domain; panel C, 50 nmol/L for the C-domain, and 50 μmol/L for the N-domain; panel D, 150 nmol/L for the C-domain, and 50 μmol/L for the N-domain. Please note that the RXP407 curves in DD and II subjects are identical, whereas the RXPA380 curve has shifted to the right in II vs. DD subjects.*

purified human ACE (Figure 7).[193] The curve consisted of two parts, reflecting the titration of the N- and C-domain, respectively. The RXPA380 curve in II and DD subjects also consisted of two parts, now reflecting the titration of the C- and N-domain, respectively (Figure 7). However, with this inhibitor, the bottom part of the curve (resembling C-domain inhibition) was shifted 3-fold to the right (IC50 150 vs. 50 nmol/L) in II subjects as compared to DD subjects. Thus, a C-domain-selective inhibitor is able to distinguish DD and II subjects.

Discussion

This study shows that, in porcine membrane-bound ACE, the C-domain is the predominant Ang I-II converting domain, being responsible for both the generation of Ang II that acts locally[31] and the release of Ang II from tissue sites. Secondly, this study raises the possibility that the N-domain contributes to Ang II generation in I allele carriers, thereby offering an explanation for the lack of effect of the D allele-related rise in ACE on Ang II generation. A further difference between II and DD subjects was observed when studying the inhibitory effect of the C-domain-selective inhibitor RXPA380. Finally, we detected the highest degree of Ang II generation in sera with the highest ACE levels. This suggests that the often quoted concept that ACE is non-rate limiting with regard to Ang II generation is incorrect.

In agreement with our previous in vivo studies,[184] no D allele-related increase in Ang I degradation was detected in serum. This conclusion contrasts with the observation in mice that Ace gene titration is accompanied by changes in the metabolism of Ang I by non-ACE enzymes.[188] However, these titration studies, unlike the D allele, did result in changes in Ang II generation, as evidenced by compensatory changes in renin[187,188] and, thus, they may not be truly representative for the functional consequences of the ACE I/D polymorphism. Based on the present data, it appears that such studies should also incorporate the varying contribution of the two ACE domains. The underlying assumption for this conclusion is that the current results (obtained with soluble ACE) also apply to membrane-bound ACE. If indeed the I allele, but not the D allele, allows Ang I-II conversion by the N-domain of membrane-bound ACE, it is obvious that the (on average) 60% higher ACE levels in DD's will not result in a higher Ang II-generating capacity than in II's. Rather, the capacity might be somewhat lower, because 100 I-allele ACE molecules maximally posses 200 Ang I-converting sites, as opposed to 160 Ang I-converting sites for 160 molecules of D-allele ACE. Only by studying DD subjects with ACE levels that are much (i.e, far more than 60%) higher than in II's, an increase in Ang I-II conversion will be found. However, such extreme differences occur rarely, as demonstrated by the fact that it took several hundred recruits to select the DD's and II's for the present study. Consequently, it is not surprising that previous studies investigating ACE I/D-related changes in Ang I-II conversion [183-185] did not find differences.

A further difference between II and DD subjects was noted when studying the inhibitory effect of the C-domain-selective inhibitor RXPA380, using the C-domain

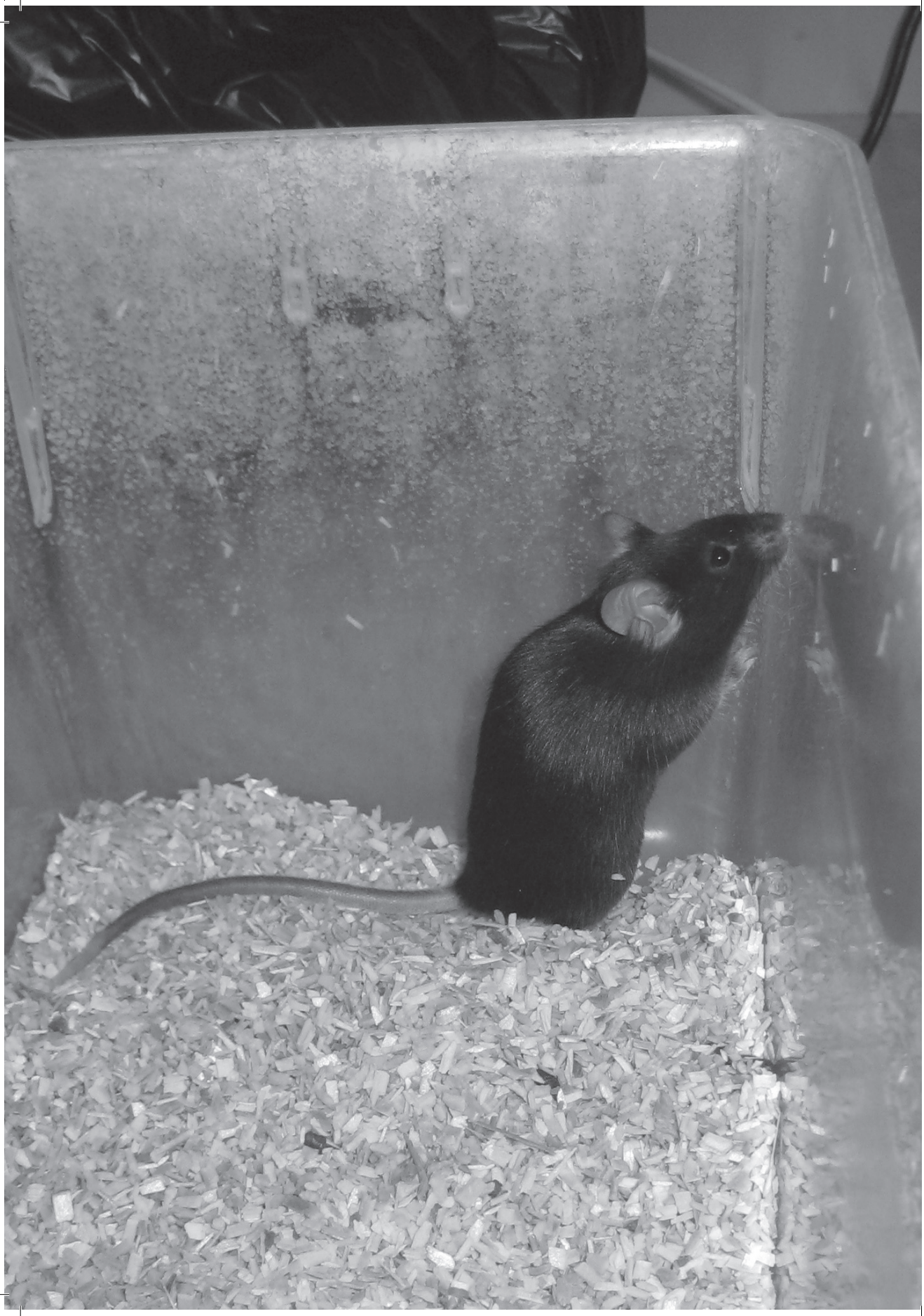
selective substrate Mca-BK(1-8). The IC50 for C-domain inhibition under these circumstances was 3-fold higher in II's than in DD's. No such difference was observed when studying the inhibitory effect of the N-domain-selective inhibitor RXP407 in combination with the N-domain-selective substrate Mca-Ala, nor when studying the inhibitory effect of quinaprilat in combination with HHL as substrate. In the latter case inhibition was observed only at quinaprilat concentrations that were no longer C-domain-selective (i.e., they also blocked the N-domain). Thus, it seems that the contributions of the C- and N-domain do differ between DD's and II's. Differences can only be observed under specific conditions, and most optimally when using C-domain-selective inhibitors in combination with C-domain-selective substrates.

What might underlie the contribution of the N-domain to Ang II generation in II's but not DD's? Based on the insertion (which concerns a 287 base pair Alu repeat in intron 16 of the ACE gene), no molecular differences are to be expected between I and D ACE. Complete genomic sequencing of the ACE gene has revealed 17 sites that are in absolute linkage disequilibrium with the I/D polymorphism, 15 of which were noncoding sites and 2 were synonymous coding variants.[194] Thus, further genomic studies are required to identify other polymorphisms that may cause this difference. The mechanism may involve the 14-residue bridge responsible for interdomain cooperativity[178,195] in view of the fact that the N-domain negatively regulates the catalytic activity of the C-domain.[177,178] Alternatively, the difference may be due to posttranslational modification (involving differences in glycosylation) and/or a factor in serum capable of interfering with ACE activity, e.g., an endogenous N-domain inhibitor. Such natural inhibitors have also been described for other enzymes, e.g. neutral endopeptidase[196] and NO synthase.[197]

Interestingly, whereas the C-domain appears to be the principal Ang I-converting domain, both domains contributed equally to bradykinin hydrolysis in II and DD subjects, in full agreement with previous studies.[32,157,188] Therefore, with regard to bradykinin, ACE I/D polymorphism-related differences are to be expected, even in populations where the ACE levels in DD's are on average only 60% higher than in II's. Small studies demonstrating increased bradykinin metabolism in DD subjects, in vitro as well as in vivo, support this view[198,199]. Importantly, the latter data are also in agreement with the findings on bradykinin levels in mice with varying ACE concentrations,[187] in contrast with the opposite findings on angiotensins in humans and mice described above.

Finally, our data in serum of hyperACE subjects largely resembled those in II's. This is due to the fact that the majority of our hyperACE subjects was of the ID genotype, thus allowing the contribution of both ACE domains to Ang I-II conversion. Importantly, the ACE levels in these subjects were even higher than in DD's, and this resulted in the highest degree of Ang I conversion of all groups. Thus, contrary to the concept that ACE is non-rate limiting with regard to Ang I conversion, our data show clearly that the higher ACE, the more Ang I is converted to Ang II, and the higher [Ang II] will be. It is important to realize that the high serum ACE levels in hyperACE subjects are the consequence of increased shedding. Unlike the situation in DD subjects[181] they are not accompanied by similarly high tissue ACE levels.

Thus, given the fact that circulating Ang II is generated by membrane-bound (and not circulating) ACE,[163] hyperACE subjects do not display increased Ang II levels in blood plasma.[190]
Future studies investigating the ACE I/D polymorphism should take into account that the contributions of both ACE domains to Ang I-II conversion may differ between I and D allele carriers. As a result, regional Ang I-II conversion, plasma renin and Ang II levels will be identical in II and DD subjects, and animal models with varying ACE expression are unsuitable to study the consequences of the ACE I/D polymorphism. Since the two domains do not make a distinction in bradykinin hydrolysis, it seems reasonable to propose that differences in kinin levels are more likely to underlie the observed association of the ACE I/D polymorphism with cardiovascular disorders.[200,201] Since the polymorphism also determines the effectiveness of ACE inhibitor therapy,[202] it is important to test this concept on a large-scale basis.



Chapter 4

AT_2 receptor-mediated vasodilation in the mouse heart depends on AT_{1A} receptor activation

Based on:
Joep H. M. van Esch, Martin P. Schuijt, Jilani Sayed, Yawar Choudhry, Thomas Walther, A. H. Jan Danser. AT_2 receptor-mediated vasodilation in the mouse heart depends on AT_{1A} receptor activation.

British Journal of Pharmacology. 2006; 148: 452-458.

Abstract

Angiotensin (Ang) II type 2 (AT_2) receptors are believed to counteract Ang II type 1 (AT_1) receptor-mediated effects. Here we investigated AT_2 receptor-mediated effects on coronary and cardiac contractility in C57BL/6 mice.

Hearts were perfused according to Langendorff. Baseline coronary flow (CF) and left ventricular systolic pressure (LVSP) were 2.7±0.1 ml 1/min and 111±3 mm Hg (n=50), respectively.

Ang II (n=14) concentration-dependently decreased CF and LVSP, by maximally 41±4% and 25±3%, respectively (pEC50's 7.41±0.12 and 7.65±0.12). The AT_1 receptor antagonist irbesartan (n=4) abolished all Ang II-induced changes, whereas the AT_2 receptor antagonist PD123319 (n=6) enhanced ($P<0.05$) the effect of Ang II on CF (to 59±1%) and LVSP (to 44±2%), without altering its potency. A similar enhancement was observed in the presence of NO synthase inhibitor Nω-nitro-L-arginine methyl ester HCl (L-NAME; n=4). On top of L-NAME, PD123319 no longer affected the response to Ang II (n=4).

The AT_2 receptor agonist CGP42112A (n=4) did not affect CF or LVSP, nor did CGP42112A (n=4) alter the constrictor response to the α_1-adrenoceptor agonist phenylephrine. Furthermore, Ang II exerted no effects in hearts of $AT_{1A}^{-/-}$ mice (n=5), whereas its effects in hearts of $AT_{1A}^{+/+}$ wildtype control mice (n=7) were indistinguishable from those in hearts of C57BL/6 mice.

In conclusion, Ang II exerts opposite effects on coronary and cardiac contractility in the mouse heart via activation of AT_{1A} and AT_2 receptors. AT_2 receptor-mediated effects depend on NO and occur only in conjunction with AT_{1A} receptor activation.

Introduction

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure, cardiovascular remodelling and maintaining body fluid volume. The main effector peptide of the RAS, angiotensin (Ang) II, activates Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. Two subtypes of AT_1 receptors have been identified in rodents (AT_{1A} and AT_{1B}) which share 94% sequence homology, whereas the AT_2 receptor only shares 34% sequence homology with these subtypes.[43,65,203] AT_1 receptors mediate the well-known vasoconstrictor, inotropic, chronotropic, aldosterone-releasing, noradrenaline-releasing and growth-stimulatory effects of Ang II, and AT_2 receptors are generally assumed to counteract these actions.[57-60,204-207] It is believed that AT_2 receptor-mediated vasodilation is an endothelium-dependent phenomenon, involving bradykinin type 2 receptors, nitric oxide (NO) and guanosine cyclic 3', 5' –monophosphate (cGMP).[74-76,208,209]
However, not all studies confirm the counterregulatory actions of AT_2 receptors.[136,210-212] Findings on AT_2 receptor-mediated effects that contrasted with the above concept have been attributed to disparities in genetic background[138] or blood pressure.[212] Furthermore, in many studies conclusions on AT_2 receptor-mediated counteracting effects were drawn based on indirect evidence, i.e., the occurrence of an enhanced response to Ang II following AT_2 receptor antagonism or gene disruption.[57,59,60,208]. The interpretation of data obtained in the absence of AT_2 receptors is complex, because AT_2 receptors downregulate AT_1 receptors in a ligand-independent manner[81], and AT_2 receptor-null mice display increased AT_1 receptor expression.[82]
In the present study we set out to characterize AT_2 receptor-mediated effects in the coronary vascular bed of the mouse heart, using the Langendorff model. Despite the many AT_2 receptor-related studies in transgenic mice, such data are currently not available. AT_2 receptor-mediated responses were studied by comparing Ang II-induced responses in the absence and presence of the AT_2 receptor antagonist PD123319, and by selectively stimulating AT_2 receptors. The latter was accomplished in three ways. First, we investigated the effects of Ang II in the presence of the AT_1 receptor antagonist irbesartan. Second, we studied the effects of the AT_2 receptor agonist CGP42112A[63,213,214], both at baseline and during phenylephrine-induced vasoconstriction. Third, we evaluated the response to Ang II in $AT_{1A}^{-/-}$ mice, i.e., mice lacking Ang II-induced vasoconstriction.[45] We also investigated whether NO mediated the AT_2 receptor-dependent responses, using the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME).

Material and methods

Animals

Male C57BL/6 mice (26±0.6 gram; n=50) were obtained from Harlan (Zeist, The Netherlands). Male $AT_{1A}^{+/+}$ (31±1 gram; n=7) and $AT_{1A}^{-/-}$ mice (27±2 g; n=5) were bred on a 129xC57BL/6 background at the animal facilities of the Charité, Campus Benjamin Franklin, Berlin, Germany.[49] All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Drugs

Ang II, CGP42112A, PD123319, bradykinin, endothelin-1, phenylephrine, and L-NAME were purchased from Sigma (Zwijndrecht, The Netherlands). Irbesartan was a kind gift of Sanofi-Synthelabo BV (Gouda, The Netherlands). Irbesartan (10 mmol/L) was dissolved in ethanol whereas all other chemicals were dissolved in water. Stock solutions were stored in aliquots at -80ºC and diluted in modified Krebs-Henseleit (KH) perfusion buffer (composition in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2,$CaCl_2$ 1.2, D-glucose 11, $NaHCO_3$ 25, pyruvic acid 2) on the day of the experiment. All perfusion solutions were passed through a 0.46 μm cellulose acetate filter (Millipore, Billerica, Massachusetts, USA) prior to their application in the Langendorff setup.

Langendorff preparation

Mice were heparinized using heparin (200 IU; i.p) and subsequently killed by cervical dislocation.[215] The heart was rapidly excised and placed in ice-cold modified KH buffer, gassed with 95% O_2 and 5% CO_2.[215,216] The aorta was immediately cannulated with a 19G needle (with a small circumferential grove close to the blunt tip) and perfused with gassed KH buffer according to Langendorff at a constant perfusion pressure of 80 mm Hg[217]. Two needle electrodes were placed at the right atrium and the hearts were paced at ~600 bpm (5 Hz, 4 ms duration, 4 Volt) using a Grass stimulator (Grass Instruments Co., Quincy, Massachusetts, USA).
Left ventricular systolic pressure (LVSP) was measured with a water-filled balloon (made of domestic food wrap) connected to a disposable pressure transducer (Braun, Melsungen, Germany). The left atrium was removed and the balloon was inserted into the left ventricle.[217,218] The left ventricular end-diastolic pressure was set at 3-5 mmHg by adjusting the balloon volume. Coronary flow (CF) was measured with a flow probe (Transonic systems, Ithaca, New York, USA).

Experimental protocol

After a stabilization period of 10-15 minutes, baseline values of CF and LVSP were obtained. Next, bolus injections (100 μL) of modified KH buffer were applied three times to determine injection-induced changes in CF and LVSP. Subsequently, bolus injections (100 μL) of Ang II, CGP42112A, bradykinin or endothelin-1 (concentration range in the injection fluid 0.1 nmol/L – 0.1 mmol/L) were applied, in the absence

or presence of irbesartan (1 μmol/L in the perfusion buffer), PD123319 (1 μmol/L) and/or the NOS inhibitor L-NAME (10 mmol/L). All blockers were present in the perfusion buffer starting 15 minutes before the first bolus injection. CGP42112A-induced effects were also studied in combination with the α_1-adrenoceptor agonist phenylephrine, by injecting 1 mmol/L phenylephrine alone or simultaneously with 0.1 μmol/L CGP42112A.

Data analysis

CF and LVSP data were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, Ohio, USA). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc, Natick, Massachusetts, USA). Six consecutive beats were selected for determination of CF and LVSP.

Data are given as mean±SEM and represent percentage change from baseline. Concentration-response curves (CRCs) were analysed as described[219], using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, California, USA), to obtain pEC_{50} ($-^{10}logEC_{50}$) and E_{max} values. The pEC_{50} values refer to the agonist concentration in the injection fluid and do not reflect the actual concentrations seen by the receptor. Statistical analysis between groups was performed by Student's *t* test or one-way analysis of variance (ANOVA), followed by post hoc evaluation according to Dunnet. $P<0.05$ was considered significant.

Results

Baseline hemodynamic values and effect of KH buffer injection

Baseline CF values were 2.7±0.1 (n=50), 2.7±0.2 (n=5) and 2.5±0.2 (n=8) ml/min in C57BL/6 mice, $AT_{1A}^{-/-}$ mice and $AT_{1A}^{+/+}$ wild type control mice, respectively. Baseline LVSP values were 111±3, 114±8 and 99±4 mm Hg, respectively. KH buffer injections did not significantly affect these baseline parameters (Figures 1-4).

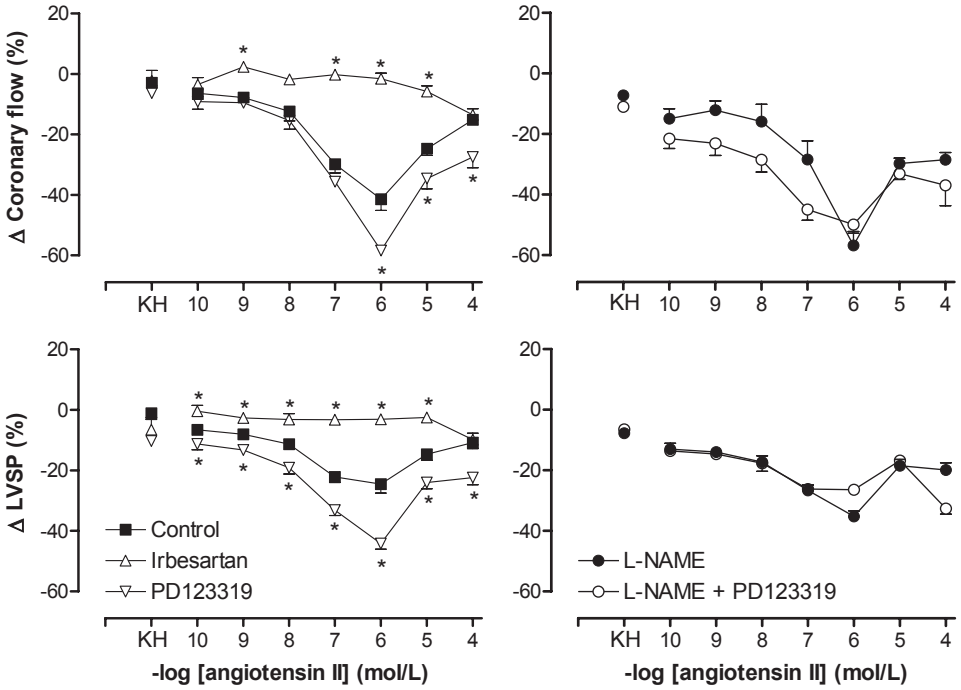


Figure 1. *Left panels, effects of Ang II bolus injections (100 μL) on coronary flow and LVSP in the mouse Langendorff heart in the absence (control; n=14) or presence of 1 μmol/L irbesartan (n=4) or 1 μmol. PD123319 (n=6). Right panels, Effects of Ang II bolus injections on coronary flow and LVSP in the mouse Langendorff heart in the presence of 1 μmol/L L-NAME with or without 1 μmol/L PD123319 (n=4 for both conditions). The x-axis displays the Ang II concentration in the injection fluid. Data are mean±SEM and represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer. *P<0.05 vs. control.*

Studies in C57BL/6 mice

Ang II (n=14) concentration-dependently decreased CF and LVSP, by maximally 41±4% and 25±3%, respectively (pEC_{50}'s 7.41±0.12 and 7.65±0.12; Figure 1). Ang II concentrations >1 μmol/L did not result in effects that were larger than those observed at 1 μmol/L, in agreement with the concept of receptor desensitization[220-222]. The Ang II effects were maximal within 10-20 and 20-30 seconds for CF and LVSP, respectively. Values returned to baseline after 0.5-1 minute.

Irbesartan (n=4) abolished all Ang II-induced changes. In contrast, PD123319 (n=6) enhanced the effect of Ang II on CF (to 59±1%; P<0.05 vs. control) and LVSP (to 44±2%; P<0.05 vs. control), without altering its potency (pEC_{50}'s 7.41±0.12 and 7.49±0.20, respectively). L-NAME (n=4) similarly enhanced (P<0.05) the effect of Ang II on CF (to 57±1%) and LVSP (to 35±2%), without altering its potency (pEC_{50}'s 6.95±0.34 and 7.22±0.13, respectively; Figure 1). PD123319 (n=4) no longer enhanced the effect of Ang II on top of L-NAME, thereby indicating that its effect depends on NO.

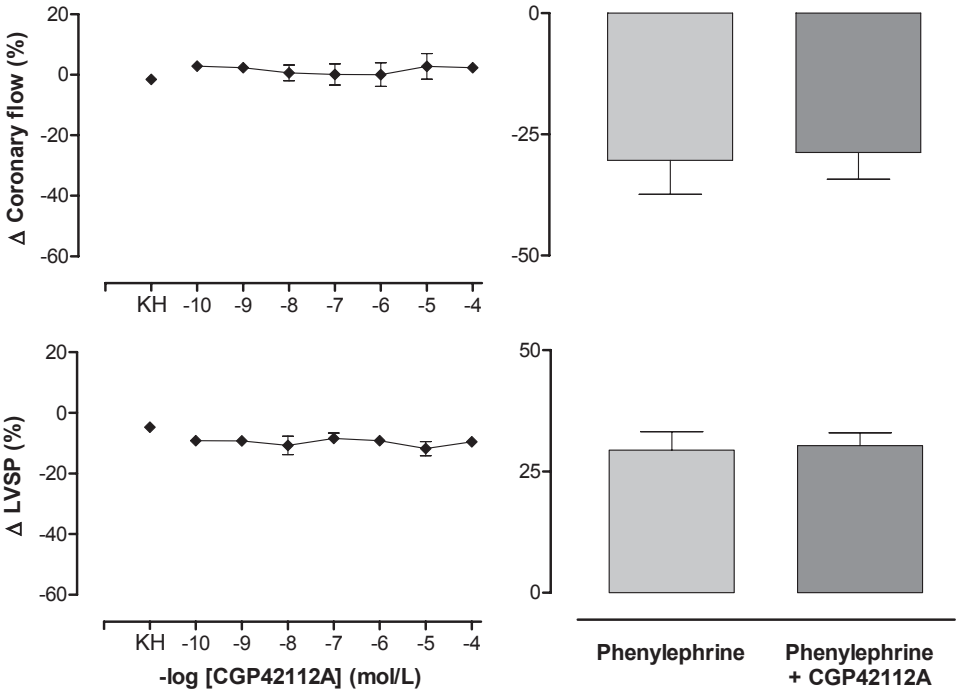


Figure 2. *Left panels, effects of CGP42112A (n=4) bolus injections (100 μL) on coronary flow and LVSP in C57BL/6 mice. The x-axis displays the concentration in the injection fluid. KH, bolus injection of Krebs-Henseleit buffer. Right panels, effects of a phenylephrine bolus injection (100 μL of a solution containing 1 mmol/L phenylephrine), with or without 0.1 μmol/L CGP42112A (n=4 for both conditions), on coronary flow and LVSP in the mouse Langendorff heart. Data are mean±SEM and represent percentage change from baseline.*

Phenylephrine (n=4) decreased CF and LVSP (Figure 2, P<0.05). CGP42112A (n=4) did not diminish the constrictor and inotropic response to phenylephrine, nor did this AT_2 receptor agonist (n=4) exert constrictor or inotropic effects of its own (Figure 2). Bradykinin (n=6) increased CF by maximally 42±6% and marginally affected LVSP (Figure 3).

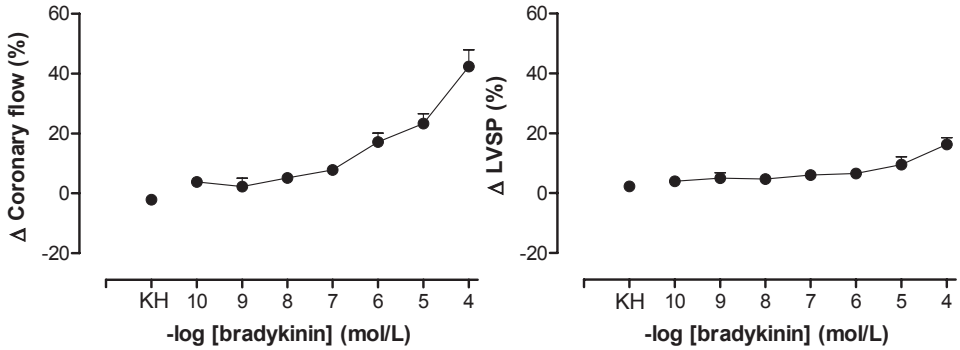


Figure 3. *Effect of bradykinin bolus injections (100 μl) on coronary flow and LVSP in the mouse Langendorff heart. Data are mean±SEM of 6 experiments and represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer.*

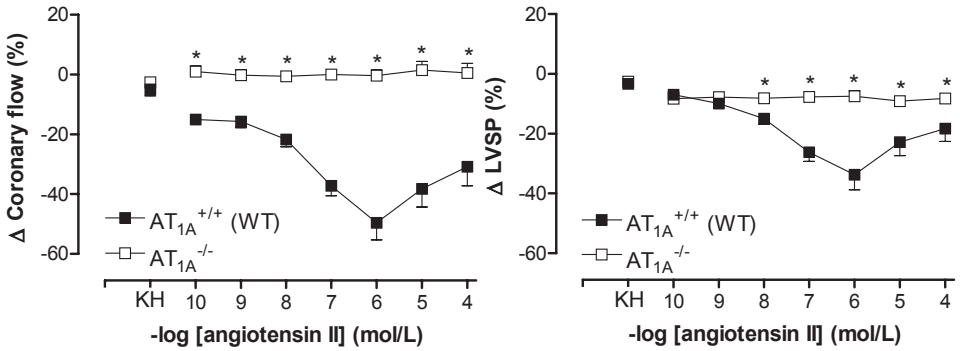


Figure 4. *Effects of Ang II bolus injections on coronary flow and LVSP in $AT_{1A}^{-/-}$ (n=5) and the corresponding $AT_{1A}^{+/+}$ (wildtype control; n=7) mice. The x-axis displays the concentration in the injection fluid. Data are mean±SEM and represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer. *P<0.05 vs. $AT_{1A}^{+/+}$.*

Studies in $AT_{1A}^{-/-}$ mice

Ang II (n=5) did not affect CF or LVSP in $AT_{1A}^{-/-}$ mice, whereas the Ang II (n=7) response in $AT_{1A}^{+/+}$ wild type control mice was indistinguishable from that in C57BL/6 mice (Figures 1, 4 and 5). Endothelin-1 (0.1 nM) decreased CF in both $AT_{1A}^{-/-}$ and $AT_{1A}^{+/+}$ wild type control mice (Figure 5). The endothelin-1-induced decreases in CF and LVSP (47±15% and 41±10%, respectively) were comparable to those induced by 1 mmol/L phenylephrine (Figure 4).

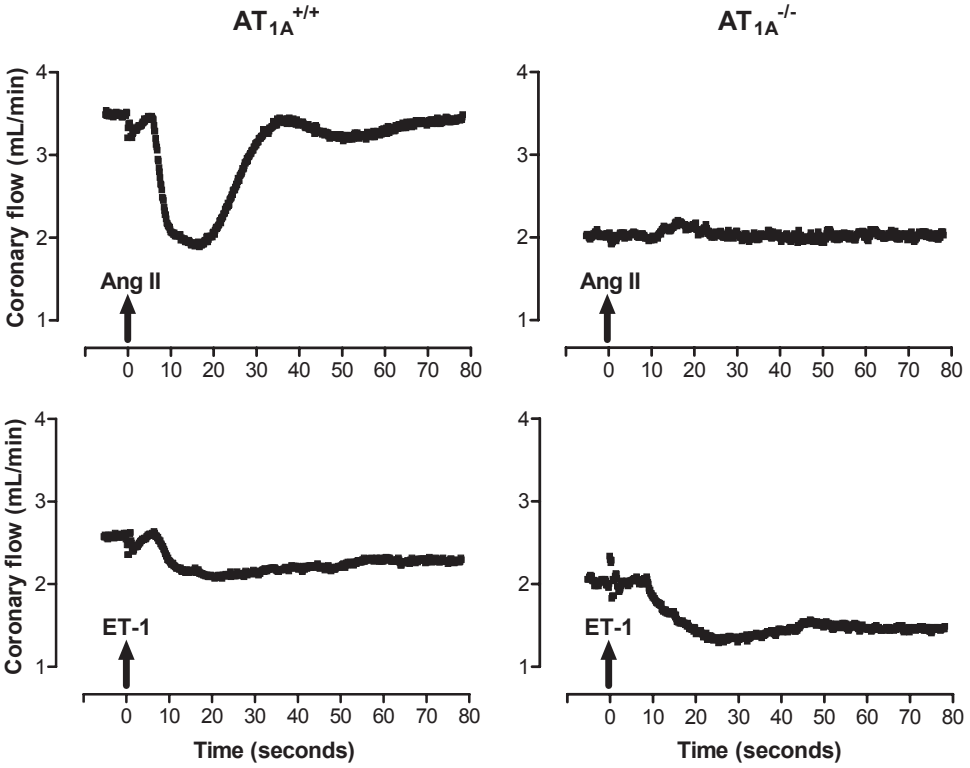


Figure 5. *Representative tracings showing the effects of a bolus injection (100 μl; arrow) containing 1 μM Ang II or 0.1 nM endothelin-1 (ET-1) on coronary flow in $AT_{1A}^{+/+}$ (wildtype control; left panels) and $AT_{1A}^{-/-}$ (right panels) mice.*

Discussion

This study is the first to support the concept of AT_2 receptor-mediated vasodilation in the mouse coronary vascular bed. Evidence for such vasodilation was obtained indirectly, i.e., as an enhanced constrictor response to Ang II in the presence of the AT_2 receptor antagonist PD123319. Data are in full agreement with previous studies on this matter in human[58], porcine[223], rabbit[224] and rat[59] coronary arteries.

Vasodilation did not occur when exposing the mouse heart to Ang II in the presence of irbesartan (a condition allowing selective AT_2 receptor stimulation, which has been used successfully in previous studies[58,64,206]), nor during exposure of the heart to the AT_2 receptor agonist CGP42112A. This was not due to an inability to detect vasodilation, since bradykinin exerted its well-known vasodilator effects in our Langendorff setup. Furthermore, Ang II exerted no effect in hearts of $AT_{1A}^{-/-}$ mice, although these mice do express AT_2 receptors.[225,226] This demonstrates first that the AT_{1A} receptor is the receptor responsible for coronary vasoconstriction, in agreement

with the observation that deletion of the AT_{1A} receptor[45], but not of the AT_{1B} receptor[47], virtually abolishes the in vivo vasoconstrictor response to Ang II and reduces blood pressure. Secondly, it demonstrates that stimulation of AT_2 receptors in the absence of AT_{1A} receptors also does not result in vasodilation.

It has been suggested that AT_2 receptor-mediated vasodilatation can only be observed in hypertensive (and not normotensive) animals.[63] Thus, concomitant vasoconstriction might be a prerequisite to observe AT_2 receptor-mediated vasodilatation. However, in contrast with this concept, CGP42112A did not affect the vasoconstrictor response to the α_1-adrenoceptor agonist phenylephrine in the mouse heart.

It appears therefore that AT_2 receptor-mediated effects depend on simultaneous AT_{1A} receptor activation, for instance because both receptors heterodimerize[61], or because interaction occurs at the post-receptor level. Heterodimerization would require the simultaneous occurrence of both receptors in the same (smooth muscle) cell, and although this concept has been tested in smooth muscle cells of transgenic mice[75,227], most studies suggest that AT_2 receptors are restricted to endothelial cells[58,228,229], whereas AT_1 receptors are mainly located on smooth muscle cells.

AT_2 receptor-mediated responses, in contrast with AT_1 receptor-mediated constriction, depend on both endothelial and smooth muscle cells, and involve a cascade starting with endothelial bradykinin type 2 receptor activation and subsequent NO synthesis, and finally resulting in guanylyl cyclase activation in smooth muscle cells.[75,208,209] In agreement with the important contribution of NO to the vasodilator effect of the AT_2 receptor, L-NAME enhanced the Ang II response to the same degree as PD123319, and PD123319 no longer enhanced the effect of Ang II in the presence of L-NAME.

The question then arises why direct, AT_2 receptor-mediated vasodilation could not be observed in the present study. First, although several groups have demonstrated such vasodilation in various species (including humans), both in vitro and in vivo[58,64,76,230,231], we are not aware of studies in mice showing Ang II-induced vasodilation. Thus, the simplest explanation is that mice differ from other species, in that their coronary AT_2 receptors are not limited to endothelial cells. Indeed, Utsunomiya et al. observed abundant AT_2 receptor protein immunoreactivity in the media of mouse coronary arteries.[232] Second, Widdop et al. have suggested that the sensitivity of some experimental preparations is too low to observe AT_2 receptor-induced vasodilation.[66] However, based on the robust (≈50%) PD123319-induced increase of the coronary constrictor response to Ang II, a considerable degree of vasodilation should have occurred in our preparation during selective AT_2 receptor stimulation, and such robust vasodilation was in fact present when exposing the heart to bradykinin, the putative mediator of the AT_2 receptor-mediated relaxation. Finally, application of Ang II via bolus injections differs greatly from the local production of Ang II in close proximity of AT_1 and AT_2 receptors[118,159,233] that occurs in vivo, and thus, one further possibility is that arterial Ang II delivery is an inappropriate tool to observe AT_2 receptor-mediated vasorelaxation.

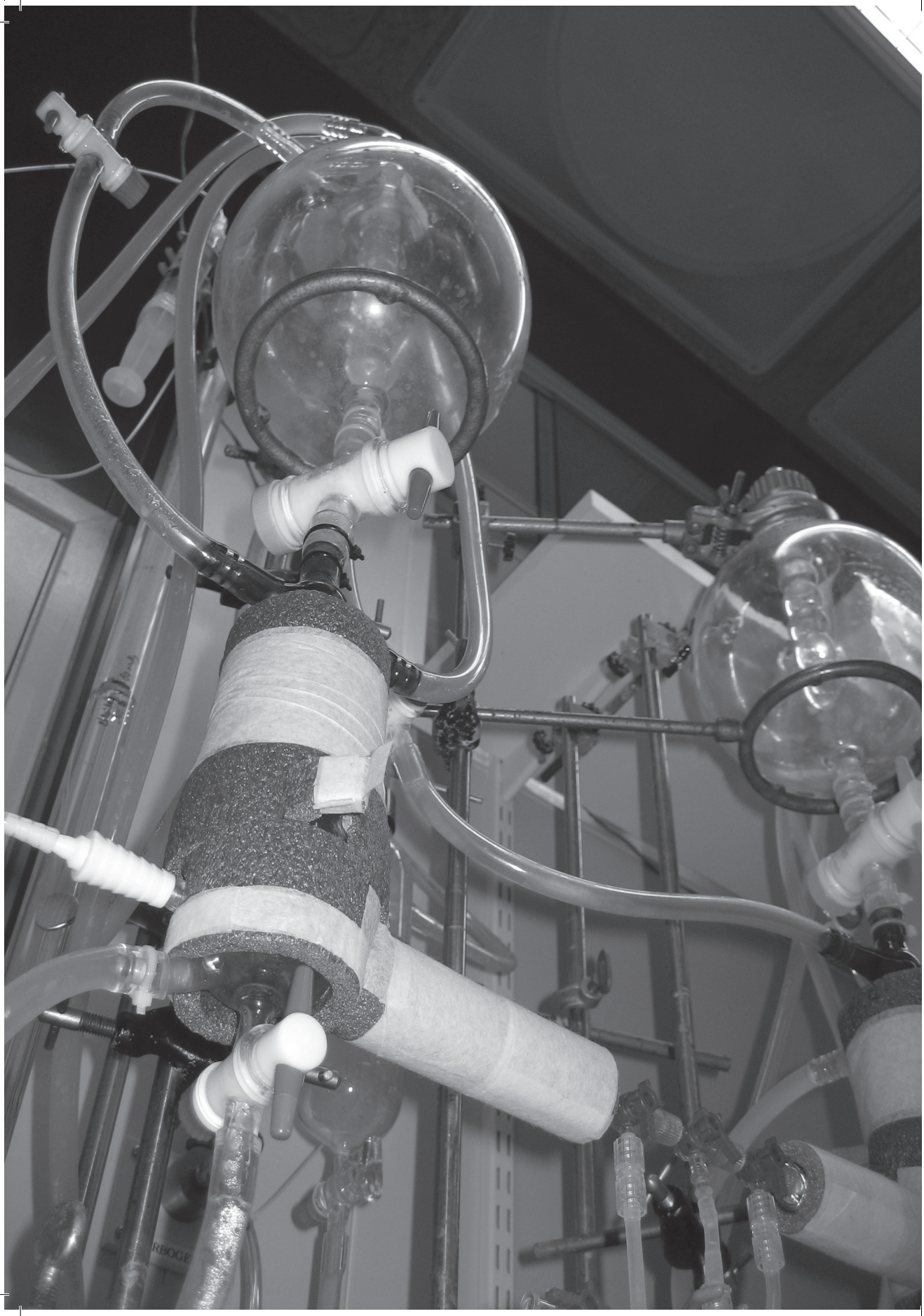
PD123319 enhanced the negative inotropic response to Ang II in the mouse heart. This suggests that AT_2 receptors counteract the AT_1 receptor-mediated negative

inotropic effects in mouse cardiomyocytes. However, selective AT_2 receptor stimulation did not affect cardiac inotropy. Combined with the observation that AT_2 receptors do not occur in cardiomyocytes[232], a more likely explanation is that the inotropic effects of Ang II in the mouse heart are a consequence of its effects on coronary flow rather than the consequence of direct stimulation of AT_1 and/or AT_2 receptor on cardiomyocytes. This may be different under pathological conditions, e.g. following myocardial infarction, when AT_2 receptors improve left ventricular systolic function.[133]

In summary, our study provides evidence for opposite effects of AT_{1A} and AT_2 receptors in the coronary vascular bed of normotensive mice. AT_2 receptor-mediated effects depended on NO and occurred only in conjunction with AT_{1A} receptor activation. The latter observation suggests that AT_{1A} and AT_2 receptors display an interaction, either directly (due to receptor heterodimerization) or at the post-receptor level. Such interaction might be of particular importance under conditions where the RAS is stimulated, e.g., during sodium depletion or in subjects with renovascular hypertension. A similar functional interaction has been described between the angiotensin-(1-7) receptor Mas and AT_1 and AT_2 receptors.[92] Future investigations should elucidate the exact site of AT_{1A}-AT_2 receptor interaction and whether the interaction is altered under pathological conditions.

Acknowledgements

We thank Rob H. van Bremen and Dr. Daphne Merkus of the Department of Experimental Cardiology (Erasmus MC, Rotterdam, The Netherlands) for their help with the analysis of the data.



Chapter 5

Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors

Based on:
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Submitted, 2008

Abstract

Angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system, activates two distinct receptors, the Ang II receptors type 1 (AT_1) and type 2 (AT_2). In rodents, two AT_1 subtypes have been identified (AT_{1A}, AT_{1B}). To determine receptor-specific functions and possible Ang II effects independent of its three known receptors we generated mice, which are deficient in either one of the Ang II receptors, in two, or in all three (triple-knockouts). Triple-knockouts were vital and fertile, but survival was impaired and plasma Ang II level was increased similarly to the elevated one observed in AT_{1A}-deficient mice. AT_2 deletion increased baseline mean arterial pressure (MAP), while mice lacking AT_{1A} were hypotensive. Blood pressure further dropped in mice lacking both AT_1 subtypes to values comparable to that in triple-knockouts. All combinations lacking the AT_{1A} were distinguished by a reduced heart rate. AT_{1A} deletion impaired the in vivo pressor response to Ang II bolus injection, whereas deficiency for AT_{1B} and/or AT_2 had no impact. However, the additional lack of AT_{1B} in AT_{1A}-deficient mice further impaired the vasoconstrictive capacity of Ang II. While general vasoconstrictor properties were not changed as shown by bolus injection of endothelin-1 and phenylephrine, Ang II failed to alter MAP in triple-knockouts, indicating that there are no other receptors involved in Ang II-mediated pressor effects. Our data identify mice deficient in all three Ang II receptors as an ideal tool to better understand structure and function of the renin-angiotensin system and to search for Ang II effects independent of AT_1 and AT_2.

Introduction

The renin-angiotensin system (RAS) plays a vital role in regulating the physiological function of the cardiovascular system. The primary effector of the RAS is the octapeptide angiotensin (Ang) II. Ang II is a potent regulator of blood pressure and water and electrolyte homeostasis.[234] Ang II mediates its functions through stimulation of two pharmacologically defined receptors, the Ang II receptor type 1 (AT_1) and type 2 (AT_2).[235] Molecular cloning of AT_1[41] and AT_2[236] has shown that both receptors belong to the family of G-protein-coupled receptors (GPCR). The two Ang II receptor subtypes have a low sequence homology (~35%), with highest similarity in the 7 predicted trans-membrane domains.[236] In rodents, two isoforms of the AT_1, AT_{1A} and AT_{1B}, have been identified. The two murine AT_1 isoforms are products of two independent genes, but share substantial sequence homology (~95% identity).[237] The AT_1 is present in almost all tissues and most of the known actions of Ang II are mediated through its stimulation. The AT_{1A} isoform is predominating in most of the organs, including e.g. heart, aorta, kidney, lung, and brain,[44] whereas the AT_{1B} is abundantly expressed in pituitary gland and adrenal cortex.[44,238] AT_2 expression in adult tissue is restricted to a few organs, as brain, adrenal, heart, vascular endothelium, kidney, and ovary,[239] whereas it is widely distributed in fetal tissues.[240] The predominant expression of AT_2 in fetal tissues suggests a role of these receptors in differentiation and developmental processes.[241]

It was demonstrated in genetically modified mice, lacking either the AT_{1A} or AT_{1A}, that blood pressure control is dominated by the AT_{1A} isoform. The baseline blood pressure was significantly decreased in mice lacking the AT_{1A},[45] whereas AT_{1B}-deficient animals were normotensive.[47,242] Interestingly, AT_2-knockout mice showed an increase in blood pressure under basal conditions,[60] supporting the hypothesis of its AT_1-counteracting effects in blood pressure regulation.[62] The vasoconstrictor effect of exogenous Ang II was almost completely blunted in AT_{1A}-knockout mice,[45] while animals lacking AT_{1B} showed normal responsiveness to Ang II.[242] In AT_2-knockout mice, Ang II stimulation led to a significant pronounced vasoconstriction, a further indicator for AT_1-counteracting effects of AT_2 on blood pressure regulation.[60] The kidney abnormalities observed in mice lacking angiotensinogen (Agt)[26,243] or angiotensin-converting enzyme (ACE),[121,175] like cortical thinning, focal areas of atrophy, and thickened walls of renal vessels, were less pronounced in AT_{1A}-knockout mice[45,244] and absent in AT_{1B} and AT_2-knockout mice,[47,60] whereas mice lacking AT_{1A} and AT_{1A} showed a similar phenotype as Agt- and ACE-knockout mice.[48,242]

In the present study we generated mice, deficient for one, two, or all three Ang II receptor subtypes, to investigate the relative influence of each subtype on blood pressure regulation, coronary contractility and normal renal development. Furthermore, we wanted to clarify if there are Ang II effects on blood pressure control and cardiac function not related to one of the known Ang II receptor subtypes.

Material and methods

Animals

AT_{1A} (-/-//+/+//+/+),[45] AT_{1B} (+/+//-/-//+/+),[242] and AT_2 (+/+//+/+//-/-) [60]single-knockout mice were used to generate the recently described AT_{1A}/AT_{1A}-double-knockout mice (-/-//-/-//+/+),[242] the herein newly described mice exclusively expressing AT_{1A} (+/+//-/-//-/-) or AT_{1B} (-/-//+/+//-/-), or mice deficient for all three Ang II receptors (triple-knockouts; -/-//-/-//-/-). To generate homozygous wild-type (+/+//+/+//+/+), double-knockout, and triple-knockout mice, the heterozygous offspring was intercrossed and bred on a 129xC57BL/6 background at the animal facilities of the Charité, Campus Benjamin Franklin, Berlin, Germany. For basal cardiac phenotyping and Ang II infusion a set of seven-month old animals was used. A second set of 6-month old male mice was used for measurement of plasma levels of Ang II. A third set was used for morphohistological analysis at the endpoint of twelve months. In addition, age-matched animals lacking Agt[120] were used for histological comparison. Experiments were performed according to the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the Guiding Principles of the American Physiological Society or according to the guidelines of the Federal Law on the Use of Experimental Animals in Germany and were approved by the local authorities.

Polymerase chain reaction (Genotyping)

Genotyping was performed by polymerase chain reaction (PCR) with specific primers for each genotype. PCR for AT_{1A} and AT_2 were described earlier.[245] For AT_{1B}-specific PCR two pairs of primers that amplified products specific for the AT_{1B} wild-type (P1: 5´ CCA GGG CAA GAT TCA GAA GG 3´ and P2: 5´ CCA ACA AAG AGA CAT GAT C 3´) or knockout (P3: 5´ CCT GCG TGC AAT CCA TCT TGT TCA ATG 3´ and P2) allele were designed and used. Agt-deficient animals were genotyped as described previously.[246]

RNA isolation and RNase protection assay

RNA of frozen organs was isolated using TRIzol reagent following the manufacturers protocol, as described previously.[247,248] AT_{1A}, AT_{1A}, and AT_2 expression was analyzed by RNase protection assays using commercially available Ambion RPA II kit (Ambion (Europe) Ltd., Huntingdon, UK), according to the protocol of the manufacturer. Thirty µg total RNA were hybridized with one of the following probes: Probe MMAT1A: A recently described vector[249] was used to transcribe a radioactive probe complementary to a 352-bp fragment specific for AT_{1A} mRNA. Probe MMAT1: A 255-bp fragment, from genomic mouse DNA amplified by PCR using the primers 5´ CTC AGC ATC GAC CGC TAC C 3´ and 5´ GAA CAG GAA GCC CAG G 3´, was subcloned in a T-vector (Promega GmbH, Mannheim, Germany). A T7 polymerase transcribed a radioactive probe complementary to a 170-bp fragment specific for AT_{1A} mRNA and a 255-bp fragment specific for AT_{1B} mRNA. Probe MMAT2: A 468-bp fragment from genomic mouse DNA, amplified by PCR using the genotyping

primers,[245] was subcloned in a T-vector. SP6 polymerase transcribed a radioactive probe complementary to a 258-bp-mRNA fragment specific for AT_2. Probe rL32: A commercially available rL32 probe template (PharMingen International, USA), complementary to a 127-bp fragment of rL32 mRNA, was used as a housekeeping control as described earlier.[247,250] RNA samples were hybridized with 40,000 cpm of the radio labeled probe for MMAT1A, MMAT1, or MMAT2 and 20,000 cpm of the radio labeled probe for rL32 as the housekeeping gene. The hybridized fragments protected from RNase A + T1 digestion, were separated by electrophoresis on a denaturing gel (5% polyacrylamid, 8 mol/L urea) and analyzed using a FUJIX BAS 2000 Phospho-Imager system (Raytest GmbH, Straubenhardt, Germany).

Blood pressure measurement

Mice of all genotypes were weighed and anesthetized with pentobarbital (60 mg/kg; i.p.). The left carotid artery and right jugular vein were cannulated using a PE-10 (0.011"IDx0.025"OD) catheter. Heart rate and mean arterial pressure (MAP) were monitored continuously with a disposable pressure transducer (TSE Systems GmbH, Bad Homburg, Germany) connected to the catheter situated in the left carotid artery. After a stabilization period of 5-15 minutes, hemodynamic baseline values were determined. Heart rate and MAP data were recorded and digitalized using the Invasive Blood Pressure Monitoring System recording software (TSE Systems GmbH, Bad Homburg, Germany). The hemodynamic response to Ang II (0.5 μmol/L in a 100 μL bolus injection) was evaluated in animals of all eight genotypes. After heart rate and MAP had returned to baseline (within ~10 minutes), the response to 0.5 mmol/L phenylephrine (PE; 100 μL) was determined, and this protocol was repeated for 2 μmol/L endothelin-1 (ET-1; 100 μL). At the end of the hemodynamic studies, kidneys and hearts were rapidly excised and weighed. Ratios of kidney weight/body weight (KW/BW) and heart weight/body weight (HW/BW) were used as indices of organ hypertrophy.

Langendorff heart

Mice were killed by cervical dislocation.[215] The heart was rapidly excised and placed in ice-cold modified Krebs-Henseleit (KH) buffer (composition in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2,$CaCl_2$ 1.2, D-glucose 11, $NaHCO_3$ 25, pyruvic acid 2), gassed with 95% O2 and 5% CO2.[62,215,216] The aorta was immediately cannulated with a 19G needle (with a small circumferential grove close to the blunt tip) and perfused with gassed KH buffer according to Langendorff at a constant perfusion pressure of 80 mmHg.[217] Two needle electrodes were placed at the right atrium and the hearts were paced at ~600 bpm (5 Hz, 4 ms duration, 4 Volt) using a Grass stimulator (Grass Instruments Co., Quincy, Massachusetts, USA). Left ventricular systolic pressure (LVSP) was measured with a water-filled balloon (made of domestic food wrap) connected to a disposable pressure transducer (Braun, Melsungen, Germany). The left atrium was removed and the balloon was inserted into the left ventricle.[217,218] The left ventricular end-diastolic pressure was set at 3-5 mmHg by adjusting the balloon volume. Coronary flow (CF) was measured with a flow probe

(Transonic systems, Ithaca, New York, USA). After a stabilization period of 10-15 minutes, baseline values of CF and LVSP were obtained. Next, bolus injections (100 μL) of modified KH buffer were applied three times to determine injection-induced changes in CF and LVSP. Subsequently, bolus injections (100 μL) of Ang II (concentration range 0.1 nmol/L-0.1 mmol/L) were applied.

Angiotensin II levels in blood plasma

Blood (130-610 μL, mean 278 μL) was collected from the abdominal aorta in 2.5 mL of 4 mol/L guanine thiocyanate.[187] Both were stored at -80°C until further processing. Ang II was determined by radioimmunoassay following SepPak extraction and high-performance liquid chromatography separation as described before.[106]

Histology

Kidneys were isolated and fixed in 10% formalin. The kidneys were embedded in paraffin and sectioned at 2 μm. Serial sections of kidneys were stained with haematoxylin-eosin (HE) or periodic acid-Schiff (PAS) as described previously.[251]

Statistics

Results are represented as mean±SEM or median and interquartile range. For determination of intergroup differences, Kruskal-Wallis test was used (Graph Pad Prism 3.01; Graph Pad Software Inc., San Diego, California, USA). The Mann-Whitney U test was employed to analyze the differences in parameters among the groups. Significance was considered from a value of $P<0.05$.

Results

Generation of animals lacking one, two, or all three known Ang II receptors

Crossbreeding of animals deficient for AT_{1A} (-/-//+/+//+/+), AT_{1B} (+/+//-/-//+/+), or AT_2 (+/+//+/+//-/-) was used to generate mice lacking two (AT_{1A}/AT_{1B}: -/-//-/-//+/+, AT_{1A}/AT_2: -/-//+/+//-/-, and AT_{1B}/AT_2: +/+//-/-//-/-) or all three (-/-//-/-//-/-) known Ang II receptors. Homozygosity in the eight genotype combinations possible has been proven by PCR after weaning and before experiments. Figure 1A shows exemplarily PCR results identifying a wild-type (+/+//+/+//+/+) mouse (upper panel) and a triple-knockout (lower panel). All eight receptor combinations were vital and fertile, although survival in AT_{1A}/AT_{1B}-double- and in the triple-knockout mice was significantly impaired until weaning (Figure 1B). However, mortality was not increased in these mice from weaning to the defined endpoint of twelve months (data not shown).

Regulation of Ang II receptors in different knockout variants

To clarify the impact of the genetic modifications on the expression level of the three Ang II receptors, we measured receptor-specific mRNA in the heart. Figure 1C shows representative RPA blots of heart RNA using an AT_{1A}-specific probe (MMAT1A). All

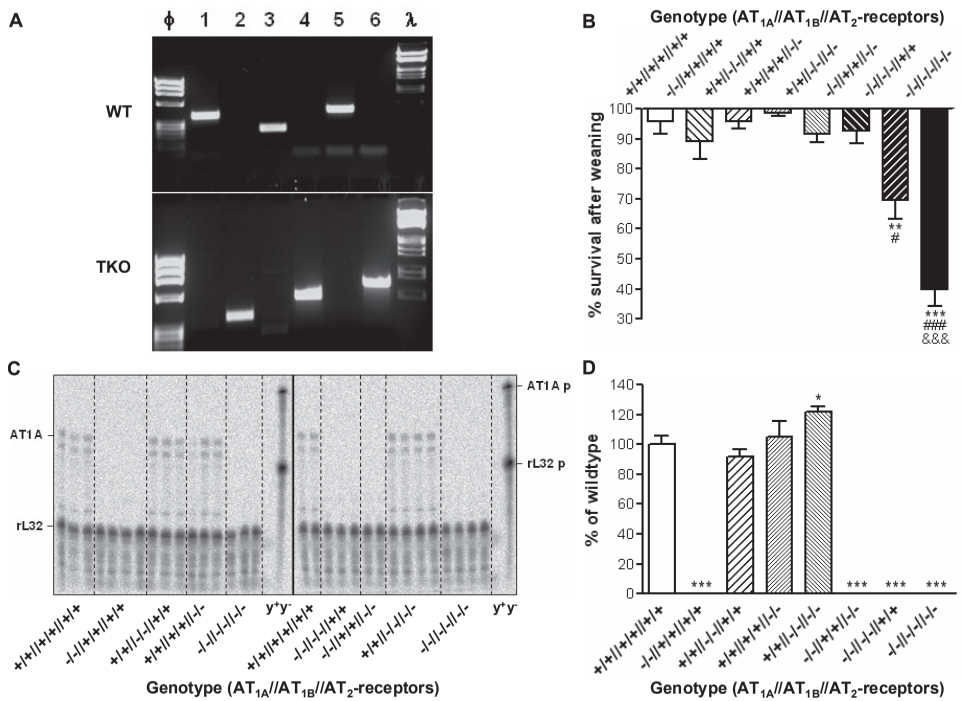


Figure 1. *The genotyping PCRs for AT_{1A} (lanes 1 and 2), AT_{1B} (lanes 3 and 4), and AT_2 (lanes 5 and 6) produced distinct bands for wild-type (upper row) and knockout (lower row) alleles depending on the appearance or absence of the Ang II receptors. WT: wild-type; TKO: triple-knockout; ϕ: ϕX174 DNA (BsuRI digested) size marker; λ: λDNA (EcoRI+HindIII digested) size marker (A). Survival of pups until weaning for all eight genotypes. Data are shown as survived mice per litter in percent. ** P<0.01; *** P<0.001 vs. +/+//+/+//+/+; # P<0.05; ### P<0.001 vs. -/-//+/+//+/+; &&& P<0.001 vs. -/-//-/-//+/+; n≥8. (B) Representative RNase-protection assays showing AT1A receptor (352 bp) and rL32 (127 bp) expression in the heart. The undigested AT1A and rL32 probes are shown in y- control lane. y+: yeast RNA with RNase A; y-: yeast RNA without RNase A (C). Quantification of AT_{1A} expression in mouse hearts of all eight genotypes after autoradiographic signal analysis. Data are shown as multiples after normalization to rL32 mRNA levels. AT_{1A} expression levels in WT were set to 100%. * P<0.05; *** P<0.001 vs. +/+//+/+//+/+; n≥6.Model of angiotensin generation at cardiac tissue sites (D).*

Table 1. *Data summary of the basal characterization of animals deficient for one, two, or all three Ang II receptor subtypes (BW: body weight; HW: heart weight; KW: kidney weight; MAP: mean arterial pressure; HR: heart rate; CF: coronary flow; LVSP: left ventricular systolic pressure; * P<0.05; ** P<0.01; *** P<0.001 vs. +/+//+/+//+/+; # P<0.05; ## P<0.01 vs. -/-//+/+//+/+; & P<0.05 vs. -/-//-/-//+/+; n≥6).*

Genotype	+/+//+/+//+/+	-/-//+/+//+/+	+/+//-/-//+/+	+/+//+/+//-/-	+/+//-/-//-/-	-/-//+/+//-/-	-/-//-/-//+/+	-/-//-/-//-/-
BW (g)	35.15±0.86	37.10±1.26	36.17±0.98	36.39±0.89	39.61±1.14**	37.26±1.05	28.47±0.70***	31.31±0.62** &
HW/BW (mg/g)	4.82±0.06	4.34±0.07***	4.66±0.14	4.53±0.07*	4.84±0.10	4.30±0.09***	4.03±0.10*** #	4.33±0.16**
KW/BW (mg/g)	7.42±0.27	7.40±0.36	6.77±0.19	6.34±0.31*	6.58±0.17**	6.10±0.34**	6.13±0.22*	5.30±0.18*** &
MAP (mmHg)	61.82±2.87	44.91±1.83***	65.30±2.23	78.09±3.70**	72.60±3.00*	43.50±1.34***	34.38±0.75*** ###	34.29±1.67*** ##
HR (1/min)	385.6±15.6	349.7±21,71***	404.5±22.6	389.8±17.3	372.2±17.5	312.9±25.4**	293.7±11.0***	259.3±8.7*** ##
CF (mL/min)	2.5±0.2	2.7±0.1	3.0±0.3	2.8±0.1	3.1±0.1	3.0±0.2	2.4±0.3	2.5±0.3
LVSP (mm Hg)	98.5±3.7	114.3±6.8	98.2±6.3	96.5±3.5	98.9±8.3	112.6±7.1	86.0±6.2	72.3±6.7

animals not deficient for AT_{1A} (+/+//++//+/+, +/+//-/-//+/+, +/+//+/+//-/-, and +/+//-/-//-/-) have been characterized by detectable AT_{1A}-specific bands, whereby AT_{1A}-RNA expression was significantly increased only in animals exclusively expressing AT_{1A} (Figure 1D; +/+//-/-//-/-). Investigating the samples with a probe discriminating between AT_{1A} and AT_{1B} (MMAT1) revealed the same pattern of regulation for AT_{1A} mRNA. However, AT_{1B}- and AT_2-specific mRNA expression was below RPA detection level, independent of the receptor combination used (data not shown) as proven with this probe and the specific one for AT_2 mRNA (MMAT2).

Basal phenotyping

Body weight recording confirmed previous findings that the lack of just one Ang II receptor did not influence body weight (Table 1).[47,60,242] The newly generated double-knockout variants (+/+//-/-//-/- and -/-//+/+//-/-) showed no change in BW, whereas AT_{1A}/AT_{1B}-double-knockout mice were characterized by a significant weight reduction, as described previously.[242] Interestingly, this body weight reduction was partially compensated in triple-knockouts. In all variants deficient for AT_{1A} the HW/BW was decreased (Table 1) with the lowest heart weight in AT_{1A}/AT_{1B}-double-knockouts. A less pronounced heart weight reduction was seen in AT_2-deficient animals. KW/BW ratio was unaltered in AT_{1A}- and AT_{1A}-single-knockout mice (Table 1). In contrast to them animals deficient for AT_2 showed a reduced KW/BW ratio. Kidney weight was also reduced in our newly generated mice exclusively expressing either AT_{1A} (+/+//-/-//-/-) or AT_{1B} (-/-//+/+//-/-). As described previously, in animals lacking both AT_1 subtypes KW/BW ratio was significantly reduced.[242] However, we observed a further, profound reduction of KW/BW ratio in triple-knockout animals. To investigate the regulation of endogenous Ang II, depending on expression of its receptors, we determined Ang II levels in plasma. In mice lacking only one Ang II receptor, independently of the subtype, plasma Ang II levels were not changed significantly (Figure 2). In mice only expressing AT_{1A}, Ang II plasma level was unchanged (98.8±34.0 fmol/mL), compared to WT (81.0±9.8 fmol/mL), whereas mice exclusively expressing AT_{1B} showed a rise in plasma Ang II (270.7±80.7 fmol/mL). Mice deficient for both AT_1 subtypes had the highest Ang

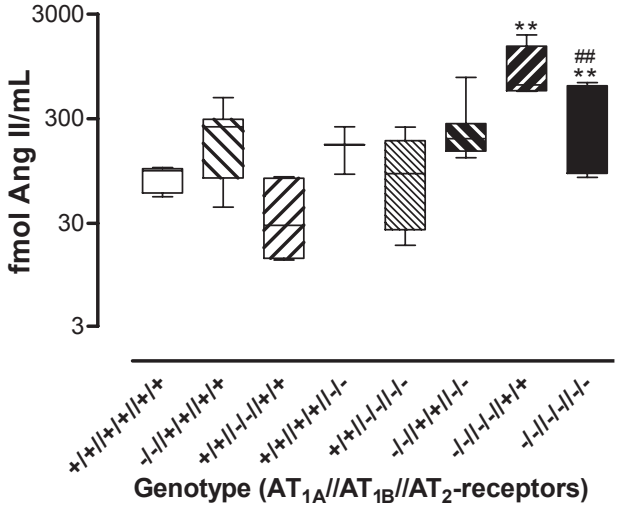


Figure 2. *Ang II levels in blood plasma of mice of all eight genotypes. Boxes show median with interquartile range. **P<0.01 vs. +/+//+/+//+/+; ## P<0.01 vs. -/-//+/+//+/+; n=2 for +/+//+/+//-/- and n≥5 for all other groups.*

II plasma concentration (885.8±217.2 fmol/mL). Notably, Ang II levels in triple-knockouts (315.8±104.7 fmol/mL) ranged between WT and AT_{1A}/AT_{1B}-double-knockouts without significant differences compared to any other group.

Renal histopathological examination

As described earlier, kidneys of AT_2-deficient animals showed comparable morphology to wild-type (Figure 3; upper panel, left) and were characterized by inconspicuous histology (Figure 3; middle panel, left).[204] Investigations on renal pathology also confirmed the previously described renal phenotype of the double-knockouts deficient for the two AT_1 subtypes, including cystic dilated cavities, partially dilated tubular lumina, multifocal and focally extensive glomeruli atrophy with a severely dilated Bowman's space (Figure 3; lower panel, left), and media hyperplasia of small and medium sized vessels.[242] Furthermore, kidneys of these AT_{1A}/AT_{1B}-deficient mice showed prominent renal mononuclear infiltrates, partially with blastic appearance and a high partially mitotic rate including bizarre mitotic figures (data not shown). To test whether the renal phenotype in triple-knockouts could be discriminated from that of kidneys deficient in both AT_1 subtypes, comparative histological analyses were accomplished. The additional deficiency in AT_2 did not impact the renal phenotype as the histological phenotype was comparable between both genotypes. Nevertheless, the number of kidneys with severe hydronephrosis was significantly higher in triple-knockout mice (data not shown).

We then examined the kidneys of the newly generated double-knockout animals expressing exclusively either AT_{1A} or AT_{1B} to investigate if one of the AT_1 subtypes alone is capable to restore the kidney morphology. Kidneys of mice expressing only AT_{1A} had hyperaemic capillary loops, but their glomeruli were well perfused and showed distinct basal membranes (Figure 3; middle panel, middle). In contrast, kidneys of mice harboring only the AT_{1b} showed a multifocal glomeruli atrophy, partially with cystic dilated Bowman's space (Figure 3; middle panel, right). Nevertheless, compared to the severely changed phenotype observed in kidneys of AT_{1A}/AT_{1B}-double-knockouts and triple-knockouts, kidneys harboring only the AT_{1B} had less damaged glomeruli and the majority was without pathological findings. To finally investigate whether the renal phenotype in mice lacking all three Ang II receptors was comparable to that of mice keeping all receptors but lacking the ligand (Ang II), we also analyzed kidneys of mice lacking the angiotensinogen gene (Agt) coding for the angiotensin precursor. Morphohistological investigations revealed that kidneys of Agt-deficient mice showed the same malformations (Figure 3, lower panel, right) as mice lacking both AT_1 subtypes and as the triple-knockouts.

Impact of Ang II receptors on basal hemodynamic parameters

Baseline MAP in AT_{1A}-deficient mice was markedly reduced (Table 1) compared to wild-type animals. The lack of only the AT_{1B} did not have an influence on basal MAP, whereas the additional AT_{1B} deficiency in AT_{1A}-knockout mice led to a further drop in blood pressure as described previously.[242] We also confirmed the rise in blood pressure in AT_2-knockout mice.[60] However, in contrast to the AT_{1B}, the lack of AT_2 on

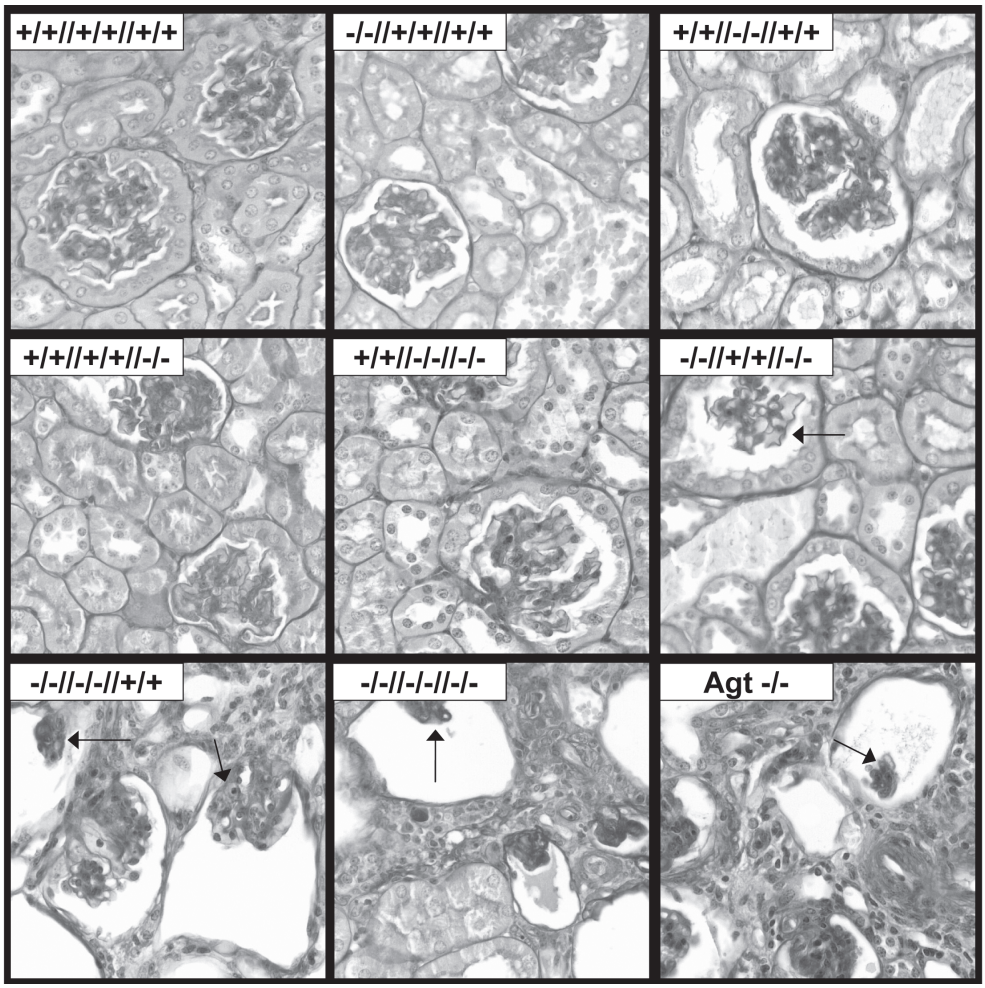


Figure 3. *PAS-stained sections of kidneys of WT (+/+//+/+//+/+), AT_{1A}- (-/-//+/+//+/+), AT_{1B}- (+/+//-/-//+/+), and AT_2-single-knockout mice, mice exclusively expressing AT_{1A} (+/+//-/-//-/-), AT_{1B} (-/-//+/+//-/-) or AT_2 (-/-//-/-//+/+), triple-knockouts (-/-//-/-//-/-), and angiotensinogen-deficient (Agt -/-) mice. Atrophic glomeruli are indicated by an arrow.*

top of AT_{1A} deficiency did not modify the hypotensive phenotype. Interestingly, also the AT_{1B} deficiency did not alter the elevated blood pressure observed in AT_2-single-knockouts. The ineffectiveness of AT_2 on blood pressure control in animals lacking AT_1 was further confirmed in triple-knockouts that did not differ in their MAP from AT_{1A}/AT_{1B}-double-knockout mice.

Heart rate in all genotypes lacking AT_{1A} was significantly reduced (Table 1). Deficiency for both AT_{1A} and AT_{1B} led to a further, but not significant reduction in heart rate, whereas this reduction reached significance in animals lacking all three receptors.

Effects of infusions of vasoactive substances

To clarify the impact of the three Ang II receptors on blood pressure and heart rate control, we administered a single bolus injection of Ang II. In wild-type mice, Ang II injection caused a fulminant increase in MAP and heart rate (Figure 4A and 4B). In AT_{1A}-single-knockout mice, Ang II effects on MAP and heart rate were reduced by almost 85% and 65% respectively. In AT_{1A}/AT_{1B}-double-knockout and triple-knockout mice the Ang II-stimulated effect on MAP was almost completely blunted, whereas in both genotypes the decrease in heart rate was unaffected compared to AT_{1A}-single-knockouts.

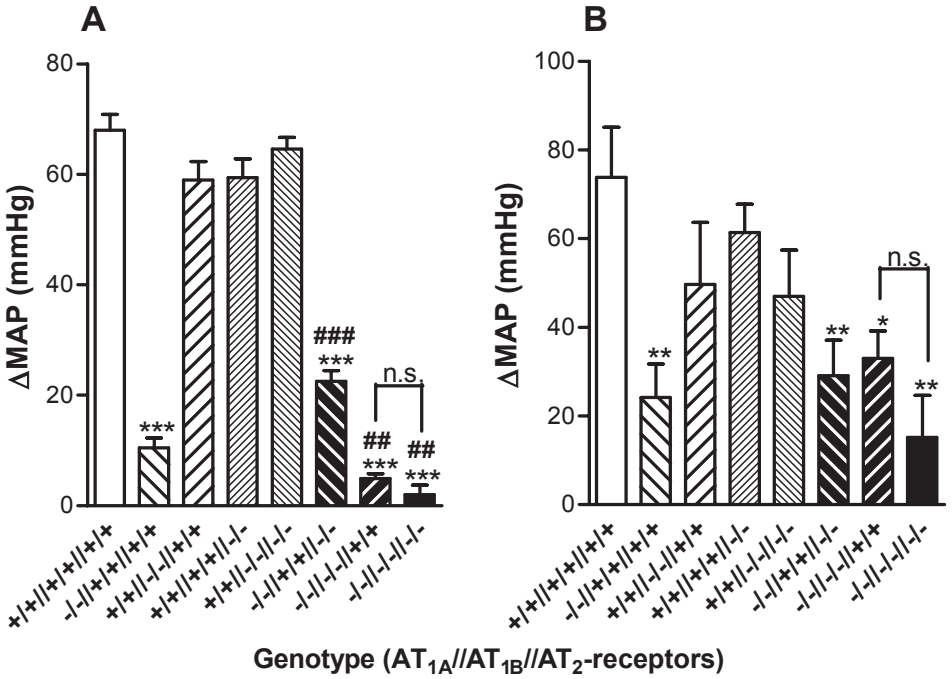


Figure 4. *Changes in MAP (A) and HR (B) after a bolus injection of Ang II. MAP: mean arterial pressure; HR: heart rate; ΔMAP: change in MAP after injection; ΔHR: change in HR after injection. * P<0.05; ** P<0.01; *** P<0.001 vs. +/+//+/+//+/+; # P<0.05; ## P<0.01; ### P<0.001 vs. -/-//+/+//+/+; n≥6.*

To prove whether the deletion of AT_{1A} alone or in combination with either AT_{1B} or AT_2 or both led to a general impairment in vasoconstrictive properties in those animals we infused two other vasoactive compounds. Firstly, we administered the peptide ET-1 as an Ang II-independent vasoconstrictor. ET-1 mediates vasoconstriction by stimulation of its own receptors, the endothelin type A and type B receptors. ET-1 infusion led to a rise in MAP in all eight genotypes without significant differences (Figure 5A). Interestingly, the negative chronotropic effect of ET-1 in wild-type animals (Figure 5B) changed inversely to a positive chronotropic effect in animals deficient for either AT_{1A} or AT_2. In AT_{1A}/AT_{1B}-double-knockout mice ET-1 effects were

not inverted but heart rate reduction was less pronounced than in wild-type controls. Nevertheless, ET-1 infusion again mediated a positive chronotropic effect in animals deficient for all three Ang II receptor subtypes.

Secondly we used the non-peptidic sympathicomimetic compound PE. In contrast to ET-1, PE-stimulated α_1-adrenoceptor-mediated increase in MAP was comparably reduced in AT_{1A}/AT_{1B}-double- (Figure 6A) and triple-knockout animals compared to controls. In wild-type mice, administration of PE reduced heart rate by 12 % (Figure 6B). PE effect on heart rate was inverted in AT_{1A}-single-knockout mice to a positive chronotropic response, while deletion of AT_{1B} and AT_2 alone did not influence changes in heart rate observed in wild-type animals. Notably, additional AT_{1b} deficiency reversed the positive chronotropic effects of PE infusion seen in AT_{1A}-single-knockouts. In contrast to all other genotype variants, PE infusion did not mediate any chronotropic effect in animals lacking all three receptor subtypes.

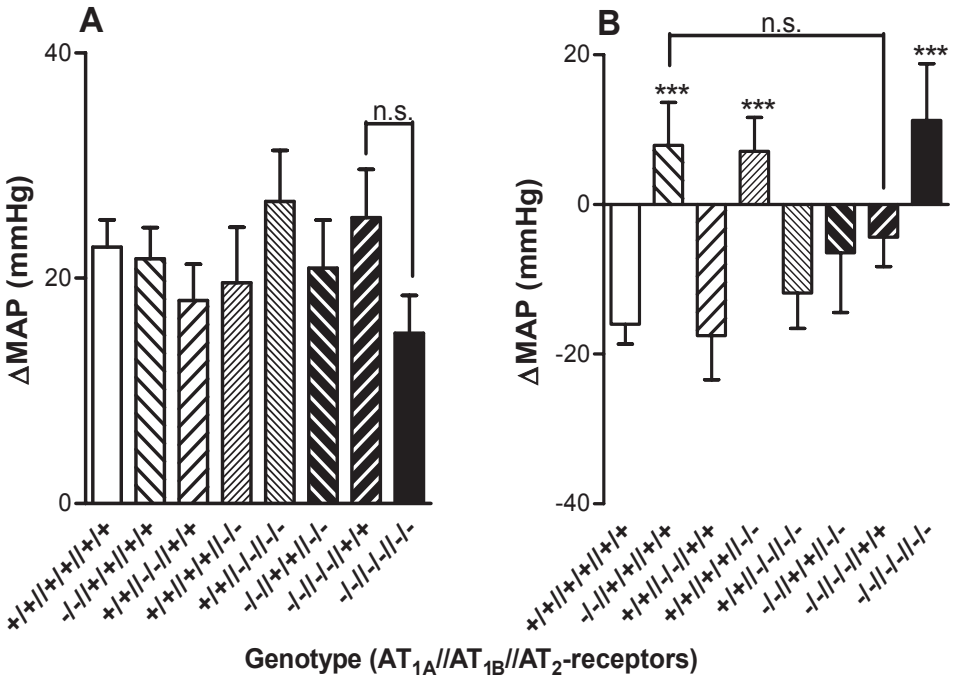


Figure 5. *Changes in MAP (A) and HR (B) after a bolus injection of ET-1. MAP: mean arterial pressure; HR: heart rate; ΔMAP: change in MAP after injection; ΔHR: change in HR after injection. *** P<0.001 vs. +/+//+/+//+/+; n≥6.*

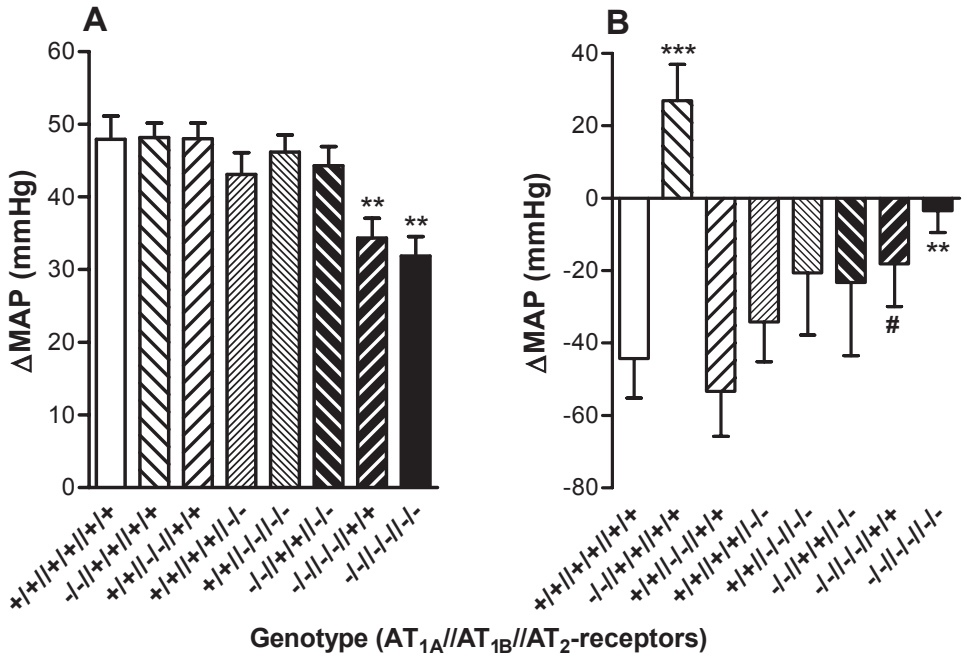


Figure 6. *Changes in MAP (A) and HR (B) after a bolus injection of PE. MAP: mean arterial pressure; HR: heart rate; ΔMAP: change in MAP after injection; ΔHR: change in HR after injection. * P<0.05; ** P<0.01; *** P<0.001 vs. +/+//+/+//+/+; # P<0.05 vs. -/-//+/+//+/+ n≥6.*

Langendorff heart

No genotype-related differences in baseline CF and LVSP were found (Table 1). In hearts of wildtype (+/+//+/+//+/+) mice, bolus injections of Ang II dose-dependently decreased CF and LVSP (Figure 7) by maximally 56±5% and 39±4%, respectively (pEC_{50}'s 7.51±0.17 and 7.35±0.13). Ang II concentrations >1 μmol/L did not result in effects that were larger than those observed at 1 μmol/L, in agreement with the concept of receptor desensitization.[220-222]

Bolus injections of Ang II exerted no effects in hearts of mice deficient for AT_{1A} (Figure 7), whereas effects in mice lacking only AT_{1B} or AT_2 were identical to those in wild-type.Effects in hearts lacking both AT_{1B} and AT_2 were also unaltered. These data indicate that the cardiac effects of Ang II are entirely AT_{1A} receptor-mediated.

Discussion

Targeting the RAS, specifically the effector peptide Ang II, represents a major opportunity for delaying or even preventing the progression of cardiovascular diseases and, in turn, reducing the risk of morbidity and mortality.[252,253] Inhibition of Ang II actions is achieved clinically by either blocking with antagonists against

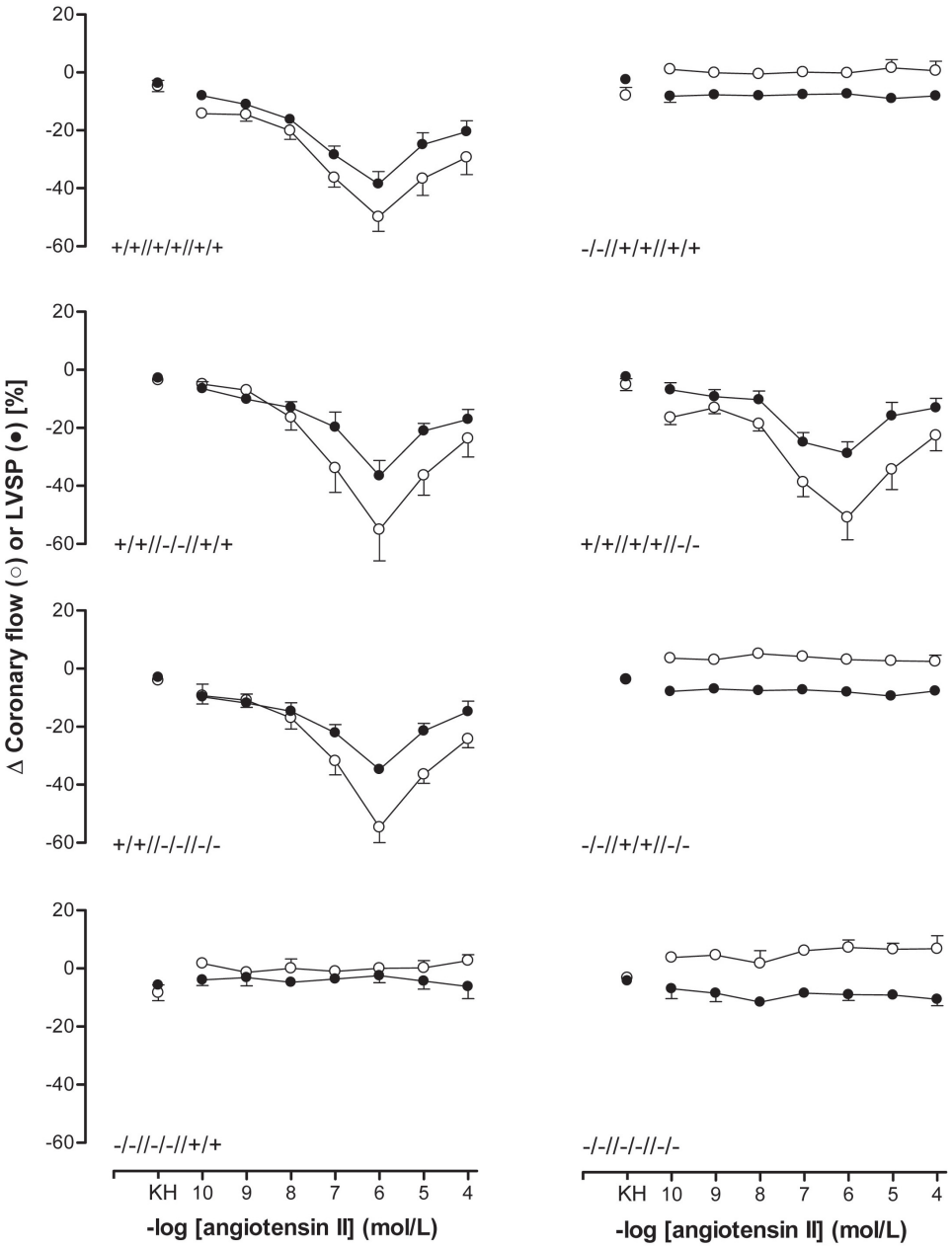


Figure 7. *Changes in CF (open symbols) and LVSP (closed symbols) after a bolus injection of Ang II in the Langendorff heart according to genotype. Data (mean+SEM of 4-8 experiments) represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer. The x-axis displays the Ang II concentration in the injection fluid.*

its type 1 receptors or preventing Ang II generation with ACE inhibitors.[254-256] Both pharmacological interventions are primarily used for blood pressure control, but research of the last decade has demonstrated that they also provide cardiovascular protective effects that are independent of blood pressure lowering, as e.g. evidence arose that the anti-inflammatory component of both drugs is part of their success.[257]
The generation of AT_1- and AT_2-specific antagonists gave a first tool that allowed identifying further pathways both drugs may benefit by, also due to defining functions specific for either AT_1 or AT_2. Nevertheless, the generation of animals overexpressing Ang II type 1 receptors lacking intracellular signaling via heterotrimeric G proteins has shown that AT_1 can mediate effects, e.g. induction of cardiac hypertrophy and bradycardia, independent of Ang II.[258] Therefore, animals deficient for AT_{1A} or AT_{1B} or AT_2 have been generated also to better discriminate between those ectopic and/or Ang II-independent receptor effects and receptor effects requiring the stimulation by Ang II. However, none of the single-receptor-knockouts is capable to reproduce the severe kidney malformations, pronounced blood pressure lowering, and reduced vitality seen in mice lacking Agt and hence lacking the ligand-specific receptor stimulation. Furthermore, using these animal models could also not fully answer the question regarding receptor specifity, since it could not be discriminated whether the observed phenotype was related to a lack in receptor stimulation due to the deficiency or due to an overstimulation of the residual two receptors. Therefore, we firstly generated new transgenic mice exclusively expressing either AT_{1A} or AT_{1B} for verification of Ang II-mediated AT_1 subtype-specific effects. Utilizing these animals and the previously described mice deficient for both AT_1 subtypes,[242] exclusively expressing AT_2, we could demonstrate that basal blood pressure and heart rate are mainly affected by AT_{1A}, while the role of AT_{1B} differs between both parameters. In mice exclusively expressing AT_{1A}, basal blood pressure is slightly increased (~118% of wild-type) probably due to a higher expression of AT_{1A} (Figure 1) and/or the missing inhibitory properties of AT_2 on AT_{1A} signalling.[61,259] The mice solely expressing AT_{1B} show a less pronounced decrease in blood pressure (~70% of wild-type) than the mice only expressing AT_2 (~55% of wild-type), indicating that AT_{1B} is partially capable to compensate the lack of AT_{1A}. Although, the reduced blood pressure in all groups lacking AT_{1A} may account for the reduced HW/BW-ratio, yet the lower HW/BW ratio in AT_2 deficient mice, which have elevated blood pressure, makes this conclusion less probable. In animals exclusively expressing AT_{1A} heart rate is unchanged compared to wild-type, whereas in mice lacking AT_{1A} and AT_{1B} it is reduced (~-23%). Mice uniquely expressing AT_{1B} show a similar reduction in heart rate (~-19%) to animals lacking only AT_{1A} (~-15%) or both AT_1 subtypes, suggesting that regarding the heart rate, the lack of AT_{1A} cannot be compensated by AT_{1B} in contrast to partial normalization of blood pressure. This difference may be best explained by the lack in valuable level of AT_{1B} in the heart but a significant expression of AT_{1B} in vessels.[44]
We also identified the formerly described blood pressure rising[260] and positive chronotropic[261] effects of exogenous Ang II to be primarily mediated by AT_{1A}. Ang II effects on blood pressure are normal in animals exclusively expressing AT_{1A}. However, the vasoconstrictive properties of Ang II are almost blunted in mice

lacking both AT_1 subtypes (~7% of wild-type), while the responsiveness to Ang II is partially restored in animals exclusively expressing AT_{1B} (~30% of wild-type). The Ang II-induced increase in heart rate in wild-type is not altered in animals harboring only AT_{1A} compared to wild-type. In mice exclusively expressing either AT_{1B} or AT_2, and thus lacking the AT_{1A}, the positive chronotropic effect of Ang II is substantially reduced (to ~40% and ~44% of wild-type, respectively). The inability of the AT_{1B} in compensating the lack in AT_{1A} expression on Ang II-induced positive chronotropic actions is similar to the effect described under basal conditions and may also be due to the aforementioned reasons.

The importance of the AT_{1A} subtype was further confirmed by our studies using the Langendorff model. Deletion of one, two or all three AT receptors did not affect baseline CF or LVSP in comparison to wild-type. The dose-dependent decrease of both CF and LVSP in response to Ang II was similar in all genotypes expressing the AT_{1A} receptor independently of presence or absence of AT_{1B} and/or AT_2. These effects were completely abolished in all genotypes deficient for AT_{1A}. Thus, AT_{1A} exclusively mediates the Ang II-induced negative inotropy and vasoconstriction in the mouse heart, in full agreement with previously published data.[62]

In recent investigations it was shown that the chronic infusion of Ang II[262] and the lack of both AT_1 subtypes[263] are regulators of the endothelin system, e.g. affecting expression levels of ET-1 and its receptors. However, to our knowledge we are the first administrating ET-1 to mice lacking Ang II receptors. The ET-1-mediated rise in blood pressure is similar in all eight genotypes. Thus, the previously described up-regulation of the two ET-1 receptors in mice deficient for AT_{1A} and AT_{1B}[263] may not have an effect on blood pressure in our *in vivo* experiments. Interestingly, we have identified ET-1 effects on heart rate depending on the combination of expressed/lacking Ang II receptors. However, these distinct regulatory effects of the Ang II receptor subtypes on the endothelin system and their clinical relevance need further investigation.

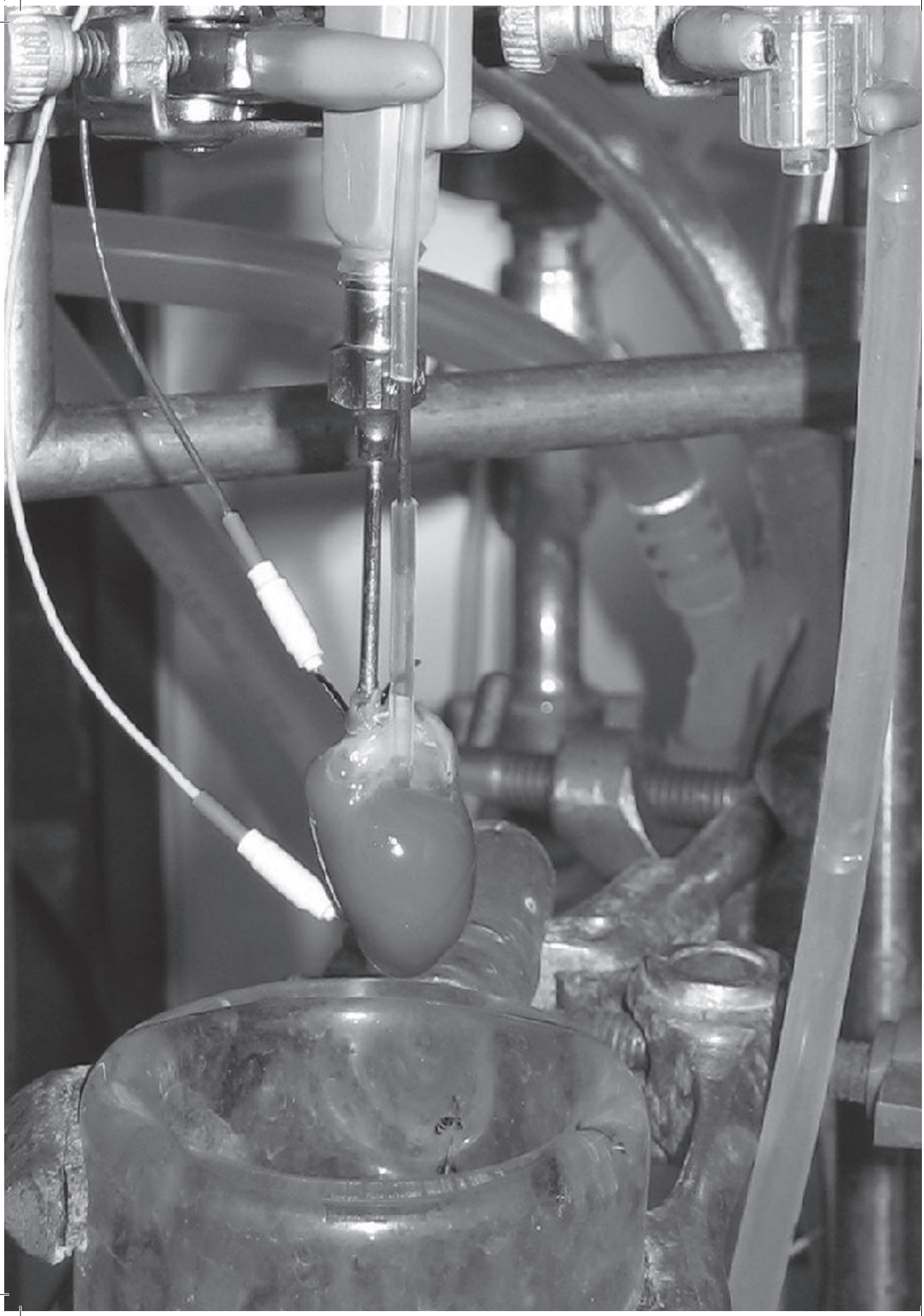
This may also count for the regulation of the sympathetic system which can be influenced by the RAS as it has been described previously.[264-267] Our data illustrates the importance of AT_1 receptors on the regulation of the sympathetic system.

Our findings also show that the previously described abnormal kidney structure, including cystic dilatations, atrophic glomeruli, and hydronephrosis, in mice lacking either both AT_1 subtypes[242] or Agt[26] is primarily caused by the lack of Ang II stimulation of AT_{1A}. This is best illustrated as the expression of only AT_{1A} preserves a normally working, healthy kidney, whereas expression of AT_{1B} alone leads to an intermediate phenotype, anticipating the development of hydronephrosis, interstitial infiltrates, and partially the atrophy of glomeruli.

To finally discriminate Ang II effects independent of its known receptors AT_1 and AT_2 and receptor effects that are Ang II independent, we also generated mice lacking all three receptor subtypes. Infusion of Ang II in the triple-knockouts generates the same effects on blood pressure and heart rate as in mice lacking both AT_1 subtypes, indicating that the AT_2 has no direct effect on these parameters and that no unknown Ang II receptors are directly involved in blood pressure and heart rate

regulation effected by Ang II. The lack of all three Ang II receptors also results in a comparable kidney morphology as seen in Agt-deficient animals, indicating that AT_1 stimulation by Ang II is a necessity for normal development of the kidney and that the lack of other bioactive Ang fragments in Agt-deficient mice, as e.g. Ang-(1-7) and Ang IV, does not influence renal pathology and thus may not play a role in kidney development. However, the more frequent occurrence of hydronephrosis in triple-knockouts compared to AT_{1A}/AT_{1B}-double-deficient animals may suggest a kidney-protective role for AT_2.

Beside their AT_1-depending effects on blood pressure reduction, several AT_1 antagonists have postulated pleiotropic effects, e.g. acting as a partial agonist on peroxisome proliferator-activated receptor γ, influencing platelet aggregation, and reducing uric acid levels in plasma.[264] Our generated animals lacking all three Ang II receptors are an excellent tool for investigation of these additional effects that may not involve AT_1- or AT_2-mediated actions. Thus, the newly generated triple-knockout mice are a very promising resource for further RAS-related research, since they can visualize effects of Ang II independent of its known receptors and identify effects of other Ang metabolites being dependent or independent of the AT_1 and AT_2.



Chapter 6

Effects of angiotensin II and its metabolites in the rat coronary vascular bed: is angiotensin III the preferred ligand of the AT_2 receptor?

Based on:

Joep H. M. van Esch, Chantal R. Oosterveer, Wendy W. Batenburg, Richard van Veghel, A. H. Jan Danser. Effects of angiotensin II and its metabolites in the rat coronary vascular bed: is angiotensin III the preferred ligand of the AT_2 receptor?

Submitted, 2008

Abstract

Aminopeptidases metabolize Angiotensin (Ang) II to Ang-(2-8) (=Ang III) and Ang-(3-8) (=Ang IV), and carboxypeptidases generate Ang-(1-7) from Ang I and II. Angiotensin-converting enzyme (ACE) inhibitors and/or Ang II type 1 (AT_1) receptor blockers affect the concentrations of these metabolites, and they may thus contribute to the beneficial effects of these drugs, possibly through stimulation of non-classical AT receptors. Here, we investigated the effects of Ang II, Ang III, Ang IV and Ang-(1-7) in the rat coronary vascular bed, with or without AT_1 - or Ang II type 2 (AT_2) receptor blockade. Results were compared to those in rat iliac arteries and abdominal aortas. Ang II, Ang III and Ang IV constricted coronary arteries via AT_1 receptor stimulation, Ang III and Ang IV being ≈20- and ≈8000-fold less potent than Ang II. The AT_2 receptor antagonist PD123319 greatly enhanced the constrictor effects of Ang III, starting at Ang III concentrations in the low nanomolar range. PD123319 enhanced the Ang II-induced constriction at submicromolar Ang II concentrations only. Ang-(1-7) exerted no effects in the coronary circulation, although, at micromolar concentrations, it blocked AT_1 receptor-induced constriction. AT_2 receptor-mediated relaxation did not occur in iliac arteries and abdominal aortas, and the constrictor effects of the Ang metabolites in these vessels were identical to those in the coronary vascular bed. In conclusion, AT_2 receptor activation in the rat coronary vascular bed results in vasodilation, and Ang III rather than Ang II is the preferred agonist of these receptors. Ang II, Ang III, Ang IV and Ang-(1-7) do not exert effects through non-classical AT receptors in the rat coronary vascular bed, iliac artery or aorta.

Introduction

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and body fluid homeostasis. The main effector peptide of the RAS, the octapeptide angiotensin (Ang) II (Ang-(1-8)), formed from the decapeptide Ang I (Ang-(1-10)) by Ang-converting enzyme (ACE), exerts its effect through activation of Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. AT_1 receptors mediate the well-known vasoconstrictor and growth-stimulatory effects of Ang II, and AT_2 receptors are generally assumed to counteract these actions[57-60,205,206].

Several Ang metabolites other than Ang II are currently believed to have effects of their own[94], in particular Ang III (Ang-(2-8)), Ang IV (Ang-(3-8)) and Ang-(1-7). These metabolites stimulate the two above Ang receptors and/or newly discovered receptors[85,98]. Ang III and Ang IV are formed from Ang II by the aminopeptidases A and N, respectively[94]. Ang-(1-7) is formed from Ang I by neutral endopeptidase or prolyl endopeptidase[84], and from Ang II by propyl endopeptidase, prolyl carboxypeptidase or ACE2[37,84].

Ang III stimulates both AT_1 and AT_2 receptors. Its in-vivo potency is lower than that of Ang II[268,269], either because it binds with lower affinity[95], and/or because it is metabolized faster[270]. Ang IV is a weak agonist of AT_1 receptors [90] and occurs at low levels[271]. It was therefore initially thought to be biologically irrelevant. The discovery of a distinct Ang IV binding site, which was designated as the AT_4 receptor because of its specificity for Ang IV, changed this view[98]. AT_4 receptors are expressed in brain, heart, kidney and vascular smooth muscle cells[68,95], and might mediate the Ang IV-induced vasorelaxation. Recently, Ang IV was also reported to reverse endothelial dysfunction in ApoE-deficient mice via AT_4 and/or AT_2 receptor stimulation[272].

Ang-(1-7) induces relaxation in several vascular beds. The fact that A-779 [D-Ala^7-Ang-(1-7)], a selective Ang-(1-7) antagonist, was able to block this relaxation, led to the discovery of a specific Ang-(1-7) receptor, the Mas receptor[84,85]. As a slow substrate for ACE, Ang-(1-7) also functions as an ACE inhibitor, resulting in decreased Ang II formation and potentiation of bradykinin-induced vasodilatation[32]. Furthermore, depending upon the presence of Ang II, Ang-(1-7) may act both as an AT_1 receptor agonist [90] and antagonist[89].

ACE inhibitors and/or AT_1 receptor antagonists affect the levels of Ang-(1-7), Ang III and/or Ang IV[107,273,274], and part of the beneficial effect of RAS blockade might actually be due to these metabolites[275]. A careful evaluation of their effects in the cardiovascular system is therefore of great interest. In the present study we investigated the effects of Ang II, Ang III, Ang IV and Ang-(1-7) in the rat coronary vascular bed, using selective AT_1 and AT_2 receptor blockers (irbesartan and PD123319, respectively). This bed is of major cardiovascular importance, and expresses both AT_1 and AT_2 receptors[58,62]. Results were compared to those in isolated vessels (iliac artery and abdominal aorta), in order to allow a comparison with previously published data on this topic[268,269].

Materials and methods

Animals

Male Wistar rats (329±3 gram; *n*=57) were obtained from Harlan (Zeist, The Netherlands). All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Drugs

Ang II, Ang III, Ang IV, Ang-(1-7), (S)-1-{ [4-(di-methylamino)-3-methylphenyl]methyl}-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol[4,5-c]pyridine-6-carboxylic acid (PD123319; K_i = 12 nmol/L)[276], 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) and N$^{\omega}$-nitro-L-arginine methyl ester HCl (L-NAME) were purchased from Sigma (Zwijndrecht, The Netherlands). 2-Butyl-3-[2'-(1H-terazol-5-yl)biphenyl-4-yl)methyl)]-1,3-diazospironon-1-en-4-one (irbesartan; K_i = 1.6 nM) [277]was a kind gift of Sanofi-Synthelabo BV (Gouda, The Netherlands). Irbesartan was dissolved in 100% ethanol (10 mmol/L), whereas all other chemicals were dissolved in water. Stock solutions were stored in aliquots at -80ºC. On the day of the experiments stock solutions used for the Langendorff preparation were diluted with Tyrode's buffer (composition in mmol/L: NaCl 125, KCl 4.7, $NaH_2PO_4.2H_2O$ 0.43, $MgCl_2$ 1.0, $CaCl_2.H_2O$ 1.3, glucose.H_2O 9.1, $NaHCO_3$ 20; pH 7.4). This implies that, at an irbesartan concentration of 1 µM (see below), the perfusion fluid contained 0.01% ethanol. Previous studies have shown that such concentrations do not affect cardiac hemodynamics[278]. Stock solutions used for the Mulvany myographs were diluted in water.

Tissue collection

Male Wistar rats were anesthetized with pentobarbital (60 mg/kg i.p.). Hearts were rapidly excised and placed in ice-cold Tyrode's buffer, gassed with 95% O_2 and 5% CO_2. Subsequently, the iliac arteries and abdominal aorta were removed and either used directly or stored overnight in cold, oxygenated Krebs-Henseleit solution (composition in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25; pH 7.4). Such overnight storage does not affect vascular responsiveness[162,279].

Langendorff preparation

The aorta was immediately cannulated with a 21G needle with a small circumferential grove close to the blunt tip, and perfused with gassed (95% O_2 and 5% CO_2) Tyrode's buffer according to Langendorff at a constant perfusion pressure of 80 mm Hg. The hearts were paced at ~480 bpm (8 Hz, 4 ms duration, 6 Volt) using a Grass stimulator (Grass Instruments Co., Quincy, Massachusetts, USA) by placing one needle electrode at the right atrium and one at the apex.

After removal of the left atrium, a water-filled latex balloon (Durex, London, UK) was inserted into the left ventricle. The left ventricular end-diastolic pressure was set at 3-5 mmHg by adjusting the balloon volume. Coronary flow was measured with a flow

probe (Transonic systems, Ithaca, New York, USA). After a stabilization period of 30 min, baseline values of coronary flow were obtained. Next, bolus injections (100 μL) of Tyrode's buffer were applied three times to determine injection-induced changes in coronary flow. Subsequently, concentration-response curves to Ang II, Ang III, Ang IV or Ang-(1-7) (concentration range in the injection fluid 0.1 nmol/L – 0.1 mmol/L) were constructed by applying bolus injections (100 μL), in the absence or presence of 1 μmol/L irbesartan, PD123319 or Ang-(1-7) in the perfusion buffer[58,62]. All blockers were present in the perfusion buffer starting 15 min before the first bolus injection.

Mulvany myographs

Iliac arteries (diameter: 992±15 μm, *n*=148) and abdominal aortas (diameter: 1541±21 μm, *n*=145) were cut into ring segments of approximately 2 mm length and mounted in a Mulvany myograph (Danish Myograph Technology, Aarhus, Denmark) with separated 6-ml organ baths containing gassed (95% O_2 and 5% CO_2) Krebs-Henseleit buffer at 37°C. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure[164]. Following a 30-min stabilization period, the normalized vessels were exposed to 30 mmol/L KCl. The maximal contractile response was determined by exposing the vessels to 100 mmol/L KCl. Thereafter, the vessels were pre-incubated for 30 min in fresh buffer in absence or presence of 1 μmol/L irbesartan, PD123319 or Ang-(1-7). All contraction studies, except those involving PD123319, were performed after a pre-incubation of 30 min with the NO synthase inhibitor L-NAME (100 μmol/L), to increase the contractile response[162]. In order to study vasorelaxation, vessels were preconstricted with U46619 (10-100 nmol/L) in the absence or presence of 1 μmol/L irbesartan. Subsequently concentration-response curves (0.1 nmol/L – 1 μmol/L) were constructed to either Ang II, Ang III, Ang IV or Ang-(1-7).

Data analysis

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, Ohio, USA). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc, Natick, Massachusetts, USA). Six consecutive beats were selected for determination of coronary flow. Ang-induced changes in left ventricular systolic pressure were not evaluated, since they were within the range induced by Tyrode's buffer injection. Data are given as mean±standard error of the mean (SEM) and represent percentage change from baseline. The n number reflects the number of animals. In the Mulvany myographs, contractile responses were expressed as a percentage of the contraction to 100 mmol/L KCl, whereas dilatory responses were expressed as a percentage of the preconstriction with U46619.

Concentration-response curves were analysed as described[219], using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, California, USA), to determine the maximum effect (E_{max}) and pEC_{50} ($=-^{10}\log EC_{50}$) values. The pEC_{50} values refer to the agonist concentration in injection fluid of the Langendorff model and do not reflect the actual concentrations seen by the receptor. In the Mulvany myograph studies, Ang

III and Ang IV did not reach E_{max} at the highest concentrations used. We therefore determined the concentration required to obtain 5% of the K^+-induced contraction ($EC_{5\%K}^+$) in order to calculate $pEC_{5\%K}^+$ values[162]. Concentration-response curves were compared by two-way ANOVA, followed by post hoc evaluation according to Dunnet. E_{max}, EC_{50} or $pEC_{5\%K}^+$ values were compared by Student's t-test in case n=2, or when n>2, by one-way ANOVA, followed by post hoc evaluation according to Dunnet. P<0.05 was considered significant.

Results

Langendorff preparation

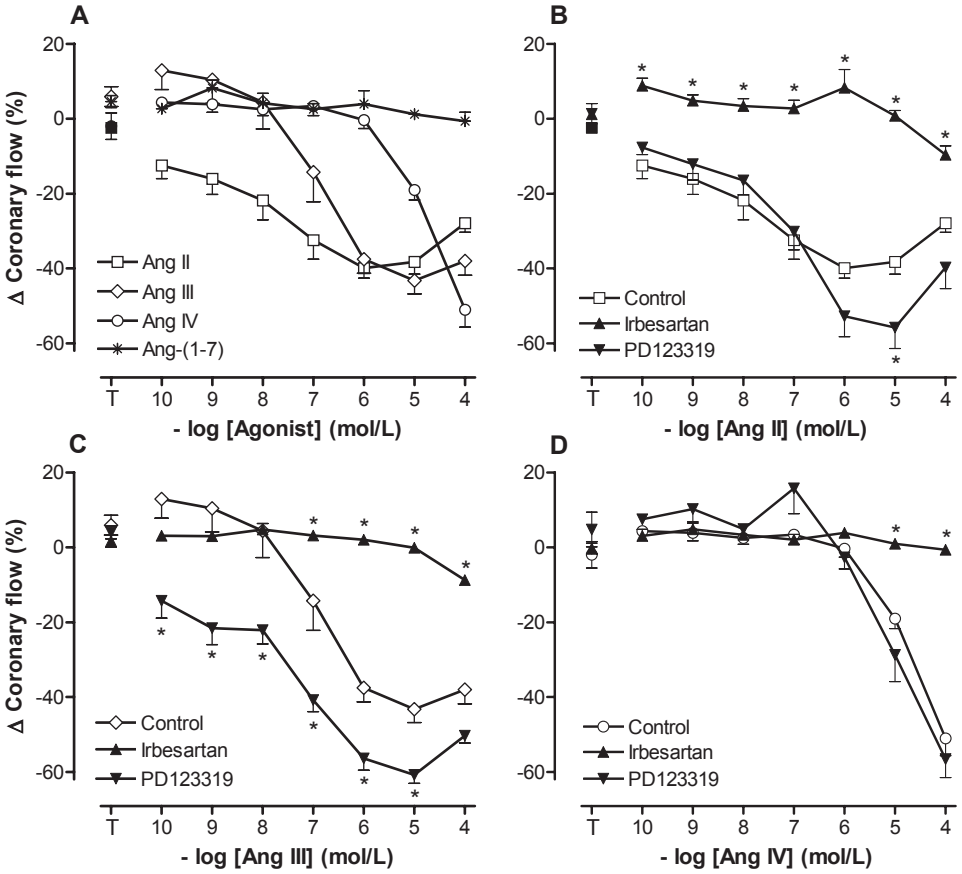


Figure 1. *Effects of Ang II , Ang III, Ang IV and Ang-(1-7) bolus injections (100 μL) on coronary flow in the rat Langendorff heart (A). Effects of Ang II (B), Ang III (C) and Ang IV bolus injections (100 μL), respectively, on coronary flow in the rat Langendorff heart in the absence or presence of 1 μmol/L irbesartan or 1 μM PD123319. The x-axis displays the concentration of the agonist in the injection fluid. Data are mean±SEM of 4-6 experiments and represent percentage change from baseline. T, bolus injection of Tyrode's buffer. *P<0.05 vs. control.*

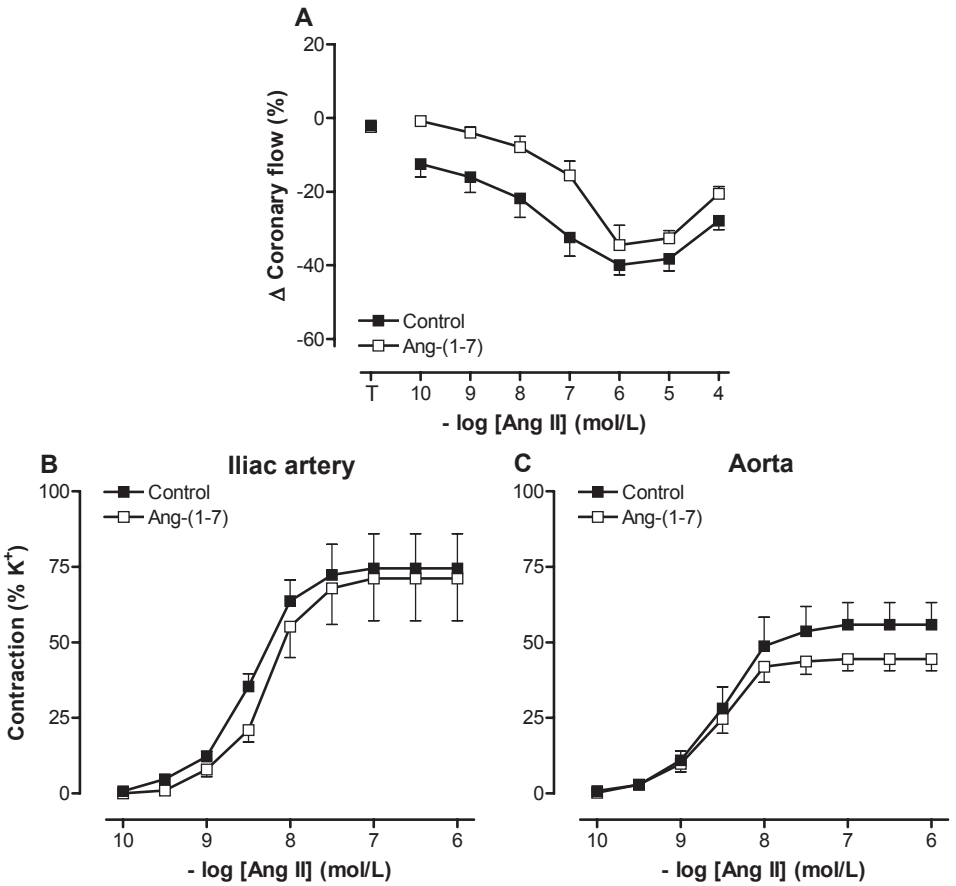


Figure 2. *Effects of Ang II bolus injections (100 μL) on coronary flow in the rat Langendorff heart in the absence or presence of 1 μmol/L Ang-(1-7) (A). The x-axis displays the Ang II concentration in the injection fluid. Data are mean±SEM of 4-5 experiments and represent percentage change from baseline. T, bolus injection of Tyrode's buffer. Effects of Ang II in L-NAME (100 μmol/L)-pretreated rat iliac arteries (B) and abdominal aortas (C), respectively, in the absence or presence of 1 μmol/L Ang-(1-7). The x-axis displays the concentration of the agonist in the organ bath fluid. Data are mean±SEM of 5-6 experiments and have been expressed as a percentage of the response to 100 mmol/L K^+.*

Baseline coronary flow (9.2±0.3 mL/min; *n*=54) was similar in all groups. Bolus injections with Tyrode's buffer injections did not significantly affect coronary flow (Fig. 1). Ang II, Ang III and Ang IV concentration-dependently decreased coronary flow, by maximally 42±3%, 43±4% and 51±5%, respectively, whereas Ang-(1-7) had no effect on coronary flow (Figure 1A). Ang II and Ang III concentrations >1 μmol/L did not result in effects that were larger than those observed at 1 μmol/L, in agreement with the concept of receptor desensitization[220-222]. Ang III (pEC_{50} 7.0±0.2, *n*=5) and Ang IV (pEC_{50} 4.7±0.1, *n*=5) were respectively ~20 and ~8000-fold less potent than Ang II (pEC_{50} 8.3±0.6, *n*=5). Irbesartan abolished all Ang II, Ang III and

Ang IV-induced flow changes (Figure 1B-D) and did not unmask effects of Ang-(1-7) (*n*=3, data not shown). PD123319 enhanced the effect of 10 μmol/L Ang II on coronary flow (P<0.05; *n*=6). PD123319 (P<0.05) enhanced the effect of Ang III on coronary flow at all Ang III concentrations (E_{max} 61±2%), and the potency of Ang III in the presence of PD123319 (pEC_{50} 7.3±0.1, *n*=5) was no longer different from that of Ang II. The effect of Ang IV on coronary flow was unaltered in presence of PD123319 (*n*=4). Ang-(1-7) (1 μmol/L) significantly (P<0.05) shifted the Ang II curve to the right (pEC_{50} 6.9±0.3, *n*=5; Figure. 2), without changing E_{max}.

Mulvany myographs

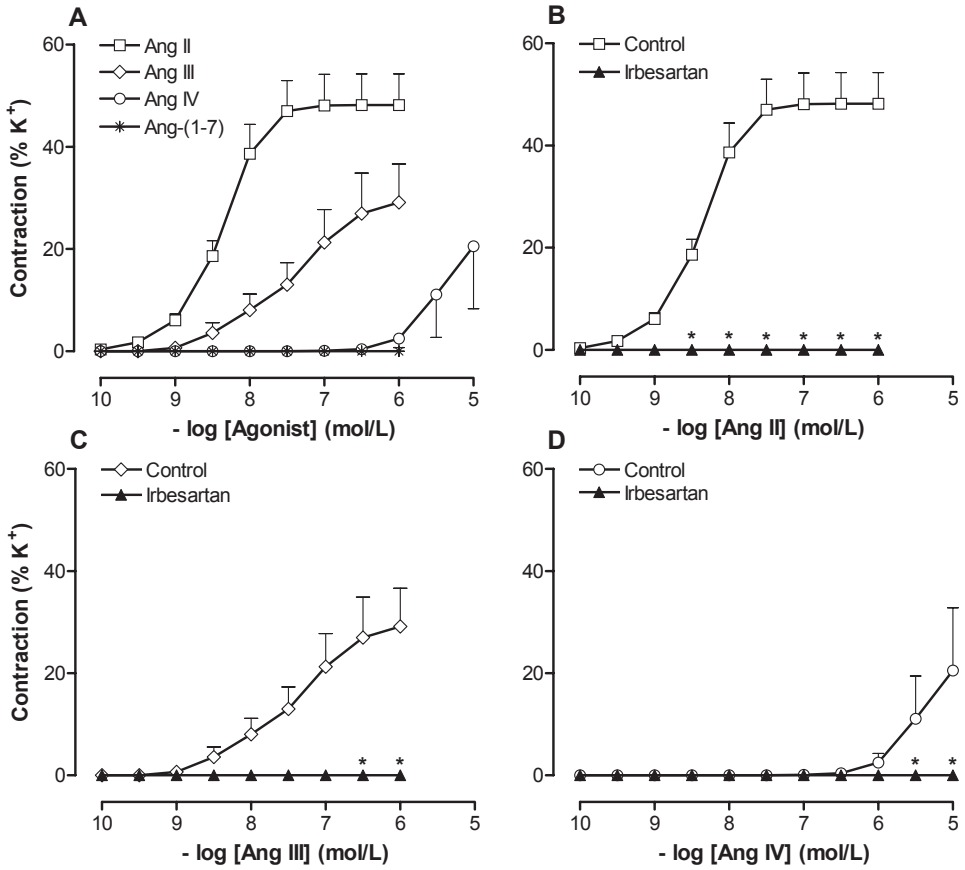


Figure 3. *Effects of Ang II, Ang III, Ang IV and Ang-(1-7) in L-NAME (100 μmol/L)-pretreated rat iliac arteries (A). Effects of Ang II (B), Ang III (C) and Ang IV (D), respectively, in L-NAME-pretreated rat iliac arteries in the absence or presence of 1 μmol/L irbesartan. The x-axis displays the concentration of the agonist in the organ bath fluid. Data are mean±SEM of 4-31 experiments and have been expressed as a percentage of the response to 100 mmol/L K^+. *P<0.05 vs. control.*

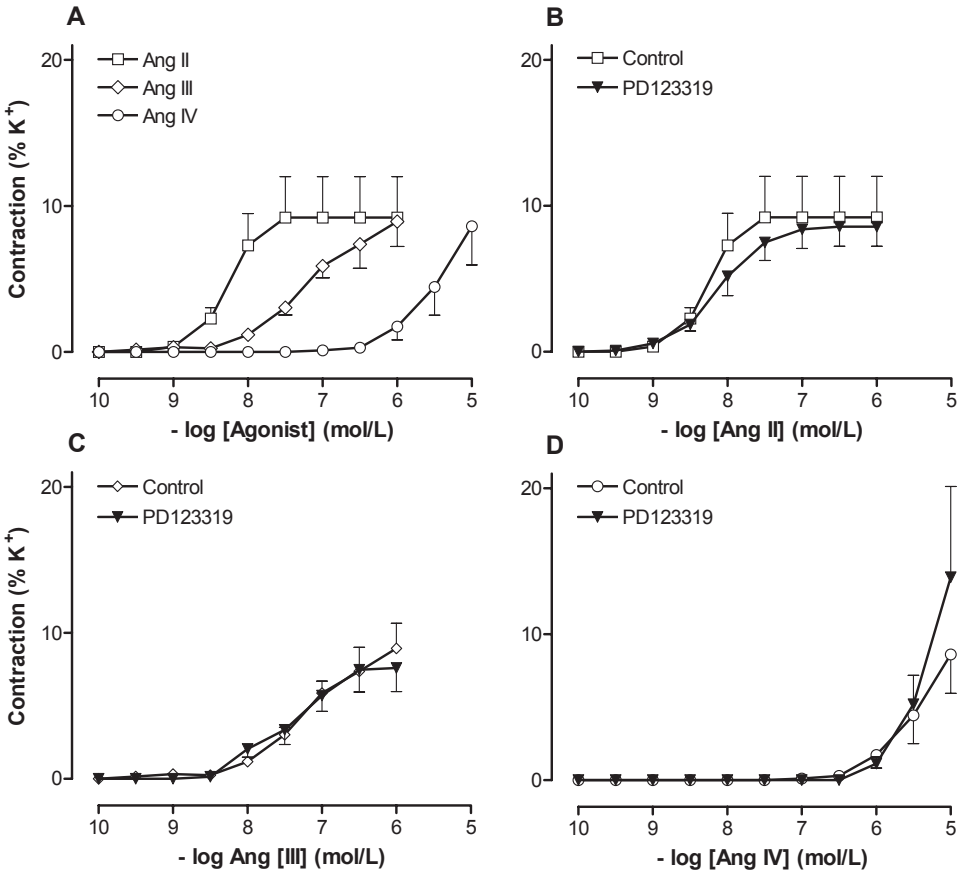


Figure 4. *Effects of Ang II, Ang III, Ang IV and Ang-(1-7) in rat iliac arteries (A). Effects of Ang II (B), Ang III (C) and Ang IV (D), respectively, in rat iliac arteries in the absence or presence of 1 μM PD123319. The x-axis displays the concentration of the agonist in the organ bath fluid. Data are mean±SEM of 5-6 experiments and have been expressed as a percentage of the response to 100 mmol/L K^+.*

Ang II, Ang III and Ang IV constricted iliac arteries (Figure 3A + 4A) and abdominal aortas (Figure. 5A + 6A) in a concentration-dependent manner, whereas Ang-(1-7) had no effect. As expected[162], the contractile responses in the presence of L-NAME (Figure. 3 and 5) were much larger than those in the absence of L-NAME (Figure 4 and 6). In the iliac arteries, in the presence of L-NAME, Ang III and Ang IV ($pEC_{5\%K}^{+}$'s: 7.8±0.3, *n*=8 and 5.6±0.2, *n*=5) were respectively ~12 (P<0.001) and ~2000-fold (P<0.0001) less potent than Ang II ($pEC_{5\%K}^{+}$: 8.9±0.1, *n*=30). In the abdominal aorta, in the presence of L-NAME, Ang III and Ang IV ($pEC_{5\%K}^{+}$'s: 7.6±0.3, *n*=10 and 5.9±0.2, *n*=5) were respectively ~12 (P<0.0001) and ~600-fold (P<0.0001) less potent than Ang II ($pEC_{5\%K}^{+}$: 8.7±0.1, *n*=25). Potencies of Ang II, Ang III and Ang IV in the absence of L-NAME were identical to those in the presence of L-NAME.
The effects of PD123319 in the two vessel types were studied in the absence of L-

NAME, since these are most likely NO-dependent[71]. The effects of irbesartan were studied in the presence of L-NAME, i.e., when contractions were largest. Irbesartan abolished all Ang II, Ang III and Ang IV-induced contractions in both vessel types (Figure 3B-D and 5B-D, *n*=5-6), but did not unmask any Ang-(1-7) effects in either iliac arteries (*n*=5, data not shown) or abdominal aortas (*n*=6; data not shown). Ang-(1-7) shifted the Ang II concentration-response curves to the right in the iliac artery ($P<0.05$), and decreased the E_{max} to Ang II in the abdominal aorta, although the latter was not significant (Figure 2B and C). PD123319 did not affect the concentration-response curves to Ang II, Ang III or Ang IV in either iliac arteries or abdominal aortas (Figure 4B-D and 6B-D, *n*=4-6).

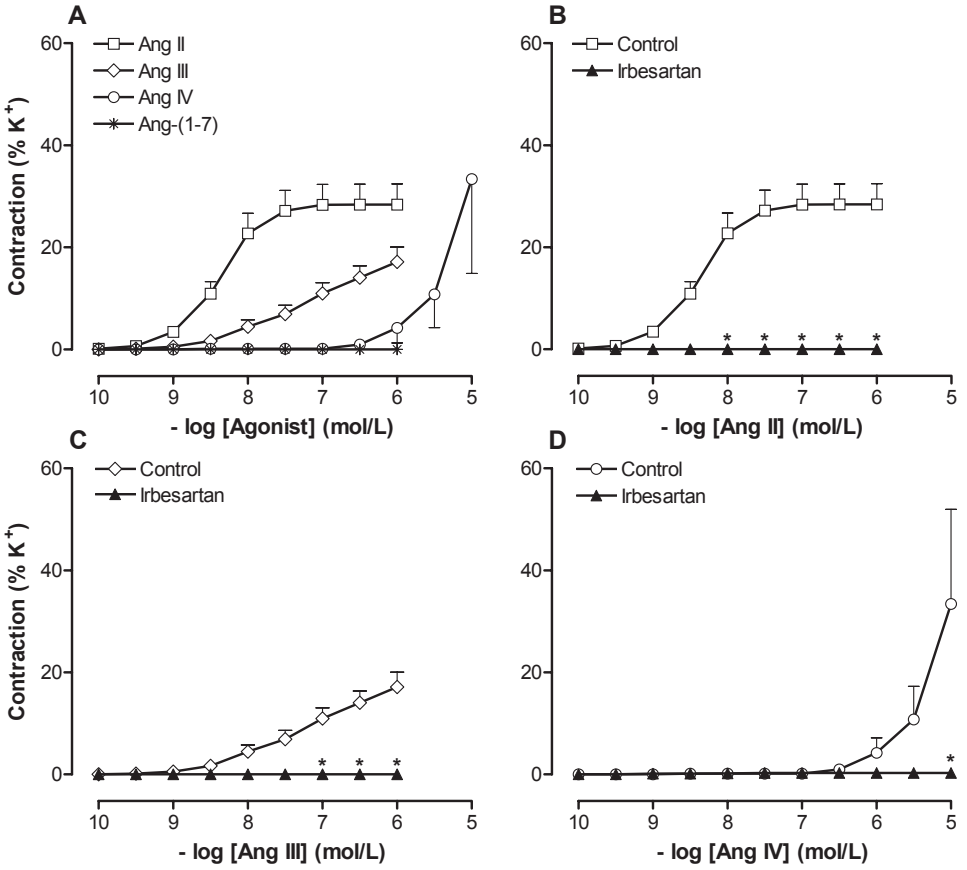


Figure 5. *Effects of Ang III, Ang IV and Ang-(1-7) in L-NAME (100 μmol/L)-pretreated rat abdominal aortas (A). Effects of Ang II (B), Ang III (C) and Ang IV (D), respectively, in L-NAME-pretreated rat abdominal aortas in the absence or presence of 1 μmol/L irbesartan. The x-axis displays the concentration of the agonist in the organ bath fluid. Data are mean±SEM of 4-28 experiments and have been expressed as a percentage of the response to 100 mmol/L K^+. *P<0.05 vs. control.*

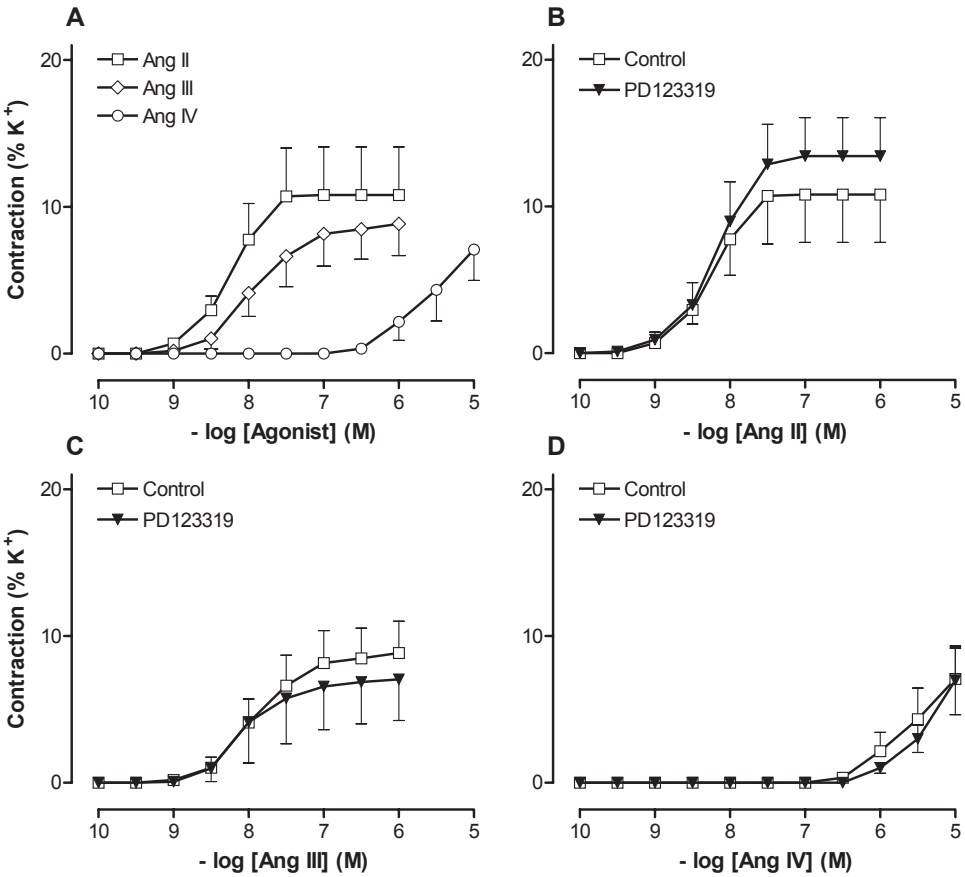


Figure 6. *Effects of Ang II, Ang III, Ang IV and Ang-(1-7) in rat abdominal aortas (A). Effects of Ang II (B), Ang III (C) and Ang IV (D), respectively, in rat abdominal aortas in the absence or presence of 1 μmol/L PD123319. The x-axis displays the concentration of the agonist in the organ bath fluid. Data are mean±SEM of 4-6 experiments and have been expressed as a percentage of the response to 100 mmol/L K^+.*

No relaxant response to Ang II, Ang III, Ang IV or Ang-(1-7) were observed in preconstricted iliac arteries and abdominal aortas in the absence or presence of irbesartan (n=3-5; data not shown).

Discussion

This study is the first to compare the effects of Ang II and its metabolites Ang III, Ang IV and Ang-(1-7) in the rat coronary circulation. Ang III and Ang IV caused coronary constriction via AT_1 receptor stimulation, although at lower potency than Ang II. Ang III, but not Ang IV, simultaneously induced vasorelaxation via AT_2 receptors, and

this was already apparent at subnanomolar Ang III concentrations, as opposed to the submicromolar concentrations of Ang II that were required to observe such relaxation. Ang-(1-7) did not exert effects of its own in the coronary circulation, but blocked AT_1 receptor-induced constriction at high concentrations. The relative constrictor potencies of the 3 angiotensin metabolites in iliac arteries and abdominal aortas were identical to those in the coronary vascular bed, and agree with previous findings in the rat thoracic aorta and the human saphenous vein[268,269]. No AT_2 receptor-mediated relaxant effects occurred in these vessels.

The reduced potency of Ang III and Ang IV as compared to Ang II towards AT_1 receptors can be explained on the basis of the important role of the Asp^{1} and Arg^{2} residues of Ang II in the 'pre-activation' process[280]. These residues interact with His^{183} and Asp^{281} of the AT_1 receptor, stabilizing its conformation, and thus allowing optimal binding of the remaining 5 C-terminal residues[281,282]. Because the Arg^{2}-Asp^{281} interaction is of much greater importance than the Asp^{1}-His^{183} interaction[281], Ang III is only slightly less potent than Ang II, while Ang IV was up to 8000-fold less potent than Ang II in the present study. Studies with constitutively active human AT_1 receptors (N111G mutant), i.e. receptors which are already in the pre-activated state, have shown that, without the need for pre-activation, Ang IV is as potent as Ang II[282].

PD123319 increased the constrictor response to Ang III in the coronary vascular bed at all Ang III concentrations tested, whereas it increased the response to Ang II at concentrations in the low micromolar range only. In addition, in the presence of PD123319, Ang III was as potent as Ang II. This suggests that the 20-fold difference in potency between Ang III and Ang II in the coronary vascular bed in the absence of PD123319 (as compared to the ≈10-fold difference in iliac arteries and aortas) is largely due to Ang III-selective stimulation of dilator AT_2 receptors, which counteract its AT_1 receptor-mediated constrictor effects. Since the PD123319-induced potentiation became apparent already at Ang III concentrations <1 nmol/L, Ang III rather than Ang II appears to be the preferred AT_2 receptor agonist. Similar observations have been made in the kidney, where Ang III (and not Ang II) induced natriuresis through AT_2 receptor activation during AT_1 receptor blockade[97]. This natriuretic effect was enhanced during aminopeptidase N blockade, which further supports the concept of Ang III-dependent Ang AT_2 receptor stimulation[283]. In spontaneously hypertensive rats, Ang III induced a depressor response during AT_1 receptor blockade[284]. This effect was blocked by PD123319, L-NAME and the bradykinin type 2 receptor antagonist Hoe140, suggesting that it depended on AT_2 receptor stimulation, NO and bradykinin type 2 receptors. Endothelial B_2 receptors and the NO/cGMP pathway have also been linked to coronary AT_2 receptor stimulation in rats[59], mice[62] and humans[58].

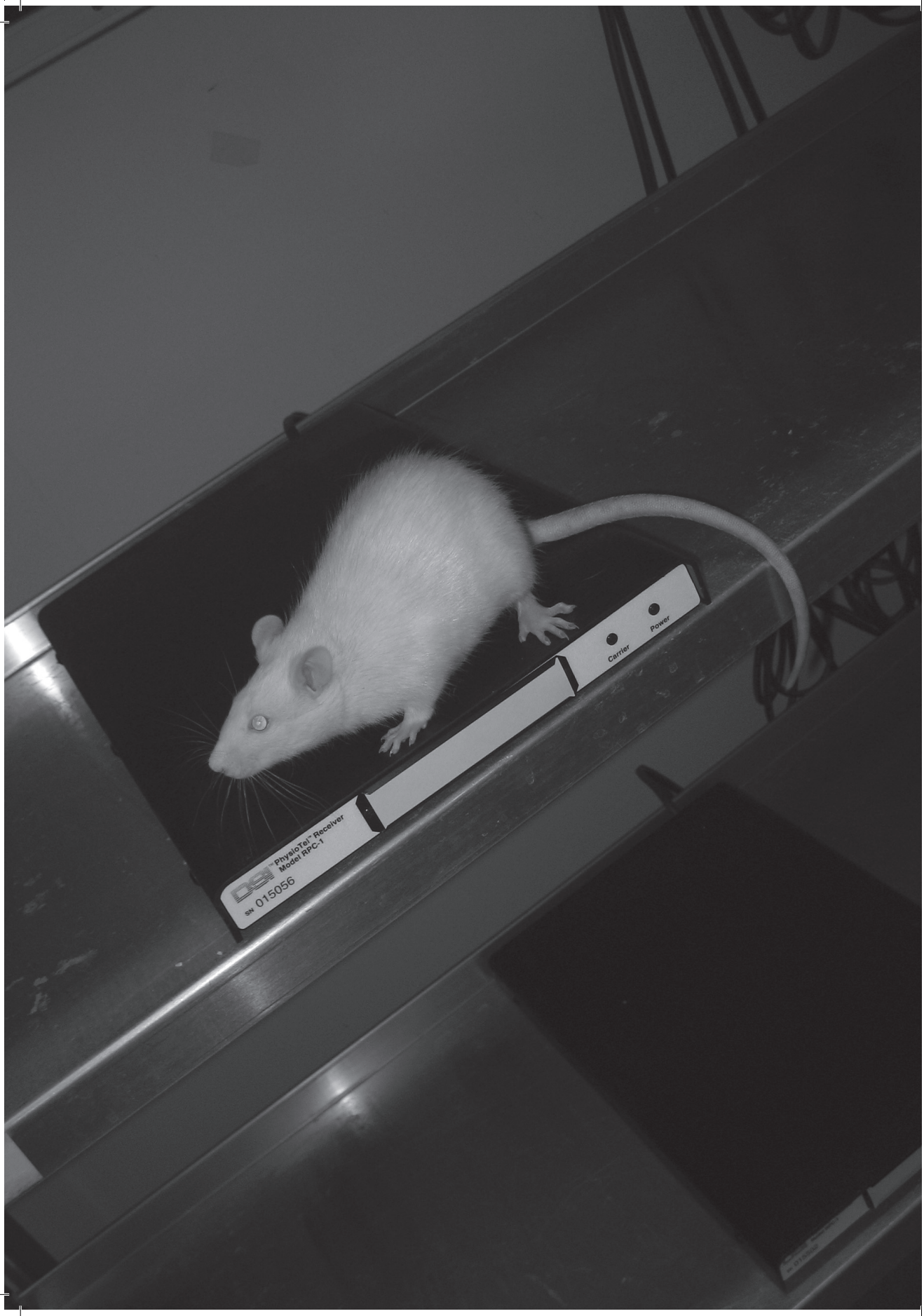
In our study, selective AT_2 receptor stimulation during AT_1 receptor blockade with irbesartan did not result in vasodilation. Similar observations were made in mice during AT_1 receptor blockade, as well as in AT_{1a} knockout mice[62], and collectively, these data suggest that AT_2 receptor-mediated effects occur only in conjunction with AT_1 receptor activation, possibly because both receptors interact, either directly (due to heterodimerization)[61] or indirectly, at the post-receptor level.

We were unable to observe Ang IV-induced vasorelaxant effects in any of our

preparations, suggesting that such effects are limited to certain vascular beds, e.g., in the brain and the kidney[102]. Ang IV did induce vasoconstriction. Since this occurred at pharmacological concentrations only, it is unlikely to have physiological relevance.

Unexpectedly, Ang-(1-7) did not cause vasodilation via Mas receptor activation in any of our preparations, neither at baseline, nor after preconstriction, or in the presence of irbesartan. At micromolar concentrations, Ang-(1-7) did shift the Ang II concentration-response curve to the right in the coronary circulation and iliac arteries, and a similar trend was observed in the abdominal aorta. This mimics previous findings in the rat renal vascular bed [89,285] and the human forearm[169], and suggests that Ang-(1-7), at pharmacological concentrations, acts as an AT_1 receptor antagonist. Ang-(1-7) also exerts ACE-inhibitory effects[32], and thus the beneficial effects of Ang-(1-7) infusions in rats [174] may be due, at least in part, to RAS blockade. Clearly, the attenuation of heart failure induced by the Mas receptor agonist AVE-0991 [286] does not involve Mas receptor-dependent coronary vasorelaxation. Indeed, in the mouse heart, the Mas receptor was mainly localized in cardiomyocytes[88].

In conclusion, AT_2 receptor activation in the rat coronary vascular bed results in vasodilation, and Ang III rather than Ang II appears to be the preferred agonist of these receptors. Due to the difficulties of measuring Ang metabolites in a reliable manner[176,287], epidemiological data linking Ang III levels to cardiovascular outcome parameters in humans are currently not available.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Chapter 7

Renin inhibition improves coronary function in spontaneously hypertensive rats

Based on:

Joep H. M. van Esch, Richard van Veghel, Ingrid M. Garrelds, A. H. Jan Danser. Renin inhibition improves coronary endothelial function in spontaneously hypertensive rats.

In preparation, 2008

Abstract

Animal and clinical data suggest that the blood pressure-lowering effect of renin inhibition equals that of ACE inhibition and angiotensin (Ang) type 1 (AT_1) receptor blockade. Whether blocking the initial, rate-limiting step of the renin-angiotensin system also offers end-organ protection still needs to be investigated. In this study, we compared the cardiovascular efficacy of the renin inhibitor aliskiren (100 mg/kg/day), the AT_1 receptor blocker irbesartan (15 and 30 mg/kg/day) and the ACE inhibitor captopril (3 and 6 mg/kg/day) in spontaneously hypertensive rats (SHR). All treatments lasted 3 weeks, and during this period mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored by radiotelemetry. After 3 weeks, the animals were sacrificed to determine (1) plasma renin activity (PRA), (2) coronary reactivity to bradykinin and Ang II, and 3) cardiac hypertrophy (heart weight (HW)/body weight (BW) ratio and cardiomyocyte area). Aliskiren, irbesartan and captopril lowered MAP in a dose-dependent manner, without affecting HR. Aliskiren decreased PRA by ≈70%, whereas irbesartan and captopril increased PRA in a dose-dependent manner. The blood pressure-lowering effects of irbesartan 15 mg/kg/day and captopril 3 mg/kg/day were comparable to those of aliskiren 100 mg/kg/day, and at these doses all drugs increased coronary reactivity to bradykinin, whereas only aliskiren and irbesartan decreased the response to Ang II. Simultaneously, at these doses, all drugs decreased HW/BW ratio and cardiomyocyte area, although significance was reached for the latter only. In conclusion, for a given decrease in blood pressure, aliskiren is as least as effective as AT_1 receptor blockade and ACE inhibition with regard to improvement of endothelial function and cardiac hypertrophy.

Introduction

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and body fluid homeostasis. The RAS is not only active in the circulation ("circulating RAS") but also in many tissues, functioning as so-called "local" RAS.[103] Angiotensin (Ang) II, the main effector peptide of the RAS, when generated from Ang I at tissue sites, stimulates both Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. This local synthesis depends on angiotensinogen, renin and/or prorenin (the inactive precursor of renin) taken up from blood, the latter uptake possibly involving the recently discovered (pro)renin receptor.[186,288] In contrast, the angiotensin-converting enzyme (ACE) required to convert Ang I into Ang II, is generated locally.

Under pathological conditions excessive RAS activity may lead to a rise in blood pressure and cardiac/renal hypertrophy, resulting in end-organ damage. ACE inhibitors are believed to exert their beneficial effects through blockade of Ang II formation from Ang I at tissue sites. However, compensatory feedback loops within the RAS will result in an increased release of renin from the kidneys and an upregulation of ACE and non-ACE Ang II-generating enzymes, thus making it very difficult to suppress Ang II levels during continuous ACE inhibition.[1,118] Treatment with AT_1 receptor blockers will also cause a rise in renin, resulting in increased Ang I and II levels in blood and tissues.[1] It is believed that during AT_1 receptor blockade the unopposed AT_2 receptors will be stimulated excessively due to the rise in Ang II levels. In fact, animal data suggest that part of the beneficial effect of AT_1 receptor blockers is due to AT_2 receptor stimulation.[64] AT_2 receptors exert effects that directly oppose the effects of AT_1 receptors, and, as they form heterodimers with AT_1 receptors, AT_2 receptor might even act as natural AT_1 receptor antagonists.[61] Since the AT_2 receptor-induced effects could not be observed in the absence of AT_1 receptors,[62] the net beneficial effect of AT_2 receptor stimulation may actually be a more complete AT_1 receptor blockade.

Renin inhibitors prevent the formation of Ang I and II by targeting the initial and rate-limiting step of the RAS. They bind to both renin and prorenin.[8] Renin, unlike ACE, has only one known substrate, and without renin, there are no angiotensins.[106] Renin inhibitors have a pharmacokinetic advantage over other RAS blockers in that they may already bind to renin and/or prorenin on their way to tissue sites (i.e., in blood), whereas the other blockers first have to penetrate into tissues in order to exert their effect. Although renin will rise during renin inhibitor treatment, like it does during any type of RAS blockade, this renin cannot be enzymatically active due to the presence of the renin inhibitor. Therefore, renin inhibitors are expected to provide a more complete suppression of the RAS with a low likelihood of side-effects.

The putative beneficial effects mediated by AT_2 receptors will not occur during renin inhibition. So far, this does not appear to diminish the effects of renin inhibitors, at least on blood pressure,[152] possibly because more complete RAS blockade induces the same effect as AT_2 receptor stimulation, as discussed above.

Despite its promise, renin inhibition was held back for a long time due to a number

of technical problems.[289] Early renin inhibitors had limited bioavailability and efficacy. In contrast, the recently developed renin inhibitor aliskiren is a potent and selective inhibitor of human renin, with an improved bioavailability as compared to the early renin inhibitors.[290] Aliskiren lowers both plasma Ang I and II in humans, and prevents the rise in plasma Ang I and II during AT_1 receptor blockade[150,151].[150,151] In short-term studies, it reduced blood pressure to the same degree as other RAS blockers.[152] Oparil and co-workers showed that aliskiren treatment on top of AT_1 receptor blockade led to an additional lowering in blood pressure.[291]

The long-term effects of aliskiren (e.g., on cardiac hypertrophy) as well as its effects on tissue Ang I and II are not yet known. This relates to the fact that aliskiren primarily blocks human renin and is less effective towards renin of other species, thus hampering its use in animals. Aliskiren is highly effective in double transgenic rats expressing both human renin and angiotensinogen,[292] but given the exceptionally high Ang II levels in these animals (up to 20 times normal), causing them to die within 7 weeks without treatment, it is uncertain to what degree such data reflect “normal” in vivo conditions.

At high doses (100 mg/kg/day), aliskiren does lower blood pressure in spontaneously hypertensive rats (SHR).[293] In the present study we therefore compared the cardiovascular efficacy of aliskiren with that of the AT_1 receptor blocker irbesartan and the ACE inhibitor captopril in SHR. First, the effects of these RAS blockers at various doses on blood pressure and heart rate were measured by telemetry in conscious animals. Based on these data we determined which dose of irbesartan and captopril induced a comparable blood pressure-lowering effect as a high dose of aliskiren. Next, applying such equi-hypotensive doses of the 3 drugs, we determined, after a 3-week treatment, coronary reactivity to bradykinin and Ang II, and the degree of cardiac hypertrophy (heart weight (HW)/body weight (BW) ratio and cardiomyocyte area).

Materials and methods

Animals

Male SHR (280-300 gram; n=33) and their normotensive Wistar Kyoto (WKY: 280-300 gram; n=2) controls were obtained from Harlan (Zeist, The Netherlands). SHR and WKY rats were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water *ad libitum*. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Drugs

Captopril, Ang II, bradykinin and sodium nitroprusside (SNP) were purchased from Sigma (Zwijndrecht, The Netherlands). Irbesartan was a kind gift of Sanofi-Synthelabo BV (Gouda, The Netherlands), and aliskiren (hydrochloride salt) was a kind gift of Novartis Pharmaceuticals (Basel, Switzerland).

The drugs were prepared for administration via osmotic minipumps as follows: Irbesartan (87.5 mg/mL or 175 mg/mL) was dissolved in saline in the presence of 4% vol/vol NaOH.[294] The pH was subsequently adjusted to 7-9 by equilibration with carbogen (95% O_2, 5% CO_2). Captopril (17.5 mg/mL or 35 mg/mL) and aliskiren (583.3 mg/mL) were dissolved in saline.
Ang II and bradykinin for in-vitro use were dissolved in water, and stock solutions were stored in aliquots at -80ºC. On the day of the experiments stock solutions were further diluted with Tyrode's buffer (composition in mmol/L: NaCl 125, KCl 4.7, $NaH_2PO_4.2H_2O$ 0.43, $MgCl_2$ 1.0, $CaCl_2.H_2O$ 1.3, glucose.H_2O 9.1, $NaHCO_3$ 20; pH 7.4).

Telemetry and treatment

Rats were anesthetized by inhalation of isoflurane (Rhodia Organique Fine Limited, Avonmouth, Bristol, UK) in air. A radio-telemetry transmitter (TA11PA-C40, Datascience Inc., St. Paul, Minnesota, USA) was implanted into the abdominal cavity with the fluid-filled catheter placed into the lower abdominal aorta. After surgery rats were treated with Temgesic® (buprenorphine, Reckitt and Colman, Hull, UK) for 2 days (0.1 and 0.05 mg/kg during the first and second day, respectively). After 11 days of recovery, telemetry measurement was started to obtain baseline hemodynamic parameters. Two days later, osmotic minipumps (2mL4 Alzet, California, USA) were implanted subcutaneously under isoflurane anesthesia to infuse either vehicle (saline), irbesartan (15 or 30 mg/kg/day), captopril (3 or 6 mg/kg/day) or aliskiren (100 mg/kg/day). Three weeks after implantation of the osmotic pumps, telemetry measurement was stopped.

Collection of tissue and blood

In the third to fourth week after implantation of the osmotic pump, animals were anesthetized by inhalation of isoflurane. The hepatic portal vein was cannulated to collect 1 ml of blood, which was centrifuged for 5 minutes at 13000 rpm. Subsequently, plasma was obtained and stored at -80ºC. The hearts were rapidly excised and placed in ice-cold Tyrode's buffer, gassed with 95% O_2 and 5% CO_2.

Langendorff preparation

The aorta was immediately cannulated with a 21G needle (with a small circumferential grove close to the blunt tip) and perfused with gassed (95% O_2 and 5% CO_2) Tyrode's buffer according to Langendorff at a constant perfusion pressure of 80 mm Hg. Two needle electrodes were placed at the right atrium and the hearts were paced at ~480 bpm (8 Hz, 4 ms duration, 6 Volt) using a Grass stimulator (Grass Instruments Co., Quincy, Massachusetts, USA). After removal of the left atrium, a water-filled latex balloon (Durex, London, UK) was inserted into the left ventricle. The left ventricular end-diastolic pressure was set at 3-5 mmHg by adjusting the balloon volume. Coronary flow (CF) was measured with a flow probe (Transonic systems, Ithaca, New York, USA). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 μL) of Tyrode's buffer were applied three

times to determine injection-induced changes in CF. First a concentration-response curve to BK (concentration range in the injection fluid 0.1 nmol/L - 0.1 mmol/L) was constructed by bolus injections (100 μL). Ten minutes later, a concentration-response curve was constructed to Ang II (concentration range in the injection fluid 0.1 nmol/L - 0.1 mmol/L). Finally, the maximum coronary flow was determined by an injection of SNP (10 mmol/L)

Cardiac hypertrophy

Following completion of the measurements in the Langendorff model, the hearts were collected, and the ventricular heart weight was determined after removal of the atria and large vessels. Ventricular hypertrophy was defined as the ratio of the ventricular HW to BW. Subsequently, ventricles were cut into 3 transversal slices and fixed in a 3.5-4% formaldehyde solution (Boom, Meppel, the Netherlands). After fixation, the slices were dehydrated and paraffin-embedded. Deparaffinized 5 μm thick sections were stained with Gomori's silver staining in order to visualize individual cardiomyocytes.[295]. Only transversally cut cells showing a nucleus were used to determine the cardiomyocyte area.

Plasma renin activity (PRA)

PRA was measured by incubating plasma for 60 minutes at pH 7.4 and 37°C in the presence of a mixture of inhibitors to block Ang I degradation, Ang I-II conversion, and prorenin-renin conversion, and to prevent bacterial growth.[296] The generated Ang I was measured by radioimmunoassay.

Data analysis

Telemetric data were recorded and digitalized using the Dataquest Acquisition & Analysis system (DQ ART 3.1 Silver, Datascience Inc., St. Paul, Minnesota, USA). Each animal was sampled for 10 seconds at 10-minute intervals for a period of 23 days. All recordings were averaged per day and baseline values were calculated using the data from the first 2 days of measurement before treatment was started. Changes in MAP from baseline were analyzed by comparison of the areas over the curve (AOC), as calculated by the trapezoidal method (mm Hg x days).[293,297]

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, Ohio, USA). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc, Natick, Massachusetts, USA). Six consecutive beats were selected for determination of CF.

Statistical analysis between groups was performed by Student's *t* test or one-way analysis of variance (ANOVA), followed by post hoc evaluation according to Dunnet. $P<0.05$ was considered significant.

Table. *Heart rate (HR) and mean arterial blood pressure (MAP) at baseline, the maximum decrease in MAP (ΔMAP) and ΔMAP at day 7, 14 and 21 after the start of treatment, the area over the curve (AOC), and body weight (BW), heart weight (HW) and the HW/BW ratio at th end of the treatment period, in WKY and SHR rats treated for 3 weeks with vehicle, aliskiren, irbesartan or captopril. Data are mean±SEM. †P<0.05 vs. WKY vehicle control; *P<0.05 vs. SHR vehicle control; ΨP<0.05 vs. SHR treated with aliskiren 100 mg/kg/day.*

		WKY	SHR					
		Vehicle Control	Vehicle Control	Aliskiren 100 mg/kg/day	Irbesartan 15 mg/kg/day	Irbesartan 30 mg/kg/day	Captopril 3 mg/kg/day	Captopril 6 mg/kg/day
N		2	6-8	5-8	5-7	5	3	2
Baseline HR (bpm)		366±5	321±3†	320±2	328±6	332±8	332±7	341±4
Baseline MAP (mm Hg)		104±4	149±3†	156±4	146±4	149±5	140±7	158±22
ΔMAP	Maximum	-2±2	-3±3	-31±3*	-33±4*	-53±2*Ψ	-20±4*	-29±7*
	Day 7	-1±0	4±1	-25±2*	-20±4*	-46±7*Ψ	-14±5*	-28±8*
	Day 14	-1±1	2±2	-17±2*	-11±3*	-43±9*Ψ	-12±3	-28±6*
	Day 21	-1±1	3±2	-12±3*	-8±2*	-45±2*Ψ	-10±3*	-25±5*
AOC MAP (mmHg x days)		-33±6	28±26	-424±36*	-352±54*	-922±100*Ψ	-272±68*	-543±145*
BW (kg)		463±21	391±4†	373±4	376±5	371±7	366±17	369±3
HW (g)		1.3±0.0	1.5±0.0†	1.2±0.1	1.5±0.0	1.2±0.1*	1.3±0.1	1.4±0.0
HW/BW (g/kg)		2.8±0.0	3.9±0.1†	3.4±0.1	3.9±0.1	3.5±0.3	3.7±0.1	3.8±0.1

Results

Baseline parameters and vehicle treatment

At baseline, the body weights of SHR (278±2 gram, n=26) and WKY rats (260±1, n=2) were identical, and SHR displayed a higher MAP and a lower HR than WKY rats (Table). Vehicle did not affect MAP or HR in either strain. At the end of the 21 day-treatment period WKY rats had gained more weight than SHR, in full agreement with previous findings.[298,299]

RAS blockade in SHR

Aliskiren 100 mg/kg/day lowered MAP in comparison to vehicle control (Table and Figure 1), without affecting HR (data not shown). MAP was maximally reduced at the 4th day after the start of infusion. Thereafter, the effect of aliskiren leveled off, but MAP remained reduced at day 7, 14 and 21. Aliskiren reduced PRA on day 7 of treatment, and this effect was unaltered on day 21 (Figure 1).

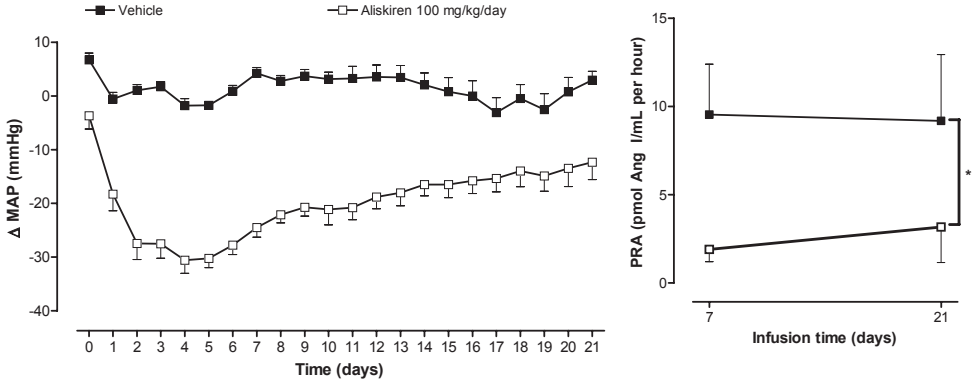


Figure 1. *Left panel, ΔMAP during a 3-week infusion of aliskiren or vehicle in SHR (n=8). Right panel, plasma renin activity (PRA) on day 7 and 21 after the start of treatment (n=6-7). Data are mean±SEM. *P<0.05 vs. control.*

Irbesartan and captopril reduced MAP at all tested doses, and both drugs increased PRA in a dose-dependent manner (Figures 2 and 3). The blood pressure-lowering effects of the two highest doses of irbesartan and captopril, estimated from the AOC values and the MAP decreases at day 7, 14 and 21 (Table), were larger than that of aliskiren, whereas the effects of the two lowest doses of both drugs were comparable to that of aliskiren. HR was unaltered during treatment with these lower doses (data not shown). On the basis of these findings, all further studies were performed with aliskiren 100 mg/kg/day, irbesartan 15 mg/kg/day and captopril 3 mg/kg/day.

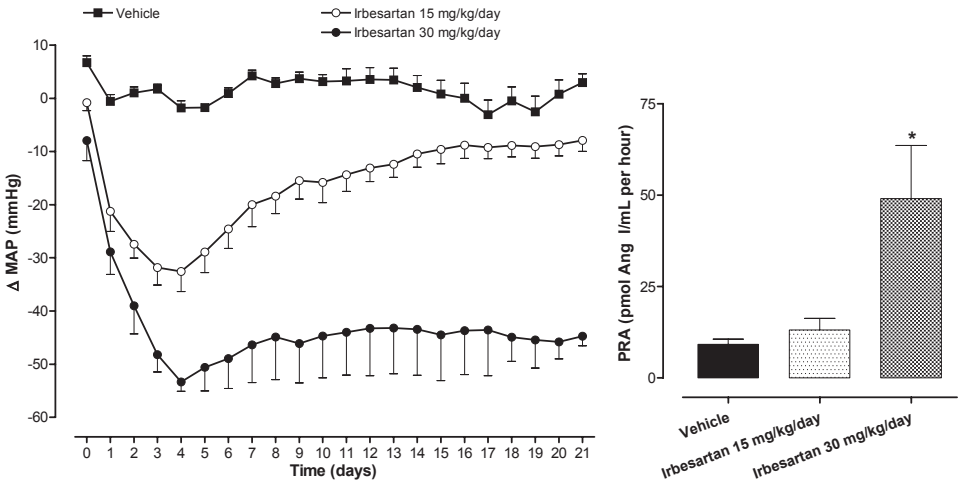


Figure 2. *Left panel, ΔMAP during a 3-week infusion of irbesartan or vehicle in SHR (n=5-8). Right panel, PRA on day 21 after the start of treatment (n=4-7). Data are mean±SEM. *P<0.05 vs. control.*

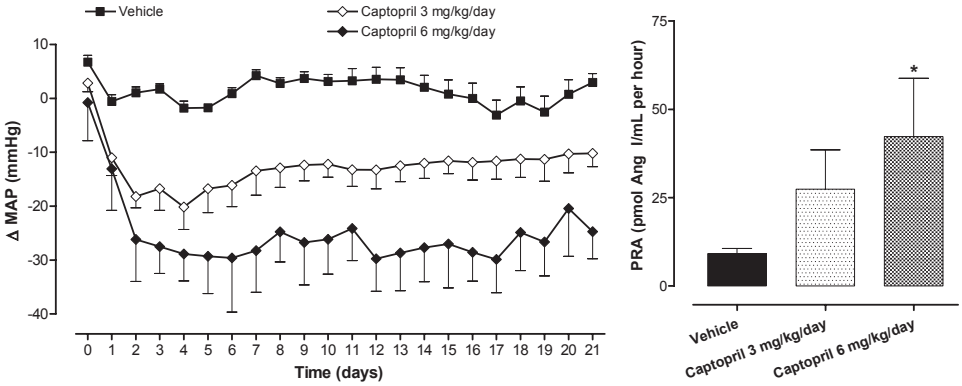


Figure 3. *Left panel, ΔMAP during a 3-week infusion of captopril or vehicle in SHR (n=2-8). Right panel, PRA on day 21 after the start of treatment (n=2-7). Data are mean±SEM. *P<0.05 vs. control.*

Langendorff studies

Baseline CF of vehicle-treated SHR (11.9±0.8 mL/min, n=9), vehicle-treated WKY rats (8.6±0.2 mL/min, n=2), aliskiren-treated SHR (9.7±0.5 mL/min, n=7), irbesartan-treated SHR (8.9±0.6 mL/min, n=4) and captopril-treated SHR (9.7±0.6 mL/min, n=4) were identical. Bolus injections with Tyrode's buffer injections did not significantly affect CF (Figures 4 and 5). Bradykinin increased CF in vehicle-treated WKY rats and SHR (Figure 4), but its effects were much larger in the former (E_{max}: +254±20% vs. +73±20%, P<0.05). Treatment of SHR with aliskiren, irbesartan or captopril increased the effect of bradykinin to WKY values (P<0.05 for all).

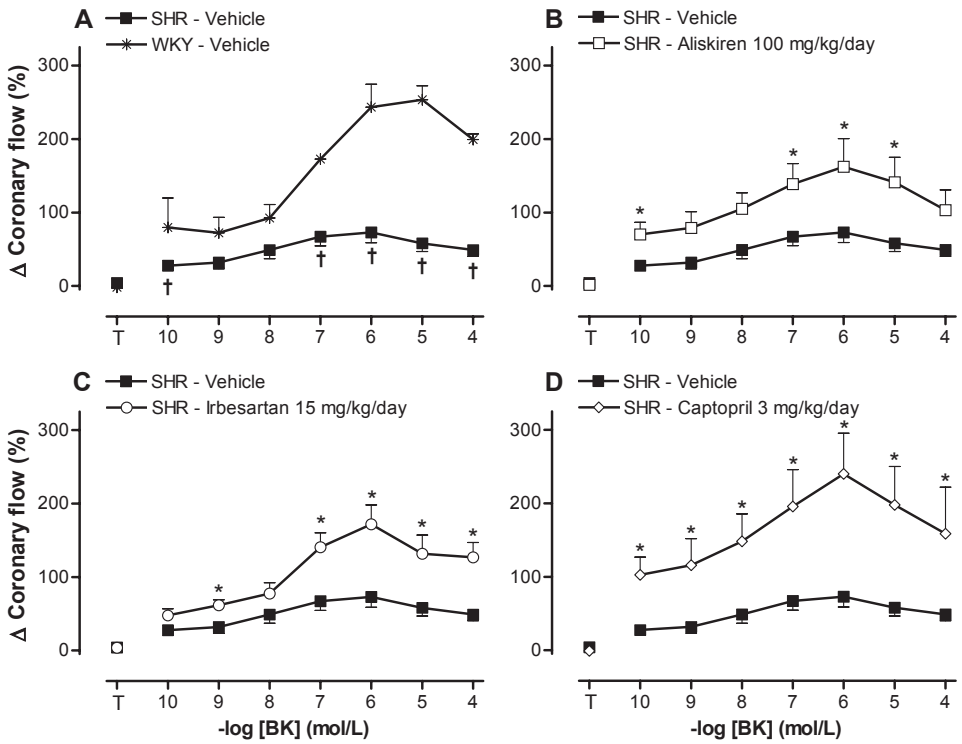


Figure 4. *Effect of bradykinin (BK) bolus injections (100 μL) on coronary flow in SHR (panels A-D) or WKY rats (panel A) after a 3-week treatment with vehicle, aliskiren, irbesartan or captopril. Data (mean±SEM of 2-7 experiments) were obtained using the Langendorff heart preparation, and represent % change from baseline. The concentrations on the x-axis represent the concentration in the injection fluid. T represents the effect of a bolus injection with Tyrode's buffer. †P<0.05 vs. WKY; *P<0.05 vs. SHR vehicle control.*

Ang II concentration-dependently decreased CF in vehicle-treated WKY rats and SHR (Figure 5), and its effects were larger in the latter (E_{max}: -62±0.1% vs. -73±4%, P<0.05). Treatment of SHR with aliskiren or irbesartan decreased the effect of Ang II to WKY values (P<0.05 for both). Captopril treatment of SHR did not alter the effect of Ang II. The maximum CF values (determined with SNP) were identical in vehicle-treated WKY rats and SHR (34.8±3.1 mL/min vs. 31.2±5.8 mL/min; n=2 for both), and treatment of SHR with aliskiren, irbesartan or captopril did not alter these values (data not shown).

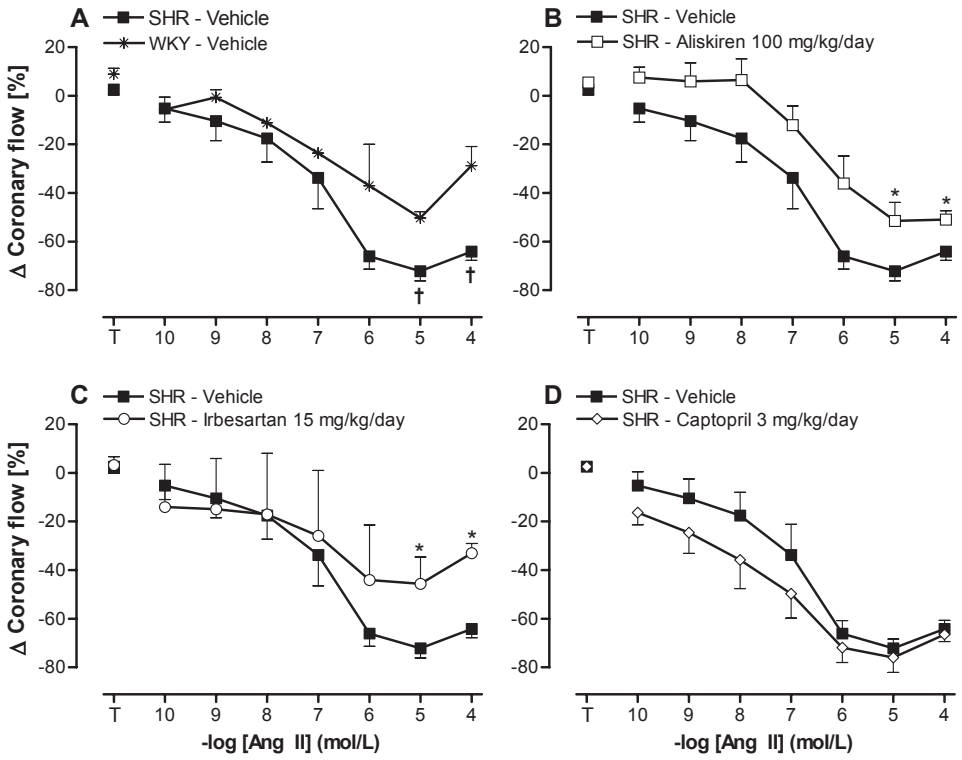


Figure 5. *Effects of angiotensin (Ang) II bolus injections (100 μL) on coronary flow in SHR (panels A-D) or WKY rats (panel A) after a 3-week treatment with vehicle, aliskiren, irbesartan or captopril. Data (mean±SEM of 2-6 experiments) were obtained using the Langendorff heart preparation, and represent % change from baseline. The concentrations on the x-axis represent the concentration in the injection fluid. T represents the effect of a bolus injection with Tyrode's buffer. †P<0.05 vs. WKY; *P<0.05 vs. SHR vehicle control.*

Cardiac hypertrophy

The HW/BW ratio (Figure 6) and cardiomyocyte area (Figure 7) were much larger (P<0.0001) in vehicle-treated SHR than in vehicle-treated WKY rats. Treatment of SHR with aliskiren, irbesartan or captopril did not significantly affect the HW/BW ratio, but reduced the cardiomyocyte area (P<0.05).

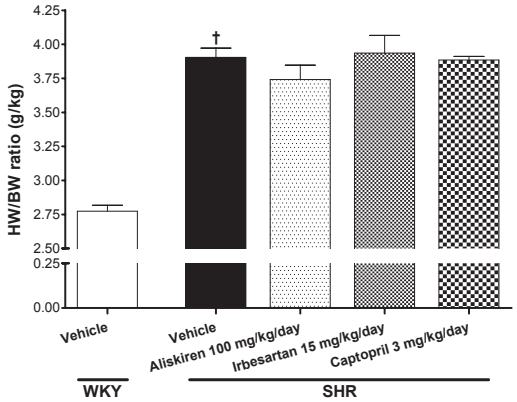


Figure 6. *Ventricular heart weight (HW)/body weight (BW) ratio of SHR and WKY rats after a 3-week treatment with vehicle, aliskiren, irbesartan or captopril. Data are mean±SEM of 2-7 experiments. †P<0.05 vs. WKY.*

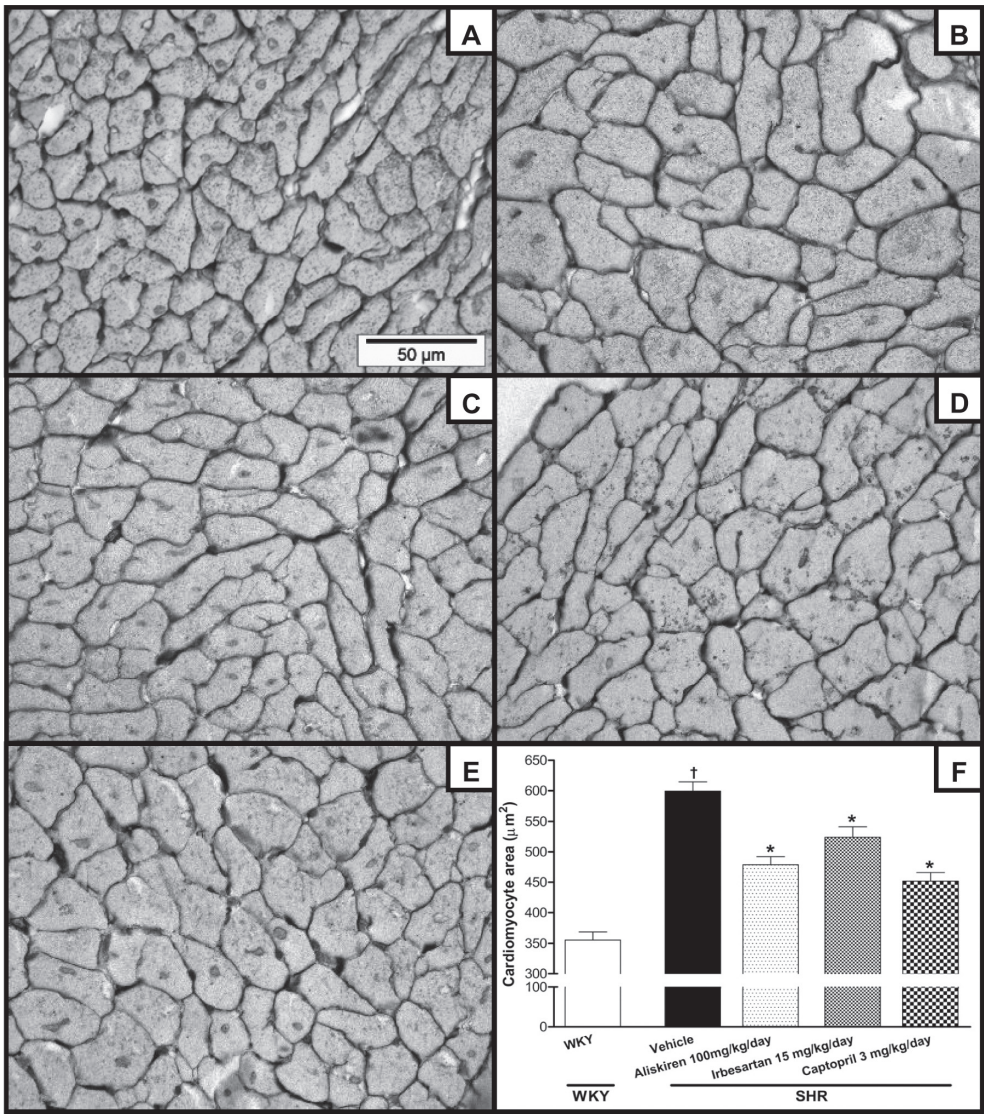


Figure 7. *Gomori-stained sections showing cardiomyocytes in the left ventricular wall of hearts from vehicle-treated WKY rats (A), vehicle-treated SHR (B), aliskiren-treated SHR (C), irbesartan-treated SHR (D) and captopril-treated SHR (E). The bar in the in picture A represents 50 µm. Panel F summarizes the findings on the cardiomyocyte area (mean±SEM of 2-5 experiments) in the 5 groups. †P<0.05 vs. WKY; *P<0.05 vs. SHR vehicle control.*

Discussion

This study is the first to compare, head-to-head, the 3 types of RAS inhibition in a well-established model, the SHR. This is of importance now that the renin inhibitor

aliskiren is entering the clinical arena. The data show that, for a given decrease in blood pressure, aliskiren improves endothelial function and decreases cardiac hypertrophy to minimally the same degree as captopril and irbesartan. In addition, it decreases the contractile effects of Ang II to the same degree as irbesartan, although it is not an AT_1 receptor blocker.

The aliskiren-induced decrease in blood pressure in SHR in the present study was comparable to that observed by Wood et al. in SHR.[293] The blood pressure effect leveled off at the end of the infusion period, although PRA at that time was still suppressed to the same degree as after 7 days of treatment. This indicates that the levels of the renin inhibitor, at least in blood, were still sufficiently high to block plasma renin. Future measurements of renin activity and Ang II levels at tissue sites should reveal to what degree renin inhibition was also effective at the tissue level. A similar degree of leveling off was observed during treatment with the lower doses of irbesartan and captopril (which decreased blood pressure to the same degree as aliskiren) but not during treatment with the higher doses of these 2 drugs. Clearly therefore, RAS blockade was incomplete at the 3 equi-hypotensive doses of aliskiren, irbesartan and captopril. The decreases in MAP at these doses were not accompanied by changes in HR, in full agreement with previous studies on RAS blockade.[293,297,300].

As expected, coronary endothelial function (determined as the response to bradykinin) was greatly impaired in SHR as compared to WKY.[301,302] All 3 RAS blockers, despite their modest blood pressure-lowering effects, normalized coronary endothelial function in SHR. In the case of captopril, part of this effect may relate to its bradykinin-potentiating properties. However, given the much smaller maximum effect of bradykinin in SHR, this potentiation cannot fully explain the beneficial effects of the ACE inhibitors. Clearly therefore, RAS blockade per se translates into an improved endothelial function, for instance because Ang II suppression diminishes oxidative stress.[279,303-306]

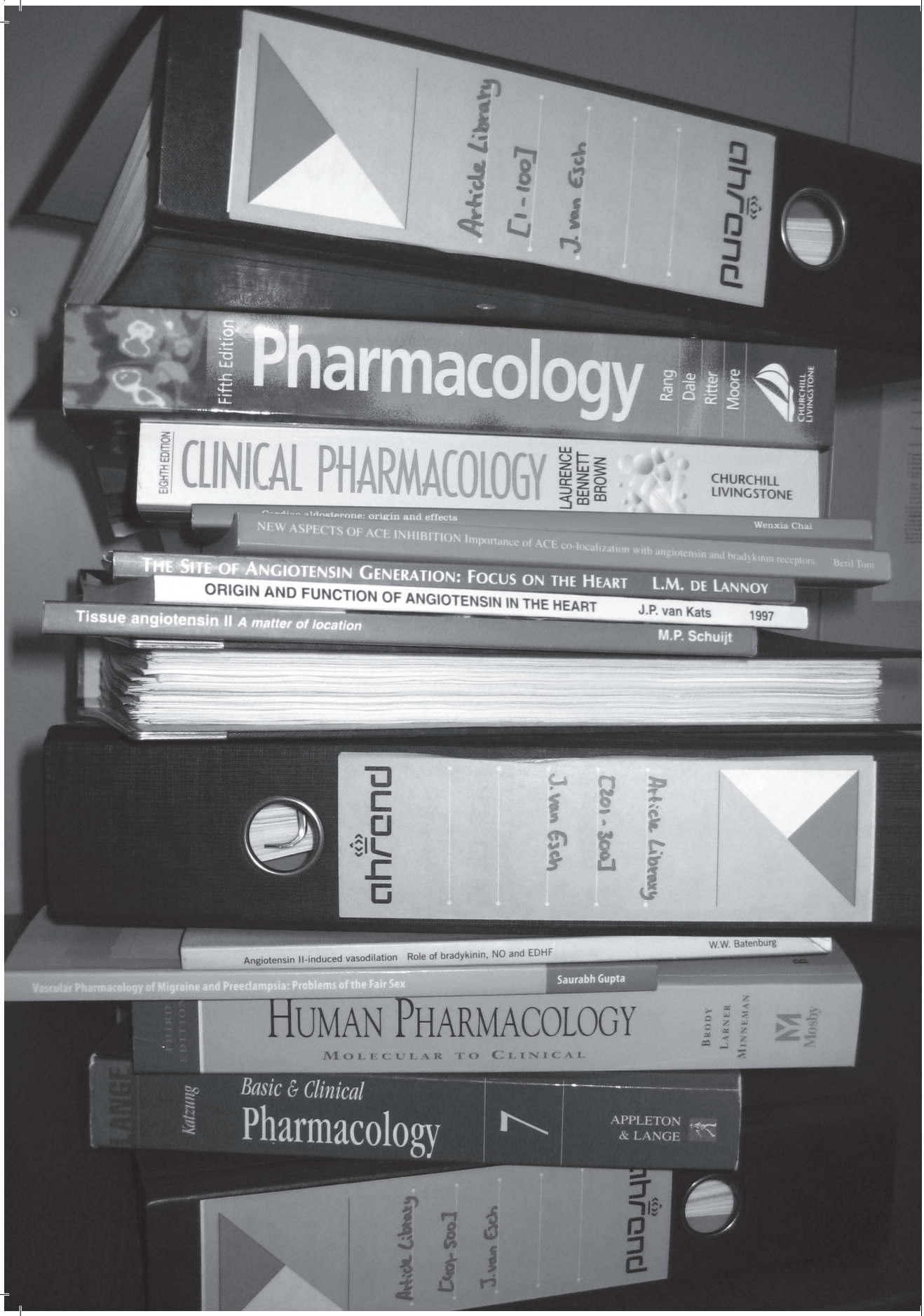
Surprisingly, aliskiren, like irbesartan, decreased the coronary responsiveness to Ang II. Theoretically, based on the assumption that a renin inhibitor will suppress Ang II, one would have expected an upregulation of AT_1 receptors during aliskiren treatment, and thus, an increased responsiveness to exogenously applied Ang II. Indeed, a tendency for such a leftward shift of the Ang II dose-response curve was observed during captopril treatment, which will also lower Ang II. The fact that this did not occur during aliskiren treatment may suggest that renin inhibition affects AT_1 receptor expression independently of its effect on Ang II. Indeed, a decrease in renal AT_1 receptor expression has also been observed in aliskiren-treated diabetic rats.[307] The mechanism underlying this phenomenon is currently unknown, but it is tempting to speculate that it relates to stimulation of the (pro)renin receptor by renin and/or prorenin.

Finally, the equi-hypotensive doses of the 3 RAS blockers did not significantly affect the HW/BW ratio. Since this parameter is largely determined by blood pressure (ref), it is not surprising, given the modest blood pressure-lowering effects of the 3 drugs, that no significant decrease occurred in this study. Nevertheless, cardiomyocyte

area did decrease during treatment with aliskiren, irbesartan and captopril, and thus measurements of cardiac Ang II and collagen content are required to get a more complete picture of the putative antihypertrophic effects of these drugs.
In summary, for a given decrease in blood pressure, aliskiren is as least as effective as AT_1 receptor blockade and ACE inhibition with regard to improvement of endothelial function and cardiac hypertrophy.

Acknowledgements

This study was financially supported by a grant of the Netherlands Heart Foundation (NHF-2007B019).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Chapter 8

Summary and general discussion

Summary

Introduction and aim of this thesis (Chapter 1)

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) but it is now well-established that angiotensin (Ang) generation also occurs at tissue sites ("tissue" RAS). Angiotensinogen (liver-derived) is cleaved by renin (kidney-derived) into Ang I which in turn can be converted into Ang II by angiotensin-converting enzyme (ACE). As main biological component of the RAS, Ang II mediates it effects through the angiotensin type 1 (AT_1) and type 2 (AT_2) receptors. In rodents, two AT_1 receptor subtypes have been identified: AT_{1A} and AT_{1B}. AT_1 receptors mediate the well-known vasoconstrictor, inotropic, chronotropic, aldosterone-releasing, noradrenaline-releasing and growth-stimulatory effects of Ang II, and AT_2 receptors are generally assumed to counteract these actions. Initially it was thought that all metabolites other than Ang II were inactive, but now it is clear that several of these metabolites such as Ang III, Ang IV and Ang-(1-7) have functions of their own which are possibly mediated by non-AT_1/AT_2 receptors. Under pathophysiological conditions excessive RAS activity may lead to an increased blood pressure and cardiac/renal hypertrophy, resulting in end-organ damage. By now the RAS can be blocked at three levels: renin, ACE and the AT_1 receptor. ACE inhibitors and AT_1 receptor blockers already have proven their effectiveness in the treatment of hypertension and cardiovascular diseases. ACE inhibitors block the formation of Ang II whereas AT_1 receptor blockers prevent Ang II to mediate effects through the AT_1 receptor. It is feasible that at least part of the beneficial effect of AT_1 receptor blockers is due to stimulation of the unblocked AT_2 receptor. It is thought that the newly developed renin inhibitors will provide a more complete suppression of the RAS as they block the first rate-limiting step. Clearly, the RAS has not yet revealed all of its secrets. Step by step the pieces of the puzzle fall into place and a better understanding of the RAS and its components eventually might lead to the discovery of novel therapeutic targets and improved treatment strategies.

ACE (Chapter 2 and 3)

Somatic ACE contains two homologous domains (the C- and N-domain) capable of hydrolyzing Ang I and bradykinin. Selective C-domain inhibition induced a rightward shift of the Ang I concentration-response curve (CRC) in porcine femoral arteries (PFAs) whereas selective N-domain inhibition did not affect Ang I-induced constriction. In human blood plasma blockade of both domains fully blocked the Ang I-II conversion. In contrast, bradykinin degradation by membrane-bound ACE depended on both domains, as both selective C- and N-domain blockade shifted the bradykinin CRC to the left, with a maximum leftward shift being observed when both domains were blocked. Thus, tissue Ang I-II conversion depends exclusively on the C-domain, whereas both domains contribute to conversion by soluble ACE and to bradykinin degradation at tissue sites.

The ACE insertion/deletion (I/D) polymorphism determines part of the interindividual

ACE variability, carriers of the D allele having higher tissue and plasma ACE levels. Unexpectedly, the higher ACE concentrations found in DD subjects do not result in a higher regional Ang I-II conversion rate and/or higher Ang II levels. Using plasma of DD and II subjects, it was observed that selective C-domain blockade fully blocked Ang I-II conversion in plasma of DD subjects whereas additional N-domain blockade was required to fully block this conversion in plasma of II subjects. Both domains contributed equally to the hydrolysis of bradykinin in plasma of DD and II subjects. The lack of Ang II accumulation in DD subjects might thus relate to the fact that in II subjects both the C- and N-domain generate Ang II whereas in DD subjects only the C-domain converts Ang I. As a consequence, the increase in ACE observed in DD subjects will not result in an increase in Ang I-II conversion, although it may result in enhanced bradykinin degradation.

Angiotensin receptors (Chapter 4, 5 and 6)

Ang II concentration-dependently decreased coronary flow (CF) and left ventricular systolic pressure (LVSP) in the mouse coronary vascular bed. Blockade of the AT_2 receptor and/or nitric oxide synthase (NOS) enhanced the Ang II-induced constrictor and inotropic responses, suggesting that Ang II exerts opposite effects on coronary and cardiac contractility via activation of AT_{1A} and AT_2 receptors. No Ang II-induced effects were however found during AT_1 receptor blockade or in hearts of mice deficient for the AT_{1A} receptor. Thus, the AT_2 receptor-induced, nitric oxide (NO)-mediated effects occur only in conjunction with AT_{1A} receptor activation.

Knock-out (KO) mice deficient in either one, two or all three Ang II receptors are an ideal tool to study the receptor-specific functions and the putative AT receptor-independent effects of Ang II and its metabolites. Mice lacking the AT_{1A} receptor were characterized by a reduced heart rate, mean arterial blood pressure (MAP) and increased Ang II plasma levels. Triple KO mice showed an impaired survival and increased Ang II levels in plasma similar to that found in AT_{1A}-deficient mice whereas deletion of the AT_2 receptor increased MAP. MAP of mice lacking both AT_1 receptor subtypes dropped comparable to that observed in triple KO mice. *In vivo*, the Ang II pressor response in AT_{1A} deficient mice was impaired whereas deficiency for the AT_{1B} and/or AT_2 receptor alone had no impact. The additional deletion of AT_{1B} in AT_{1A}-deficient mice further impaired the vasoconstrictive capacity of Ang II. Despite the fact that the general vasoconstrictor responses to endothelin-1 and phenylephrine were unchanged in triple KO mice, Ang II failed to affect MAP in these mice. This indicates that receptors other than the three known AT receptors are not involved in the Ang II-mediated pressor responses.

In the rat coronary circulation, Ang III and Ang IV caused constriction via AT_1 receptor stimulation, although at lower potency than Ang II. Ang III, but not Ang IV, simultaneously induced vasorelaxation via AT_2 receptors, and this was already apparent at subnanomolar Ang III concentrations, as opposed to the submicromolar concentrations of Ang II that were required to observe such relaxation. Ang-(1-7) did not exert effects of its own in the coronary circulation, but blocked AT_1 receptor-induced constriction at high concentrations. The relative constrictor potencies of the

3 angiotensin metabolites in rat iliac arteries and abdominal aortas were identical to those in the coronary vascular bed. These findings suggest that in the rat coronary vascular bed AT_2 receptor activation results in vasodilation, and that Ang III rather than Ang II is the endogenous agonist of these receptors. Ang III, Ang IV and Ang-(1-7) do not exert effects through non-classical AT receptors in the rat coronary vascular bed, iliac artery or abdominal aorta.

Renin inhibition (Chapter 7)

Spontaneously hypertensive rats (SHR) were treated with the renin inhibitor aliskiren, the AT_1 receptor blocker irbesartan or the ACE inhibitor captopril at various doses, and monitored for a period of 3 weeks using radiotelemetry. All three RAS blockers lowered MAP in a dose-dependent manner without affecting heart rate. Irbesartan 15 mg/kg/day and captopril 3 mg/kg/day were equi-potent to aliskiren 100 mg/kg/day in their ability to lower blood pressure. Treatment with the three RAS blockers at these doses increased coronary endothelial function (i.e., reactivity to bradykinin), whereas only aliskiren and irbesartan decreased the coronary response to Ang II. Simultaneously, at these doses, all 3 drugs decreased HW/BW ratio and cardiomyocyte area, although significance was reached for the latter only. In conclusion, for a given decrease in blood pressure, aliskiren is as least as effective as AT_1 receptor blockade and ACE inhibition with regard to improvement of endothelial function and cardiac hypertrophy.

General Discussion

ACE

The active centers of the ACE C- and N-domain contribute equally to bradykinin hydrolysis.[32,153] In contrast, selective inhibition of the N-domain did not affect the blood pressure responses to angiotensin (Ang) I bolus injections in mice,[154] nor did targeted inactivation of the N-domain in mice affect blood pressure or plasma Ang II levels.[155] This suggests that the ACE C-domain is the predominant site of Ang I conversion. The data in this thesis fully support this view, and a recent study in mice with targeted inactivation of the C-domain further confirmed our findings.[308] Carriers of the ACE D allele display higher ACE levels than ACE I allele carriers. This is most likely due to the fact that the D allele leads to higher ACE mRNA expression.[182] Remarkably, the higher ACE levels in subjects with the DD genotype (as compared to II's) do not result in a higher regional conversion rate nor in an alteration in the levels of circulating Ang II in DD's.[183-185,309] Ang II levels also did not differ between mice with different numbers of *Ace* gene copies,[187,188] and compensatory renin regulation, as well as changes in the levels of Ang I-degrading enzymes, have been shown to explain the lack of effect of ACE titration on Ang II in mice. D allele-related changes in renin or differences in Ang I degradation have however not been observed in humans[184,189] and, thus, alternative explanations must be put forward for the absence of a D allele-related change in Ang II in man. Our observation in serum

that the N-domain contributes to Ang II generation in I allele carriers, on top of the C-domain, offers an explanation for this phenomenon. This finding, if confirmed *in vivo*, has multiple implications.
Clearly, I allele carriers should be treated with ACE inhibitors that block both domains. Since virtually all of the currently marketed ACE inhibitors do not make a distinction between the C- and N-domain (i.e., they block both domains when used at regular doses), this probably is already the case. For DD subjects, a C-domain selective ACE inhibitor might be sufficient. Such selective ACE inhibitors most likely have less bradykinin-related side-effects.
Furthermore, given the absence of an effect of the D allele on Ang II generation, it is now understandable that no conclusive data have been obtained with regard to ACE genotyping and ACE inhibitor efficacy. Future studies should therefore either make use of haplotypes,[310,311] or apply an even simpler approach, i.e. perform ACE 'phenotyping'[191] as a first step toward personalized medicine for ACE inhibitors. In other words: to measure the concentration of ACE in circulating blood as a starting point. Plasma (or serum) ACE concentrations are stable within one individual,[312] and reasonably reflect tissue ACE.[181] Measuring ACE allows one to take into consideration the full variation range of ACE, and not just the <20% that is accounted for by the ACE I/D polymorphism.[309] By measuring the response (e.g., the decrease in blood pressure) to a standard dose of an ACE inhibitor in patients with a wide range of ACE concentrations, one should be able to finally settle the question whether patients with high ACE levels respond better or worse than patients with low ACE levels.
Ace gene titration studies in mice do not necessarily reflect the functional consequences of the ACE I/D polymorphism. Based on the present data, it appears that such studies should also incorporate the varying contribution of the two ACE domains. Finally, the molecular mechanism underlying the contributions of the 2 ACE domains in I- and D-allele carriers should be unraveled. Since the insertion concerns a 287 base pair *Alu* repeat in intron 16 of the ACE gene, no molecular differences are to be expected between I and D ACE, and thus a factor in serum capable of interfering with ACE activity, e.g., an endogenous N-domain inhibitor, might be involved.

Angiotensin receptors

In concordance with previous studies,[45,46] our data show that mice lacking the AT_{1A} receptor are hypotensive and display a reduced cardiac weight/body weight ratio.[313] *In vivo*, the pressure response to Ang II was almost completely abolished in AT_{1A} receptor deficient mice, whereas selective deletion of the AT_{1B} - or AT_2 receptor did not alter the response to Ang II. These findings are in line with our (*ex-vivo*) Langendorff studies which demonstrated that the AT_{1A} subtype is the only receptor subtype mediating vasoconstriction in the mouse coronary vascular bed.[62] *In vivo*, deletion of the AT_{1B} receptor on top of AT_{1A} receptor deficiency further decreased baseline blood pressure, and in such animals the pressor response to Ang II was absent. *In-vitro* studies by others have demonstrated that the AT_{1B} receptor is the

most important regulator of Ang II-induced contractile responses in the mouse aorta and femoral artery.[50] The fact that we did not find a role for the AT_{1B} receptor in the mouse coronary vascular bed can be explained by the lack of AT_{1B} receptor expression in the heart.[44] Humans express only one AT_1 receptor subtype, and thus knowledge on the relative roles of both subtypes in rats and mice is of limited clinical importance.

In vivo, deletion of the AT_2 receptor increased blood pressure, whereas *ex vivo,* blockade of the AT_2 receptor in the mouse and rat heart enhanced the AT_1 receptor-mediated vasoconstrictor response to Ang II. This effect was NO-dependent. These data confirm that the AT_2 receptor mediates vasodilatation, thus counteracting the contractile effects of the AT_1 receptor.[57-60] Enhanced AT_1 receptor-mediated effects, either in the coronary vascular bed or the systemic circulation, did not occur in AT_2 receptor KO mice, nor did we observe AT_2 receptor-mediated vasodilatation in mice lacking AT_{1A} and/or AT_{1B} receptors. The former was not due to a selective upregulation of AT_{1A} receptors in AT_2 receptor KO mice, as suggested by others.[82,314,315] It could relate, at least in the systemic vascular bed, to the fact that only one (relatively high) dose of Ang II was tested, whereas an enhanced response will of course only be seen at lower, submaximal doses. However, in the coronary vascular bed, a whole range of Ang II concentrations was tested, and still no enhanced effect occurred in the absence of AT_2 receptors. The most likely explanation of these findings is therefore that AT_2 receptor-mediated effects occur only in conjunction with AT_{1A} receptor activation.[62] Data supporting the existence of AT_1/AT_2 receptor heterodimers supports this idea.[61] Further studies, preferably under pathological conditions (when AT_2 receptors are upregulated[316]) are required to fully unravel this phenomenon.

Our studies in triple KO mice provided no evidence for hemodynamic effects of Ang II that are mediated via non-AT_1/AT_2 receptors, e.g., the Mas receptor or the AT_4 receptor. Given the rapid *in-vivo* metabolism of Ang II to metabolites like Ang III, Ang IV and Ang-(1-7), these data suggest that also these metabolites do not induce (major) hemodynamic effects via non-AT_1/AT_2 receptors, although obviously additional studies infusing these metabolites *in vivo* are required to further substantiate this view. *In-vitro* studies with Ang III, Ang IV and Ang-(1-7) in both isolated rat hearts and blood vessels indeed did not provide evidence for non-AT_1/AT_2 receptor-mediated effects of these metabolites. Both Ang III and Ang IV induced constrictor effects in an AT_1 receptor-dependent manner, although at much lower potency than Ang II. This is not surprising given the current knowledge on Ang II-AT_1 receptor interaction.[280-282] In particular the concentrations of Ang IV that were required to obtain contractile responses were many orders of magnitude above its *in-vivo* concentrations, thus making it unlikely that this metabolite exerts hemodynamic effects under normal physiological conditions. Unexpectedly, Ang III appeared to be a stronger AT_2 receptor agonist than Ang II, with dilator effects already occurring at concentrations in the low nanomolar range. Possibly therefore, Ang III rather than Ang II is the preferred ligand for the AT_2 receptor. Recent studies by others on Ang III-induced natriuresis via AT_2 receptor stimulation support this view.[97,283,317]

Ang-(1-7) exerted no effects in the coronary circulation, iliac artery or abdominal

aorta of the rat, but did act as an AT_1 receptor agonist at micromolar concentrations. This mimics previous findings in the rat renal vascular bed[89,285] and the human forearm[169], and suggests that Ang-(1-7), at pharmacological concentrations, acts as an AT_1 receptor antagonist. Ang-(1-7) also exerts ACE-inhibitory effects,[32] and thus the beneficial effects of Ang-(1-7) infusions in rats[174] may be due, at least in part, to combined ACE inhibition and AT_1 receptor blockade.

The question remains whether AT_2 receptor stimulation is of clinical relevance. The animal work in this thesis, as well as human *in-vitro* data[58] support the idea of AT_2 receptor-mediated vasodilation. A wide range of animal studies suggest that the beneficial effects of AT_1 receptor antagonists actually depend on the AT_2 receptor stimulation that will occur during such treatment,[64,211] possibly even involving Ang-(1-7) as an agonist.[91] Given the lack of AT_2 receptor-induced effects in the absence of AT_1 receptors, and taking into consideration that the effectiveness of ACE inhibitors and AT_1 receptor blockers is identical, one might speculate that, at most, AT_2 receptor stimulation results in additional AT_1 receptor blockade, and that no AT_2 receptor-induced effects will be observed once complete AT_1 receptor blockade is obtained.

Renin inhibition

Due to the species-specifity of the renin-angiotensinogen reaction, human renin inhibitors like aliskiren are relatively weak inhibitors of renin isoforms from non-primate species, including rat, rabbit, and pig.[290] Thus, testing aliskiren in rats requires the use of much higher doses (per kg body weight) than in humans. Yet, in order to get a better understanding of the mechanism of action of renin inhibitors, in particular of its effects on tissue angiotensin production, animal studies are required. Indeed, high doses do lower blood pressure effectively in spontaneously hypertensive rats (SHR).[293] Obviously, much lower (human-like) doses can be applied successfully when using so-called double transgenic rats, i.e., rats that express both the human renin and angiotensinogen genes.[318] These rats were actually designed to test human renin inhibitors, as they develop severe hypertension and end-organ damage due to the human renin-human angiotensinogen reaction. Thus, it is not surprising that renin inhibitors are highly effective in this model.[292,319] In fact, without treatment these rats die within 6-7 weeks. The advantage of using SHR is that this is a well-established physiological model displaying the normal feedback mechanisms during RAS inhibition. Moreover, the use of SHR has contributed greatly to a better understanding of the mechanisms behind ACE inhibition and AT_1 receptor blockade.[297,300,320] It is for this reason that we have started to make a comparison between the 3 types of RAS blockade in SHR. Theoretically, various differences are expected to exist between the 3 groups of drugs.

First, renin inhibitors might have a pharmacokinetic advantage as compared to ACE inhibitors and AT_1 receptor blockers, related to the fact that tissue angiotensin production depends on renin of renal origin. Thus, renin inhibitors may already bind to renin in the circulation, on its way to tissue sites, whereas the other blockers first have to penetrate into the tissues to exert their effects. As a consequence of its binding to circulating renin, the renin-inhibitor complex will eventually replace

unbound “free” renin at tissue sites, thereby preventing angiotensin generation. Thus, the blood pressure-lowering effect of aliskiren may last for a considerable amount of time after stopping treatment (i.e., the time it takes to replace all aliskiren-bound tissue renin by free renin), a phenomenon which has indeed been observed in humans[321] and animals[293]. However, other mechanisms explaining this long-lasting effect should also be considered. Aliskiren has a long half-life, of more than 30 hours.[322,323] Furthermore, it is conceivable that aliskiren accumulates at renal tissue sites, possibly even in renin storage granules, thus allowing the release of blocked renin even when treatment has stopped. This concept can be tested using renin-synthesizing cells *in vitro*. Finally, aliskiren may bind to prorenin, the inactive precursor of renin, either in blood (i.e., on its way to tissue sites) or at the tissue level. Recent studies suggest that prorenin gets activated at tissue sites, through its binding to the (pro)renin receptor. In fact, given its high levels, it might be prorenin rather than renin which is responsible for local angiotensin generation.[110,324] Thus, a blocker which prevents prorenin-induced angiotensin generation might be an ideal tool to suppress tissue angiotensin generation.

Second, it is well-known that during RAS blockade renin levels will rise due to compensatory feedback mechanisms.[1,147,325,326] Of course, such a rise in renin will also occur during renin inhibition. However, because of the presence of the renin inhibitor this renin cannot be enzymatically active, and thus the circulating angiotensin levels will remain low.[289,327] Whether this is also true for tissue angiotensin levels remains to be proven. It has been suggested that the renin rise during renin inhibition with aliskiren is larger than during other types of RAS blockade.[328] Given the aliskiren/renin stoichiometry during aliskiren therapy,[329] it is highly unlikely that this renin is enzymatically active. However, such elevated renin levels might activate the (pro)renin receptor,[15,186,330] and thus induce direct, angiotensin-independent effects. The same applies to prorenin, which will also rise during renin inhibition. (Pro)renin receptor-induced effects include an increase in DNA synthesis, activation of the mitogen-activated protein kinase p38, p42 and p44, stimulation of the release of transforming-growth factor-β_1 and plasminogen activator-inhibitor-1, and phosphorylation of heat shock protein 27.[14,288,331] *In-vitro* studies have already indicated that renin inhibitors do not interfere with (pro)renin-(pro)renin receptor interaction.[332] The consequence of such (pro)renin receptor overstimulation is currently unknown. However, since such a (pro)renin rise also occurs during other types of RAS blockade, its detrimental effects, if present, should have been observed by now. A possible explanation comes from the work of Schefe et al.,[333] who showed that, on activation of the receptor, the transcription factor promyelocytic zinc finger is translocated to the nucleus and represses transcription of the (pro)renin receptor itself, thus creating a short negative feedback loop. In other words: high (pro)renin levels, as occurring during RAS blockade, will suppress (pro)renin receptor expression, thereby preventing excessive receptor activation. Furthermore, the members of a family with a mutated renin allele, resulting in high plasma prorenin levels, were phenotypically normal.[334] Future studies, applying (pro)renin receptor antagonists on top of aliskiren, should settle this issue.

Third, during ACE inhibition and AT_1 receptor blockade, the levels of various angiotensin metabolites (in particular Ang-(1-7) and Ang II) will rise, and a series of preclinical studies suggests that part of the beneficial effects of these drugs involves either Mas receptor activation by Ang-(1-7) or AT_2 receptor stimulation by Ang II.[335,336] Obviously, given the decrease in the levels of all angiotensin metabolites during renin inhibition, such effects cannot occur during aliskiren treatment. So far, this does not appear to diminish the effects of renin inhibitors, at least on blood pressure,[152] possibly because a more complete RAS blockade induces the same effect as AT_2 receptor stimulation during AT_1 receptor blockade, as discussed above. A similar explanation may apply to the potential beneficial effects of Ang-(1-7) mediated by the Mas receptor, which is also known to form heterodimers with the AT_1 receptor.[92,93,337] Finally, given the many feedback mechanisms within the RAS, it seems unlikely that renin inhibitors will fully suppress the RAS. Thus, a combination of aliskiren with other RAS blockers might induce an even better blockade. With regard to blood pressure, this has already been observed[150,291,338], and future studies should now address to what degree this is true with regard to end-organ damage. In this aspect, one might also consider aliskiren combination therapies with beta-adrenoceptor antagonists (which lower renin release) and/or (pro)renin receptor blockers.

Nederlandse samenvatting

Introductie en doel (Hoofdstuk 1)

Het renine-angiotensine systeem (RAS) speelt een belangrijke rol bij de regulatie van de water- en zouthuishouding en de bloeddruk. Vroeger werd het RAS gezien als een circulerend systeem ("circulerend" RAS) maar het is inmiddels algemeen bekend dat angiotensine (Ang) produktie ook plaatsvindt in weefsels ("weefsel" RAS). Angiotensinogeen (een voorloper gevormd in de lever) wordt door renine (enzym gevormd in de nieren) omgezet tot Ang I wat op zijn beurt kan worden omgezet in Ang II door het angiotensine I-converterend enzym (ACE). Als belangrijkste component van het RAS medieert Ang II zijn effecten via de angiotensine type 1 (AT_1) en type 2 (AT_2) receptoren. In knaagdieren zijn er twee subtypen van de AT_1 receptor geïdentificeerd: AT_{1A} en AT_{1B}. AT_1 receptoren mediëren de welbekende effecten van Ang II zoals vasoconstrictie (vernauwing van bloedvaten), inotropie (beïnvloeding van de kracht van de samentrekking van de hartspier), chronotropie (beïnvloeding van de frequentie van de samentrekking van de hartspier), groeistimulatie en de afgifte van onder andere aldosteron en noradrenaline, en van AT_2 receptoren wordt aangenomen dat deze over het algemeen een tegenovergesteld effect bewerkstelligen. Oorspronkelijk dacht men dat alle Ang II metabolieten inactief waren maar inmiddels is het duidelijk dat verschillende metabolieten, zoals Ang III, Ang IV en Ang-(1-7), zelf ook een functie hebben die mogelijk wordt gemedieerd door non-AT_1/AT_2 receptoren. Onder pathofysiologische condities kan een overmatige RAS activiteit leiden tot een verhoogde bloeddruk en cardiale/renale hypertrofie wat kan resulteren in eind-orgaanschade. Het RAS kan tegenwoordig worden geblokkeerd op drie verschillende niveaus: renine, ACE en de AT_1 receptor. ACE remmers en AT_1 receptor blokkers hebben hun effectiviteit al bewezen bij de behandeling van hypertensie (hoge bloeddruk) en andere cardiovasculaire ziekten. ACE remmers blokkeren de formatie van Ang II terwijl AT_1 receptor blokkers voorkomen dat Ang II zijn effecten medieert via de AT_1 receptor. Het is aannemelijk dat tenminste een deel van de voordelige effecten van AT_1 receptor blokkers is te wijten aan stimulatie van de ongeblokkeerde AT_2 receptor. Men denkt dat behandeling met de nieuw ontwikkelde renine remmers zal leiden tot een meer complete onderdrukking van het RAS omdat deze remmers aangrijpen op de eerste, snelheids-beperkende stap. Het is duidelijk dat het RAS nog niet al zijn geheimen heeft geopenbaard. Stap voor stap vallen de stukjes van de puzzel op zijn plaats en een meer complete kennis van het RAS en zijn componenten zou kunnen leiden tot de ontdekking van nieuwe therapeutische doelen en verbeterde behandelingsmethoden.

ACE (Hoofdstukken 2 en 3)

Somatisch ACE bevat twee homologe domeinen (het C- en N-domein) die in staat zijn om Ang I en bradykinine te hydrolyseren. Selectieve remming van het C-domein leidde tot een rechtsverschuiving van de Ang I concentratie-respons curve (CRC) in de arteriën van varkens, terwijl selectieve remming van het N-domein de door Ang I-geïnduceerde constrictie niet beïnvloedde. In humaan bloed werd de Ang

I-II conversie pas volledig geblokkeerd door remming van beide domeinen. De degradatie van bradykinine door membraan-gebonden ACE daarentegen was afhankelijk van beide domeinen; omdat zowel selectieve remming van het C- als N-domein leidde tot een verschuiving naar links van de bradykinine CRC, waarbij een maximale verschuiving naar links werd waargenomen na blokkade van beide domeinen. Dit betekent dat de Ang I-II conversie in weefsels afhankelijk is van het C-domein terwijl beide domeinen een rol spelen bij de conversie door ongebonden "soluble" ACE en bij de degradatie van bradykinine in weefsel.
Het ACE insertie/deletie (I/D) polymorfisme bepaalt een deel van de variabiliteit in ACE tussen verschillende individuen: dragers van het D allel hebben hogere ACE concnetraties in weefsel en bloed. Tegen de verwachtingen in, leiden de hogere ACE concentraties die worden gevonden in DD individuen niet tot een hogere regionale Ang I-II conversie snelheid en/of hogere Ang II levels. Met behulp van bloed van DD en II personen kon worden aangetoond dat selectieve remming van het C-domein de Ang I-II conversie in het bloed van DD individuen volledig blokkeerde terwijl additionele remming van het N-domein nodig was om de conversie in bloed van II individuen volledig te remmen. Beide domeinen zijn gelijk in hun bijdrage aan de hydrolyse van bradykinine in plasma van DD en II individuen. Het uitblijven van een Ang II ophoping in DD personen wordt mogelijk veroorzaakt door het feit dat bij II individuen zowel het C- als N-domein Ang II genereren terwijl alleen het C-domein Ang I converteert in DD individuen. Dit heeft tot het gevolg dat de toename in ACE die wordt gevonden in DD individuen niet zal resulteren in een toename van Ang I-II conversie, alhoewel deze wel kan zorgen voor een verhoogde bradykinine degradatie.

Angiotensine receptoren (Hoofdstukken 4, 5 en 6)

Ang II zorgde voor een concentratie-afhankelijke verlaging van de coronaire flow (CF) en linker ventrikel systolische druk (LVSP) in het hart van de muis. Blokkade van de AT_2 receptor en/of stikstof monoxide synthase verhoogde de Ang II-geïnduceerde constrictoire en inotrope effecten wat de suggestie wekt dat Ang II tegenovergestelde effecten medieert op coronaire - en hartcontractiliteit via activatie van AT_{1A} en AT_2 receptoren. Er werden echter geen Ang II-geïnduceerde effecten gevonden tijden AT_1 receptor blokkade of in harten van muizen die deficiënt waren voor de AT_{1A} receptor. Dus de AT_2 receptor-geïnduceerde, stikstof monoxide-gemedieerde effecten kunnen alleen plaatsvinden na activatie van de AT_{1A}receptor.
Knock-out (KO) muizen deficiënt voor een, twee of alle drie de Ang II receptoren zijn ideaal voor het bestuderen van de receptor-specifieke functies en mogelijke AT receptor-onafhankelijke effecten van Ang II en zijn metabolieten. Muizen deficiënt voor de AT_{1A} receptor werden gekarakteriseerd door een lagere hartslag en bloeddruk, en een verhoogde Ang II concentratie in hun bloed. De overleving van triple KO muizen was afgenomen en deze dieren hadden een toegenomen Ang II concentratie in bloed, vergelijkbaar met die in AT_{1A}-defiënte muizen. Uitschakeling van de AT_2 receptor verhoogde de bloeddruk. De daling in bloeddruk van muizen deficiënt voor beide AT_1 receptor subtypen was vergelijkbaar met die in triple KO

muizen. *In vivo* was het bloeddrukverhogende effect van Ang II afgenomen in AT_{1A} deficiënte muizen, maar niet in muizen met een deficiëntie voor de AT_{1B} en/of AT_2 receptor. De additionele deletie van de AT_{1B} receptor in AT_{1A}-deficiënte muizen leidde tot een verdere afname van de vasoconstrictoire effecten van Ang II. Ondanks het feit dat de algemene vasoconstrictoire effecten op endotheline-1 en fenylefrine onveranderd waren in triple KO muizen, was Ang II niet in staat de bloeddruk te beïnvloeden in deze muizen. Dit geeft aan dat er geen andere receptoren dan de drie bekende AT receptoren betrokken zijn bij de Ang II-gemedieerde pressor effecten.
Zowel Ang III als Ang IV zorgden voor een vasoconstrictoire response in de coronaire circulatie van de rat, hoewel hun potentie lager was dan die van Ang II. Tegelijkertijd induceerde Ang III, maar niet Ang IV, ook een vasorelaxatie via de AT_2 receptoren en dit was al duidelijk zichtbaar bij subnanomolaire concentraties van Ang III in tegenstelling tot de submicromolaire concentraties van Ang II die nodig waren om een dergelijke relaxatie waar te nemen. Ang-(1-7) medieerde geen effecten in de coronaire circulatie maar blokkerde wel de AT_1 receptor-geïnduceerde constrictie bij hoge concentraties. De relatieve constrictoire eigenschappen van de drie angiotensine metabolieten in de arteria iliaca en abdominale aorta van de rat waren identiek aan de effecten die werden gevonden in de coronaire circulatie. Deze bevindingen suggereren dat AT_2 receptor activatie in het coronaire vasculaire bed resulteert in vasodilatatie en dat Ang III in plaats van Ang II de endogene agonist is van deze receptoren. Ang III, Ang IV en Ang-(1-7) medieerden geen effecten door niet-klassieke AT receptoren in het coronaire vaatbed, arteria iliaca en abdominale aorta van de rat.

Renine remming (Hoofdstuk 7)

Spontaan hypertensieve ratten (SHR) werden behandeld met verschillende doses van de renine remmer aliskiren, de AT_1 receptor blokker irbesartan of de ACE remmer captopril, en gevolgd voor een periode van 3 weken met behulp van radiotelemetrie. Alle RAS blokkers verlaagden de MAP op een dosis-afhankelijke manier zonder hierbij de hartslag te beïnvloeden. Irbesartan 15 mg/kg/dag en captopril 3 mg/kg/dag waren equi-potent in vergelijking met aliskiren 100 mg/kg/dag in hun vermogen de bloeddruk te verlagen. Behandeling met deze doses van de drie RAS blokkers verbeterde de coronaire endotheel functie (de reactiviteit op bradykinine), terwijl alleen aliskiren en irbesartan leidden tot een verlaagde coronaire respons op Ang II. Tegelijkertijd verlaagden alle drie de medicijnen de hartgewicht/lichaamsgewicht ratio en cardiomyocyt grootte, alhoewel significantie alleen voor de laatste parameter werd bereikt. In conclusie kunnen we stellen dat, voor een gegeven daling in bloeddruk, aliskiren tenminste zo effectief is als AT_1 receptor blokkade en ACE remming met betrekking tot de verbetering van de endotheel functie en de cardiale hypertrofie.

Publications

Full papers:

van Helden H.P.M, van de Meent D., Oostdijk J.P., Joosen M.J.A, van Esch J.H.M., Hammer A.H., Diemel R.V., Protection of Rats Against Perfluorisobutene (PFIB)-Induced Pulmonary Edema by Curosurf and N-Acetylcysteine. *Inhalation Toxicology*. 2004; 16; 549-564.

van Esch J.H.M., Tom B., Batenburg W.W., Georgiadis D., Yiokatis, A., van Gool J.M., de Bruin R.J., de Vries R., Danser A.H.J., Selective Angiotensin-Converting Enzyme C-Domain Inhibition is Sufficient to Prevent Angiotensin I-Induced Vasoconstriction. *Hypertension*. 2005; 45: 120-125.

van Esch J.H.M., Schuijt M.P., Sayed J., Choudhry Y., Walther T., Danser A.H.J., AT_2 Receptor-Mediated Vasodilation in the Mouse Heart Depends on AT_{1A} Receptor Activation. *British Journal of Pharmacology*. 2006; 148: 452-8.

Batenburg W.W., van Esch J.H.M., Garrelds I.M., Jorde U., Lamers J.M., Dekkers D.H., Walther T., Kellett E., Milligan G., van Kats J.P., Danser, A.H.J, Carvedilol-induced antagonism of angiotensin II: a matter of $alpha_1$-adrenoceptor blockade. *Journal of Hypertension*. 2006; 24:1355-1363.

Danser A.H.J. Batenburg W.W., van Esch J.H.M., Prorenin and the (pro)renin receptor – an update. *Neprhology Dialysis Transplantation*. 2007; 22: 1288-1292.

van Esch J.H.M. van Gool M.G., de Bruin R.J.A., Payne J.R., Montgomery H.E., Hectors M., Deinum J., Dive V., Danser A.H.J., Different contributions of the ACE C- and N-domain in subjects with the ACE II and DD genotype. *Journal of Hypertension.* 2008; 26: 706-713.

Gembardt F., Heringer-Walther S., van Esch J.H.M., Sterner-Cock A., van Veghel R., Le T.H., Garrelds I.M., Coffman T.M., Danser A.H.J., Schultheiss H.P., Walther T., Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors. Submitted, 2008.

van Esch J.H.M., Oosterveer C.R., Batenburg W.W., van Veghel R., Danser A.H.J., Effects of Angiotensin and its metabolites in the rat coronary vascular bed: is angiotensin III the endogenous ligand of the AT_2 receptor? Submitted, 2008

Bookchapters

van Esch J.H.M., Danser A.H.J., Local angiotensin generation and AT_2 receptor activation. In: Proteases in biology and disease – Frontiers in research of the RAS on human disease, Lueng P.S. (Ed): 2007; Vol 7, Chapter 12.

Letter

Krop M., van Esch J.H.M., Danser A.H.J., Letter Regarding Article by Itoh et al, "Role of p90 Ribosomal S6 Kinase-Mediated Prorenin-Converting Enzyme in Ischemic and Diabetic Myocardium", *Circulation*. 2006; 114: e556.

Other

van Esch J.H.M., Renine-angiotensinesysteem blokkeren met renineremmers: nieuwe cardiovasculaire aanpak. *Pharmaceutisch weekblad*. 2007; 24-25.

Abstracts

Batenburg W.W., van Esch J.H.M., Garrelds I.M., Jorde U., van Kats J.P., Saxena P.R., Danser A.H.J., Carvedilol-induced antagonism of angiotensin II: focus on AT_1 receptor-α_1 adrenoceptor crosstalk. *Hypertension*. 2004; 44: 498.

van Esch J.H.M., Dive V., Georgiadis D., Yiokatis, A., van Gool J.M.G., Danser A.H.J., Tissue angiotensin I-II conversion is exclusively mediated by the ACE C-domain. *Circulation.* 2004; 110: 54: 255.

Batenburg W.W., van Esch J.H.M., Danser A.H.J., Carvedilol-induced antagonism of angiotensin II: focus on AT_1 receptor-α_1-adrenoceptor crosstalk. *Naunyn-Schmiedeberg's Archives for Pharmacology*. 2005; 371: R3.

van Esch J.H.M., Dive V., Georgiadis D., Yiokatis, A., van Gool J.M.G., Danser A.H.J., Tissue angiotensin I-II conversion is exclusively mediated by the ACE C-domain. *Naunyn-Schmiedeberg's Archives for Pharmacology*. 2005; 371: R4.

van Esch J.H.M., Schuijt M.P., Sayed J., Choudry Y., Walther T., Danser A.H.J., Characterization of angiotensin II receptor subtypes in the isolated perfused mouse Langendorff heart. *Journal of Hypertension*. 2005; 23 (suppl 4): S260.

Batenburg W.W., van Esch J.H.M., Jorde U., Lamers J.M.J., Dekkers D.H.W., van Kats J.P., Danser A.H.J., Carvedilol antagonizes angiotensin II: A mechanism explaining the beneficial metabolic effects of non-selective beta-adrenoceptor blockade. *Journal of Hypertension*. 2005; 23 (suppl 4): S364.

van Esch J.H.M., Sterner-Kock A., Garrelds I.M., Heringer-Walther S., van Veghel R., Danser A.H.J., Walther T., Angiotensin II-mediated cardiac effects in AT_{1A}-, AT_{1B}- and AT_2-receptor single, double and triple knockout mice. *Journal of Hypertension*. 2006; 24 (suppl 6): S86.

van Esch J.H.M., van Veghel R., Garrelds I.M., Danser A.H.J., Walther T., Angiotensin II-mediated cardiac effects in AT_{1A}-, AT_{1B}- and AT_2-receptor single, double and triple knockout mice. *Journal of Hypertension*. 2007; 25 (suppl 2): S18.

Gembardt F., van Esch J.H.M., Sterner-Cock van Veghel R., Garrelds I., Heringer-Walther S., Coffman T., Danser A.H.J., Walther T., Angiotensin II-induced effects in AT_{1A}-, AT_{1B}- and AT_2-receptor single, double and triple knockout mice. *Hypertension*. 2007; 50: E78.

van Esch J.H.M., van Veghel R., Garrelds I.M., Danser A.H.J., Renin inhibition improves coronary endothelial function in spontaneously hypertensive rats. *Hypertension*. 2007; 50: E100-E101.

van Esch J.H.M., Oosterveer C.R., Batenburg W.W., Danser A.H.J., Evaluation of cardiac AT_2 receptor stimulation by angiotensin II and its metabolites. *Naunyn-Schmiedeberg's Archives for Pharmacology*. 2007; In press.

van Esch J.H.M., van Veghel R., Garrelds I.M., Danser A.H.J., Renin inhibition improves coronary endothelial function in spontaneously hypertensive rats. *Naunyn-Schmiedeberg's Archives for Pharmacology.* 2007; In press.

Dankwoord

Na lange tijd van data verzamelen, analyseren en proberen te beredeneren wat dit dan wel allemaal betekent is het er dan toch van gekomen, mijn proefschrift! Dit gehele proces was uiteraard absoluut onmogelijk geweest zonder de hulp van collega's, vrienden en familie. Ik wil daarom de laatste bladzijden van dit proefschrift graag gebruiken om deze mensen, en ik hoop dat ik niemand vergeet, te bedanken. Daarom zijn we dan nu aangeland bij het, vaak eerst en meest gelezen hoofdstuk van het proefschrift, het dankwoord.

Jan, als mijn promotor (en "RAS-goeroe") ben jij natuurlijk de eerste die ik wil bedanken. Ze zeggen wel eens "alle begin is moeilijk" en ik denk dat dit ook zeker opgaat voor de eerste twee jaar van mijn AIO carriere. Je vertrouwen, positieve kijk en verhelderende blik hebben er mede toe bijgedragen dat ik vandaag hier sta en dit boekje heb kunnen afronden. Indrukwekkend dat jij in een oog opslag altijd meer uit een grafiek weet te halen dan ik na uren staren. Bedankt dat ik altijd bij je aan kon kloppen voor steun, discussie of gewoon een praatje.

Prof.dr. Jaap. Verweij, Prof.dr. Cock van Duijn, Prof.dr. Gerjan Navis wil ik bedanken voor bereidheid deel te nemen in de kleine commissie en de beoordeling van mijn proefschrift. Prof.dr. Carlos Villalon, Dr. Ton van den Meiracker en Prof.dr. Maarten Schalekamp wil ik bedanken deelname in de grote commissie. Carlos, I am very happy that you were willing to combine your trip to Europe with being a member of my committee. Although we never really worked together we had some very interesting discussions (both work as non-work related) during your visits to our department. Ton, bedankt voor de interessante discussies en natuurlijk het gezelschap tijdens het camper avontuur/congres in Arizona. Prof.dr. Maarten Schalekamp, indrukwekkend hoe u alles binnen het RAS kunt verklaren met ingewikkelde formules die u zo uit uw mouw weet te schudden.

Ik ben blij dat ik tijdens mijn promotie word bijgestaan door mijn twee kamergenoten van het eerste uur, Richard en Saurabh. Richard, je hebt een hele belangrijke rol gespeeld bij de tot standkoming van dit proefschrift. Je hebt me de afgelopen jaren ontzettend geholpen bij het opzetten en uitvoeren van de vele experimenten, mijn computerproblemen maar ook als ik een keertje op zoek was naar een leuke film of wat muziek. Je bent altijd in voor een geintje en ik denk niet dat er nog ooit een weekje cursus komt dat kan tippen aan ons avontuur in Center Parcs. Als er ooit nog een vervolg komt rij jij! Saurabh, after I managed to clear the confusion that Saurabh and "Amu" in fact were one and the same person sitting in the same room with me, we quickly became good friends. You always have been there for me during good and bad times. We had a great time both in- as outside the lab and I am happy that we are still in such good contact after you moved to Denmark to continue your career. I am convinced I will have to call you Professor Gupta one day.

Wendy, vanaf dag een heb je me wegwijs gemaakt op het lab. Zeg nou zelf, van wie kon ik nu beter les krijgen dan de “Empress of Mulvany”? Ik kon altijd bij je terecht voor raad, advies of gewoon een praatje over van alles en nog wat. We hebben samen inmiddels heel wat congressen bezocht en naast de wetenschappelijke lading was er altijd tijd voor ontspanning en gezelligheid, ik hoop dat er nog vele zullen volgen! Trouwens, je mag het nu best eens vertellen hoor van die vingerhoedjes.....
Manne (“Mr. Money” voor Arizona campertrip veteranen), jij hebt het strijken van congres-bloesjes toch wel echt tot een hogere dimensie verheven. Bedankt voor de gezellige tijd zowel binnen als buiten het werk. Ik denk dat je zelf inmiddels lekker op weg bent met het vullen van je eigen boekje en na mijn promotie ben jij de volgende, succes!

“Mijn studenten”, wil ik bedanken voor het vertrouwen, geduld en natuurlijk de gezelligheid. Voor mij was dit een hele leerzame periode en ik hoop dat jullie iets van mij hebben kunnen opsteken. Beste Chantal, hoofdstuk 6 is zeker “jouw ding”. Ik weet zeker dat dit hoofdstuk geen onderdeel van mijn boekje zou zijn geweest zonder je inzet en enthousiasme. Succes met het afronden van je studie Farmacie en ik hoop dat deze stage je toch nog een beetje warm heeft gemaakt voor het onderzoek. Els, ik vind het harstikke leuk dat ik de kans heb gekregen je op weg te helpen met je onderzoek tijdens je master-opleiding. Als sportfanaten onder elkaar hebben we altijd wel iets om over te kletsen. Ik vind het super dat we inmiddels collega’s en kamergenoten zijn geworden.

Ingrid, ik ben altijd weer verbaasd wat er is te vinden in de zakken van jouw labjas; rekenmachientjes, buisjes, dopjes, roervlootjes, alles is daar te vinden! Harstikke bedankt voor het meten van al die bloed en orgaan “prutjes”. Ik weet dat hiervoor soms heel wat spierballen nodig waren!
Kayi (“ontzettende vrouw”), ik hoop dat 3 juni voor jou een onvergetelijke dag word en veel succes met je verdere promotie onderzoek.
De rest van mijn (ex-)collegas wil ik bedanken voor hun adviezen, hulp en gezellige tijd; Birgitte, Ria, René (de Vries), Wenxia, Antoinette, Anton, Mieke Suneet, Jan Heiligers† Jasper, Aloys, Hari, Thomas, Sylvia, Florian, Wang Yong, Jialin, Michaela, Vijay, Uday, Irene, Serge, Mellisa, Martin, Beril en Prof.dr. Saxena. Dit geld ook voor de “nieuwe” collegas van het hypertensie lab van Interne Geneeskunde; René (de Bruin), Pieter, Mariette, Joost, Leon, Gooitzen, Jeanette, Usha, Angelique, Marcel, Bianca, Evelien, Marjolein, Joyce, Edith en Frans Boomsma.

Mijn “schoonfamilie” Peter, Anja, Boet en Lobke wil ik bedanken voor al hun hulp en interesse tijdens mijn promotietraject. Peter en Anja, super dat jullie je reisschema konden aanpassen om hier vandaag bij te kunnen zijn. Boet veel succes met het afronden van je promotie onderzoek.

En dan natuurlijk ons pap en ons mam! Jullie hebben me vrij gelaten en gestimuleerd om te gaan studeren wat ik zelf wilde en me geleerd dingen “geen halve bak” te doen. Bedankt voor jullie onvoorwaardelijke steun en vertrouwen. Inhoudelijk was het misschien een beetje moeilijk uitleggen wat ik nou precies uitvoerde op het lab, maar ik hoop dat dit boekje hier toch een beetje duidelijkheid in kan scheppen. Mijn “kleine” zusje Caroline en Maurice wil bedanken voor al hun steun en interesse. Voor jullie is juni, net als voor mij, een belangrijke maand in jullie leven. Ik weet zeker dat jullie op 13 juni een geweldig mooie dag gaan beleven!

Als laatste wil ik eindigen met mijn lieve vriendinnetje, Debby. Mijn dank aan jou is moeilijk in woorden uit te drukken. Ondanks je eigen drukke programma, stond je als geen ander altijd voor me klaar. Bedankt dat je het hebt volgehouden met mijn niet altijd even makkelijke persoontje. Zonder jouw steun en liefde had dit boekje er nooit gelegen. Ik weet zeker dat we heel erg gelukkig gaan worden in ons nieuwe droom-huisje in Schiedam.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 3 mei 1979 te Oisterwijk, waar hij zijn HAVO diploma behaalde aan het Durendael College. In 1996 begon hij aan de studie Hoger Laboratorium Onderwijs aan Hogeschool Brabant te Etten-leur. In het laatste jaar liep hij 12 maanden stage op de afdeling Farmacologie bij TNO Prins Maurits Laborarium te Rijswijk. Onder supervisie van Dr. H.P.M. van Helden deed hij hier onderzoek naar surfactant-schade en longoedeem veroorzaakt door het longtoxische agens perfluorisobutylene (PFIB). Na het behalen van zijn diploma in de specialisatie biochemie in 2000, vervolgde hij zijn studietraject datzelfde jaar met de opleiding medische Biologie aan de Katholieke Universiteit Nijmegen. Zijn eerste stage liep hij op de afdeling Moleculaire biologie te Nijmegen waar hij onder leiding van Prof.dr. H.G. Stunnenberg en Dr. A. Vermunt onderzoek deed naar de expressie van de malaria vaccin kandidaat pfs48/45 door middel van het eukaryote vaccinia expressie systeem. Zijn tweede stage liep hij op de afdeling psychoneurofarmacologie te Nijmegen waar hij de hartslag, bloeddruk, lichaamstemperatuur en locomotor activiteit in APO-SUS en APO-UNSUS ratten bestudeerde met behulp van radiotelemetrie onder leiding van Prof.dr. A.R. Cools en Dr. A. Ellenbroek. In zijn doctoraalscriptie behandelde hij de rol van dopamine in de synaps, waarna hij in 2003 zijn diploma behaalde.
Vanaf november 2003, is hij werkzaam geweest als assistent in opleiding op de afdeling Inwendige geneeskunde - sector Farmacologie, vasculaire en metabole ziekten (voorheen afdeling Farmacologie) van het Erasmus MC te Rotterdam onder supervisie van Prof.dr. A.H.J. Danser. Dit heeft uiteindelijk geresulteerd in dit proefschrift. Sinds november 2007, zet hij zijn werk voort als post-doc opdezelfde afdeling gesubsidieerd door Nederlandse Hartstichting (NHS).

Abbreviations

ACE	Angiotensin converting enzyme
ACEi	ACE inhibitors
ACE2	Angiotensin converting enzyme 2
Ang	Angiotensin
Ang I	Angiotensin I / Angiotensin-(1-10)
Ang II	Angiotensin II / Angiotensin-(1-8)
Ang III	Angiotensin III / Angiotensin-(2-8)
Ang IV	Angiotensin IV / Angiotensin-(3-8)
Ang-(1-7)	Angiotensin-(1-7)
ANOVA	Analysis of variance
AT_1	Angiotensin type 1
AT_{1A}	Angiotensin type 1A
AT_{1B}	Angiotensin type 1B
AT_2	Angiotensin type 2
AT_4	Angiotensin type 4
A-779	D-ala7-Ang (1-7)
B_2	Bradykinin type 2
BK	Bradykinin
Bpm	Beats per minute
BW	Body weight
CF	Coronary flow
cGMP	Cyclic Guanosine 3', 5' monophosphate
CRC	Concentration response curve
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
GPCR	G-protein-coupled receptor
L-NAME	Nω-nitro-L-arginine methyl ester HCl
LVSP	Left ventricular systolic pressure
HHL	Hip-His-Leu
HW	Heart weight
HR	Heart rate
IGFII	Insulin-like growth factor II
i.p.	Intraperitoneal
IU	International units
KH	Krebs Henseleit
KO	Knock-out
KW	Kidney weight
M6P	Mannose-6-phosphate
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein
mmHg	Millimetres of mercury
NO	Nitric oxide

NOS	Nitric oxide synthase
PCMA	Porcine coronary microarteries
PE	Phenylephrine
PFA	Porcine femoral artery
RAS	Renin angiotensin system
SHR	Spontaneously hypertensive rat
WKY	Wistar Kyoto
WT	Wildtype

References

1. van Kats JP, Duncker DJ, Haitsma DB, Schuijt MP, Niebuur R, Stubenitsky R, Boomsma F, Schalekamp MADH, Verdouw PD, Danser AHJ. Angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade prevent cardiac remodeling in pigs after myocardial infarction: role of tissue angiotensin II. *Circulation*. 2000;102:1556-63.
2. Morris BJ. Molecular biology of renin. I: Gene and protein structure, synthesis and processing. *J Hypertens*. 1992;10:209-14.
3. Clausmeyer S, Reinecke A, Farrenkopf R, Unger T, Peters J. Tissue-specific expression of a rat renin transcript lacking the coding sequence for the prefragment and its stimulation by myocardial infarction. *Endocrinology*. 2000;141:2963-70.
4. Shinagawa T, Do YS, Baxter J, Hsueh WA. Purification and characterization of human truncated prorenin. *Biochemistry*. 1992;31:2758-64.
5. Clark AF, Sharp MG, Morley SD, Fleming S, Peters J, Mullins JJ. Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology. *J Biol Chem*. 1997;272:18185-90.
6. Sharp MG, Fettes D, Brooker G, Clark AF, Peters J, Fleming S, Mullins JJ. Targeted inactivation of the Ren-2 gene in mice. *Hypertension*. 1996;28:1126-31.
7. Fukamizu A, Sugimura K, Takimoto E, Sugiyama F, Seo MS, Takahashi S, Hatae T, Kajiwara N, Yagami K, Murakami K. Chimeric renin-angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes. *J Biol Chem*. 1993;268:11617-21.
8. Danser AHJ, Deinum J. Renin, prorenin and the putative (pro)renin receptor. *Hypertension*. 2005;46:1069-76.
9. Suzuki F, Hayakawa M, Nakagawa T, Nasir UM, Ebihara A, Iwasawa A, Ishida Y, Nakamura Y, Murakami K. Human prorenin has “gate and handle” regions for its non-proteolytic activation. *J Biol Chem*. 2003;278:22217-22.
10. Itskovitz J, Rubattu S, Levron J, Sealey JE. Highest concentrations of prorenin and human chorionic gonadotropin in gestational sacs during early human pregnancy. *J Clin Endocrinol Metab*. 1992;75:906-10.
11. Danser AHJ, van den Dorpel MA, Deinum J, Derkx FHM, Franken AA, Peperkamp E, de Jong PT, Schalekamp MADH. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J Clin Endocrinol Metab*. 1989;68:160-7.
12. Sealey JE, Goldstein M, Pitarresi T, Kudlak TT, Glorioso N, Fiamengo SA, Laragh JH. Prorenin secretion from human testis: no evidence for secretion of active renin or angiotensinogen. *J Clin Endocrinol Metab*. 1988;66:974-8.
13. Lenz T, Sealey JE, Lappe RW, Carilli C, Oshiro GT, Baxter JD, Laragh JH. Infusion of recombinant human prorenin into rhesus monkeys. Effects on hemodynamics, renin-angiotensin-aldosterone axis and plasma testosterone. *Am J Hypertens*. 1990;3:257-61.
14. Saris JJ, t Hoen PA, Garrelds IM, Dekkers DH, den Dunnen JT, Lamers JM, Danser AHJ. Prorenin induces intracellular signaling in cardiomyocytes independently of

angiotensin II. *Hypertension*. 2006;48:564-71.

15. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest*. 2002;109:1417-27.
16. Saris JJ, Derkx FHM, de Bruin RJA, Dekkers DH, Lamers JM, Saxena PR, Schalekamp MADH, Danser AHJ. High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin. *Am J Physiol Heart Circ Physiol*. 2001;280:H1706-15.
17. Kang JX, Li Y, Leaf A. Mannose-6-phosphate/insulin-like growth factor-II receptor is a receptor for retinoic acid. *Proc Natl Acad Sci U S A*. 1997;94:13671-6.
18. Kornfeld S. Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem*. 1992;61:307-30.
19. Di Bacco A, Gill G. The secreted glycoprotein CREG inhibits cell growth dependent on the mannose-6-phosphate/insulin-like growth factor II receptor. *Oncogene*. 2003;22:5436-45.
20. Véniant M, Ménard J, Bruneval P, Morley S, Gonzales MF, Mullins JJ. Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver. *J Clin Invest*. 1996;98:1966-70.
21. Peters J, Farrenkopf R, Clausmeyer S, Zimmer J, Kantachuvesiri S, Sharp MG, Mullins JJ. Functional significance of prorenin internalization in the rat heart. *Circ Res*. 2002;90:1135-41.
22. Doolittle RF. Angiotensinogen is related to the antitrypsin-antithrombin-ovalbumin family. *Science*. 1983;222:417-9.
23. Kageyama R, Ohkubo H, Nakanishi S. Induction of rat liver angiotensinogen mRNA following acute inflammation. *Biochem Biophys Res Commun*. 1985;129:826-32.
24. Kitamura N, Ohkubo H, Nakanishi S. Molecular biology of the angiotensinogen and kininogen genes. *J Cardiovasc Pharmacol*. 1987;10 (suppl 7):S49-53.
25. Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev*. 2006;86:747-803.
26. Niimura F, Labosky PA, Kakuchi J, Okubo S, Yoshida H, Oikawa T, Ichiki T, Naftilan AJ, Fogo A, Inagami T, et al. Gene targeting in mice reveals a requirement for angiotensin in the development and maintenance of kidney morphology and growth factor regulation. *J Clin Invest*. 1995;96:2947-54.
27. Kimura S, Mullins JJ, Bunnemann B, Metzger R, Hilgenfeldt U, Zimmermann F, Jacob H, Fuxe K, Ganten D, Kaling M. High blood pressure in transgenic mice carrying the rat angiotensinogen gene. *EMBO J*. 1992;11:821-7.
28. Hubert C, Houot AM, Corvol P, Soubrier F. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. *J Biol Chem*. 1991;266:15377-83.
29. Wei L, Alhenc-Gelas F, Corvol P, Clauser E. The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *J Biol Chem*. 1991;266:9002-8.
30. Wei L, Alhenc-Gelas F, Soubrier F, Michaud A, Corvol P, Clauser E. Expression and characterization of recombinant human angiotensin I-converting enzyme. Evidence

for a C-terminal transmembrane anchor and for a proteolytic processing of the secreted recombinant and plasma enzymes. *J Biol Chem*. 1991;266:5540-6.

31. van Esch JHM, Tom B, Dive V, Batenburg WW, Georgiadis D, Yiotakis A, van Gool JM, de Bruijn RJA, de Vries R, Danser AHJ. Selective angiotensin-converting enzyme C-domain inhibition is sufficient to prevent angiotensin I-induced vasoconstriction. *Hypertension*. 2005;45:120-5.
32. Tom B, de Vries R, Saxena PR, Danser AHJ. Bradykinin potentiation by angiotensin-(1-7) and ACE inhibitors correlates with ACE C- and N-domain blockade. *Hypertension*. 2001;38:95-9.
33. Esther CR, Jr., Howard TE, Marino EM, Goddard JM, Capecchi MR, Bernstein KE. Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Lab Invest*. 1996;74:953-65.
34. Kessler SP, Senanayake PD, Gaughan C, Sen GC. Vascular expression of germinal ACE fails to maintain normal blood pressure in ACE-/- mice. *FASEB J*. 2007;21:156-166.
35. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*. 2000;87:E1-9.
36. Turner AJ, Hooper NM. The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol Sci*. 2002;23:177-83.
37. Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*. 2002;277:14838-43.
38. Gurley SB, Allred A, Le TH, Griffiths R, Mao L, Philip N, Haystead TA, Donoghue M, Breitbart RE, Acton SL, Rockman HA, Coffman TM. Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice. *J Clin Invest*. 2006;116:2218-25.
39. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature*. 2002;417:822-8.
40. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. 2003;426:450-4.
41. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature*. 1991;351:233-6.
42. Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature*. 1991;351:230-3.
43. Elton TS, Stephan CC, Taylor GR, Kimball MG, Martin MM, Durand JN, Oparil S. Isolation of two distinct type I angiotensin II receptor genes. *Biochem Biophys Res Commun*. 1992;184:1067-73.
44. Burson JM, Aguilera G, Gross KW, Sigmund CD. Differential expression of angiotensin

receptor 1A and 1B in mouse. *Am J Physiol*. 1994;267:E260-7.

45. Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci U S A*. 1995;92:3521-5.
46. Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, et al. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem*. 1995;270:18719-22.
47. Chen X, Li W, Yoshida H, Tsuchida S, Nishimura H, Takemoto F, Okubo S, Fogo A, Matsusaka T, Ichikawa I. Targeting deletion of angiotensin type 1B receptor gene in the mouse. *Am J Physiol*. 1997;272:F299-304.
48. Tsuchida S, Matsusaka T, Chen X, Okubo S, Niimura F, Nishimura H, Fogo A, Utsunomiya H, Inagami T, Ichikawa I. Murine double nullizygotes of the angiotensin type 1A and 1B receptor genes duplicate severe abnormal phenotypes of angiotensinogen nullizygotes. *J Clin Invest*. 1998;101:755-60.
49. Oliverio MI, Best CF, Kim HS, Arendshorst WJ, Smithies O, Coffman TM. Angiotensin II responses in AT1A receptor-deficient mice: a role for AT1B receptors in blood pressure regulation. *Am J Physiol*. 1997;272:F515-20.
50. Zhou Y, Chen Y, Dirksen WP, Morris M, Periasamy M. AT1b receptor predominantly mediates contractions in major mouse blood vessels. *Circ Res*. 2003;93:1089-94.
51. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82-97.
52. Thomas WG, Thekkumkara TJ, Baker KM. Molecular mechanisms of angiotensin II (AT1A) receptor endocytosis. *Clin Exp Pharmacol Physiol*. 1996;3:S74-80.
53. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, Makita N, Iwanaga K, Zhu W, Kudoh S, Toko H, Tamura K, Kihara M, Nagai T, Fukamizu A, Umemura S, Iiri T, Fujita T, Komuro I. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol*. 2004;6:499-506.
54. AbdAlla S, Lother H, Quitterer U. AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature*. 2000;407:94-8.
55. Purdy RE, Weber MA. Angiotensin II amplification of alpha-adrenergic vasoconstriction: role of receptor reserve. *Circ Res*. 1988;63:748-57.
56. Guimaraes S, Pinheiro H. Functional evidence that in the cardiovascular system AT1 angiotensin II receptors are AT1B prejunctionally and AT1A postjunctionally. *Cardiovasc Res*. 2005;67:208-15.
57. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature*. 1995;377:744-7.
58. Batenburg WW, Garrelds IM, Bernasconi CC, Juillerat-Jeanneret L, van Kats JP, Saxena PR, Danser AHJ. Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries. *Circulation*. 2004;109:2296-301.
59. Schuijt MP, Basdew M, van Veghel R, de Vries R, Saxena PR, Schoemaker RG, Danser AHJ. AT(2) receptor-mediated vasodilation in the heart: effect of myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2001;281:H2590-6.
60. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa

I, Hogan BL, Inagami T. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature*. 1995;377:748-50.

61. AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U. The angiotensin II AT2 receptor is an AT1 receptor antagonist. *J Biol Chem*. 2001;276:39721-6.
62. van Esch JHM, Schuijt MP, Sayed J, Choudhry Y, Walther T, Danser AHJ. AT(2) receptor-mediated vasodilation in the mouse heart depends on AT(1A) receptor activation. *Br J Pharmacol*. 2006;148:452-8.
63. Li XC, Widdop RE. AT2 receptor-mediated vasodilatation is unmasked by AT1 receptor blockade in conscious SHR. *Br J Pharmacol*. 2004;142:821-30.
64. Widdop RE, Matrougui K, Levy BI, Henrion D. AT2 receptor-mediated relaxation is preserved after long-term AT1 receptor blockade. *Hypertension*. 2002;40:516-20.
65. Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, Dzau VJ. Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. *J Biol Chem*. 1993;268:24539-42.
66. Widdop RE, Jones ES, Hannan RE, Gaspari TA. Angiotensin AT2 receptors: cardiovascular hope or hype? *Br J Pharmacol*. 2003;140:809-24.
67. Bottari SP, de Gasparo M, Steckelings UM, Levens NR. Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications. *Front Neuroendocrinol*. 1993;14:123-71.
68. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev*. 2000;52:415-72.
69. Yamada H, Akishita M, Ito M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ, Horiuchi M. AT2 receptor and vascular smooth muscle cell differentiation in vascular development. *Hypertension*. 1999;33:1414-9.
70. Perlegas D, Xie H, Sinha S, Somlyo AV, Owens GK. ANG II type 2 receptor regulates smooth muscle growth and force generation in late fetal mouse development. *Am J Physiol Heart Circ Physiol*. 2005;288:H96-102.
71. Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab*. 2000;11:1-6.
72. Horiuchi M, Akishita M, Dzau VJ. Molecular and cellular mechanism of angiotensin II-mediated apoptosis. *Endocr Res*. 1998;24:307-14.
73. Wiemer G, Schölkens BA, Wagner A, Heitsch H, Linz W. The possible role of angiotensin II subtype AT2 receptors in endothelial cells and isolated ischemic rat hearts. *J Hypertens Suppl*. 1993;11:S234-5.
74. Siragy HM, Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*. 1997;100:264-9.
75. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest*. 1999;104:925-35.
76. Katada J, Majima M. AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. *Br J Pharmacol*. 2002;136:484-91.

77. Cervenka L, Maly J, Karasova L, Simova M, Vitko S, Hellerova S, Heller J, El-Dahr SS. Angiotensin II-induced hypertension in bradykinin B2 receptor knockout mice. *Hypertension*. 2001;37:967-73.
78. Abadir PM, Carey RM, Siragy HM. Angiotensin AT2 receptors directly stimulate renal nitric oxide in bradykinin B2-receptor-null mice. *Hypertension*. 2003;42:600-4.
79. Ritter O, Schuh K, Brede M, Rothlein N, Burkard N, Hein L, Neyses L. AT2 receptor activation regulates myocardial eNOS expression via the calcineurin-NF-AT pathway. *Faseb J*. 2003;17:283-5.
80. Abadir PM, Periasamy A, Carey RM, Siragy HM. Angiotensin II type 2 receptor-bradykinin B2 receptor functional heterodimerization. *Hypertension*. 2006;48:316-322.
81. Jin XQ, Fukuda N, Su JZ, Lai YM, Suzuki R, Tahira Y, Takagi H, Ikeda Y, Kanmatsuse K, Miyazaki H. Angiotensin II type 2 receptor gene transfer downregulates angiotensin II type 1a receptor in vascular smooth muscle cells. *Hypertension*. 2002;39:1021-7.
82. Tanaka M, Tsuchida S, Imai T, Fujii N, Miyazaki H, Ichiki T, Naruse M, Inagami T. Vascular response to angiotensin II is exaggerated through an upregulation of AT1 receptor in AT2 knockout mice. *Biochem Biophys Res Commun*. 1999;258:194-8.
83. Siragy HM, Xue C, Abadir P, Carey RM. Angiotensin subtype-2 receptors inhibit renin biosynthesis and angiotensin II formation. *Hypertension*. 2005;45:133-7.
84. Santos RAS, Campagnole-Santos MJ, Andrade SP. Angiotensin-(1-7): an update. *Regul Pept*. 2000;91:45-62.
85. Santos RAS, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A*. 2003;100:8258-63.
86. Brosnihan KB, Li P, Ferrario CM. Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension*. 1996;27:523-8.
87. Metzger R, Bader M, Ludwig T, Berberich C, Bunnemann B, Ganten D. Expression of the mouse and rat mas proto-oncogene in the brain and peripheral tissues. *FEBS Lett*. 1995;357:27-32.
88. Santos RAS, Castro CH, Gava E, Pinheiro SV, Almeida AP, Paula RD, Cruz JS, Ramos AS, Rosa KT, Irigoyen MC, Bader M, Alenina N, Kitten GT, Ferreira AJ. Impairment of in vitro and in vivo heart function in angiotensin-(1-7) receptor MAS knockout mice. *Hypertension*. 2006;47:996-1002.
89. Stegbauer J, Vonend O, Oberhauser V, Rump LC. Effects of angiotensin-(1-7) and other bioactive components of the renin-angiotensin system on vascular resistance and noradrenaline release in rat kidney. *J Hypertens*. 2003;21:1391-9.
90. van Rodijnen WF, van Lambalgen TA, van Wijhe MH, Tangelder GJ, Ter Wee PM. Renal microvascular actions of angiotensin II fragments. *Am J Physiol Renal Physiol*. 2002;283:F86-92.
91. Walters PE, Gaspari TA, Widdop RE. Angiotensin-(1-7) acts as a vasodepressor agent via angiotensin II type 2 receptors in conscious rats. *Hypertension*. 2005;45:960-6.
92. Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Almeida AP. Evidence

for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension*. 2005;46:937-42.

93. Lemos VS, Silva DM, Walther T, Alenina N, Bader M, Santos RA. The endothelium-dependent vasodilator effect of the nonpeptide Ang(1-7) mimic AVE 0991 is abolished in the aorta of mas-knockout mice. *J Cardiovasc Pharmacol*. 2005;46:274-9.
94. Ardaillou R, Chansel D. Synthesis and effects of active fragments of angiotensin II. *Kidney Int*. 1997;52:1458-68.
95. Wright JW, Harding JW. Brain angiotensin receptor subtypes AT1, AT2, and AT4 and their functions. *Regul Pept*. 1995;59:269-95.
96. Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, Llorens-Cortes C. Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A*. 1996;93:11968-73.
97. Padia SH, Howell NL, Siragy HM, Carey RM. Renal angiotensin type 2 receptors mediate natriuresis via angiotensin III in the angiotensin II type 1 receptor-blocked rat. *Hypertension*. 2006;47:537-44.
98. Swanson GN, Hanesworth JM, Sardinia MF, Coleman JK, Wright JW, Hall KL, Miller-Wing AV, Stobb JW, Cook VI, Harding EC, et al. Discovery of a distinct binding site for angiotensin II (3-8), a putative angiotensin IV receptor. *Regul Pept*. 1992;40:409-19.
99. Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, Mustafa T, Lee J, Mendelsohn FA, Simpson RJ, Connolly LM, Chai SY. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J Biol Chem*. 2001;276:48623-6.
100. Keller SR, Scott HM, Mastick CC, Aebersold R, Lienhard GE. Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. *J Biol Chem*. 1995;270:23612-8.
101. Wright JW, Stubley L, Pederson ES, Kramar EA, Hanesworth JM, Harding JW. Contributions of the brain angiotensin IV-AT4 receptor subtype system to spatial learning. *J Neurosci*. 1999;19:3952-61.
102. Patel JM, Martens JR, Li YD, Gelband CH, Raizada MK, Block ER. Angiotensin IV receptor-mediated activation of lung endothelial NOS is associated with vasorelaxation. *Am J Physiol*. 1998;275:L1061-8.
103. Schuijt MP, Danser AHJ. Cardiac angiotensin II: an intracrine hormone? *Am J Hypertens*. 2002;15:1109-16.
104. de Lannoy LM, Danser AHJ, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MADH. Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension*. 1997;29:1240-51.
105. Klett C, Nobiling R, Gierschik P, Hackenthal E. Angiotensin II stimulates the synthesis of angiotensinogen in hepatocytes by inhibiting adenylylcyclase activity and stabilizing angiotensinogen mRNA. *J Biol Chem*. 1993;268:25095-107.
106. Danser AHJ, van Kats JP, Admiraal PJ, Derkx FHM, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MADH. Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension*. 1994;24:37-48.

107. Campbell DJ, Kladis A, Duncan AM. Nephrectomy, converting enzyme inhibition, and angiotensin peptides. *Hypertension*. 1993;22:513-22.
108. Katz SA, Opsahl JA, Lunzer MM, Forbis LM, Hirsch AT. Effect of bilateral nephrectomy on active renin, angiotensinogen, and renin glycoforms in plasma and myocardium. *Hypertension*. 1997;30:259-66.
109. van den Eijnden MMED, de Bruin RJA, de Wit E, Sluiter W, Deinum J, Reudelhuber TL, Danser AHJ. Transendothelial transport of renin-angiotensin system components. *J Hypertens*. 2002;20:2029-37.
110. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AH, Ishida Y, Inagami T, Saruta T. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the “handle” region for nonproteolytic activation of prorenin. *J Clin Invest*. 2004;114:1128-35.
111. Müller DN, Fischli W, Clozel JP, Hilgers KF, Bohlender J, Menard J, Busjahn A, Ganten D, Luft FC. Local angiotensin II generation in the rat heart: role of renin uptake. *Circ Res*. 1998;82:13-20.
112. de Lannoy LM, Danser AHJ, Bouhuizen AM, Saxena PR, Schalekamp MADH. Localization and production of angiotensin II in the isolated perfused rat heart. *Hypertension*. 1998;31:1111-7.
113. Schuijt MP, van Kats JP, de Zeeuw S, Duncker DJ, Verdouw PD, Schalekamp MADH, Danser AHJ. Cardiac interstitial fluid levels of angiotensin I and II in the pig. *J Hypertens*. 1999;17:1885-91.
114. van Kats JP, van Meegen JR, Verdouw PD, Duncker DJ, Schalekamp MADH, Danser AHJ. Subcellular localization of angiotensin II in kidney and adrenal. *J Hypertens*. 2001;19:583-9.
115. van Kats JP, de Lannoy LM, Danser AHJ, van Meegen JR, Verdouw PD, Schalekamp MADH. Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo. *Hypertension*. 1997;30:42-9.
116. Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem*. 1990;265:22348-57.
117. Hackenthal E, Hackenthal R, Hilgenfeldt U. Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes. *Biochim Biophys Acta*. 1978;522:574-88.
118. Tom B, Garrelds IM, Scalbert E, Stegmann AP, Boomsma F, Saxena PR, Danser AHJ. ACE-versus chymase-dependent angiotensin II generation in human coronary arteries: a matter of efficiency? *Arterioscler Thromb Vasc Biol*. 2003;23:251-6.
119. Saris JJ, van Dijk MA, Kroon I, Schalekamp MADH, Danser AHJ. Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension*. 2000;35:764-8.
120. Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K, Fukamizu A, Murakami K. Angiotensinogen-deficient mice with hypotension. *J Biol Chem*. 1994;269:31334-7.
121. Krege JH, John SW, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O’Brien DA, Smithies O. Male-female differences in fertility and blood

pressure in ACE-deficient mice. *Nature*. 1995;375:146-8.

122. Campbell DJ, Alexiou T, Xiao HD, Fuchs S, McKinley MJ, Corvol P, Bernstein KE. Effect of reduced angiotensin-converting enzyme gene expression and angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptide levels in mice. *Hypertension*. 2004;43:854-9.
123. Ruiz-Ortega M, Esteban V, Suzuki Y, Ruperez M, Mezzano S, Ardiles L, Justo P, Ortiz A, Egido J. Renal expression of angiotensin type 2 (AT2) receptors during kidney damage. *Kidney Int*. 2003;86:S21-26.
124. Hashimoto N, Maeshima Y, Satoh M, Odawara M, Sugiyama H, Kashihara N, Matsubara H, Yamasaki Y, Makino H. Overexpression of angiotensin type 2 receptor ameliorates glomerular injury in a mouse remnant kidney model. *Am J Physiol Renal Physiol*. 2004;286:F516-25.
125. Hiyoshi H, Yayama K, Takano M, Okamoto H. Angiotensin type 2 receptor-mediated phosphorylation of eNOS in the aortas of mice with 2-kidney, 1-clip hypertension. *Hypertension*. 2005;45:967-73.
126. Duke LM, Widdop RE, Kett MM, Evans RG. AT(2) receptors mediate tonic renal medullary vasoconstriction in renovascular hypertension. *Br J Pharmacol*. 2005;144:486-92.
127. Wu JN, Edwards D, Berecek KH. Changes in renal angiotensin II receptors in spontaneously hypertensive rats by early treatment with the angiotensin-converting enzyme inhibitor captopril. *Hypertension*. 1994;23:819-22.
128. Yayama K, Horii M, Hiyoshi H, Takano M, Okamoto H, Kagota S, Kunitomo M. Up-regulation of angiotensin II type 2 receptor in rat thoracic aorta by pressure-overload. *J Pharmacol Exp Ther*. 2004;308:736-43.
129. Wharton J, Morgan K, Rutherford RA, Catravas JD, Chester A, Whitehead BF, De Leval MR, Yacoub MH, Polak JM. Differential distribution of angiotensin AT2 receptors in the normal and failing human heart. *J Pharmacol Exp Ther*. 1998;284:323-36.
130. Asano K, Dutcher DL, Port JD, Minobe WA, Tremmel KD, Roden RL, Bohlmeyer TJ, Bush EW, Jenkin MJ, Abraham WT, Raynolds MV, Zisman LS, Perryman MB, Bristow MR. Selective downregulation of the angiotensin II AT1-receptor subtype in failing human ventricular myocardium. *Circulation*. 1997;95:1193-200.
131. Schieffer B, Wirger A, Meybrunn M, Seitz S, Holtz J, Riede UN, Drexler H. Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. *Circulation*. 1994;89:2273-82.
132. Xu J, Carretero OA, Liu YH, Shesely EG, Yang F, Kapke A, Yang XP. Role of AT2 receptors in the cardioprotective effect of AT1 antagonists in mice. *Hypertension*. 2002;40:244-50.
133. Yang Z, Bove CM, French BA, Epstein FH, Berr SS, DiMaria JM, Gibson JJ, Carey RM, Kramer CM. Angiotensin II type 2 receptor overexpression preserves left ventricular function after myocardial infarction. *Circulation*. 2002;106:106-11.
134. Bove CM, Yang Z, Gilson WD, Epstein FH, French BA, Berr SS, Bishop SP, Matsubara H, Carey RM, Kramer CM. Nitric oxide mediates benefits of angiotensin II type 2 receptor overexpression during post-infarct remodeling. *Hypertension*. 2004;43:680-

5.
135. Liu YH, Xu J, Yang XP, Yang F, Shesely E, Carretero OA. Effect of ACE inhibitors and angiotensin II type 1 receptor antagonists on endothelial NO synthase knockout mice with heart failure. *Hypertension*. 2002;39:375-81.
136. Ichihara S, Senbonmatsu T, Price E, Jr., Ichiki T, Gaffney FA, Inagami T. Angiotensin II type 2 receptor is essential for left ventricular hypertrophy and cardiac fibrosis in chronic angiotensin II-induced hypertension. *Circulation*. 2001;104:346-51.
137. Senbonmatsu T, Ichihara S, Price E, Jr., Gaffney FA, Inagami T. Evidence for angiotensin II type 2 receptor-mediated cardiac myocyte enlargement during in vivo pressure overload. *J Clin Invest*. 2000;106:R25-9.
138. Schneider MD, Lorell BH. AT(2), judgment day: which angiotensin receptor is the culprit in cardiac hypertrophy? *Circulation*. 2001;104:247-8.
139. Schmieder RE, Erdmann J, Delles C, Jacobi J, Fleck E, Hilgers K, Regitz-Zagrosek V. Effect of the angiotensin II type 2-receptor gene (+1675 G/A) on left ventricular structure in humans. *J Am Coll Cardiol*. 2001;37:175-82.
140. Herrmann SM, Nicaud V, Schmidt-Petersen K, Pfeifer J, Erdmann J, McDonagh T, Dargie HJ, Paul M, Regitz-Zagrosek V. Angiotensin II type 2 receptor gene polymorphism and cardiovascular phenotypes: the GLAECO and GLAOLD studies. *Eur J Heart Fail*. 2002;4:707-12.
141. Alfakih K, Lawrance RA, Maqbool A, Walters K, Ball SG, Balmforth AJ, Hall AS. The clinical significance of a common, functional, X-linked angiotensin II type 2-receptor gene polymorphism (-1332 G/A) in a cohort of 509 families with premature coronary artery disease. *Eur Heart J*. 2005;26:584-9.
142. Savoia C, Touyz RM, Volpe M, Schiffrin EL. Angiotensin type 2 receptor in resistance arteries of type 2 diabetic hypertensive patients. *Hypertension*. 2007;49:341-346.
143. Deshayes F, Nahmias C. Angiotensin receptors: a new role in cancer? *Trends Endocrinol Metab*. 2005;16:293-9.
144. Fujita M, Hayashi I, Yamashina S, Itoman M, Majima M. Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. *Biochem Biophys Res Commun*. 2002;294:441-7.
145. Silvestre JS, Tamarat R, Senbonmatsu T, Icchiki T, Ebrahimian T, Iglarz M, Besnard S, Duriez M, Inagami T, Levy BI. Antiangiogenic effect of angiotensin II type 2 receptor in ischemia-induced angiogenesis in mice hindlimb. *Circ Res*. 2002;90:1072-9.
146. Walther T, Menrad A, Orzechowski HD, Siemeister G, Paul M, Schirner M. Differential regulation of in vivo angiogenesis by angiotensin II receptors. *FASEB J*. 2003;17:2061-7.
147. Campbell DJ, Aggarwal A, Esler M, Kaye D. beta-blockers, angiotensin II, and ACE inhibitors in patients with heart failure. *Lancet*. 2001;358:1609-10.
148. van Kats JP, Chai W, Duncker DJ, Schalekamp MADH, Danser AHJ. Adrenal angiotensin: origin and site of generation. *Am J Hypertens*. 2005;18:1104-10.
149. de Lannoy LM, Schuijt MP, Saxena PR, Schalekamp MADH, Danser AHJ. Angiotensin converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of the isolated perfused rat heart. *J Hypertens*. 2001;19:959-65.
150. Azizi M, Menard J, Bissery A, Guyenne TT, Bura-Riviere A, Vaidyanathan S, Camisasca

RP. Pharmacologic demonstration of the synergistic effects of a combination of the renin inhibitor aliskiren and the AT1 receptor antagonist valsartan on the angiotensin II-renin feedback interruption. *J Am Soc Nephrol*. 2004;15:3126-33.

151. Nussberger J, Wuerzner G, Jensen C, Brunner HR. Angiotensin II suppression in humans by the orally active renin inhibitor Aliskiren (SPP100): comparison with enalapril. *Hypertension*. 2002;39:E1-8.
152. Gradman AH, Schmieder RE, Lins RL, Nussberger J, Chiang Y, Bedigian MP. Aliskiren, a novel orally effective renin inhibitor, provides dose-dependent antihypertensive efficacy and placebo-like tolerability in hypertensive patients. *Circulation*. 2005;111:1012-8.
153. Georgiadis D, Beau F, Czarny B, Cotton J, Yiotakis A, Dive V. Roles of the two active sites of somatic angiotensin-converting enzyme in the cleavage of angiotensin I and bradykinin: insights from selective inhibitors. *Circ Res*. 2003;93:148-54.
154. Junot C, Gonzales MF, Ezan E, Cotton J, Vazeux G, Michaud A, Azizi M, Vassiliou S, Yiotakis A, Corvol P, Dive V. RXP 407, a selective inhibitor of the N-domain of angiotensin I-converting enzyme, blocks in vivo the degradation of hemoregulatory peptide acetyl-Ser-Asp-Lys-Pro with no effect on angiotensin I hydrolysis. *J Pharmacol Exp Ther*. 2001;297:606-11.
155. Fuchs S, Xiao HD, Cole JM, Adams JW, Frenzel K, Michaud A, Zhao H, Keshelava G, Capecchi MR, Corvol P, Bernstein KE. Role of the N-terminal catalytic domain of angiotensin-converting enzyme investigated by targeted inactivation in mice. *J Biol Chem*. 2004;279:15946-53.
156. Perich RB, Jackson B, Johnston CI. Structural constraints of inhibitors for binding at two active sites on somatic angiotensin converting enzyme. *Eur J Pharmacol*. 1994;266:201-11.
157. Deddish PA, Marcic B, Jackman HL, Wang HZ, Skidgel RA, Erdos EG. N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1-7) and keto-ACE. *Hypertension*. 1998;31:912-7.
158. Michaud A, Williams TA, Chauvet MT, Corvol P. Substrate dependence of angiotensin I-converting enzyme inhibition: captopril displays a partial selectivity for inhibition of N-acetyl-seryl-aspartyl-lysyl-proline hydrolysis compared with that of angiotensin I. *Mol Pharmacol*. 1997;51:1070-6.
159. Schuijt MP, de Vries R, Saxena PR, Schalekamp MADH, Danser AHJ. Vasoconstriction is determined by interstitial rather than circulating angiotensin II. *Br J Pharmacol*. 2002;135:275-83.
160. Danser AHJ, Chowdury S, de Lannoy LM, van der Giessen WJ, Saxena PR, Schalekamp MADH. Conversion and degradation of [125I] labelled angiotensin I in isolated perfused porcine coronary and carotid arteries. *Cardiovasc Res*. 1995;29:789-95.
161. Tom B, Dendorfer A, de Vries R, Saxena PR, Danser AHJ. Bradykinin potentiation by ACE inhibitors: a matter of metabolism. *Br J Pharmacol*. 2002;137:276-84.
162. MaassenVanDenBrink A, de Vries R, Saxena PR, Schalekamp MADH, Danser AHJ. Vasoconstriction by in situ formed angiotensin II: role of ACE and chymase. *Cardiovasc Res*. 1999;44:407-15.

163. Admiraal PJ, Danser AH, Jong MS, Pieterman H, Derkx FH, Schalekamp MA. Regional angiotensin II production in essential hypertension and renal artery stenosis. *Hypertension*. 1993;21:173-84.
164. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res*. 1977;41:19-26.
165. Batenburg WW, Garrelds IM, van Kats JP, Saxena PR, Danser AHJ. Mediators of bradykinin-induced vasorelaxation in human coronary microarteries. *Hypertension*. 2004;43:488-92.
166. van Kesteren CA, Saris JJ, Dekkers DH, Lamers JM, Saxena PR, Schalekamp MA, Danser AH. Cultured neonatal rat cardiac myocytes and fibroblasts do not synthesize renin or angiotensinogen: evidence for stretch-induced cardiomyocyte hypertrophy independent of angiotensin II. *Cardiovasc Res*. 1999;43:148-56.
167. Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension*. 2002;39:799-802.
168. Roks AJ, van Geel PP, Pinto YM, Buikema H, Henning RH, de Zeeuw D, van Gilst WH. Angiotensin-(1-7) is a modulator of the human renin-angiotensin system. *Hypertension*. 1999;34:296-301.
169. Ueda S, Masumori-Maemoto S, Ashino K, Nagahara T, Gotoh E, Umemura S, Ishii M. Angiotensin-(1-7) attenuates vasoconstriction evoked by angiotensin II but not by noradrenaline in man. *Hypertension*. 2000;35:998-1001.
170. Danser AHJ, Admiraal PJ, Derkx FH, Schalekamp MADH. Angiotensin I-to-II conversion in the human renal vascular bed. *J Hypertens*. 1998;16:2051-6.
171. Kohlstedt K, Brandes RP, Muller-Esterl W, Busse R, Fleming I. Angiotensin-converting enzyme is involved in outside-in signaling in endothelial cells. *Circ Res*. 2004;94:60-7.
172. Linz W, Schaper J, Wiemer G, Albus U, Scholkens BA. Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats. *Br J Pharmacol*. 1992;107:970-5.
173. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med*. 2000;342:145-53.
174. Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*. 2002;105:1548-50.
175. Esther CR, Marino EM, Howard TE, Machaud A, Corvol P, Capecchi MR, Bernstein KE. The critical role of tissue angiotensin-converting enzyme as revealed by gene targeting in mice. *J Clin Invest*. 1997;99:2375-85.
176. Danser AHJ, Koning MM, Admiraal PJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH. Production of angiotensins I and II at tissue sites in intact pigs. *Am J Physiol*. 1992;263:H429-37.
177. Marcic B, Deddish PA, Jackman HL, Erdos EG, Tan F. Effects of the N-terminal sequence of ACE on the properties of its C-domain. *Hypertension*. 2000;36:116-21.
178. Woodman ZL, Schwager SL, Redelinghuys P, Carmona AK, Ehlers MR, Sturrock

ED. The N domain of somatic angiotensin-converting enzyme negatively regulates ectodomain shedding and catalytic activity. *Biochem J*. 2005;389:739-44.

179. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest*. 1990;86:1343-6.
180. Mizuiri S, Hemmi H, Kumanomidou H, Iwamoto M, Miyagi M, Sakai K, Aikawa A, Ohara T, Yamada K, Shimatake H, Hasegawa A. Angiotensin-converting enzyme (ACE) I/D genotype and renal ACE gene expression. *Kidney Int*. 2001;60:1124-30.
181. Danser AHJ, Schalekamp MADH, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation*. 1995;92:1387-8.
182. Suehiro T, Morita T, Inoue M, Kumon Y, Ikeda Y, Hashimoto K. Increased amount of the angiotensin-converting enzyme (ACE) mRNA originating from the ACE allele with deletion. *Hum Genet*. 2004;115:91-6.
183. Lachurie ML, Azizi M, Guyene TT, Alhenc-Gelas F, Menard J. Angiotensin-converting enzyme gene polymorphism has no influence on the circulating renin-angiotensin-aldosterone system or blood pressure in normotensive subjects. *Circulation*. 1995;91:2933-42.
184. Danser AHJ, Deinum J, Osterop AP, Admiraal PJ, Schalekamp MADH. Angiotensin I to angiotensin II conversion in the human forearm and leg. Effect of the angiotensin converting enzyme gene insertion/deletion polymorphism. *J Hypertens*. 1999;17:1867-72.
185. Azizi M, Junot C, Ezan E, Hallouin MC, Guyene T, Menard J. Functional consequences of angiotensin-converting enzyme gene polymorphism on N-acetyl-Ser-Asp-Lys-Pro degradation and angiotensin II production. *J Mol Med*. 2002;80:492-8.
186. Danser AHJ, Batenburg WW, van Esch JHM. Prorenin and the (pro)renin receptor--an update. *Nephrol Dial Transplant*. 2007;22:1288–1292.
187. Alexiou T, Boon WM, Denton DA, Nicolantonio RD, Walker LL, McKinley MJ, Campbell DJ. Angiotensinogen and angiotensin-converting enzyme gene copy number and angiotensin and bradykinin peptide levels in mice. *J Hypertens*. 2005;23:945-54.
188. Takahashi N, Hagaman JR, Kim HS, Smithies O. Minireview: computer simulations of blood pressure regulation by the renin-angiotensin system. *Endocrinology*. 2003;144:2184-90.
189. Danser AHJ, Derkx FHM, Hense HW, Jeunemaitre X, Riegger GA, Schunkert H. Angiotensinogen (M235T) and angiotensin-converting enzyme (I/D) polymorphisms in association with plasma renin and prorenin levels. *J Hypertens*. 1998;16:1879-83.
190. Kramers C, Danilov SM, Deinum J, Balyasnikova IV, Scharenborg N, Looman M, Boomsma F, de Keijzer MH, van Duijn C, Martin S, Soubrier F, Adema GJ. Point mutation in the stalk of angiotensin-converting enzyme causes a dramatic increase in serum angiotensin-converting enzyme but no cardiovascular disease. *Circulation*. 2001;104:1236-40.
191. Montgomery HE, Clarkson P, Dollery CM, Prasad K, Losi MA, Hemingway H, Statters D, Jubb M, Girvain M, Varnava A, World M, Deanfield J, Talmud P, McEwan JR, McKenna WJ, Humphries S. Association of angiotensin-converting enzyme gene I/D

polymorphism with change in left ventricular mass in response to physical training. *Circulation*. 1997;96:741-7.

192. Kuoppala A, Lindstedt KA, Saarinen J, Kovanen PT, Kokkonen JO. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. *Am J Physiol Heart Circ Physiol*. 2000;278:H1069-74.
193. Jullien ND, Cuniasse P, Georgiadis D, Yiotakis A, Dive V. Combined use of selective inhibitors and fluorogenic substrates to study the specificity of somatic wild-type angiotensin-converting enzyme. *Febs J*. 2006;273:1772-81.
194. Rieder MJ, Taylor SL, Clark AG, Nickerson DA. Sequence variation in the human angiotensin converting enzyme. *Nat Genet*. 1999;22:59-62.
195. Sturrock ED, Danilov SM, Riordan JF. Limited proteolysis of human kidney angiotensin-converting enzyme and generation of catalytically active N- and C-terminal domains. *Biochem Biophys Res Commun*. 1997;236:16-9.
196. Rougeot C, Messaoudi M, Hermitte V, Rigault AG, Blisnick T, Dugave C, Desor D, Rougeon F. Sialorphin, a natural inhibitor of rat membrane-bound neutral endopeptidase that displays analgesic activity. *Proc Natl Acad Sci U S A*. 2003;100:8549-54.
197. Leiper J, Vallance P. Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. *Cardiovasc Res*. 1999;43:542-8.
198. Brown NJ, Blais C, Jr., Gandhi SK, Adam A. ACE insertion/deletion genotype affects bradykinin metabolism. *J Cardiovasc Pharmacol*. 1998;32:373-7.
199. Murphey LJ, Gainer JV, Vaughan DE, Brown NJ. Angiotensin-converting enzyme insertion/deletion polymorphism modulates the human in vivo metabolism of bradykinin. *Circulation*. 2000;102:829-32.
200. Myerson SG, Montgomery HE, Whittingham M, Jubb M, World MJ, Humphries SE, Pennell DJ. Left ventricular hypertrophy with exercise and ACE gene insertion/deletion polymorphism: a randomized controlled trial with losartan. *Circulation*. 2001;103:226-30.
201. Brull D, Dhamrait S, Myerson S, Erdmann J, Woods D, World M, Pennell D, Humphries S, Regitz-Zagrosek V, Montgomery H. Bradykinin B2BKR receptor polymorphism and left-ventricular growth response. *Lancet*. 2001;358:1155-6.
202. McNamara DM, Holubkov R, Postava L, Janosko K, MacGowan GA, Mathier M, Murali S, Feldman AM, London B. Pharmacogenetic interactions between angiotensin-converting enzyme inhibitor therapy and the angiotensin-converting enzyme deletion polymorphism in patients with congestive heart failure. *J Am Coll Cardiol*. 2004;44:2019-26.
203. Iwai N, Inagami T. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett*. 1992;298:257-60.
204. Siragy HM, Inagami T, Ichiki T, Carey RM. Sustained hypersensitivity to angiotensin II and its mechanism in mice lacking the subtype-2 (AT2) angiotensin receptor. *Proc Natl Acad Sci U S A*. 1999;96:6506-10.
205. van Kesteren CAM, van Heugten HAA, Lamers JMJ, Saxena PR, Schalekamp MADH, Danser AHJ. Angiotensin II-mediated growth and antigrowth effects in cultured neonatal rat cardiac myocytes and fibroblasts. *J Mol Cell Cardiol*. 1997;29:2147-57.

206. Munzenmaier DH, Greene AS. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension*. 1996;27:760-5.
207. Masaki H, Kurihara T, Yamaki A, Inomata N, Nozawa Y, Mori Y, Murasawa S, Kizima K, Maruyama K, Horiuchi M, Dzau VJ, Takahashi H, Iwasaka T, Inada M, Matsubara H. Cardiac-specific overexpression of angiotensin II AT2 receptor causes attenuated response to AT1 receptor-mediated pressor and chronotropic effects. *J Clin Invest*. 1998;101:527-35.
208. Hannan RE, Davis EA, Widdop RE. Functional role of angiotensin II AT2 receptor in modulation of AT1 receptor-mediated contraction in rat uterine artery: involvement of bradykinin and nitric oxide. *Br J Pharmacol*. 2003;140:987-95.
209. Batenburg WW, Tom B, Schuijt MP, Danser AHJ. Angiotensin II type 2 receptor-mediated vasodilation. Focus on bradykinin, NO and endothelium-derived hyperpolarizing factor(s). *Vascul Pharmacol*. 2005;42:109-18.
210. Levy BI, Benessiano J, Henrion D, Caputo L, Heymes C, Duriez M, Poitevin P, Samuel JL. Chronic blockade of AT2-subtype receptors prevents the effect of angiotensin II on the rat vascular structure. *J Clin Invest*. 1996;98:418-25.
211. Duke LM, Evans RG, Widdop RE. AT2 receptors contribute to acute blood pressure-lowering and vasodilator effects of AT1 receptor antagonism in conscious normotensive but not hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2005;288: H2289-97.
212. You D, Loufrani L, Baron C, Levy BI, Widdop RE, Henrion D. High blood pressure reduction reverses angiotensin II type 2 receptor-mediated vasoconstriction into vasodilation in spontaneously hypertensive rats. *Circulation*. 2005;111:1006-11.
213. Barber MN, Sampey DB, Widdop RE. AT(2) receptor stimulation enhances antihypertensive effect of AT(1) receptor antagonist in hypertensive rats. *Hypertension*. 1999;34:1112-6.
214. Yee DK, Heerding JN, Krichavsky MZ, Fluharty SJ. Role of the amino terminus in ligand binding for the angiotensin II type 2 receptor. *Brain Res Mol Brain Res*. 1998;57:325-9.
215. Gustafson LA, Van Beek JH. Measurement of the activation time of oxidative phosphorylation in isolated mouse hearts. *Am J Physiol Heart Circ Physiol*. 2000;279: H3118-23.
216. Wang QD, Tokuno S, Valen G, Sjoquist PO, Thoren P. Cyclic fluctuations in the cardiac performance of the isolated Langendorff-perfused mouse heart: pyruvate abolishes the fluctuations and has an anti-ischaemic effect. *Acta Physiol Scand*. 2002;175:279-87.
217. Sutherland FJ, Shattock MJ, Baker KE, Hearse DJ. Mouse isolated perfused heart: characteristics and cautions. *Clin Exp Pharmacol Physiol*. 2003;30:867-78.
218. Curtis MJ, Macleod BA, Tabrizchi R, Walker MJ. An improved perfusion apparatus for small animal hearts. *J Pharmacol Methods*. 1986;15:87-94.
219. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol*. 1978;235:E97-102.
220. Abdellatif MM, Neubauer CF, Lederer WJ, Rogers TB. Angiotensin-induced

desensitization of the phosphoinositide pathway in cardiac cells occurs at the level of the receptor. *Circ Res*. 1991;69:800-9.

221. Reagan LP, Ye X, Maretzski CH, Fluharty SJ. Down-regulation of angiotensin II receptor subtypes and desensitization of cyclic GMP production in neuroblastoma N1E-115 cells. *J Neurochem*. 1993;60:24-31.
222. Iglesias AG, Suarez C, Feierstein C, Diaz-Torga G, Becu-Villalobos D. Desensitization of angiotensin II: effect on [Ca2+]i, inositol triphosphate, and prolactin in pituitary cells. *Am J Physiol Endocrinol Metab*. 2001;280:E462-70.
223. Zhang C, Hein TW, Wang W, Kuo L. Divergent roles of angiotensin II AT1 and AT2 receptors in modulating coronary microvascular function. *Circ Res*. 2003;92:322-9.
224. Pörsti I, Hecker M, Bassenge E, Busse R. Dual action of angiotensin II on coronary resistance in the isolated perfused rabbit heart. *Naunyn Schmiedebergs Arch Pharmacol*. 1993;348:650-8.
225. Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation*. 1998;97:1952-9.
226. Ryan MJ, Didion SP, Mathur S, Faraci FM, Sigmund CD. Angiotensin II-induced vascular dysfunction is mediated by the AT1A receptor in mice. *Hypertension*. 2004;43:1074-9.
227. Nakajima M, Hutchinson HG, Fujinaga M, Hayashida W, Morishita R, Zhang L, Horiuchi M, Pratt RE, Dzau VJ. The angiotensin II type 2 (AT2) receptor antagonizes the growth effects of the AT1 receptor: gain-of-function study using gene transfer. *Proc Natl Acad Sci U S A*. 1995;92:10663-7.
228. Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest*. 1995;95:651-7.
229. Müller C, Endlich K, Helwig JJ. AT2 antagonist-sensitive potentiation of angiotensin II-induced constriction by NO blockade and its dependence on endothelium and P450 eicosanoids in rat renal vasculature. *Br J Pharmacol*. 1998;124:946-52.
230. Dimitropoulou C, White RE, Fuchs L, Zhang H, Catravas JD, Carrier GO. Angiotensin II relaxes microvessels via the AT(2) receptor and Ca(2+)-activated K(+) (BK(Ca)) channels. *Hypertension*. 2001;37:301-7.
231. Endo Y, Arima S, Yaoita H, Tsunoda K, Omata K, Ito S. Vasodilation mediated by angiotensin II type 2 receptor is impaired in afferent arterioles of young spontaneously hypertensive rats. *J Vasc Res*. 1998;35:421-7.
232. Utsunomiya H, Nakamura M, Kakudo K, Inagami T, Tamura M. Angiotensin II AT2 receptor localization in cardiovascular tissues by its antibody developed in AT2 gene-deleted mice. *Regul Pept*. 2005;126:155-61.
233. van Kats JP, Danser AHJ, van Meegen JR, Sassen LMA, Verdouw PD, Schalekamp MADH. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation*. 1998;98:73-81.
234. Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev*. 1993;45:205-51.

235. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, et al. Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun*. 1989;165:196-203.
236. Kambayashi Y, Bardhan S, Takahashi K, Tsuzuki S, Inui H, Hamakubo T, Inagami T. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem*. 1993;268:24543-6.
237. Iwai N, Inagami T, Ohmichi N, Nakamura Y, Saeki Y, Kinoshita M. Differential regulation of rat AT1a and AT1b receptor mRNA. *Biochem Biophys Res Commun*. 1992;188:298-303.
238. Gasc JM, Shanmugam S, Sibony M, Corvol P. Tissue-specific expression of type 1 angiotensin II receptor subtypes. An in situ hybridization study. *Hypertension*. 1994;24:531-7.
239. Steckelings UM, Kaschina E, Unger T. The AT2 receptor--a matter of love and hate. *Peptides*. 2005;26:1401-9.
240. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT2 receptors in the developing rat fetus. *J Clin Invest*. 1991;88:921-33.
241. Shanmugam S, Lenkei ZG, Gasc JM, Corvol PL, Llorens-Cortes CM. Ontogeny of angiotensin II type 2 (AT2) receptor mRNA in the rat. *Kidney Int*. 1995;47:1095-100.
242. Oliverio MI, Kim HS, Ito M, Le T, Audoly L, Best CF, Hiller S, Kluckman K, Maeda N, Smithies O, Coffman TM. Reduced growth, abnormal kidney structure, and type 2 (AT2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT1A and AT1B receptors for angiotensin II. *Proc Natl Acad Sci U S A*. 1998;95:15496-501.
243. Kim HS, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, Jennette JC, Coffman TM, Maeda N, Smithies O. Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A*. 1995;92:2735-9.
244. Matsusaka T, Nishimura H, Utsunomiya H, Kakuchi J, Niimura F, Inagami T, Fogo A, Ichikawa I. Chimeric mice carrying 'regional' targeted deletion of the angiotensin type 1A receptor gene. Evidence against the role for local angiotensin in the in vivo feedback regulation of renin synthesis in juxtaglomerular cells. *J Clin Invest*. 1996;98:1867-77.
245. von Bohlen und Halbach O, Walther T, Bader M, Albrecht D. Genetic deletion of angiotensin AT2 receptor leads to increased cell numbers in different brain structures of mice. *Regul Pept*. 2001;99:209-16.
246. Walther T, Steendijk P, Westermann D, Hohmann C, Schulze K, Heringer-Walther S, Schultheiss HP, Tschope C. Angiotensin deficiency in mice leads to dilated cardiomyopathy. *Eur J Pharmacol*. 2004;493:161-5.
247. Walther T, Tschope C, Sterner-Kock A, Westermann D, Heringer-Walther S, Riad A, Nikolic A, Wang Y, Ebermann L, Siems WE, Bader M, Shakibaei M, Schultheiss HP, Dorner A. Accelerated mitochondrial adenosine diphosphate/adenosine triphosphate transport improves hypertension-induced heart disease. *Circulation*. 2007;115:333-44.
248. Tschope C, Heringer-Walther S, Koch M, Spillmann F, Wendorf M, Hauke D, Bader M, Schultheiss HP, Walther T. Myocardial bradykinin B2-receptor expression at different

time points after induction of myocardial infarction. *J Hypertens*. 2000;18:223-8.
249. Gross V, Schunck WH, Honeck H, Milia AF, Kargel E, Walther T, Bader M, Inagami T, Schneider W, Luft FC. Inhibition of pressure natriuresis in mice lacking the AT2 receptor. *Kidney Int*. 2000;57:191-202.
250. Gembardt F, Sterner-Kock A, Imboden H, Spalteholz M, Reibitz F, Schultheiss HP, Siems WE, Walther T. Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents. *Peptides*. 2005;26:1270-7.
251. Padi SS, Chopra K. Selective angiotensin II type 1 receptor blockade ameliorates cyclosporine nephrotoxicity. *Pharmacol Res*. 2002;45:413-20.
252. Konstam MA, Neaton JD, Poole-Wilson PA, Pitt B, Segal R, Sharma D, Dasbach EJ, Carides GW, Dickstein K, Riegger G, Camm AJ, Martinez FA, Bradstreet DC, Ikeda LS, Santoro EP. Comparison of losartan and captopril on heart failure-related outcomes and symptoms from the losartan heart failure survival study (ELITE II). *Am Heart J*. 2005;150:123-31.
253. Bernal J, Pitta SR, Thatai D. Role of the renin-angiotensin-aldosterone system in diastolic heart failure: potential for pharmacologic intervention. *Am J Cardiovasc Drugs*. 2006;6:373-81.
254. Dendorfer A, Dominiak P, Schunkert H. ACE inhibitors and angiotensin II receptor antagonists. *Handb Exp Pharmacol*. 2005:407-42.
255. Ribeiro AB. Angiotensin II antagonists--therapeutic benefits spanning the cardiovascular disease continuum from hypertension to heart failure and diabetic nephropathy. *Curr Med Res Opin*. 2006;22:1-16.
256. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM. Renin-angiotensin system and cardiovascular risk. *Lancet*. 2007;369:1208-19.
257. Tokuyama H, Kelly DJ, Zhang Y, Gow RM, Gilbert RE. Macrophage infiltration and cellular proliferation in the non-ischemic kidney and heart following prolonged unilateral renal ischemia. *Nephron Physiol*. 2007;106:p54-62.
258. Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupershmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF, Sadoshima J. Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest*. 2005;115:3045-56.
259. Andresen BT, Shome K, Jackson EK, Romero GG. AT2 receptors cross talk with AT1 receptors through a nitric oxide- and RhoA-dependent mechanism resulting in decreased phospholipase D activity. *Am J Physiol Renal Physiol*. 2005;288:F763-70.
260. Crowley SD, Gurley SB, Coffman TM. AT(1) receptors and control of blood pressure: the kidney and more. *Trends Cardiovasc Med*. 2007;17:30-4.
261. Li Q, Zhang J, Pfaffendorf M, van Zwieten PA. Direct positive chronotropic effects of angiotensin II and angiotensin III in pithed rats and in rat isolated atria. *Br J Pharmacol*. 1996;118:1653-8.
262. McEwan PE, Sherry L, Kenyon CJ, Webb DJ, Gray GA. Regulation of the myocardial endothelin system by angiotensin-II and losartan. *J Cardiovasc Pharmacol*. 2000;36: S144-7.

263. Ouyang X, Le TH, Roncal C, Gersch C, Herrera-Acosta J, Rodriguez-Iturbe B, Coffman TM, Johnson RJ, Mu W. Th1 inflammatory response with altered expression of profibrotic and vasoactive mediators in AT1A and AT1B double-knockout mice. *Am J Physiol Renal Physiol*. 2005;289:F902-10.
264. Reid JL. Molecular-specific effects of angiotensin II antagonists: clinical relevance to treating hypertension? *J Renin Angiotensin Aldosterone Syst*. 2005;6:15-24.
265. Moreau N, Richer C, Vincent MP, Giudicelli JF. Sympathoinhibitory effects of losartan in spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 1993;22:126-34.
266. Dendorfer A, Raasch W, Tempel K, Dominiak P. Interactions between the renin-angiotensin system (RAS) and the sympathetic system. *Basic Res Cardiol*. 1998;93 Suppl 2:24-9.
267. Batenburg WW, van Esch JHM, Garrelds IM, Jorde U, Lamers JMJ, Dekkers DH, Walther T, Kellett E, Milligan G, van Kats JP, Danser AHJ. Carvedilol-induced antagonism of angiotensin II: a matter of alpha1-adrenoceptor blockade. *J Hypertens*. 2006;24:1355-63.
268. Li Q, Zhang L, Pfaffendorf M, van Zwieten PA. Comparative effects of angiotensin II and its degradation products angiotensin III and angiotensin IV in rat aorta. *Br J Pharmacol*. 1995;116:2963-70.
269. Li Q, Feenstra M, Pfaffendorf M, Eijsman L, van Zwieten PA. Comparative vasoconstrictor effects of angiotensin II, III, and IV in human isolated saphenous vein. *J Cardiovasc Pharmacol*. 1997;29:451-6.
270. Robertson MJ, Cunoosamy MP, Clark KL. Effects of peptidase inhibition on angiotensin receptor agonist and antagonist potency in rabbit isolated thoracic aorta. *Br J Pharmacol*. 1992;106:166-72.
271. Campbell DJ, Kladis A. Simultaneous radioimmunoassay of six angiotensin peptides in arterial and venous plasma of man. *J Hypertens*. 1990;8:165-72.
272. Vinh A, Widdop RE, Drummond GR, Gaspari TA. Chronic angiotensin IV treatment reverses endothelial dysfunction in ApoE-deficient mice. *Cardiovasc Res*. 2008;77:178-87.
273. Shibasaki Y, Mori Y, Tsutumi Y, Masaki H, Sakamoto K, Murasawa S, Maruyama K, Moriguchi Y, Tanaka Y, Iwasaka T, Inada M, Matsubara H. Differential kinetics of circulating angiotensin IV and II after treatment with angiotensin II type 1 receptor antagonist and their plasma levels in patients with chronic renal failure. *Clin Nephrol*. 1999;51:83-91.
274. Campbell DJ, Lawrence AC, Towrie A, Kladis A, Valentijn AJ. Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension*. 1991;18:763-73.
275. Schindler C, Bramlage P, Kirch W, Ferrario CM. Role of the vasodilator peptide angiotensin-(1-7) in cardiovascular drug therapy. *Vasc Health Risk Manag*. 2007;3:125-37.
276. Macari D, Bottari S, Whitebread S, De Gasparo M, Levens N. Renal actions of the selective angiotensin AT2 receptor ligands CGP 42112B and PD 123319 in the sodium-depleted rat. *Eur J Pharmacol*. 1993;249:85-93.
277. Cazaubon C, Gougat J, Bousquet F, Guiraudou P, Gayraud R, Lacour C, Roccon

A, Galindo G, Barthelemy G, Gautret B, et al. Pharmacological characterization of SR 47436, a new nonpeptide AT1 subtype angiotensin II receptor antagonist. *J Pharmacol Exp Ther*. 1993;265:826-34.

278. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JMJ, Danser AHJ. Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective? *Br J Pharmacol*. 2005;145:664-71.
279. Schuijt MP, Tom B, de Vries R, Saxena PR, Sluiter W, van Kats JP, Danser AHJ. Superoxide does not mediate the acute vasoconstrictor effects of angiotensin II: a study in human and porcine arteries. *J Hypertens*. 2003;21:2335-44.
280. Noda K, Feng YH, Liu XP, Saad Y, Husain A, Karnik SS. The active state of the AT1 angiotensin receptor is generated by angiotensin II induction. *Biochemistry*. 1996;35:16435-42.
281. Feng YH, Noda K, Saad Y, Liu XP, Husain A, Karnik SS. The docking of Arg2 of angiotensin II with Asp281 of AT1 receptor is essential for full agonism. *J Biol Chem*. 1995;270:12846-50.
282. Le MT, Vanderheyden PM, Szaszak M, Hunyady L, Vauquelin G. Angiotensin IV is a potent agonist for constitutive active human AT1 receptors. Distinct roles of the N-and C-terminal residues of angiotensin II during AT1 receptor activation. *J Biol Chem*. 2002;277:23107-10.
283. Padia SH, Kemp BA, Howell NL, Siragy HM, Fournie-Zaluski MC, Roques BP, Carey RM. Intrarenal aminopeptidase N inhibition augments natriuretic responses to angiotensin III in angiotensin type 1 receptor-blocked rats. *Hypertension*. 2007;49:625-30.
284. Widdop RE, Walters PE, Gaspari TA. Are angiotensin peptide fragments endogenous ligands for the AT2 receptor? *J Hypertens (Suppl 6)*. 2006;24:S186.
285. van der Wouden EA, Ochodnicky P, van Dokkum RP, Roks AJ, Deelman LE, de Zeeuw D, Henning RH. The role of angiotensin(1-7) in renal vasculature of the rat. *J Hypertens*. 2006;24:1971-8.
286. Ferreira AJ, Jacoby BA, Araujo CA, Macedo FA, Silva GA, Almeida AP, Caliari MV, Santos RA. The nonpeptide angiotensin-(1-7) receptor Mas agonist AVE-0991 attenuates heart failure induced by myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2007;292:H1113-9.
287. Danser AHJ, Koning MM, Admiraal PJJ, Derkx FHM, Verdouw PD, Schalekamp MADH. Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol*. 1992;263:H418-28.
288. Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer JD. Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int*. 1996;50:1897-903.
289. Azizi M, Webb R, Nussberger J, Hollenberg NK. Renin inhibition with aliskiren: where are we now, and where are we going? *J Hypertens*. 2006;24:243-56.
290. Wood JM, Maibaum J, Rahuel J, Grutter MG, Cohen NC, Rasetti V, Ruger H, Goschke R, Stutz S, Fuhrer W, Schilling W, Rigollier P, Yamaguchi Y, Cumin F, Baum HP, Schnell CR, Herold P, Mah R, Jensen C, O'Brien E, Stanton A, Bedigian MP. Structure-based design of aliskiren, a novel orally effective renin inhibitor. *Biochem*

Biophys Res Commun. 2003;308:698-705.

291. Oparil S, Yarows SA, Patel S, Fang H, Zhang J, Satlin A. Efficacy and safety of combined use of aliskiren and valsartan in patients with hypertension: a randomised, double-blind trial. *Lancet*. 2007;370:221-9.
292. Pilz B, Shagdarsuren E, Wellner M, Fiebeler A, Dechend R, Gratze P, Meiners S, Feldman DL, Webb RL, Garrelds IM, Jan Danser AH, Luft FC, Muller DN. Aliskiren, a human renin inhibitor, ameliorates cardiac and renal damage in double-transgenic rats. *Hypertension*. 2005;46:569-76.
293. Wood JM, Schnell CR, Cumin F, Menard J, Webb RL. Aliskiren, a novel, orally effective renin inhibitor, lowers blood pressure in marmosets and spontaneously hypertensive rats. *J Hypertens*. 2005;23:417-26.
294. Dendorfer A, Dominiak P, Tempel K, Raasch W. Peripheral sympatholytic actions of four AT1 antagonists: are they relevant for long-term antihypertensive efficacy? *J Hypertens*. 2005;23:1861-7.
295. Van Kerckhoven R, Saxena PR, Schoemaker RG. Restored capillary density in spared myocardium of infarcted rats improves ischemic tolerance. *J Cardiovasc Pharmacol*. 2002;40:370-80.
296. Derkx FH, Steunkel C, Schalekamp MP, Visser W, Huisveld IH, Schalekamp MA. Immunoreactive renin, prorenin, and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. *J Clin Endocrinol Metab*. 1986;63:1008-15.
297. Webb RL, Navarrete AE, Davis S, de Gasparo M. Synergistic effects of combined converting enzyme inhibition and angiotensin II antagonism on blood pressure in conscious telemetered spontaneously hypertensive rats. *J Hypertens*. 1998;16:843-52.
298. El-Mas MM, Abdel-Rahman AA. Longitudinal studies on the effect of hypertension on circadian hemodynamic and autonomic rhythms in telemetered rats. *Life Sci*. 2005;76:901-15.
299. Kurtz TW, Morris RC, Jr. Biological variability in Wistar-Kyoto rats. Implications for research with the spontaneously hypertensive rat. *Hypertension*. 1987;10:127-31.
300. Bunkenburg B, Schnell C, Baum HP, Cumin F, Wood JM. Prolonged angiotensin II antagonism in spontaneously hypertensive rats. Hemodynamic and biochemical consequences. *Hypertension*. 1991;18:278-88.
301. Konishi M, Su C. Role of endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension*. 1983;5:881-6.
302. Tesfamariam B, Halpern W. Endothelium-dependent and endothelium-independent vasodilation in resistance arteries from hypertensive rats. *Hypertension*. 1988;11:440-4.
303. Héctor Polizio A, Pena C. Effects of angiotensin II type 1 receptor blockade on the oxidative stress in spontaneously hypertensive rat tissues. *Regul Pept*. 2005;128:1-5.
304. Sola S, Mir MQ, Cheema FA, Khan-Merchant N, Menon RG, Parthasarathy S, Khan BV. Irbesartan and lipoic acid improve endothelial function and reduce markers of inflammation in the metabolic syndrome: results of the Irbesartan and Lipoic Acid in

Endothelial Dysfunction (ISLAND) study. *Circulation*. 2005;111:343-8.

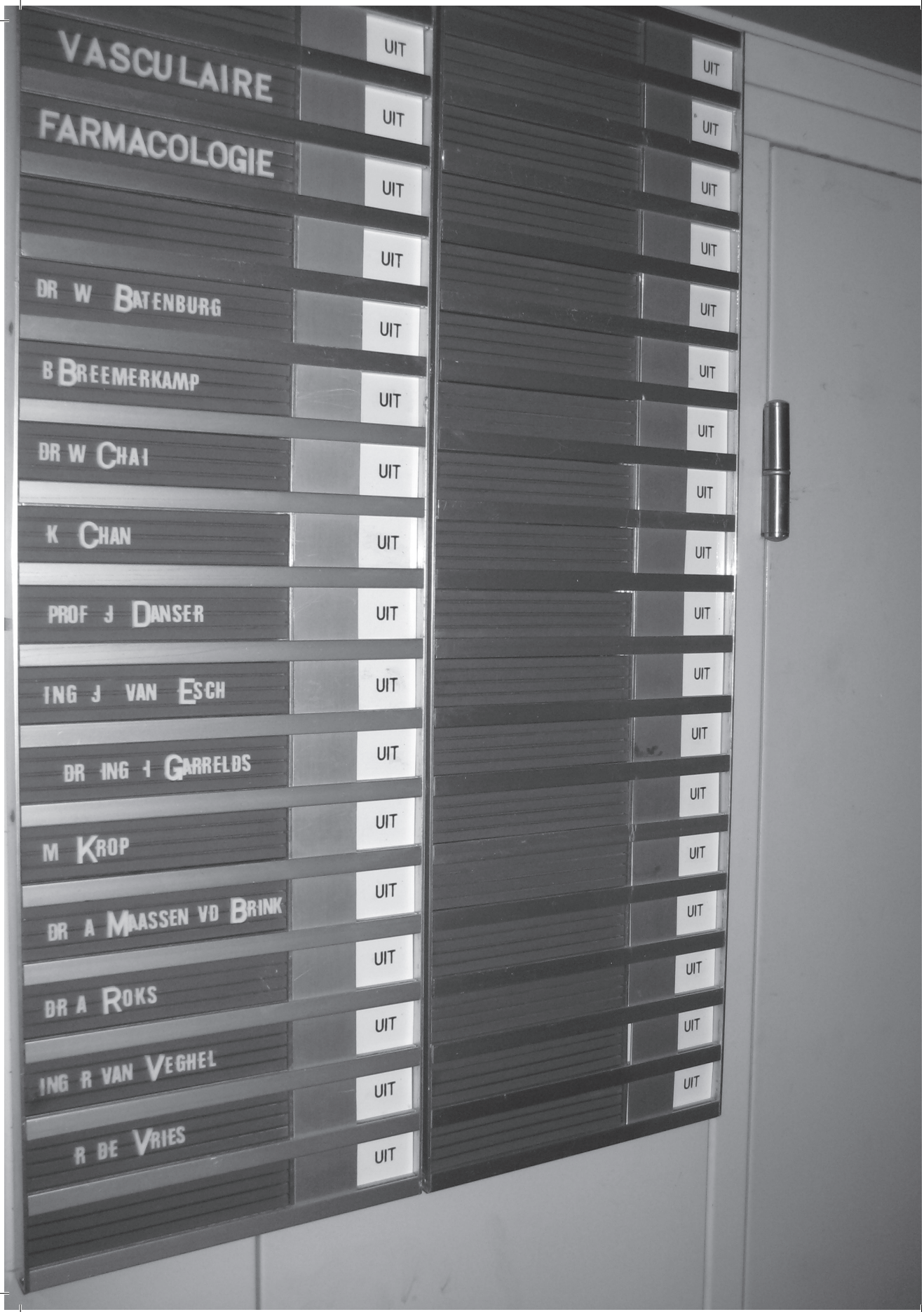
305. Hornig B, Landmesser U, Kohler C, Ahlersmann D, Spiekermann S, Christoph A, Tatge H, Drexler H. Comparative effect of ace inhibition and angiotensin II type 1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. *Circulation*. 2001;103:799-805.
306. Püntmann VO, Hussain MB, Mayr M, Xu Q, Singer DR. Role of oxidative stress in angiotensin-II mediated contraction of human conduit arteries in patients with cardiovascular disease. *Vascul Pharmacol*. 2005;43:277-82.
307. Feldman DL, Jin L, Xuan H, Mirserendino-Molteni R. Effect of the direct renin inhibitor (DRI) aliskiren on renal gene expression of pro-fibrotic molecules in experimental hypertensive diabetic nephropathy. *J Am Soc Nephrol*. 2007;18:168A.
308. Fuchs S, Xiao HD, Hubert C, Michaud A, Campbell DJ, Adams JW, Capecchi MR, Corvol P, Bernstein KE. Angiotensin-converting enzyme C-terminal catalytic domain is the main site of angiotensin I cleavage in vivo. *Hypertension*. 2008;51:In press.
309. Danser AHJ, Batenburg WW, van den Meiracker AH, Danilov SM. ACE phenotyping as a first step toward personalized medicine for ACE inhibitors. Why does ACE genotyping not predict the therapeutic efficacy of ACE inhibition? *Pharmacol Ther*. 2007;113:607-18.
310. Katzov H, Bennet AM, Kehoe P, Wiman B, Gatz M, Blennow K, Lenhard B, Pedersen NL, de Faire U, Prince JA. A cladistic model of ACE sequence variation with implications for myocardial infarction, Alzheimer disease and obesity. *Hum Mol Genet*. 2004;13:2647-57.
311. Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JC. ACE polymorphisms. *Circ Res*. 2006;98:1123-33.
312. Alhenc-Gelas F, Richard J, Courbon D, Warnet JM, Corvol P. Distribution of plasma angiotensin I-converting enzyme levels in healthy men: relationship to environmental and hormonal parameters. *J Lab Clin Med*. 1991;117:33-9.
313. Hamawaki M, Coffman TM, Lashus A, Koide M, Zile MR, Oliverio MI, DeFreyte G, Cooper Gt, Carabello BA. Pressure-overload hypertrophy is unabated in mice devoid of AT1A receptors. *Am J Physiol*. 1998;274:H868-73.
314. Stegbauer J, Vonend O, Habbel S, Quack I, Sellin L, Gross V, Rump LC. Angiotensin II modulates renal sympathetic neurotransmission through nitric oxide in AT2 receptor knockout mice. *J Hypertens*. 2005;23:1691-8.
315. Gross V, Walther T, Milia AF, Walter K, Schneider W, Luft FC. Left ventricular function in mice lacking the AT2 receptor. *J Hypertens*. 2001;19:967-76.
316. Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res*. 1998;83:1182-91.
317. Padia SH, Kemp BA, Howell NL, Fournie-Zaluski MC, Roques BP, Carey RM. Conversion of Renal Angiotensin II to Angiotensin III Is Critical for AT2 Receptor Mediated Natriuresis In Rats. *Hypertension*. 2008;51:1-6.
318. Bohlender J, Fukamizu A, Lippoldt A, Nomura T, Dietz R, Menard J, Murakami K, Luft FC, Ganten D. High human renin hypertension in transgenic rats. *Hypertension*. 1997;29:428-34.
319. Dechend R, Shagdarsuren E, Gratze P, Fiebeler A, Pilz B, Meiners S, Derer W,

Feldman DL, Webb R, Muller DN. Low-dose renin inhibitor and low-dose AT(1)-receptor blocker therapy ameliorate target-organ damage in rats harbouring human renin and angiotensinogen genes. *J Renin Angiotensin Aldosterone Syst*. 2007;8:81-4.

320. Bolterman RJ, Manriquez MC, Ortiz Ruiz MC, Juncos LA, Romero JC. Effects of captopril on the renin angiotensin system, oxidative stress, and endothelin in normal and hypertensive rats. *Hypertension*. 2005;46:943-7.
321. Oh BH, Mitchell J, Herron JR, Chung J, Khan M, Keefe DL. Aliskiren, an oral renin inhibitor, provides dose-dependent efficacy and sustained 24-hour blood pressure control in patients with hypertension. *J Am Coll Cardiol*. 2007;49:1157-63.
322. Vaidyanathan S, Jermany J, Yeh C, Bizot MN, Camisasca R. Aliskiren, a novel orally effective renin inhibitor, exhibits similar pharmacokinetics and pharmacodynamics in Japanese and Caucasian subjects. *Br J Clin Pharmacol*. 2006;62:690-8.
323. Vaidyanathan S, Reynolds C, Yeh CM, Bizot MN, Dieterich HA, Howard D, Dole WP. Pharmacokinetics, safety, and tolerability of the novel oral direct renin inhibitor aliskiren in elderly healthy subjects. *J Clin Pharmacol*. 2007;47:453-60.
324. Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJ, Burckle CA, Muller DN, Bader M, Nguyen G, Danser AHJ. Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. *J Hypertens*. 2007;25:2441-53.
325. Jones MR, Sealey JE, Laragh JH. Effects of angiotensin receptor blockers on ambulatory plasma Renin activity in healthy, normal subjects during unrestricted sodium intake. *Am J Hypertens*. 2007;20:907-16.
326. Belz GG, Butzer R, Kober S, Mutschler E. Pharmacodynamic studies on the angiotensin II type 1 antagonists irbesartan and candesartan based on angiotensin II dose response in humans. *J Cardiovasc Pharmacol*. 2002;39:561-8.
327. Nussberger J, Gradman AH, Schmieder RE, Lins RL, Chiang Y, Prescott MF. Plasma renin and the antihypertensive effect of the orally active renin inhibitor aliskiren in clinical hypertension. *Int J Clin Pract*. 2007;61:1461-8.
328. Sealey JE, Laragh JH. Aliskiren, the first renin inhibitor for treating hypertension: reactive renin secretion may limit its effectiveness. *Am J Hypertens*. 2007;20:587-97.
329. Danser AHJ, Charney A, Feldman DL, Nussberger J, Fisher N, Hollenberg NK. The renin rise with aliskiren: it's simply stoiciometry. *Hypertension*. 2008:In press.
330. Nguyen G. Renin/prorenin receptors. *Kidney Int*. 2006;69:1503-6.
331. Huang Y, Wongamorntham S, Kasting J, McQuillan D, Owens RT, Yu L, Noble NA, Border W. Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. *Kidney Int*. 2006;69:105-13.
332. Feldt S, Batenburg WW, Mazak I, Maschke U, Wellner M, Kvakan H, Dechend R, Fiebeler A, Burckle C, Contrepas A, Danser AHJ, Bader M, Muller DN. Prorenin and renin-induced ERK 1/2 activation in monocytes is not blocked by aliskiren or the handle-region peptide. *Hypertension*. 2008;51:682-688.
333. Schefe JH, Menk M, Reinemund J, Effertz K, Hobbs RM, Pandolfi PP, Ruiz P, Unger

T, Funke-Kaiser H. A novel signal transduction cascade involving direct physical interaction of the renin/prorenin receptor with the transcription factor promyelocytic zinc finger protein. *Circ Res*. 2006;99:1355-66.

334. Villard E, Lalau JD, van Hooft IS, Derkx FH, Houot AM, Pinet F, Corvol P, Soubrier F. A mutant renin gene in familial elevation of prorenin. *J Biol Chem*. 1994;269:30307-12.
335. Ferrario CM, Jessup J, Gallagher PE, Averill DB, Brosnihan KB, Ann Tallant E, Smith RD, Chappell MC. Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. *Kidney Int*. 2005;68:2189-96.
336. Liu YH, Yang XP, Sharov VG, Nass O, Sabbah HN, Peterson E, Carretero OA. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. *J Clin Invest*. 1997;99:1926-35.
337. Kostenis E, Milligan G, Christopoulos A, Sanchez-Ferrer CF, Heringer-Walther S, Sexton PM, Gembardt F, Kellett E, Martini L, Vanderheyden P, Schultheiss HP, Walther T. G-protein-coupled receptor Mas is a physiological antagonist of the angiotensin II type 1 receptor. *Circulation*. 2005;111:1806-13.
338. O'Brien E, Barton J, Nussberger J, Mulcahy D, Jensen C, Dicker P, Stanton A. Aliskiren reduces blood pressure and suppresses plasma renin activity in combination with a thiazide diuretic, an angiotensin-converting enzyme inhibitor, or an angiotensin receptor blocker. *Hypertension*. 2007;49:276-84.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