The More Complex Renin-Angiotensin System: New Insights Into An Old System
Thesis, Erasmus University, Rotterdam. With summary in Dutch
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The More Complex Renin-Angiotensin System: New Insights Into An Old System

Het nog complexere renine-angiotensine systeem:
Nieuwe inzichten in een oud systeem

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ter verkrijging van de graad van doctor aan de
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For my parents and my family.

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Chapter 1

General Introduction

1.1 The 'classical' renin-angiotensin system

Since the first description of renin by Tigerstedt and Bergmann in 1898,[1] the renin-angiotensin system (RAS) has been extensively studied. In the last decades, many investigations have demonstrated the importance of the RAS for maintenance and regulation of many physiological processes. One of the most prominent functions of the RAS is the regulation of blood pressure and water homeostasis.

1.1.1 From angiotensinogen to angiotensin II

The 'classical' RAS is described by the cascade starting with the cleavage of angiotensinogen (Agt). Agt is the only known precursor for angiotensin (Ang) peptides (**Tab. 1**).[2] Human Agt is a glycoprotein composed of 452 amino acids.[3] Agt is mainly produced in the liver and is secreted into the circulation. In circulation, Agt is cleaved by the aspartyl proteinase renin to release Ang I (**Fig. 1**). Renin is predominantly synthesised by the juxtaglomerular apparatus in the kidney as a pre-prohormone. The pre-segment acts as a signal peptide and directs the prohormone to a secretory pathway. The enzymatically inactive pro-renin can be activated by proteolytic removal of the pro-segment or through a conformational change induced by acidification[4] or binding to the recently cloned (pro)-renin receptor.[5]

Peptide	Amino acid sequence									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Ang I [Ang-(1-10)]	Asp -	Arg -	Val -	Tyr -	Ile -	His -	Pro -	Phe -	His -	Leu
Ang II [Ang-(1-8)]	Asp -	Arg -	Val -	Tyr -	Ile -	His -	Pro -	Phe		
Ang III [Ang-(2-8)]		Arg -	Val -	Tyr -	Ile -	His -	Pro -	Phe		
Ang IV [Ang-(3-8)]			Val -	Tyr -	Ile -	His -	Pro -	Phe		
Ang-(1-7)	Asp -	Arg -	Val -	Tyr -	Ile -	His -	Pro			

Table 1 The amino acid sequences of biologically active angiotensin (Ang) peptides.

In the next step, the two C-terminal amino acids of Ang I are removed by angiotensin-converting enzyme (ACE) to release Ang II (**Fig. 1**).[6] ACE is a zinc metallopeptidase, which is catalysing the hydrolysis of a wide variety of peptides, besides Ang I[7-9] e.g. the vasodilator bradykinin.[7,9] In mammals, two ACE isoforms exist, the somatic ACE with two active sites (C-domain and N-domain) and a truncated (lacking the N-domain) germinal isoform which is only expressed in testes. Both isoforms are membrane-bound enzymes, but could be released by a secretase.[10] The germinal ACE plays a major role in fertility,[11] whereas the somatic isoform is crucial in maintenance of renal structure and function and blood pressure.[11-13]
It was demonstrated that Ang II can also be generated by an ACE-independent pathway. The chymotrypsin-like serine protease chymase can release Ang II from Ang I.[14] Up-to-now the physiological role of the chymase-dependent Ang II production is uncertain, but under pathophysiological conditions chymase seems to play an important role.[15]
Ang II, the main acting peptide of the RAS, is a potent regulator of blood pressure and water homeostasis.[16] Furthermore, it is stimulating cell growth and proliferation[17] and has an impact on voluntary alcohol consumption.[18] Ang II stimulates its two known receptors, the Ang II receptor subtype 1 (AT_1) and subtype 2 (AT_2). Both Ang II receptors have been cloned[19-22] and belong to the family of the seven transmembrane domain receptors, a typical characteristic of G protein-coupled receptors (GPCRs). In mice and rats, the AT_1 exists in two distinct subtypes (AT_{1a} and AT_{1b}), whereas it appears that other mammalian species do not have AT_1 subtypes. AT_{1a} and AT_{1b} exhibit similar ligand binding and signal transduction properties and cannot be distinguished pharmacologically, but differ in their tissue distribution.[23]

The activation of the RAS is a cascading process, which can be pharmacologically blocked on three levels: 1. Ang I release with renin inhibitors, e.g. aliskiren;[24] 2. Ang II generation with ACE inhibitors, e.g. enalapril;[25] 3. Ang II-mediated effects by either antibodies neutralising Ang II[26] or subtype specific antagonists (AT_1: e.g. losartan, AT_2: e.g. PD123,319).[16] The discovery of compounds inhibiting the RAS activity provided first research tools to characterise the function of the system. Furthermore, a variety of genetically modified animals lacking components of the cascade (Agt,[27] renin,[28,29] ACE,[12] AT_{1a},[30] AT_{1b},[31] and AT_2[32]) helped to reveal the complex functions of the RAS.

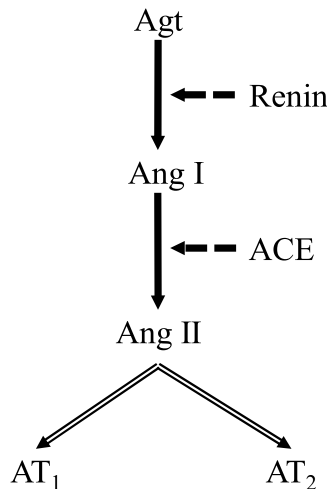

Figure 1 The cascade of the 'classical' renin-angiotensin system leading to activation of the angiotensin (Ang) II receptors, AT_1 and AT_2, is initiated by the cleavage of angiotensinogen (Agt) by renin, releasing Ang I. Ang II is then generated through metabolism of Ang I by angiotensin-converting enzyme (ACE) and can stimulate its receptors.

1.1.2 The angiotensin II receptors

Most of the known physiological effects of Ang II are mediated by AT_1, e.g. blood pressure regulation, electrolyte and water homeostasis, and renal function. The AT_1 is widely distributed and expressed in most adult tissues. Upon stimulation of the AT_1 by Ang II the receptor can activate different intracellular signalling pathways. The signalling cascades activated can be classified into two groups: G protein-dependent and G protein-independent pathways. The AT_1 is rapidly internalised upon Ang II binding. The internalised receptors can be either degraded in lysosomes or recycled to the cell surface.[33,34]

Vasoconstriction, the major acute effect of Ang II, is mediated by activation of heterotrimeric G proteins. It was shown that AT_1 can couple to two members of the G_α family ($G_{\alpha q/11}$ and $G_{\alpha i}$) and to $G_{\beta\gamma}$ complexes.[35,36] The activation of these G proteins initiates further downstream cascades including phospholipase C (PLC) and phospholipase D (PLD).[37,38] The activation of these phospholipases induces an increase in intracellular calcium, enhancement of actin and myosin interaction, and a release of leukotrienes, leading to vasoconstriction. Furthermore, AT_1-mediated signalling leads to a potent activation of mitogen-activated protein kinases (MAPK).[39,40] The MAPK pathway induces cellular protein synthesis, volume regulation, gene expression, and growth.

Beside the aforementioned G protein-dependent effects, AT_1-mediates Ang II effects independently from G protein coupling. Upon stimulation AT_1 can activate non-receptor tyrosine kinases, e.g. Src,[41,42] JAK/STAT,[43] and receptor tyrosine kinases, e.g. platelet-derived growth factor (PDGF) receptor,[44,45] and epidermal growth factor (EGF) receptor.[46,47] These signalling pathways are mainly related to the growth-promoting effects of Ang II.

The AT_2 only shares ~30% amino acid sequence identity with the AT_1,[21,48] mainly localised in the hydrophobic transmembrane domains. The residues in these helical domains are

considered to be essential for Ang II binding. In contrast to AT_1, AT_2 has a very restricted expression pattern in adult tissues, limited to certain cell types,[49] but is the dominating Ang II receptor in foetal tissues.[50] The two Ang II receptors do not only differ in their structure and expression pattern, but also in the signalling pathways triggered.

First investigations of the AT_2 did not reveal a G protein coupling,[51] questioning its membership in the GPCR family. Today it is known that the AT_2 belongs to the GPCR superfamily and can couple to $G_{\alpha i}$[52,53] and $G_{\alpha s}$ subunits,[54] but in many cases the activated signalling cascades are G protein-independent. The first described signalling pathway activated by Ang II stimulation of AT_2 is the activation of protein phosphatases.[55] Up-to-now the activation of 3 distinct phosphatases by AT_2 stimulation has been described: MAPK phosphatase 1 (MKP-1),[56] SH2 domain-containing phosphatase 1 (SHP-1),[57] and protein phosphatase 2A (PP2A).[58] These phosphatases dephosphorylate and subsequently inactivate MAPKs, most documented is the inactivation of extra-cellular signal-regulated kinases 1 and 2 (ERK1/2),[56,58,59] but other targets also have been described, e.g. signal transducers and activators of transcription (STAT)[60] and receptor tyrosine kinases.[61] The phosphatase activation seems to be the main mechanism mediating AT_2-stimulated growth inhibition and apoptosis induction.[62] A further mechanism for the pro-apoptotic properties of Ang II-stimulated AT_2 signalling is the activation of caspase 3 resulting from the *de novo* synthesis of ceramides.[63]

The vasorelaxant effects induced by AT_2 are mediated by the nitric oxide (NO)/cyclic guanosine 3',5'-monophosphate (cGMP) pathway. The AT_2 can impact the NO/cGMP pathway in two different ways. Firstly, AT_2 stimulation leads to release of bradykinin (BK) which subsequently activates its own subtype 2 receptors (B_2) inducing NO and cGMP release.[64] The AT_2-mediated BK release is still not fully understood. A possible mechanism is the AT_2-dependent acidification of the cytoplasm,[65] which can activate kininogenases to generate BK from kininogen.[64,66] Secondly, a BK-independent pathway has been described, showing a direct modulation of NO synthase (NOS) activity by AT_2.[67,68]

In *in vivo* studies, it was shown that Ang II activates phospholipase A_2 (PLA_2) involving $G_{\beta\gamma}$ complexes.[69] PLA_2 releases arachidonic acid, which can activate MAPKs and further kinases.[70,71] Interestingly, this is not only one of the few cases in which the AT_2 mediates its effects by G proteins, but also one in which the AT_2 is coupled to kinase- and not phosphatase-activation.

Both Ang II receptor subtypes can form heterodimers, resulting in reduced Ang II-stimulated, AT_1-mediated effects.[72] Furthermore, it was demonstrated that both Ang II receptor subtypes can form heterodimers with B_2.[73,74] The described heterodimers have changed signalling profiles compared to the non-dimerised receptors. At least the formation of AT_1/B_2-heterodimers has been put into question by newer investigations, which could not find changed signalling profiles or evidence for dimerisation.[75]

1.2 The 'new' renin-angiotensin system

Due to ongoing research, the relatively simple 'classical' concept of the RAS has been expanded. In addition to the 'circulating' RAS a local 'tissue' RAS exists in many organs.[76-81] Furthermore, there are indications for an intracellular RAS,[82] making the RAS not only an endocrine, but also a paracrine and an intracrine system.

Recent investigations have shown that the RAS is more complex than it was thought. Beside Ang II, shorter fragments of Ang II, namely Ang III [Ang-(2-8)],[83] Ang IV [Ang-(3-8)],[84,85] and Ang-(1-7)[86-88] (**Tab. 1**), demonstrate potent biological activity.

1.2.1 Angiotensin III

The heptapeptide Ang III (**Tab. 1**) is generated through cleavage of Ang II by aminopeptidase A (APA).[89] Ang III has the same affinity to AT_1 and AT_2 as Ang II[19,21,22] and exerts its effects via these receptors (**Fig. 2**).[90] The intracerebroventricular (i.c.v.) injection of Ang II and Ang III showed a comparable increase in blood pressure and salt intake.[91-93] However, Ang III injected i.c.v. increased the firing rate of neurons in brain regions involved in the regulation of blood pressure.[94,95] The use of specific APA inhibitors provided strong evidence that Ang III is the main regulator of blood pressure of the RAS in the central nervous system.[96,97]

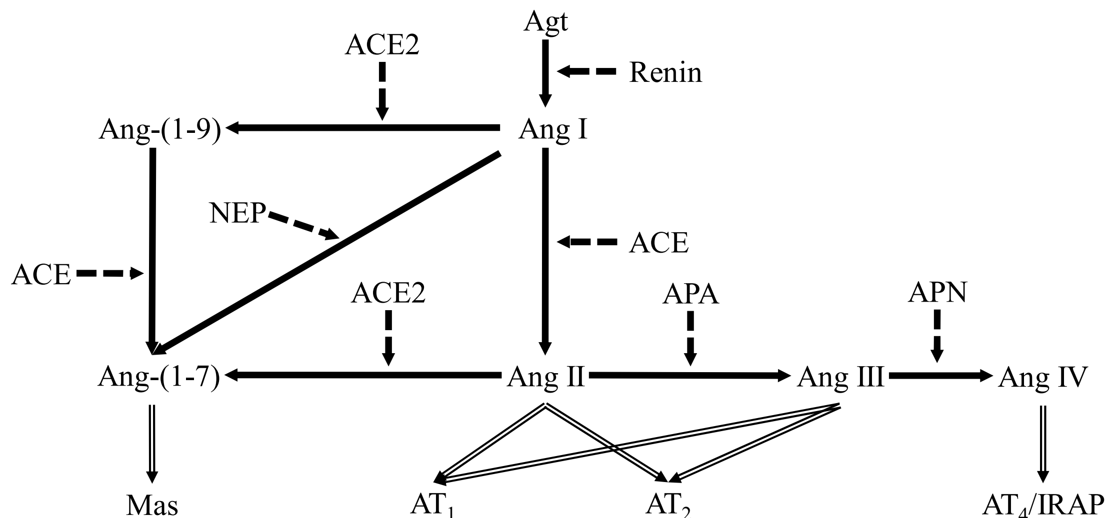

Figure 2 The 'extended' renin-angiotensin system with the cascades generating bioactive angiotensin (Ang) fragments Ang III, Ang IV, and Ang-(1-7) and their target structures. (Agt: angiotensinogen; ACE: angiotensin-converting enzyme; ACE: angiotensin-converting enzyme 2; NEP: neutral endopeptidase; APA: aminopeptidase A; APN aminopeptidase N; IRAP: insulin-regulated aminopeptidase).

1.2.2 Angiotensin IV

Further processing of Ang III by aminopeptidase N (APN) leads to the hexapeptide Ang IV (**Tab. 1**).[86] Ang IV can stimulate AT_1 and induce vasoconstriction by the same mechanism as Ang II,[98] but to a lesser extent.[99] Besides this AT_1-dependent effects, it was shown that Ang IV can also mediate effects independent of AT_1 and AT_2.[100] The membrane-associated peptidase insulin-regulated aminopeptidase (IRAP) has been identified as the potential Ang IV receptor.[101] Ang IV is an inhibitor of the enzymatic activity of IRAP. The inhibition of IRAP by Ang IV can lead to the accumulation of other substrates of IRAP, e.g. vasopressin, substance P, and somatostatin. These peptides play an important role in cognitive processes[102,103] and could be the reason for the potent effects in the central nervous system, e.g. memory promoting effects and anti-epileptogenic effects, seen after i.c.v. administration of Ang IV.[100]

1.2.3 The ACE2/angiotensin-(1-7)/Mas axis

The heptapeptide Ang-(1-7) can be generated through cleavage of Ang I or Ang II by a variety of enzymes.[104-111] To date it is thought that the major pathways of Ang-(1-7) generation are: 1) the cleavage of Ang I by neutral endopeptidase (NEP, neprilysin),[86] 2) the metabolism of Ang II by the recently discovered ACE homologue ACE2, and 3) the cleavage of Agt by ACE2 and further processing of the produced Ang-(1-9) by ACE (**Fig. 2**).[111]

NEP is a membrane-bound (type-II) metallo-enzyme and is widely distributed throughout the body.[112] Promoted by the availability of specific inhibitors[113] and of knockout mice,[114] many biochemical and physiological properties of this peptidase have been described in detail. Beside the release of Ang-(1-7) from Ang I,[105] NEP can cleave a broad variety of peptides.

Typical substrates are the enkephalines,[115] bradykinin,[116] atrial natriuretic peptide (ANP),[117] C-type natriuretic peptide (CNP),[118] the insulin β chain,[119] substance P,[120] and the „Alzheimer-peptide“ β-Amyloid (Aβ).[121]
The recently discovered ACE homologue ACE2[107,108] is an important part of the RAS, which can counteract the function of ACE by generating Ang-(1-7) from Ang II.[109,111] ACE2 is also involved in an alternative Ang-(1-7) generating pathway by metabolising Ang I to the biological inactive fragment Ang-(1-9).[109] This peptide can be further processed by ACE, removing the two C-terminal amino acids and thereby generating Ang-(1-7).[110] Beside its role in the processing of Ang peptides, ACE2 has a strong impact on the development of acute respiratory distress syndrome (ARDS).[122] Furthermore, recent investigations have revealed that ACE2 is a functional receptor for the corona virus, which causes the severe acute respiratory syndrome (SARS).[123]
Ang-(1-7) was first thought to be a biological inactive degradation product of Ang II.[124] In 1988, it was first observed that Ang-(1-7) demonstrates biological activity.[125] In recent years, Ang-(1-7) has become an Ang metabolite of high interest, since it can often counteract the detrimental effects of Ang II, e.g. its vasoconstrictive and proliferative properties.[87,126] In contrast to Ang II, Ang-(1-7) increases the sensitivity of the baroreceptor reflex.[127] The heptapeptide promotes the release of vasodilating prostaglandins[128,129] and NO.[130] Beside the release of this vasorelaxant compounds, Ang-(1-7) can potentiate BK-stimulated effects.[131,132] Additionally to its vascular effects, it was shown that Ang-(1-7) also has beneficial effects on cardiac function.[133,134] Moreover, it is opposing the growth-stimulating effects of Ang II in vascular smooth muscle cells[135] and cardiomyocytes.[136] Ang-(1-7) also demonstrates effects on renal function by participating in the control of water and electrolyte homeostasis.[137] However, research involving Ang-(1-7) is not restricted to cardiovascular and renal function. In recent years, investigations have demonstrated Ang-(1-7) effects on wound healing,[138] cancer,[139-141] and early progenitor cells.[142]
In 2003, it was demonstrated that the receptor Mas is associated with Ang-(1-7)-induced effects.[143] The receptor Mas is encoded by the *Mas* proto-oncogene, which was first detected through its tumourigenic activity in *in vivo* tumour assays.[144] It was suggested that Mas is a receptor for Ang II,[145] but further investigations revealed that Ang II-induced elevation of intracellular Ca^{2+} in *Mas*-transfected cells was only seen in cells expressing AT_1.[146,147] Despite this, clear evidence indicates a physiological role for Mas in the function of Ang II. It was shown that Mas deficiency leads to alterations of AT_1 signalling in neuronal cells.[148] After its discovery, considerable efforts have been made to elucidate signal transduction pathways stimulated by Mas. First investigations suggested a Mas-mediated activation of the small GTPase Rac1,[149] but recently it was shown that Mas activates another small GTPase, RhoA,[150] by a constitutive activation of $G_{\alpha q/11}$ subunits.[150,151] Furthermore, it was shown that Ang-(1-7) can inhibit the activation of MAPKs in cardiomyocytes by stimulation of Mas.[136]

1.3 G protein-coupled receptors encoded by the ‘Mas-related genes’ gene family

Lately, a large gene family with high sequence homology to the *Mas* proto-oncogene was identified by two independent groups.[152,153] The first group identified mouse and human members of the ‘*Mas*-related genes’ (MRG) and named the receptor family encoded by these genes Mrg.[152] The second group isolated rat and human members from small sensory neurons and suggested sensory-neuron-specific GPCRs (SNSR) as a name.[153] To prevent confusion between the two naming suggestions, this work follows the Mrg convention.
The MRGs are expressed in nociceptive sensory neurons of the dorsal root ganglia. Most of these GPCRs are orphan, but some of the Mrg members can be potently activated by peptide ligands such as RF-amide, the bovine adrenal medulla 22-like peptide (BAM22), or γ2-

melanocyte stimulating hormone (γ2-MSH).[152-154] Based on these findings, it is hypothesised that these receptors are involved in the modulation of nociceptive stimuli.[155]
In mice, the *MRG* family consists of more than 50 members,[152,156] which is comprised of 5 single-copy genes (encoding for the receptors MrgD, MrgE, MrgF/RTA, MrgG, and MrgH/GPR90) and three large subgroups (encoding for the receptor-families MrgA, MrgB, and MrgC). The family also contains several pseudo-genes. In contrast to mice, the receptor subgroups MrgA and MrgC have only one member in rats.[156] Beside MrgH, the receptors encoded by the single-copy genes have clear orthologs in humans, but none of the genes from the *MrgA*, *MrgB*, and *MrgC* subfamilies have a clear human ortholog.[152,157] However, the MrgX subfamily, consisting of 4 receptors,[152] and MRG, the first Mrg identified in humans,[158] have no known orthologs in species other than humans.

Aims of this work

It is undisputed that the blockade of the ACE/Ang II/AT_1 axis is cardioprotective. Furthermore, in the last decade new enzymes, receptors, and peptides related to the RAS and its activational state have been identified. Our group and others have shown that the stimulation of the newly described ACE2/Ang-(1-7)/Mas axis also plays an important role in cardioprotection. But still there are a lot of scientific unknowns in the regulation and even importance of RAS activity. A more complete picture of the regulation and function of the RAS shall not only lead to an improved understanding of physiological and pathophysiological processes, but shall also reveal novel therapeutic targets for the treatment of cardiovascular and other RAS-related diseases. To further elucidate the complex functionality of the RAS, this thesis addressed the following issues:

1. ACE is not the only peptidase involved in the regulation of the RAS. It was shown for several other enzymes that they can process Ang peptides. The ratio of bioactive Ang metabolites determines the activational status of the RAS. In this work, we have focused on the ACE-homologue ACE2, which mainly generates Ang-(1-7) from Ang II, and on APA, an aminopeptidase converting Ang II to Ang III. In time we started our work on ACE2, the biochemical activity of ACE2 was analysed, but little was know about tissue expression and activity, therefore we investigated the peptidase distribution in mice and rats (Chapter 2). Furthermore, to better characterise the role of Ang III as the major Ang effector peptide in the central nervous system, we investigated its role in water homeostasis and voluntary alcohol consumption using APA-knockout mice (Chapter 3).
2. During the last decades, new biologically active peptides belonging to the RAS have been identified. The discovery of these peptides also directed research projects to identify receptors mediating their effects, e.g. IRAP was identified to be a 'receptor' for Ang IV. We have identified the receptor Mas to be associated with the intracellular signalling induced by Ang-(1-7). In this work, we investigated the importance of Mas for endothelial function. We analysed the changes in tension of explanted microvessels from Mas-deficient mice and their wild-type controls after stimulation with vasorelaxant compounds (Chapter 4). Additionally, we characterised its impact on the ACE/Ang II/AT_1 axis by describing the interaction of Mas with AT_1 (Chapter 6). Recently, a whole family of receptors closely related to Mas have been identified. In this work we analysed if the MRGs could be affiliated with the RAS. To proceed in this matter, we investigated their intracellular signalling activated by different Ang peptides (Chapter 5).
3. Ang II mediates its biological effects by stimulating two distinct receptors AT_1 and AT_2. In rodents, two pharmacologically undistinguishable AT_1 subtypes have been identified, namely AT_{1a} and AT_{1b}. Therefore, we wanted to identify effects specifically mediated by one of the AT_1 subtypes generating and using mice deficient in one, two, or all three known Ang II receptors. In these mice, we also investigated if an additional receptor, beside AT_1 and AT_2, may mediate Ang II effects (Chapters 7 and 8).

Chapter 2

Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents

Based on: 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(7):1270-1277

2.1 Abstract

Biochemical analysis revealed that angiotensin-converting enzyme related carboxy-peptidase (ACE2) cleaves angiotensin (Ang) II to Ang-(1-7), a heptapeptide identified as an endogenous ligand for the G protein-coupled receptor Mas. No data are currently available that systematically describe ACE2 distribution and activity in rodents. Therefore, we analyzed the ACE2 expression in different tissues of mice and rats on mRNA (RNase protection assay) and protein levels (immunohistochemistry, ACE2 activity, western blot). Although ACE2 mRNA in both investigated species showed the highest expression in the ileum, the mouse organ exceeded rat ACE2, as also demonstrated in the kidney and colon. Corresponding to mRNA, ACE2 activity was highest in the ileum and mouse kidney but weak in the rat kidney, which was also confirmed by immunohistochemistry. Contrary to mRNA, we found weak activity in the lung of both species. Our data demonstrate a tissue- and species-specific pattern for ACE2 under physiological conditions.

2.2. Introduction

In the regulation of heart function and blood pressure, different peptide systems are involved, e.g. the renin-angiotensin system (RAS), the kallikrein-kinin system, and the natriuretic peptide system. In these systems, proteases like angiotensin-converting enzyme (ACE) or neutral endopeptidase (NEP) have the distinction of generating or catabolising biologically active peptides.[9,118,159] The newly discovered angiotensin-converting enzyme-related carboxy-peptidase (ACE2) has considerable sequence homology to ACE (40% identity and 61% similarity), contains a HE*XX*H zinc-binding domain, and conserves other critical residues typical of the ACE family.[107,108] The first step in generating angiotensin peptides is the cleavage of angiotensinogen to angiotensin (Ang) I by renin. Ang I is hydrolyzed by either ACE or chymase to Ang II, which mediates its biological actions via the AT_1 and AT_2 receptors.[88,160] Ang I is also metabolized by NEP to Ang-(1-7),[88] which mediates distinct effects through its receptor Mas.[143] Importantly, Ang-(1-7) can also be directly metabolized from Ang II by ACE2, whereas aminopeptidase A converts Ang II to Ang III.[96] ACE2 also hydrolyzes Ang I to Ang-(1-9), although there is no hydrolysis of Ang-(1-9), Ang-(1-7), and Ang-(1-5). Moreover, ACE2 hydrolysis is also specific for des-Arg^9-bradykinin and its shorter fragments, although it cleaves neither bradykinin nor bradykinin-(1-7).[109] ACE2 mRNA is expressed in many tissues but shows a less ubiquitous profile than ACE. First studies in mice detected the highest expression in the ileum by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR).[161]

ACE2 is an important part of the RAS, which counteracts the function of ACE. It was also shown that ACE2 expression can be up-regulated by blockade of AT_1-receptors.[162] The importance of ACE2 in cardiovascular regulation was confirmed by targeted disruption of ACE2 in mice. The absence of ACE2 in mice leads to a severe cardiac contractility defect, increased Ang II levels, and up-regulation of hypoxia-induced genes in the heart.[163] In addition to its peptidolytic function, recent investigations have discovered that ACE2 is a functional receptor for the corona virus, which causes the severe acute respiratory syndrome (SARS).[123]

In this investigation, we (i) measured the mRNA distribution of ACE2 through different tissues in both species. Moreover, we (ii) quantified ACE2 protein by western blot using a commercial polyclonal antibody to ACE2.We (iii) measured ACE2 activity in different tissues of mice and rats. We (iv) established a monoclonal antibody against ACE2 to complete the investigation of tissue distribution by immunohistochemistry. Finally, we compared (v) the distribution of ACE2 in both species on the mRNA and protein level.

2.3 Materials and methods

2.3.1 Animals

All experiments were done according to the guidelines of the Federal Law on the Use of Experimental Animals in Germany and were approved by the local authorities. For this investigation we used C57Bl/6 mice and Sprague-Dawley (SD) rats in an age of 3-5 months. Animals were killed by cervical dislocation. For RNase protection assay (RPA), ACE2 activity assay and western blot, the tissues were snap frozen in liquid nitrogen. The samples were stored at -80°C until further processing (all organs in total, heart divided into atria and ventricles). The tissues for immunohistochemistry were put in 4% formalin. After 24 h they were embedded and processed to paraffin sections.

2.3.2 RNase protection assay

2.3.2.1 Probe generation

The polymerase chain reaction (PCR) amplified a 358 bp fragment (probe: MMACE2) from mouse kidney cDNA using the 5'-primer CTC AGT GGA TGG GAT CTT GG (MMACE25) and the 3'-primer TGT AGC CAT CTG CTC CCT CT (MMACE23), respectively a 342 bp fragment (probe: RNACE2) from rat lung cDNA using the 5'-primer CGG GGA AAG ATG TCA AGC TCC TGC (RNACE25) and the 3'-primer CTT GTC TGG TGA CAG CGC (RNACE23), which were subcloned in a T-vector (Promega GmbH, Mannheim, Germany). A SP6 polymerase transcribed a radioactive probe complementary to MMACE2 (resp. RNACE2) mRNA, and a RNA complementary to 127 nucleotides of the rL32 mRNA was used as positive control.[164] ACE2-specific mRNA for mouse and rat were identified by RNase protection assay (RPA) using the Ambion RPA II kit (Ambion (Europe) Ltd., Huntingdon, UK).

2.3.2.2 RNase protection assay

Total RNA was isolated from tissues using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) with subsequent chloroform-isopropanol extraction according to the manufacturer's instructions. A 15 µg total RNA fraction of each sample was hybridized with approximately 50,000 cpm for ACE2 and 50,000 cpm for rL32 of the radiolabelled antisense probes in the same assay. Equal loading has been insured by mRNA measurements and mRNA gel electrophoresis using 1 µg of each sample (**not shown**). The hybridized fragments protected from RNase A+ T1 digestion were separated by electrophoresis on a denaturing gel (5%, w/v polyacrylamide, 8M urea) and analyzed using a FUJIX BAS 2000 Phospho-Imager system (Raytest GmbH, Straubenhardt, Germany) to perform quantitative analysis by measuring the intensity of the ACE2 bands. The blots of each species were calculated to ACE2 mRNA expression in kidney, which was present on both blots of each species. The expression level in the lung was set to 100%.

2.3.3 ACE2 activity assay

ACE2 activity was measured similar to the method by Vickers *et al.*[109] Tissue was homogenized in assay buffer (50 mM 2-morpholinoethanesulfonic acid, 300 mM NaCl, 10 µM $ZnCl_2$, 0.01% Brij-35, pH 6.5). Protein concentration was determined using Roti-Quant (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) by the manufacturers instruction. We used Mca-APK(Dnp) (Biosynthan GmbH, Berlin, Germany) dissolved in DMSO (50 µM, final concentration) as the ACE2 substrate. The assay was performed in assay buffer and was started by adding 10 µl of tissue homogenate. After 2 h incubation at ambient temperature (24°C), the reaction was suppressed by adding 100 µM *o*-phenanthrolin (final concentration). Parallel control tests were performed in the presence of 1 µM DX600 (**data not shown**).[165] After centrifugation (10 min, 10,000×*g*) the fluorescence was measured at 320 nm

(excitation) and 405 nm (emission) with the Perkin-Elmer fluorescence reader Lambda 5 (Perkin-Elmer LAS GmbH, Rodgau, Germany). The molecular standardization was performed with Mca-AP (Biosynthan GmbH, Berlin, Germany) and calculated per mg protein. The functionality of the assay was proven by a standardized solution with defined, recombinant ACE2 activity (R&D Systems GmbH, Wiesbaden, Germany).

2.3.4 Western blot

Tissue was homogenized in phosphate-buffered solution (PBS) containing protease inhibitor mixture (Complete, Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined with BCA Protein Assay Kit (Perbio Science GmbH, Bonn, Germany). Sample proteins (10 µg/lane) and a pre-stained protein-weight marker (Amersham Biosciences GmbH, Freiburg, Germany) were size fractionated by SDS-polyacrylamide gels (10%) and transferred to PVDF membranes with a Pegasus semidry-blotter (Phase GmbH, Lübeck, Germany). Equal loading has been insured by staining control gels with Simply-Blue Safe Stain (Invitrogen GmbH, Karlsruhe, Germany) using 10 µg of each sample (not shown). The membranes were blocked at room temperature in 5% dry milk powder (blotting grade, non-fat dry milk, Bio-Rad Laboratories GmbH, Munich, Germany) prepared with Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h, incubated with goat polyclonal antibody against ACE2 (Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:250 diluted in 5% dry milk powder TTBS, 1 h), and then washed three times with TTBS (15 min each). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-goat IgG (DakoCytomation A/S, Glostrup, Denmark, 1:1,000, 1 h) and washed three times. Specific immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences GmbH, Freiburg, Germany). The bands on the X-ray film were quantified by densitometry scanning and expressed as percentage of the kidney protein signal.

2.3.5 Immunohistochemistry

2.3.5.1 Generation of monoclonal antibodies against ACE2 (clones: 7E7 and 1D3)

Monoclonal antibodies against the synthetic peptide AVGEIMSLSAAT (AA 403-414 of murine ACE2) have been raised. For immunization of the mice peptide was cross-linked with glutaraldehyde to albumin fraction V from bovine serum. BALB/cJ female mice were injected with the conjugate. Following four booster injections the spleen lymphocytes were fused with FO myeloma cells by using polyethylene glycol 1500 (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturers' instructions.

The different hybridoma supernatants were screened for specific antibodies by using the synthetic peptide in the NC-test.[166] For production of monoclonal antibodies, positive hybridoma cells were grown in CELLine incubators (Integra Biosciences GmbH, Fernwald, Germany). The mouse monoclonal antibodies were affinity purified on a MabTrap G II column (Amersham-Pharmacia GmbH, Otelfingen, Switzerland) from cell culture supernatants. Immunoglobulin class and subclasses were determined with the Immuno Type Kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

2.3.5.2 Immunohistochemistry

Paraffin sections of mouse tissues were prepared and stained using standard histology procedures. For immunostaining, deparaffinised and rehydrated tissue slides were first treated for 30 min with 30% H_2O_2 to block the endogenous peroxidase. After rinsing in ddH_2O and soaking in PBS for 5-10 min, slides were treated with 10% (w/v) BSA in PBS to eliminate non-specific protein binding sites. The slides were then exposed (overnight, 4°C) to the monoclonal ACE2 antibodies (clone 7E7, 1D3) at concentrations of 1 and 4 µg/ml,

respectively. After removing excess antibody, slides were treated with biotin-labelled anti-mouse (Dianova GmbH, Hamburg, Germany) antibody for 30 min at 37°C and finally with horseradish peroxidase (HRP) labelled streptavidine (Zymed Laboratories Inc., San Francisco, USA) for 20 min at 37°C. After washing, slides were incubated in aminoethylcarbazol (Sigma-Aldrich Co., St. Louis, USA) for 10 min at room temperature. Slides were counterstained with haematoxylin, and cover slipped according to conventional procedures. Slides were examined under a conventional microscope after removing the excess substrate in ddH_2O. Negative controls were performed without the primary antibody, just applying dilution buffer of the primary antibody.

2.3.6 Statistics
Data were analyzed by *t*-test using SPSS11 software (SPSS Benelux BV, Gorinchem, The Netherlands). Each value was expressed as the mean±SEM, and statistical significance was accepted for $P<0.05$.

2.4 Results

2.4.1 RNase protection assay
ACE2 mRNA could be detected in all investigated organs, but with profound distinction between different organs. In both species, only a low amount was found in ventricle, liver, testis, forebrain, and spleen (**Figs. 3 and 4**), whereas in the lungs a moderate and comparable expression of ACE2 mRNA was found and set to 100%. The highest levels were found in the ileum of both species (**Fig. 5**). Between the species several differences in tissue specific expression of ACE2 mRNA were found. The expression in mouse was most pronounced higher than in rat in kidney (~31.9-fold), colon (~18.6-fold), and ileum (~12.0-fold) (**Fig. 5**), whereas in bladder (~2.5-fold) and ventricle (~2.1-fold) ACE2 expression in rat exceeded the mouse.

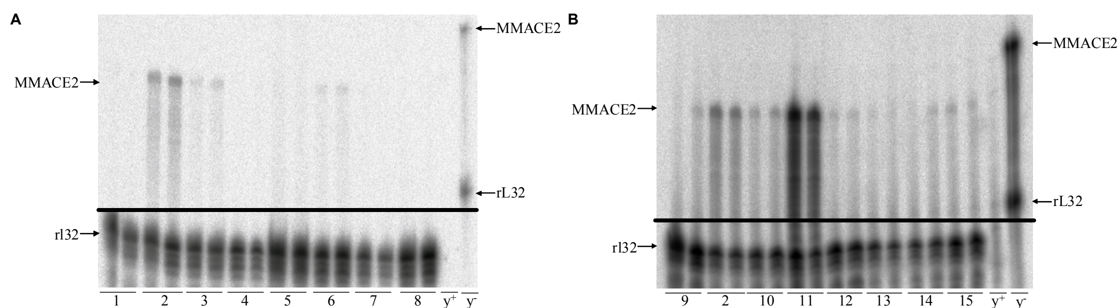

Figure 3 mRNA expression in different mouse tissues. Representative RPA of different tissues from C57Bl/6 mice. The specific bands for MMACE2 and the housekeeping mRNA rl32 are indicated with arrows on the left. The MMACE2 and rL32 probes are indicated with arrows on the right. (**A**) 1. ventricle, 2. kidney, 3. lung, 4. liver, 5. testis, 6. bladder, 7. forebrain, 8. spleen, y^+, yeast plus RNase; y^-, yeast without RNase; (**B**) 9. thymus, 2. kidney, 10. stomach, 11. ileum, 12. colon, 13. brainstem, 14. atrium, 15. adipose tissue; MM, Mus Musculus; y^+, yeast plus RNase; y^-, yeast without RNase.

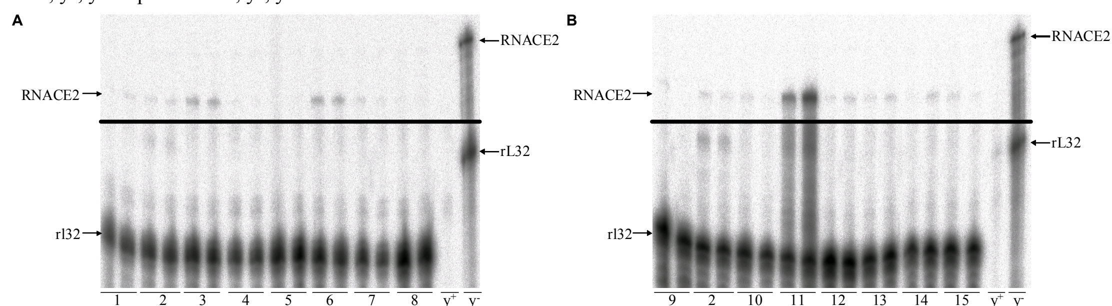

Figure 4 mRNA expression of different rat tissues. Representative RPA of different tissues from C57Bl/6 mice. The specific bands for RNACE2 and the housekeeping mRNA rl32 are indicated with arrows on the left. The RNACE2 and rL32 probes are indicated with arrows on the right. (**A**) 1. ventricle, 2. kidney, 3. lung, 4. liver, 5. testis, 6. bladder, 7. forebrain, 8. spleen, y^+, yeast plus RNase; y^-, yeast without RNase; (**B**) 9. thymus, 2. kidney, 10. stomach, 11. ileum, 12. colon, 13. brainstem, 14. atrium, 15. adipose tissue; RN, Rattus Norvegicus; y^+, yeast plus RNase; y^-, yeast without RNase.

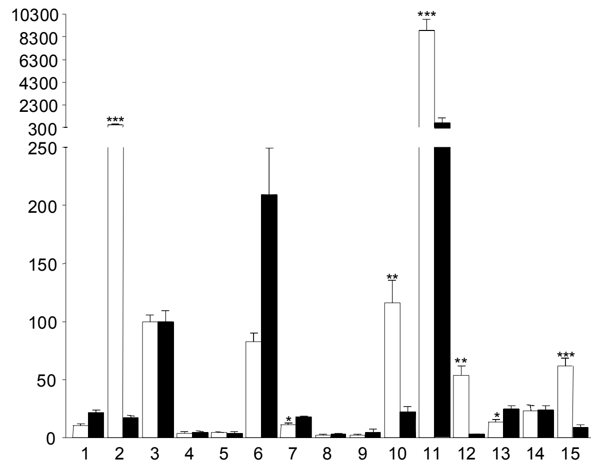

Figure 5 Quantification of the RPAs of mice (white columns) and rats (black columns). The mRNA amount of the lungs is set to 100% ($n \geq 4$). The values are shown as mean+SEM. 1. ventricle, 2. kidney, 3. lung, 4. liver, 5. testis, 6. bladder, 7. forebrain, 8. spleen, 9. thymus, 10. stomach, 11. ileum, 12. colon, 13. brainstem, 14. atrium, 15. adipose tissue. *$P<0.05$, **$P<0.01$, ***$P<0.0001$ compared mouse *vs.* rat.

2.4.2 ACE2 activity assay

In accordance with the RNA expression data, highest activity for ACE2 was found in the ileum of mouse and rat (**Tab. 2**), whereas the activity in the mouse was 3.2-fold higher. Lowest ACE2 activity was found for both species in spleen. Low activity was also found for liver of mice and thymus of rats. Corresponding to the differences on mRNA levels in the kidney the ACE2 activity was much higher in mice than in rats (~13.9-fold). The activity of ACE2 in the lung was different to mRNA and 2.6-fold higher in rats than in mice. In contrast to RPA data the activity in colon was comparable between both species.

	mmol Mca-AP-OH/mg protein/h			
Tissue	**Mouse**		**Rat**	
	Mean	SEM	Mean	SEM
Ventricle	0.1035	0.0161	0.1238	0.0156
Kidney	11.7588	0.6394	0.8585	0.1042
Lung	0.2615	0.0111	0.6690	0.1327
Liver	0.0772	0.0078	0.2065	0.0419
Testis	0.1276	0.0123	0.1946	0.0341
Bladder	0.5557	0.0758	0.7404	0.0876
Forebrain	0.4265	0.0563	0.3729	0.0098
Spleen	0.0273	0.0049	0.0236	0.0072
Thymus	0.1380	0.0453	0.0555	0.0054
Stomach	0.4447	0.0222	0.4999	0.0437
Ileum	51.5659	14.6782	16.3141	1.4390
Colon	0.1348	0.0419	0.1823	0.0339
Brainstem	0.3519	0.1396	0.3507	0.0381
Atrium	0.1076	0.0268	0.1617	0.0161
Adipose Tissue	0.3578	0.0905	0.9696	0.3500

Table 2 ACE2 activity in different tissues. It is shown the amount of Mca-AP generated from Mca-APK(Dnp) by 1 mg of purified protein from different tissues of mice and rat within 1h. ($n \geq 4$)

2.4.3 Western blot

Using a commercial polyclonal antibody in western blot for the quantification of protein levels in mouse and rat tissues (**Fig. 6**) a pattern completely different from RNA expression and ACE2 activity was found. A moderate and comparable expression could be detected in the kidney of both species and was set to 100%. Thus, the highest amount of protein could be detected in atrium of both species (mouse: 124.5%; rat: 131.5%) and ventricle (mouse: 131.7%; rat: 143.3%). For the mouse less ACE2 protein was found in lung (19.7%) and testis

(28.7%), whereas no protein was detectable in these two tissues in rat. In thymus (mouse: 44.4%; rat: 50.6%) and forebrain (mouse: 87.9%; rat: 80.7%) of both species a moderate expression was detectable, whereas no ACE2 protein was found in spleen of mouse and rat.

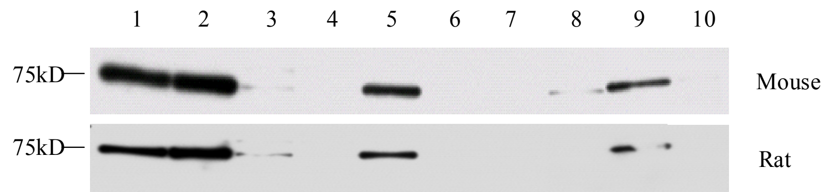

Figure 6 Representative western blot of mouse (upper panel) and rat (lower panel) tissues with a commercial polyclonal antibody against ACE2. The band at 75 kDa is indicated. 1. atrium, 2. ventricle, 3. thymus, 4. spleen, 5. kidney, 6. testis, 7. lung, 8. bladder, 9. forebrain, 10. adipose tissue.

2.4.4 Immunohistochemistry

To further clarify the discrepancy between RPA and activity on one side and western blot on the other, immunohistochemistry was performed in lung, kidney (**Fig. 7**), and testis (**data not shown**) of mice and rat with new monoclonal ACE2 antibodies (clones 7E7 and 1D3), we generated. The antibodies were determined to belong to the IgG1 subclass. In the lungs of both species alveolar macrophages and type 2 cells (**Fig. 7, upper row**) were stained with both monoclonal ACE2 antibodies (**data for clone 1D3 not shown**). The epithelium of the renal tubuli was strongly stained (**Fig. 7, lower row, left**) in the kidney of mice. In rats only a weak signal, but the same pattern as in mouse, was detected, what aligned with mRNA and ACE2 activity (**Fig. 7, lower row, right**).

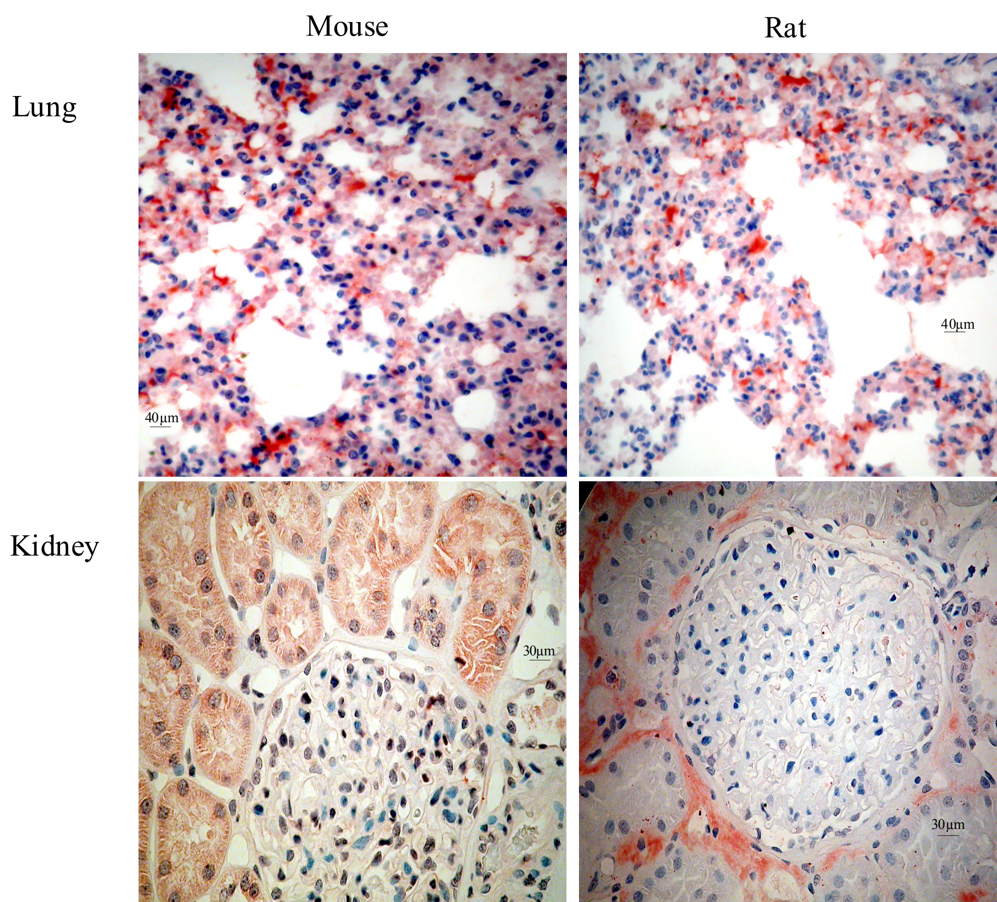

Figure 7 Immunohistochemical visualization of ACE2 positive cells. Sections of lungs (upper row) and kidneys (lower row) from mouse (left panel) and rat (right panel). In the lungs of both species alveolar macrophages and type 2 cells were stained positive. The tubulus epithelium in mouse kidney was stained positive, whereas in the rat kidney only weak staining was seen.

2.5 Discussion

In recent investigations it was shown that peptidases like ACE and NEP are important regulators of cardiovascular and endothelial function as well as myocardial remodelling.[167-170] Consequently, after its discovery in 2000, ACE2 became an enzyme of interest for scientific investigation of its impact in cardiovascular physiology and pathophysiology.[107,108,163] To elucidate some of its physiological functions we investigated the tissue distribution of mRNA and protein in a variety of tissues of C57Bl/6 mice and Sprague-Dawley rats. While we could see correlating patterns of mRNA and ACE2 activity in most of the examined tissues, we also found significant divergences between the investigated species. The huge difference between mRNA and protein levels in the lung may be due to shedding as demonstrated for ACE.[107,171,172] This shedding leads to an increased secretion of ACE2 and lowered its protein content in the lung by even high mRNA expression. The significant differences that we found between the species on ACE2 protein and mRNA levels in kidney could be explained by the varying interspecies regulation and expression of peptidases, as shown in the literature for NEP activity in rat and rabbit kidneys.[173]
Comparing our mRNA and activity data with the western blot pattern, we have to conclude that the commercial polyclonal antibody is not detecting ACE2 protein in organ homogenates and is not suitable for ACE2 staining. In contrast, using immunohistochemistry, our new monoclonal ACE2 antibodies produce staining patterns comparable to our mRNA and activity data.
We have shown that ACE2 expression in rodents is highest in ileum among the examined organs. It was shown for other peptidases of the RAS like ACE and NEP that they are also present at high levels in the intestine.[174] However, the distinct function of these peptidases in the ileum is not yet known. Further investigations have to clarify the physiological and pathophysiological functions of the peptidases in the gastrointestinal tract.
Beside its physiological function as a peptidase, ACE2 is used by corona virus as a co-receptor in severe acute respiratory syndrome (SARS).[123] It was shown that the SARS corona virus only can enter cells which express ACE2.[175] ACE2 distribution in the small intestine, lung and vascular endothelium may offer a point of entry for the SARS corona virus, but does not reflect its basic function.[123,176,177] Interestingly, the distribution patterns we found from RNA and ACE2 activity contradict investigations using a commercial northern blot for detecting mRNA[107,108] but have been confirmed by recent papers using RT-PCR.[161] This discrepancy may be a species-specific alteration of tissue distribution, since they used human tissue for northern blot, or it may be due to technique differences (commercial northern versus RPA and activity assay). The first possibility is at least supported by our finding that significant differences in ACE2 expression patterns exist between the close relatives mouse and rat.
Recent investigations revealed biological activity for angiotensin peptides other than Ang II, like Ang-(1-7).[126,178,179] ACE2 can generate Ang-(1-7) by cleaving the C-terminal amino acid from Ang II.[109] ACE2 is also involved in another pathway leading to the generation of Ang-(1-7). It cleaves Ang I to Ang-(1-9).[107] Ang-(1-9) is then hydrolyzed by ACE to Ang-(1-7). We demonstrated that Ang-(1-7) is an endogenous ligand for the G protein-coupled receptor (GPCR) Mas.[143] mRNA of the GPCR Mas was found at high levels in testis and certain brain regions and at moderate levels in kidney and heart.[164,180,181] It was shown that high concentrations of Ang-(1-7) were present in heart, kidney, and brain.[182-185] In recent investigations, it was demonstrated that ACE2, Mas, and its endogenous ligand Ang-(1-7) are present in the same cells of the kidney.[186] As we recently postulated, this indicates a relevant impact of the ACE2/ Ang-(1-7)/Mas axis on blood pressure regulation and cardioprotection.
Actual investigations indicate an up-regulation of ACE2 in heart failure, pointing to the relevance of ACE2 in cardiac function.[172,187,188] However, there was a high incidence of

sudden death in animals over-expressing ACE2. Electrophysiology revealed severe, progressive conduction and rhythm disturbances with sustained ventricular tachycardia that progressed to fibrillation and death.[189] While anti-arrhythmic actions were demonstrated for Ang-(1-7) in low concentrations (0.22 nM) by stimulating its own receptor, 100-fold higher concentrations of Ang-(1-7) lead to arrhythmias by stimulating the AT_1 receptor.[190,191] Therefore, the over-expression of ACE2 may lead to a high increase in the production of Ang-(1-7), turning its cardioprotective actions into effects causing arrhythmias by unspecific AT_1 stimulation. In future studies, the actions of Ang-(1-7) and its concentration dependency on ACE2 expression on heart rhythm have to be proven in *in vivo* experiments with AT_1- and *Mas*-deficient animals.
Our data on tissue and species-specific ACE2 expression point to the fact that the RAS becomes increasingly complex. Since we identified an expression pattern markedly different from ACE, we conclude that the expression levels of the involved peptidases like ACE, ACE2, and NEP that generate and/or degrade the bioactive peptides of the RAS are predictive of either the occurrence of vasoconstriction or dilatation or the dominance of pathophysiological stimuli over beneficial conditions.

2.6 Acknowledgements

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Chapter 3

Lack of angiotensin II conversion to angiotensin III increases water but not alcohol consumption in aminopeptidase A-deficient mice

Based on: Faber F, Gembardt F, Sun X, Mizutani S, Siems WE, Walther T. Lack of angiotensin II conversion to angiotensin III increases water but not alcohol consumption in aminopeptidase A-deficient mice. *Regul Pept.* **2006**;136(1-3):130-137

3.1 Abstract

Elevated central concentrations of the vasopressor octapeptide angiotensin (Ang) II increase the water intake in mammals. Recently, we showed that central Ang II is also crucial in alcohol-consuming behaviour. Since the heptapeptide Ang III, an Ang II metabolite, is discussed to mediate Ang II-related effects, we investigated water and alcohol consumption in mice, genetically deficient in aminopeptidase A (APA), a peptidase responsible for Ang II conversion to Ang III.

Sixteen male APA-deficient mice and their age matched wild-type controls were monitored on their water intake under basal conditions and total fluid and alcohol intake before and after social stress in a two-bottle free-choice paradigm. Alterations were connected to the regulation in activity of Ang-related peptidases (APA, ACE; ACE2) in brain regions involved in alcohol intake and peripheral organs.

In comparison to their wild-type controls, APA-deficient mice drank significantly more water but not more alcohol at all investigated time points. A reduction in water intake, as observed in wild-type animals after social stress, did not occur in knockout mice. However, the reduction in alcohol consumption after social stress was significantly reduced in both strains. Alcohol consumption was up-regulating all three peptidases in the kidney, but not in lung. Notable, renal ACE2 activity was significantly higher in APA-deficient mice under basal condition.

While the inhibition of Ang II metabolism to Ang III does not influence the alcohol intake, water consumption in mice deficient for APA was significantly elevated. These differences induced by an altered Ang II/Ang III ratio oppose the hypothesis that central Ang II and Ang III act in a congruent pattern.

3.2 Introduction

The renin-angiotensin system (RAS) is a very complex system that is essentially involved in the regulation of blood pressure, electrolyte homeostasis, and the water and sodium intake.[92,192] It further influences several body functions such as cognition, memory acquisition and recall,[193] fertilization,[194] and fat accumulation.[195] At least part of these functions may be realized via the interaction with other neurotransmitter systems like the dopaminergic system.[196-198] The major acting peptide of the RAS is angiotensin (Ang) II (Ang-(1-8)). The generation of Ang II is a multi-step procedure, depending on the activity of several peptidases. At first, the protease renin cleaves the precursor peptide angiotensinogen into Ang I. This inactive decapeptide is activated into the octapeptide Ang II by angiotensin-converting enzyme (ACE). Ang II itself can be converted into several other biologically active peptides by different enzymes. One of them is aminopeptidase A (APA) converting Ang II into Ang III (Ang-(2-8)).[199] Both peptides bind to the AT_1 receptor, and this interaction initiates the classical functions of the RAS including body water balance, maintenance of blood pressure and control of vasopressin release.[90] Finally, Ang III is cleaved by aminopeptidase N (APN) to the hexapeptide Ang IV (Ang-(3-8)), which acts as an inhibitor of insulin-regulated aminopeptidase (IRAP,[101] also named placental leucine aminopeptidase (P-LAP)[200] or oxytocinase[201]). Notable, the concentration of one of the different Ang metabolites finally depends on the concertive regulation of activities of the peptidases involved (e.g. APA, ACE, ACE2), and thus concentrations of these metabolites can be altered by regulation of one or more of these peptidases.

Ang II and Ang III are found to cause comparable pressure effects if injected into cerebral ventricles.[127] However, since the beginning of the nineties several findings have encouraged conclusions that Ang III is the most important biologically active peptide of the central RAS and of all the central Ang-mediated actions.[90,202] The pivotal role of Ang III was

demonstrated in normotensive and spontaneously hypertensive rats treated with the specific APA inhibitor EC33. In both lines the Ang II-induced pressure responses were abolished.[96] Furthermore, the inhibition of Ang III degradation by APN inhibitor PC18 leads to an increase in blood pressure suggesting that Ang III and not Ang II is the main effector peptide.[97]

Beside the RAS functions mentioned above, it has been shown that genetic and pharmacological manipulations of the brain RAS alter the voluntary alcohol consumption. We recently demonstrated in alcohol drinking experiments with a two-bottle free-choice paradigm that the consumption of alcohol strongly depends on the concentration of Ang II. Mice over-expressing the angiotensinogen gene and therefore having elevated Ang II levels showed an increased alcohol intake compared to their wild-type littermates, whereas angiotensinogen-deficient mice were characterized by a strongly reduced alcohol consumption.[18] Using transgenic rats expressing an angiotensinogen-specific antisense RNA specifically in the brain,[203] we distinguished that the Ang-evoked effects on alcohol consumption are mediated by central Ang II and that the peripheral peptide plays no role in voluntary alcohol consumption.[204]

In APA-deficient mice the conversion of Ang II to Ang III is inhibited. These animals show a modest but significant elevation of basal blood pressure and enhanced hypertensive responses due to the accumulation of Ang II.[205]

However, to strengthen the widely accepted role of Ang III as the major Ang effector peptide of the central RAS, it would be a promising tool to provide first experimental studies in transgenic animals describing the role of Ang III on water homeostasis and voluntary alcohol consumption. For this purpose, we compared the alcohol drinking behaviour of APA-knockout mice with that of their respective wild-type littermates in a two-bottle free-choice experiment under basic and stress conditions.

3.3 Materials and methods

3.3.1 Animals

All experiments were done according to the guidelines of the Federal Law on the Use of Experimental Animals in Germany or The Netherlands and were approved by the local authorities. For this investigation we used male APA-deficient mice[205] and their wild-type controls, which were obtained from breeding stocks of T.W. at the Charité - Campus Benjamin Franklin (CBF) in Berlin, Germany. The animals were on a mixed background of C57Bl/6J and 129Sv as originally described.[205] The animals were maintained on a 12 h light/dark cycle. Animals were in an age of 4-8 months. The genotype of the animals was checked by PCR using two primers that amplified a product specific for the APA wild-type allele (P1: 5′GAC AGT GAA GAT GAA AGC GG3′ and P2: 5′ATC ACC ACG TAC TCC TGC TT 3′) and two primers that amplified a product in the mutant allele only (P3: 5′GATATT CGG CAA GCA GGC AT 3′ and P4: 5′GGC AGC GCG GCTATC GTG G 3′). The PCR was performed at 59 °C for 30 s, 72 °C for 35 s for primers P1 and P2 and at 59 °C for 30 s, 72 °C for 45 s for primers P3 and P4.

3.3.2 Preference tests

APA-deficient mice (n=16) and their respective wild-type littermates (n=16) were kept in groups of two animals per cage. Each cage was divided into two equal compartments by a transparent plastic divider as described by Maul *et al.*,[204] and thus each animal had its own two bottles to make its choice. This setting allowed the animals to maintain social contact and at the same time prevented them from inflicting harm upon each other. For the first four days of experiment both bottles were filled with tap water. Subsequent, they were held in a free-

choice paradigm with a bottle of tap water and a bottle containing a 10% (v/v) ethanol solution. Food and beverage were available *ad libitum*. Water and basal alcohol consumption were recorded every third day for 7 sessions. Just as often, bottle positions were changed.
Then, social stress experiments were performed similarly to the method described by Sillaber *et al.*[206] Each mouse (intruder) was put into a cage with 3 unfamiliar male mice (residents). Before starting the stress experiment, the residents had been housed in this cage for two days. In this paradigm, the residents attacked the intruder within 2 min. After the attack intruder and residents were immediately separated by a transparent plastic divider. The intruder was kept in the smaller part of the cage for further 15 min and was then removed to its original cage where it had again free choice between tap water and alcohol. Water and alcohol consumption were recorded every third day for 5 sessions.

3.3.3 Peptidase activities

For peptidase measurements 6 animals per group were chosen randomly and killed by decapitation. Organs were rapidly removed and snap frozen in dry ice. Brains were rapidly removed and brain regions prepared as described before.[18] The samples were stored at -80°C until further processing. Tissue homogenates were prepared at 4°C in 50 mM Tris buffer, pH 7.4, and filtered through nylon gauze and stored until use at -80°C. Protein concentration was determined using Roti-Quant (Carl Roth GmbH & Co KG, Karlsruhe, Germany) by the manufacturers' instruction.

3.3.3.1 APA activity assay

APA activity was measured according to a somewhat modified method described by Wang and Cooper.[207] Briefly, homogenized tissues were incubated in a $CaCl_2$-containing, 25 mM Tris buffer, pH 8.0, at 37°C. The reaction was started by addition of 100 µM Glu-AMC and stopped by addition of perchloric acid. The specificity of APA action was proofed by adding the aminopeptidase inhibitor amastatin (Sigma Aldrich GmbH, Taufkirchen, Germany) to a final concentration of 10^{-4} M. After dilution and centrifugation the increase of fluorescence resulting from AMC was measured at 380 nm (excitation) and 460 nm (emission). The activity is expressed as nmol AMC/min/mg protein.

3.3.3.2 ACE activity assay

ACE activity was measured with a fluorimetric method as described by Maul *et al.*[18] using Hip-His-Leu as substrate and His-Leu as standard reagent. Specificity of the test was determined using 10^{-6} M of the ACE inhibitor lisinopril (Sigma Aldrich GmbH, Taufkirchen, Germany). Fluorescence arising from His-Leu after reaction with *o*-phthalaldehyde was measured at 365 nm (excitation) and 500 nm (emission). The activity is expressed as nmol His-Leu/min/mg protein.

3.3.3.3 ACE2 activity assay

ACE2 activity was measured similar to the method by Vickers *et al.*[109] We used Mca-APK (Dnp) (Biosynthan GmbH, Berlin, Germany) dissolved in DMSO (50 µM, final concentration) as the ACE2 substrate. The measurement was performed in assay buffer (50 mM 2-Morpholinoethanesulfonic acid, 300 mM NaCl, 10 µM $ZnCl_2$, 0.01% Brij-35, pH 6.5) and was started by adding 10 µl of tissue homogenate. After 2 h incubation at ambient temperature (24°C), the reaction was suppressed by adding 100 µM *o*-phenanthrolin (final concentration). Parallel control tests were performed in the presence of 1 µM DX600 (Phoenix Europe GmbH, Karlsruhe, Germany).[165] After centrifugation (10 min, 10,000× g) the fluorescence was measured at 320 nm (excitation) and 405 nm (emission). The molecular standardization was performed with Mca-AP (Biosynthan GmbH, Berlin, Germany) and calculated per mg protein. The functionality of the assay was proven by a standardized

solution with defined, recombinant ACE2 activity (R&D Systems GmbH, Wiesbaden, Germany).

3.3.4 Statistics

For statistical analysis in **Figs. 8 and 9** we performed paired *t*-test. Data for **Figs. 10-12** were analyzed by independent *t*-test using GraphPadPrism 4.00 (Graph Pad Software, Inc., San Diego, USA). Each value was expressed as the mean±SEM, and statistical significance was accepted for $P<0.05$.

3.4 Results

3.4.1 Basal drinking behaviour

After an adaptation phase to home in the new cage environment, basal water intake was measured for 4 days (two sessions). As shown in **Fig. 8a**, APA-deficient mice drank significantly more water (water/body weight ratio) whereby body weight did not differ.

3.4.2 Alcohol uptake under basal conditions

The water and alcohol consumption under basal conditions was measured over 7 consecutive drinking sessions (21 days). The total fluid intake was significantly decreased in comparison to drinking behaviour before alcohol consumption for both genotypes, whereby the level of significance between wild-type and APA-knockout mice even increased (**Fig. 8b**). This increase resulted from an elevated water intake, as already observed under basal conditions (**Fig. 8a**), while the alcohol intake and the alcohol/total fluid intake ratio were not different between both groups (**Fig. 8c-d**).

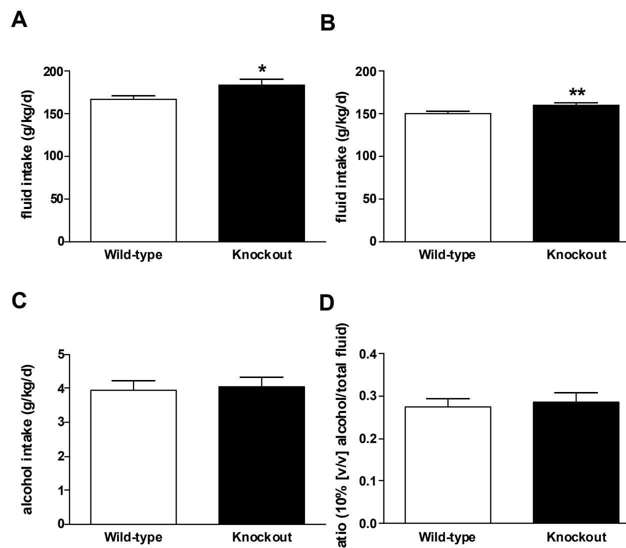

Figure 8 Water and alcohol consumption in a two-bottle free-choice paradigm (wild-type mice: white columns; APA-deficient mice: black columns). Basal conditions: (**A**) total fluid (water) intake under basal conditions is shown for two 48 h-drinking periods in which both bottles were filled with water. Two-bottle free-choice paradigm before social stress: It is shown for 7 consecutive drinking periods the (**B**) total fluid intake, (**C**) total alcohol consumption, and (**D**) mean alcohol preference ratio (part of the 10% [v/v] ethanol solution per total fluid consumption). All values are means±SEM; n=16 for both groups; *$P<0.05$, **$P<0.005$.

3.4.3 Alcohol drinking behaviour after stress

Mice of both genotypes were then confronted with short-term social stress.[206] Notable, this stress did not alter higher total fluid (**Fig. 9a**) or water consumption (**Fig. 10a**, left panel) in APA-knockout mice compared to wild-type mice as already described under basal and pre-stress conditions. As observed in the pre-stress sessions, the alcohol intake and the alcohol/total fluid intake ratio were not different between both groups (**Fig. 9b-c**). However, comparing water intake before and after stress (**Fig. 10a**, left panel), there was no intra-group

difference, while we found, analyzing the inter-group alteration, a significant different stress-induced regulation in water consumption (**Fig. 10a**, right panel; $P<0.05$) analyzing each animal before and after stress.

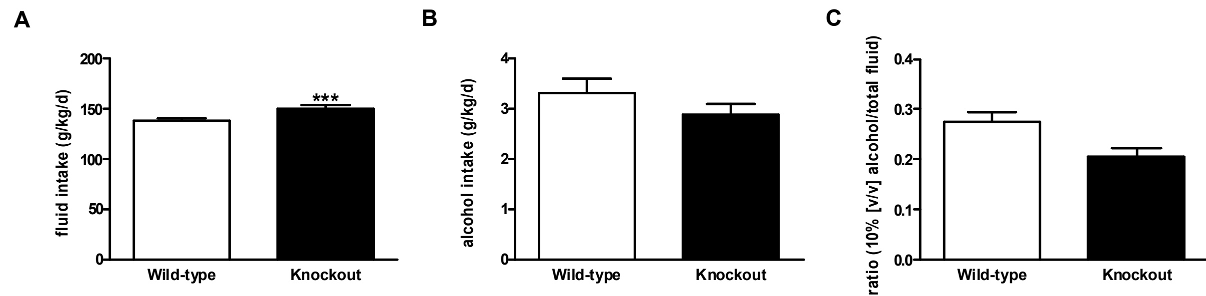

Figure 9 Drinking behavior under social stress (wild-type mice: white columns; APA-deficient mice: black columns). Two-bottle free-choice paradigm after social stress: (**A**) total fluid intake, (**B**) total alcohol consumption, and (**C**) mean alcohol preference ratio (part of the 10% [v/v] ethanol solution per total fluid consumption) are shown for 5 free-choice 72 h drinking periods. All values are means±SEM; n=16 for both groups; ****P*<0.0001.

In contrast, while the alcohol intake was significantly reduced in both lines after stress (**Fig. 10b**, left panel). This decrease did not differ between knockout mice and their wild-type controls (**Fig. 10b**, right panel).

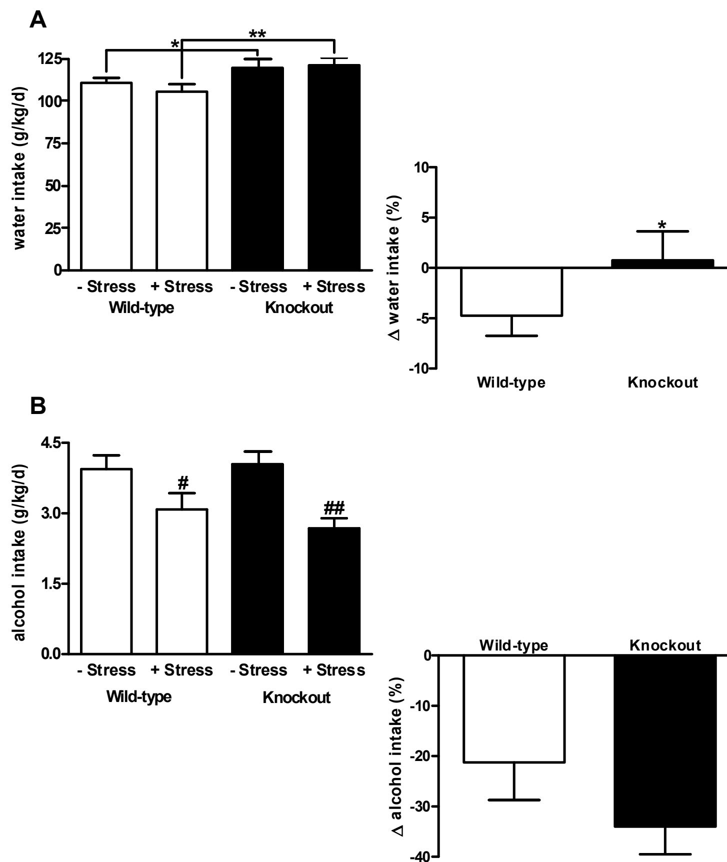

Figure 10 Stress-induced changes in drinking behavior (wild-type mice: white columns; APA-deficient mice: black columns). (**A**) Total water intake in both lines before and under stress conditions (left panel) and the corresponding percentual changes (right panel). (**B**) Total alcohol consumption in both lines before and under stress conditions (left panel) and the corresponding percentual changes (right panel). All values are means±SEM; n=16 for both groups; **P*<0.05, ***P*<0.005 (wild-type vs. knockout), #*P*<0.05, ##*P*<0.005 (no stress *vs.* stress).

3.4.4 Peptidase activities under non-alcohol and alcohol intake conditions

To investigate whether the differences between both genotypes in water intake are paralleled by alterations in peptidases playing a key role in angiotensin metabolism, we measured APA, ACE, and ACE2 activities in brain areas that are discussed to be related to alcohol drinking behaviour.[208] We especially included ACE and ACE2 to conclude on possible modifications

in Ang II generation and metabolism under APA deficiency. APA activity in wild-type mice was highest in tegmentum/colliculi and much lower, but to comparable levels, in cortex and striatum (**Fig. 11a**). Importantly, APA activity in APA-deficient mice was on a background level.

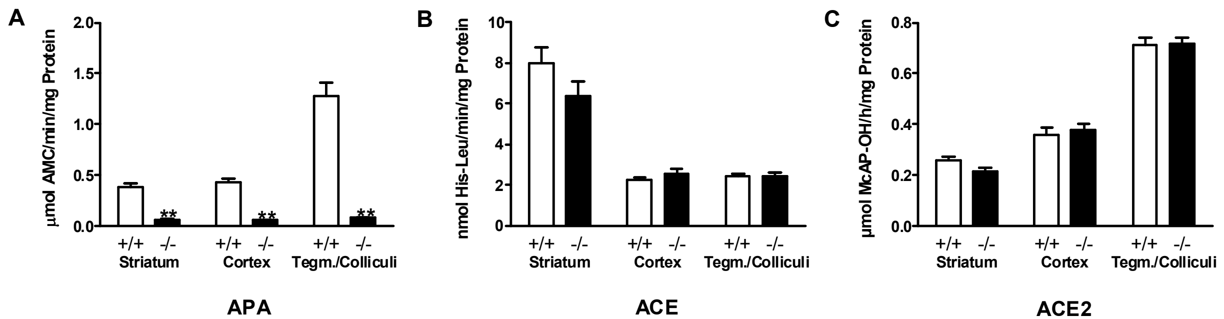

Figure 11 Activities of RAS-related peptidases in the brain at the end of the experiment (wild-type mice [+/+]: white columns; APA-deficient mice [-/-]: black columns). (**A**) APA activity is shown as the amount of AMC generated from Glu-AMC by 1 mg of purified protein from different brain regions within 1 min. (**B**) ACE activity is shown as the amount of His-Leu generated from Hip-His-Leu by 1 mg of purified protein from different brain regions within 1 min. (**C**) ACE2 activity is shown as the amount of Mca-AP generated from Mca-APK by 1 mg of purified protein from different brain regions within 1 min. All values are means±SEM; n=6 per group; $^{**}P<0.001$.

In contrast to the APA activity pattern, ACE, as described before,[209] was most active in striatum but without any difference between the both genotypes in all three investigated areas (**Fig. 11b**). Moreover, we showed for the first time, ACE2 activity in different brain regions. It was detectable in all three regions with a pattern, contrasting ACE but comparable to APA in wild-type mice (**Fig. 11c**). However, the ACE2 activity did not differ between both lines.

To investigate whether peptidase activities in peripheral organs may be altered under continuous alcohol intake, we measured the three peptidases in lung and kidney of mice after alcohol intake and in an independent set of animals that did not undergo the alcohol drinking protocol. APA activity was not detectable in knockout mice. Notable, the peripheral APA activity in wild-type animals exceeded that measured in tegmentum/colliculi (16.2-fold in kidney, 8.6-fold in lung; **Fig. 12a**). Renal, but not pulmonary APA activity increased under alcohol intake.

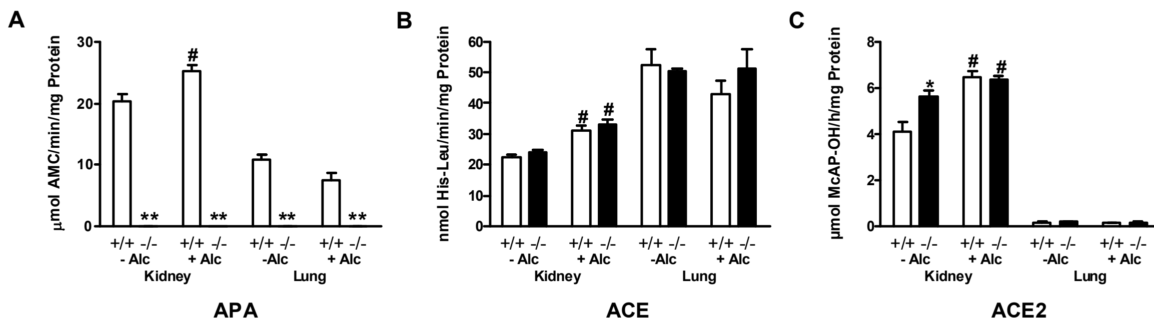

Figure 12 Activities of peptidases in kidney and lung depending on alcohol consumption (wild-type mice [+/+]: white columns; APA-deficient mice [−/−]: black columns). The activities of (**A**) APA, (**B**) ACE, and (**C**) ACE2 are displayed as the amount of their degraded substrates by 1 mg of purified protein from tissues within 1 min. The activities were measured in animals with and without access to 10% [v/v] ethanol solution. All values are means±SEM; n=6; $^{**}P<0.001$ (wild-type *vs.* knockout), $\#P<0.05$ (no alcohol *vs.* alcohol).

As described before,[13] ACE activity was higher in lung than in kidney (**Fig. 12b**). As for APA in wild-type animals, only renal ACE activity increased with alcohol intake, but with comparable increase between APA-deficient mice and their wild-type controls. Confirming our data in C57Bl6 mice,[210] renal ACE2 activity in wild-types exceeded pulmonary activity by 40-fold. Importantly, while ACE2 activity in the APA-deficient lungs did not differ from that measured in wild-type controls (under basal and alcohol intake conditions), ACE2 activity was significantly elevated in APA-deficient kidneys under basal conditions. Although ACE2 activity significantly increased in both lines with alcohol consumption (**Fig. 12c**), this increase was much more pronounced in wild-type mice and thus, the baseline difference in ACE2 activity was blunted under alcohol intake.

3.5 Discussion

It is well accepted that the actions of the RAS also include fluid homeostasis by stimulating angiotensinergic neurons.[211-213] Intercerebroventricular (i.c.v.) injections of Ang II rise blood pressure and increase water intake.[214,215] However, in previous experiments it was postulated that Ang III, the Ang II metabolite lacking the N-terminal amino acid, is the major effector of the central RAS.[90,202] i.c.v. infusion of Ang III mediates, besides blood pressure increase and a rise in sodium intake,[92,96,202] significant dipsogenic effects.[92] By performing experiments that characterize Ang effects on sodium intake and thirst by central pre-treatment with EC33, a specific APA inhibitor, or PC18, a specific inhibitor of APN, Wilson *et al*. suggested Ang III to be the dominant player on sodium and water intake. However, as the APA inhibitor failed to completely inhibit the Ang II-stimulated effects, the authors were not able to finally determine if the conversion of Ang II to Ang III is a prerequisite for the dipsogenic effects. In contrast to their results we found in APA-deficient mice, which have a lack of Ang III generation, a significant higher basal water intake as for their corresponding wild-type controls, concluding that Ang II but not Ang III determines the water intake. Interestingly, APA also catabolises cholecystokinin octapeptide (CCK-8),[216] but the injection of CCK-8 reduces the water intake,[217] and consequently, APA-deficiency would lead to decreased water-intake by CCK-8 accumulation. Thus, the Ang II-mediated dipsogenic effect may even be partly masked due to the opposing CCK-8 effects. This is in nice agreement with former experiments showing that injected CCK-8 reduces the water intake induced by Ang II injection.[218] The increase in water intake is in agreement with investigations revealing an up-regulated urinary excretion (~+14%) in APA-deficient animals.[205] Notable, this increase was in a similar proportion as the increased water uptake (~+12%) we have measured here. It could be speculated that the higher urinary excretion is primarily, since increased renal ACE2 activity measured in our APA-deficient mice, may elevate Ang-(1-7), acting as a diuretic compound.[219] Consequently, the higher diuresis would induce the increased thirst in mice deficient in APA. However, this speculation has to be rejected, as we found in wild-type and APA-deficient mice comparable renal ACE2 activity after social stress (**Fig. 11c**), but still unequal levels in water intake. Taken together, since APA deficiency causes increased Ang II and diminished Ang III levels, our results implicate that rather Ang II than Ang III is the driving force of the RAS regarding water homeostasis.

The development of alcoholism is determined by multiple genetic factors as well as environmental influences.[220-223] It is one of the most frequent addiction diseases throughout the world and therefore an enormous economical problem.[224,225] Previously, pharmacological studies at our laboratory revealed that modification of the RAS alters the alcohol drinking behaviour of mice in an Ang II-dependent manner.[18] Recent experiments with transgenic mice and rats distinguished between peripheral and central Ang II alterations and discovered the central one to be the crucial in influencing alcohol intake.[204] Regarding to the experiments by Wright *et al*.[90,202] and Maul *et al*.[18,204], we consequently wanted to elucidate the impact of Ang III on the voluntary alcohol consumption. In our approach we did not find any differences in alcohol consumption between both lines under basal conditions. Since we cannot detect differences in alcohol drinking behaviour due to an equal sum of both peptides in APA-deficient mice and their wild-type controls and only a concentration shift from Ang III to Ang II, we conclude that both Ang II and Ang III regulate the alcohol consumption.

Although, due to our experimental setup, we cannot finally differentiate between central and peripheral Ang actions, it seems that in general the changed Ang II/Ang III ratio has no influence on the alcohol consumption in APA-deficient mice. However, since it was shown that peripheral injection of Ang II reduces the alcohol intake,[226] as described by others for the depletion of central Ang II,[204] it cannot be excluded that the loss of APA leads to a decrease

in alcohol consumption, due to the accumulation of Ang II peripherally, whereas the central Ang II induces alcohol intake, and thus peripheral and central effects are nullifying each other. It was shown that stress is influencing alcohol consumption,[206,227] therefore we wanted to elucidate the role of Ang peptides under stress conditions. After exposing the mice to social stress both strains showed the same changes in alcohol consumption, in contrast to the unequal changes in water intake. These results support our conclusion that the Ang II/Ang III ratio does not determine the alcohol consumption, also under stress conditions, but the sum of Ang II/Ang III mediates the formerly described Ang-mediated effects.[18,204]
Since the ratio of Ang II/Ang III may also be influenced by activities of APA-independent peptidases involved in Ang metabolism, we measured the activities of ACE and ACE2 in peripheral and central tissues. The catalytic activities of both peptidases were on comparable levels in the examined brain regions, kidney, and lung of both strains after alcohol consumption. Interestingly, renal ACE and ACE2 are significantly up-regulated under alcohol consumption, implicating an effect of alcohol on peripheral peptide metabolism. The contrary activity pattern of ACE and ACE2 in the examined brain regions, could lead in these areas to highly different ratios of Ang peptides, like Ang II and Ang-(1-7), and therefore regulating central actions of Ang.
Finally, we can conclude from our data that the APA-deficiency in mice does not have a noticeable effect on the alcohol consumption, whereas the water intake is strongly influenced by the altered Ang II/Ang III ratio. Although our data implicate a dominating role for Ang II in water homeostasis, further experiments should be done to finally clarify the role of central Ang III.

3.6 Acknowledgements

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Chapter 4

Endothelial dysfunction through genetic deletion or inhibition of the G protein-coupled receptor Mas: a new target to improve endothelial function

Based on: Peiró C, Vallejo S, Gembardt F, Azcutia V, Heringer-Walther S, Rodríguez-Mañas L, Schultheiss HP, Sánchez-Ferrer CF, Walther T. Endothelial dysfunction through genetic deletion or inhibition of the G protein-coupled receptor Mas: a new target to improve endothelial function. *J Hypertens*. **2007**;25(12):2421-5.

4.1 Abstract

Background Endothelial dysfunction is an initial step in the pathogenesis of cardiovascular diseases. Since we previously identified the G protein-coupled receptor Mas as a receptor for angiotensin (Ang)-(1-7), a heptapeptide with endothelium-dependent vasorelaxant properties, we investigated whether alterations on the Ang-(1-7)/Mas axis alter endothelial function.

Results Ang-(1-7)-mediated relaxation of murine wild-type mesenteric arteries was equally impaired in both wild-type arteries pre-treated with the Ang-(1-7) receptor blocker, A779, and arteries isolated from *Mas*-deficient mice. Importantly, the response to the endothelium-dependent vasorelaxant, bradykinin (BK), and acetylcholine (ACh) effects were comparably inhibited, while endothelium-independent vessel relaxation by sodium nitroprusside was unaltered in these vessels. Hypothesizing endothelial dysfunction, we proved the *in vivo* relevance of the *ex vivo* findings investigating mesenteric properties after 1 week of minipump infusion of A779 in wild-type mice. Both BK- and ACh-induced relaxation were significantly impaired in wild-type vessels of pre-treated animals. A779-induced impairment of endothelial function was confirmed *in vitro*, since BK-mediated nitric oxide (NO) release was increased by Ang-(1-7) and blunted by A779 pre-treatment in primary human endothelial cell cultures.

Conclusions Our data highlight a pivotal role for the receptor Mas in preserving normal vascular relaxation. Consequently, Mas agonists arise as a promising tool in the treatment of cardiovascular diseases characterized by endothelial dysfunction

4.2 Introduction

Endothelial dysfunction is a common link for several cardiovascular diseases, such as hypertension, atherosclerosis and diabetic vasculopathy.[228] Endothelial dysfunction, usually defined as an impairment of the endothelium-dependent vasodilatatory responses, is mainly related to a diminished availability of endothelial nitric oxide (NO), which can occur through different mechanisms: a lower release of NO from endothelial cells; an enhanced inactivation of NO by increased oxidative stress; and a lower diffusion of NO towards underlying vascular smooth muscle or a reduced response of the muscle cells to NO.[229]

The renin-angiotensin system (RAS), a potent regulator of blood pressure, plays a major role in the pathogenesis of cardiovascular diseases and endothelial reactivity.[230,231] Evidence has been emerging that angiotensin (Ang) II is not the only active peptide of the RAS. Thus, besides this peptide, other RAS mediators, including Ang III [Ang-(2-8)], Ang IV [Ang-(3-8)] and Ang-(1-7) have been discovered and characterized.[87] Since most of the actions of Ang-(1-7) counteract those of Ang II,[232] this heptapeptide has become an Ang of interest in cardiovascular research in the past few years. Ang-(1-7) itself produces a potent antidiuretic effect on water-loaded rats[232] and potentiates vascular bradykinin effects.[130]

Studies using the selective Ang-(1-7) antagonist, D-Ala-Ang-(1-7) (A779), have provided evidence for the existence of an Ang-(1-7) receptor distinct from the classical Ang receptors AT_1 and AT_2.[233] We have recently identified the G protein-coupled receptor Mas as an endogenous receptor for Ang-(1-7).[143] Mice genetically deficient for this receptor were normotensive but characterized by a sustained long-term potentiation in hippocampal neurons, changes in exploratory behaviour,[234] and alterations in heart rate and blood pressure variability.[235]

In the present study, we have used *ex vivo* experiments with isolated mesenteric vessels of *Mas*-deficient and wild-type mice, *in vivo* approaches using treatment of mice with Ang-(1-7) or A779, and *in vitro* assays with human endothelial cells, to discover the impact of the Ang-(1-7)/Mas axis on endothelial function.

4.3 Material and methods

4.3.1 Animals

Six-month-old C57Bl/6 mice or *Mas*-deficient mice[234] on a C57Bl/6 background were used in the experiments. Animals were maintained under standardized conditions with an artificial 12 h dark-light cycle, with free access to food and water. All animal studies were performed according to national guidelines and approved by the institutional animal care committees.

4.3.2 Drug effects on vascular tone of mesenteric microvessels

Mice were anaesthetized with 70 mg/kg intra-peritoneal (i.p.) sodium pentobarbital and exsanguinated. The third-branch mesenteric arteries (mean internal diameter ranged between 150 and 200 mm; non-significant differences were observed among the different groups of mice) were mounted as ring preparations on a small-vessel myograph to measure isometric tension, as described previously.[236] Arteries were contracted with 125 mmol/l K^+, and then vasoactive responses to Ang-(1-7) (1 pmol/l to 1 μmol/l), bradykinin (BK, 1 nmol/l to 100 μmol/l), acetylcholine (ACh, 1 nmol/l to 100 μmol/l), or sodium nitroprusside (SNP, 1 nmol/l to 100 μmol/l) were tested by adding increasing concentrations of the drugs. In some cases, mesenteric segments were pre-incubated for 15 min with A779 (1 μmol/l). BK, ACh and SNP were from Sigma (St. Louis, Missouri, USA); Ang-(1-7) and A779 were obtained from Bachem (Bubendorf, Switzerland).

One group of wild-type mice was submitted to a continuous infusion of either saline solution or A779 (0.5 μl/h of a solution of 0.76 mg/ml) for 7 days. The drug was infused by micro-osmotic pumps (ALZET, model 1007D; DURECT Corporation, Cupertino, California, USA), which had been placed subcutaneously. At the end of the treatment, the animals were processed as indicated above.

4.3.3 Cell culture and nitric oxide quantification

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase digestion from umbilical cords provided by Hospital Universitario de Getafe, as approved by the Hospital Ethics Committee. HUVEC showed cobblestone morphology at confluence as well as positive immunostaining for Factor VIII, and were cultured in M199 medium supplemented with 10% fetal calf serum (FCS), 25 mg/ml endothelial cell growth supplement (ECGS) and 100 mg/ml heparin. For experiments, HUVEC were pre-treated with Ang-(1-7) (100 nmol/l) or A779 (1 μmol/l) or both for 72 h, with medium renewal every 12 h, and thereafter stimulated with BK (1 μmol/l) for 20 min. As an indirect measure of NO release, the nitrite/nitrate (NOx) content of HUVEC supernatants was spectrophotometrically quantified using the Griess method.[237] Cultures between passages 1 and 5 were used.

4.3.4 Statistics

Deviations from the mean regarding the drug response curves were analyzed statistically using factorial two-way analysis of variance (ANOVA). Due to the presence of biological variability in the relaxant responses, comparisons were only made in curves performed the same day in a parallel manner. For other statistical comparisons, Student's *t*-test was employed (mean±SEM). Significance was considered from a value of $P<0.05$.

4.4 Results

4.4.1 Ang-(1-7)-induced relaxation in mesenteric arteries from wild-type and Mas-deficient mice

We examined the effects of Ang-(1-7) on relaxation in a resistance vessel model; that is mesenteric arteries derived from wild-type and *Mas*-deficient mice. In wild-type-derived mesenteric arteries, Ang-(1-7) induced a concentration-dependent relaxation (1 pmol/l to 1 µmol/l), which was markedly diminished in the presence of A779 (1 µmol/l), the specific Ang-(1-7) receptor antagonist (**Fig. 13a**). In vessels derived from *Mas*-deficient animals, Ang-(1-7)-mediated relaxation was significantly reduced compared to wild-type vessels ($P<0.001$; **Fig. 13a-b**). Indeed, the vasorelaxant responses to Ang-(1-7) in knockout mice were similar to that seen in wild-type mesenteries pre-treated with A779 (**Fig. 13a**). It is noteworthy that A779 could not further inhibit the residual vasorelaxant effect of Ang-(1-7) in *Mas*-deficient vessels (**Fig. 13b**).

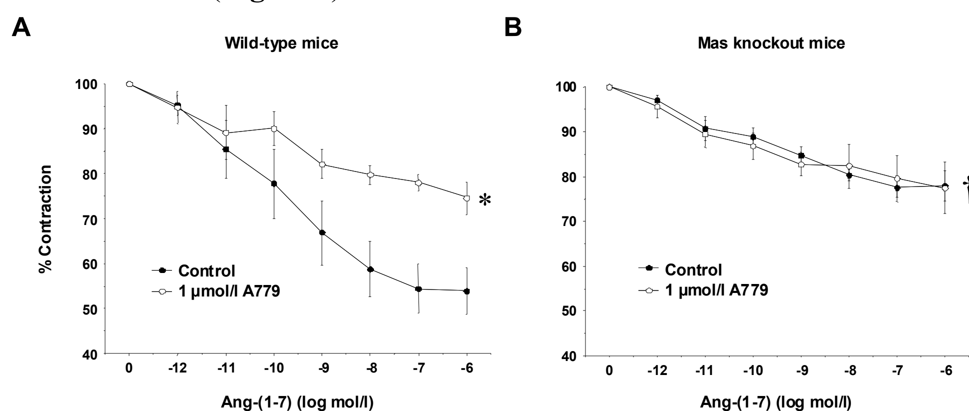

Figure 13 Relaxant responses to angiotensin-(1-7) [Ang-(1-7)] in isolated mesenteric microvessels from (**A**) wild-type and (**B**) Mas-knockout mice, either in the absence or in the presence of A779 (1 µmol/l). The vasoactive responses are expressed as percentage of a previous contraction elicited by 125 mmol/l K^+, which yielded values of 3.99±0.30 and 4.20±0.58 mN in control and A779-treated segments, respectively, from wild-type animals and 4.17±0.79 and 4.07±0.29 mN in control and A779-treated segments from *Mas*-knockout segments, respectively. For each curve, 6-12 mesenteric microvessels from at least three different animals were employed. *$P<0.05$ between control and A779-treated curves. †$P<0.05$ between control wild-type and *Mas*-knockout segments.

4.4.2 Bradykinin- and acetylcholine-induced relaxation in mesenteric arteries from wild-type and Mas-deficient mice

We next investigated the responses to the endothelium-dependent vasorelaxant BK (1 nmol/l to 30 mmol/l) in Ang-(1-7)-receptor-deficient vessels. Isolated vessels lacking *Mas* showed significantly impaired dilation in response to BK stimulation ($P<0.001$; **Fig. 14a**). We further tested a second endothelium-dependent vasorelaxant substance, ACh (1 nmol/l to 30 mmol/l), and observed that its effect was also blunted in *Mas*-deficient vessels ($P<0.001$; **Fig. 14b**). To determine whether such an impairment was endothelium dependent, or rather due to a reduced vasorelaxant capacity of *Mas*-deficient smooth muscle cells, we tested the effect of the endothelium-independent vasorelaxant SNP (1 nmol/l to 10 mmol/l). As shown in **Fig. 14c**, *Mas*-deficient and wild-type vessels responded equally to SNP, indicating normal reactivity of vascular smooth muscle cells in mesenteric arteries lacking Mas.

4.4.3 Vasorelaxant responses in mesenteric arteries from A779-infused mice

We infused wild-type animals with A779 for 7 days and then isolated the mesenteric arteries to investigate the endothelium-dependent responses to BK (1 nmol/l to 10 µmol/l). **Figure 15a** shows that BK-induced dilation was significantly impaired in A779-infused animals, as compared to wild-type mice. Again, such an effect was not due to impaired vascular smooth muscle relaxant capacity, as the SNP response was not altered (**data not**

shown). We also tested vasorelaxation in response to ACh, which was also found to be impaired in arteries from A779-infused animals (**Fig. 15b**).

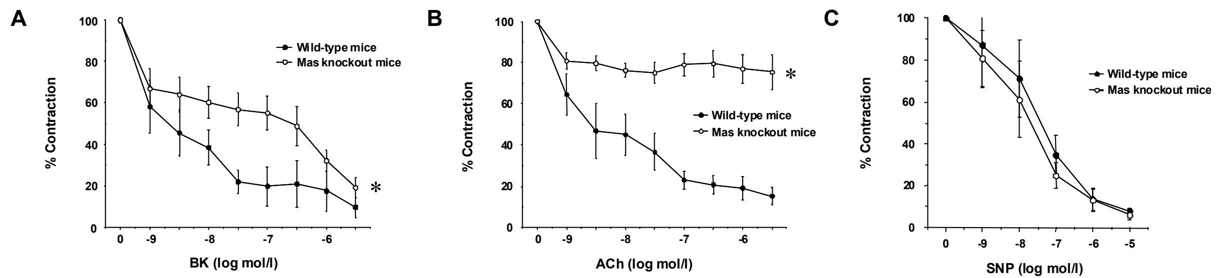

Figure 14 Relaxant responses to (**A**) bradykinin (BK), (**B**) acetylcholine (ACh), and (**C**) sodium nitroprusside (SNP), in isolated mesenteric microvessels from wild-type and Mas-knockout mice. The vasoactive responses are expressed as percentage of a previous contraction elicited by 125 mmol/l K^+, which yielded values of 3.75±0.32, 4.06±0.66 and 3.95±0.78 mN, respectively, in BK-, ACh- and SNP-treated segments from wild-type mice, and 4.04±0.47, 3.90±0.54 and 4.12±0.51 mN, respectively, in BK-, ACh- and SNP-treated segments from Mas-deficient animals, For each curve, 6-12 mesenteric microvessels from at least three different animals were employed. **P*<0.05 between wild-type and *Mas*-knockout mice.

4.4.4 Effect of Ang-(1-7) and A779 on endothelial cell-derived NO

To mechanistically understand the effects of Mas/ Ang-(1-7) on endothelial relaxation we investigated the release of endothelial NO. We used primary cultures of human endothelial cells. BK (1 µmol/l) induced NO-release, which was significantly increased in Ang-(1-7)-pre-treated cells (**Fig. 15c**). Ang-(1-7)-induced NO-release was markedly reduced by co-pre-treatment with A779. Furthermore, the pre-treatment with A779 alone abolished BK-mediated NO-release in human endothelial cultures (**Fig. 15c**).

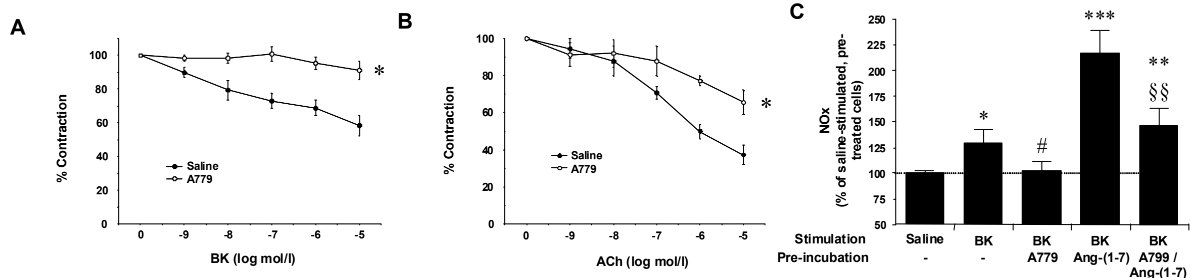

Figure 15 Relaxant responses to (**A**) bradykinin (BK) and (**B**) acetylcholine (ACh), in isolated mesenteric microvessels from wild-type mice infused for 7 days with either saline or A779. The vasoactive responses are expressed as a percentage of a previous contraction elicited by 125 mmol/l K^+, which yielded values of 4.18±0.26 and 4.08±0.41 mN, respectively, in BK- and ACh-treated segments from salineinfused mice, and 4.14±0.55 and 4.06±0.46 mN, respectively, in BK- and ACh-treated segments from A779-infused mice. For each curve, 6-12 mesenteric microvessels from at least three different animals were employed. **P*<0.05 between saline- and A779-treated mice. (**C**) Quantification of nitrate/nitrite (NOx) content in the supernatants of human umbilical vein endothelial cells (HUVEC) pretreated for 72 h with vehicle, Ang-(1-7), A779 or Ang-(1-7)/A779 and thereafter stimulated with BK for 20 min (see Methods). Results are from at least four independent experiments performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 *vs.* saline-stimulated/vehicle-pretreated; #P<0.05 versus BK-stimulated/vehicle-pretreated; §§P<0.01 *vs.* BK-stimulated/ Ang-(1-7)-pretreated.

4.5 Discussion

In rats, Ang-(1-7) causes vasorelaxation in a number of vascular beds.[232] Since we recently demonstrated that *Mas*-deficient murine aortas lose their Ang-(1-7)-induced relaxation response,[143] we examined the effects of Ang-(1-7) on relaxation in a resistance vessel model, that is mesenteric arteries derived from wild-type and *Mas*-deficient mice. The dose-dependent relaxation induced by Ang-(1-7) in vessels isolated from wild-type mice was diminished by the specific Ang-(1-7) receptor antagonist A779. Ang-(1-7)-mediated relaxation was similarly reduced in knockout vessels. These results demonstrate that Ang-(1-7) acts as a vasodilator in murine resistance vessels, the vasorelaxant response being largely mediated by interaction of the heptapeptide with the receptor Mas.

In both wild-type vessels pre-treated with A779 and *Mas*-deficient vessels, Ang-(1-7) was nevertheless still able to induce a residual relaxation. Indeed, Ang-(1-7) is a vasodilator that acts mainly by interacting with the receptor Mas,[143] as confirmed by our new data. Ang-(1-7)

may also interact with an additional specific receptor, as speculated recently,[238] or activates other vasorelaxant mechanisms, including inhibition of angiotensin-converting enzyme (ACE) or interaction with AT_1 or AT_2 receptors in the endothelial cells.[239] Although we have not explored such mechanisms in the present study, they cannot be discarded. Additionally, a small percentage (not more than 5%) of the vascular tone reduction at the end of the concentration-dependent curves could be attributed to the passive elastic properties of the vascular wall.

Since Ang-(1-7) enhances BK-induced endothelium-dependent vasorelaxation,[130,240] we investigated whether BK-mediated vasorelaxation was impaired in vessels lacking the Ang-(1-7) receptor. Indeed, isolated vessels lacking *Mas* showed significantly impaired dilation in response to either BK or ACh. A deficient functionality of vascular smooth muscle was discarded, as SNP relaxation was unaffected. These results demonstrate that Mas mediates not only Ang-(1-7)-dependent relaxation, but is also required for other endothelium-dependent vasodilators unrelated to RAS, such as ACh and BK, to fully exert their vasorelaxant action in resistance vessels. Therefore, our data indicate that *Mas* deficiency may lead to microvascular endothelial dysfunction.

Interestingly, at the highest concentrations of BK used (1 and 30 µmol/l), but not of ACh, the relaxation observed in *Mas*-deficient vessels was comparable to that of wild-type vessels. The endothelium-dependent relaxations induced by BK and ACh are due to the release of three different factors - NO (clearly the most important one), prostacyclin and EDHF (endothelium-derived hyperpolarizing factor, the nature of which is still under discussion). The relative amount released of each of these mediators depends on the agonist used and the vascular bed studied,[241] although endothelial dysfunction refers essentially to defects in the NO pathway. In our work, BK and ACh elicited very similar relaxant responses in wild-type mice, while in the *Mas*-knockout mice the impairment in relaxation was quantitatively higher in response to ACh than to BK. As Mas influences the NO pathway but may not affect other components of the vasorelaxant response (prostacyclin, EDHF), it could be hypothesized that at rather high concentrations of BK, *Mas*-deficient vessels could still effectively release NO-independent vasorelaxant mediators, through a mechanism unshared with ACh.

To determine whether impaired endothelial relaxation in isolated *Mas*-deficient resistance vessels also has in-vivo relevance and is not merely due to gene deregulation caused by life-long *Mas* deficiency, we infused wild-type animals with A779 for 1 week and then isolated the mesenteric arteries to investigate the endothelium-dependent response to BK and ACh. Again, impaired dilation to both BK and ACh could be observed, therefore confirming that prolonged pharmacological blockade of the receptor Mas also causes endothelial dysfunction.

The vasoactive mediators BK and ACh exert their relaxant action mainly through the release of endothelial NO. To investigate whether the altered vessel behaviour reported in the present study is related to an altered endothelial NO-release, we used primary cultures of human endothelial cells. Ang-(1-7)-pre-treated cells showed a significantly increased BK-induced NO-release that was significantly reduced by pre-treatment with A779. Furthermore, our *in vivo* findings were confirmed by the observation that pre-treatment with A779 alone abolished BK-mediated NO-release. This clearly implies that Ang-(1-7) can facilitate NO-release via Mas, while a pharmacological blockade of the receptor significantly inhibits NO availability.

Taken together, our findings point to a pivotal role for the receptor Mas in preserving normal endothelium-dependent relaxation. Thus, Mas agonists appear to be a promising tool for treating cardiovascular diseases characterized by endothelial dysfunction.

4.6 Acknowledgements

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There are no conflicts of interest.

Chapter 5

Angiotensin metabolites can stimulate receptors of the Mas-related genes family.

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5.1 Abstract

The *Mas* proto-oncogene encodes a G protein-coupled receptor, we identified, also by using the specific angiotensin-(1-7) antagonist A779, to be associated with intracellular signalling of the angiotensin (Ang) II metabolite Ang-(1-7). Recently, *Mas*-related genes (Mrg) have been identified coding for the Mrg-receptor family. All family members share high sequence homology to Mas. Most of them are orphan receptors. To proof whether structure similarities of the Mrg receptors with Mas turn them into potential receptors for Ang-(1-7) or other Ang metabolites, we transfected COS or HEK293 cells with an assortment of Mrg receptors and investigated arachidonic acid (AA) release and transcriptional activation by recording serum response factor (SRF) activation after stimulation with Ang II, Ang III, Ang IV, and Ang-(1-7). None of the investigated receptors activated transcription via SRF. Ang-(1-7) stimulated AA release already in control vector-transfected COS cells, indicating the existence of an endogenous receptor (A779 sensitive). Though less pronounced than for Mas, two of the six studied receptors (MrgD, MRG) initiated significant AA release after stimulation with Ang-(1-7). Interestingly, Mas, MrgD, and MRG mediated Ang IV-stimulated AA release that was highest for Mas. While Ang III activated Mas and MrgX2, Ang II stimulated AA release via Mas and MRG. Thus, we identified other receptors of the Mrg family to respond on Ang-(1-7) stimulation. Furthermore, we describe first an AT_1-independent direct Ang IV signalling and show that Ang II and Ang III mediate signalling independent of their specific receptors AT_1 and AT_2, whereby the receptor specificity differs.

5.2 Introduction

The *Mas* proto-oncogene was discovered through its tumourigenic activity in an *in vivo* tumour assay.[144] It encodes for a protein with seven transmembrane domains belonging to the class of G protein-coupled receptors (GPCR). In early studies, it was suggested to be a receptor for angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system (RAS).[145] Years later, we could show that Mas cannot directly mediate Ang II signalling but hetero-oligomerizes with the AT_1 receptor and by doing so inhibits the actions of Ang II.[242] Thus, Mas is not *per se* a receptor for Ang II but influence the signal cascades activated by the Ang II/AT_1 axis.

Furthermore, we demonstrated that Ang-(1-7), a potent endogenous effector peptide of the RAS with distinct biological functions that can be generated directly from Ang I by neprilysin (neutral endopeptidase)[243] or from Ang II via the recently described angiotensin converting enzyme-2,[107,108] binds to *Mas*-transfected COS or CHO cells and elicits arachidonic acid (AA) release. The release was completely blocked by D-Ala^7- Ang-(1-7), also named A779 (Ang-(1-7)-specific antagonist), but not affected by AT_1 or AT_2 receptor blockers.[143] Thus, we identified Mas to be also a receptor associated with Ang-(1-7) signalling. In a variety of follow-up experiments, Mas was identified to be associated with several Ang-(1-7) actions.[136,244-246] It has been shown that Ang-(1-7), involving Mas, acts as a counter-regulatory hormone to Ang II, e.g., limiting its pressor, proliferative, and angiogenic actions.[247]

Lately, a large family of GPCRs was identified to have strong sequence homology to the receptor Mas. Two independent groups have recently described this gene family. While one group named the identified receptor family sensory-neuron-specific GPCRs,[153] the other group entitled the family Mas-related genes (Mrg),[152] the nomenclature we will follow. These genes are known to be expressed in the sensory neurons of the dorsal root ganglia. It has been postulated that they are involved in the sensory perception of painful stimuli.[152,153] Most of these GPCRs are orphan, but some of the Mrg members can be activated by peptide ligands such as RF-amide, the opioid peptides BAM22, or γ2-MSH.[152-154]

The characterization of Mas as a receptor associated with Ang-(1-7) actions pointed to the question if other biologically active Ang peptides, like Ang III and Ang IV, are also interacting with the receptor Mas because all these Ang peptides have related sequences and structure. In addition, since Ang II and Ang III are interacting with two independent receptors, AT_1 and AT_2, that share more than 30% sequence homology, the question arose whether receptors with similar homology to Mas (Mrg family members share between 30% and 41% homology to Mas) may also interact with Ang-(1-7). Furthermore, we followed the hypothesis that one of the investigated members could be a potential receptor for one of the other biological active Ang metabolites.
Therefore, we stimulated COS and HEK cells with different Ang metabolites and measured the AA release or the activation of serum response factor (SRF), which regulates gene transcription by binding to the serum response element (SRE), to investigate the potential activation of two independent signalling pathways after ligand/receptor interaction.

5.3 Materials and methods

5.3.1 Materials
Cell culture media, media supplements, and antibiotics were purchased from Invitrogen GmbH (Karlsruhe, Germany). Plasmids used encoding the human receptors Mas and AT_1 were described earlier.[242] The plasmids encoding the human receptors MrgD, MrgF (previously described as RTA[248]), MrgX1, and MrgX2 and the murine receptor MrgH (previously described as GPR90[249]; no human analogue is known) were kind gifts of Sanofi-Aventis GmbH (Frankfurt/Main, Germany). All Ang peptides used for stimulation and the Ang-(1-7)-selective antagonist A779 were purchased from Bachem GmbH (Weil am Rhein, Germany).

5.3.2 Cloning of the human receptor MRG
cDNA coding for the human receptor MRG was amplified by PCR using gene-specific primers (primer 1: 5'-CCT GAC TGT GAT GCC-3'; primer 2: 5'-GTG TAC AAT TCC CCA GCT C-3'). The PCR product was subcloned in a T-Vector (Promega GmbH, Mannheim, Germany), and identity was confirmed by sequencing. Finally, the cDNA was transferred to the same expression vector as the plasmids provided by Sanofi-Aventis (pcDNA3.1; Invitrogen GmbH Karlsruhe, Germany).

5.3.3 Phylogenetic analysis of the used receptors
To analyze the homologies of the used receptors, we aligned the protein sequences of the human receptors MRG (accession number: NP 443199), MrgD (NP 944605), MrgF (NP 659452), MrgX1 (NP 671732), MrgX2 (NP 473371), AT_1 (NP 114438), and AT_2 (NP 000677) and the murine receptor MrgH (NP 109651) with the human receptor Mas (NP 002368). Further alignments were done comparing the two human Ang II receptors (AT_1, AT_2), the murine (NP 032578) and the human receptor Mas, and the murine receptors MrgH and Mas. For this purpose we used the blastp module of the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast)[250] with the BLOSUM62 matrix. The penalty for gap opening was set to 11 and the penalty for gap extension was set to 2. To generate the phylogenetic tree, the same protein sequences were aligned using CLUSTAL W (http://www.ebi.ac.uk/clustalw)[251].

5.3.4 Cell culture
HEK293 and COS[252] cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml

penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

5.3.5 Cell transfections

In COS cells stable DNA transfections were performed by the calcium phosphate co-precipitation method[253] and selected by neomycin. In HEK293 cells, transient DNA transfections were performed with Polyfect (Qiagen GmbH, Hilden, Germany) according to manufacturer's recommendations. After transfection the cells were maintained in FCS-reduced (0.5%) DMEM.

5.3.6 Arachidonic acid (AA) release

AA release assays were performed as described earlier.[143] In brief, COS cells, transfected as indicated, were preloaded with 0.2 µCi/well of [^{3}H]AA (GE Healthcare Europe GmbH, Munich, Germany) for 18 h in FCS-free DMEM. After preloading, cells were stimulated with the indicated peptides (final concentration: 10^{-8} M) for 15 min at 37°C in Hanks' balanced salt solution. The amount of [^{3}H]AA released into the medium and that remaining in the cells was measured by liquid scintillation spectrometry. The [^{3}H]AA released into the medium was expressed as percent of the released [^{3}H]AA in unstimulated cells (saline; **Fig. 16**) or as percentage changes of the released [^{3}H]AA compared to the [^{3}H]AA released by accordingly stimulated pcDNA3.1-transfected cells (**Fig. 18**).

5.3.7 Serum response factor (SRF) activation assay

Luciferase reporter gene assays were performed with the Dual Luciferase Reporter Assay System (Promega GmbH, Mannheim, Germany) according to manufactures' protocol. Briefly, HEK293 cells were seeded into 48-well plates and co-transfected with the indicated plasmids together with pSRE.L encoding firefly luciferase reporter plasmid (kindly provided by Prof. T. Wieland, Mannheim, Germany) and pRL-TK (Promega GmbH, Mannheim, Germany) encoding renilla luciferase control vector. The transfected cells were maintained in DMEM with 0.5% FCS for 18 h and than stimulated as indicated (in experiments with a fixed concentration the final concentration was 10^{-6} M) for further 8 h in the same medium. Afterward, cells were lysed with passive lysis buffer (Promega GmbH, Mannheim, Germany), and luciferase activities were determined with a microplate luminometer (Orion; Berthold Detection Systems GmbH, Pforzheim, Germany) in a white 96-well plate. The given values are firefly/renilla luciferase ratios as percent of the luciferase ratios in unstimulated cells.

5.3.8 Statistics

Results are represented as mean±SEM range, in which n is the number of separate experiments done in triplicate. Comparison of the effects of peptides on AA release and SRF activation were analyzed by a non-paired Student's *t*-test, and dose-response curves were compared by two-way ANOVA (Graph Pad Prism 3.01; Graph Pad Software Inc., San Diego, California, USA). Significance was considered from a value of $P<0.05$.

5.4 Results

To proof our hypothesis that other Ang metabolites with high sequence homology to Ang-(1-7) may also stimulate intracellular signalling requiring the receptor Mas, we investigated the peptide-stimulated AA release in COS cells transfected with either pcDNA3.1 or a Mas expression vector. As shown in **Fig. 16a** all four tested Ang peptides were able to induce a slight, but significant, increase in AA release in cells transfected with pcDNA3.1 (Ang II: ~+0.16 RU, $P<0.01$ vs. saline; Ang III: ~+0.26 RU; Ang IV: ~+0.26 RU;

Ang-(1-7): ~+0.36 RU, *P*<0.001 vs. saline). The endogenous ligand for Mas, Ang-(1-7), led to a massive release of AA (~+206%, *P*<0.001 vs. pcDNA3.1+ Ang-(1-7); **Fig. 16a**) in *Mas*-transfected cells as shown previously.[143] Ang II stimulation slightly increased AA release in *Mas*-transfected cells (~+23%, *P*<0.01 vs. pcDNA3.1+Ang II) compared to control vector-transfected cells. Interestingly, treatment with either Ang III or Ang IV of *Mas*-transfected cells stimulated significantly a robust AA release (Ang III: ~+79%, *P*<0.001 vs. pcDNA3.1+Ang III; Ang IV: ~+74%, *P*<0.001 vs. pcDNA3.1+Ang IV).

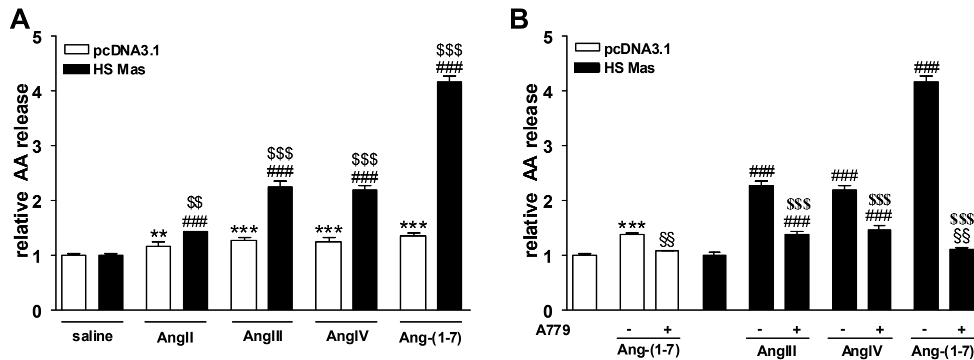

Figure 16 (**A**) [^{3}H]arachidonic acid releases in pcDNA3.1- and Mas-transfected COS cells after stimulation with different Ang peptides. Data are presented as mean±SEM. ***P*<0.01, ****P*<0.001 *vs.* pcDNA3.1-transfected COS cells treated with saline; $$*P*<0.01, $$$*P*<0.001 *vs.* pcDNA-transfected COS cells treated with the same peptide; ###*P*<0.001 *vs.* Mas-transfected COS cells treated with saline; n≥8. (**B**) Effects of A-779 on Ang peptide-stimulated [^{3}H]arachidonic acid release in pcDNA3.1- and Mas-transfected COS cells. Data are presented as mean±SEM. ****P*<0.001 *vs.* pcDNA3.1-transfected COS cells treated with saline; §§*P*<0.01 *vs.* pcDNA-transfected COS cells treated with Ang-(1-7); ###*P*<0.001 *vs.* Mas-transfected COS cells treated with saline; $$$*P*<0.001 *vs.* Mas-transfected COS cells treated with the same peptide; n≥8.

To test whether these significant increases in AA release by the Ang metabolites were mediated specifically via Mas, we pre-treated pcDNA3.1- and *Mas*-transfected cells with the Ang-(1-7)-receptor-specific antagonist A779. In *Mas*-transfected COS cells, A779-pre-treatment blocked the AA release induced by Ang-(1-7) to levels of saline-treated cells transfected with Mas (**Fig. 16b**, right panel), while A779 attenuated Ang III- (*P*<0.001 vs. Mas+saline) and Ang IV-stimulated (*P*<0.001 vs. Mas+saline) AA release to the levels of pcDNA3.1-transfected cells stimulated by the same peptides. Notably, also the AA release stimulated by Ang-(1-7) in pcDNA3.1-transfected cells is Mas-mediated, since A779-pre-treatment diminished AA release to basal levels (**Fig. 16b**, left panel).

Based on the finding that the two Ang II receptors (AT_1, AT_2) share ~30% sequence homology we tested whether receptors with sequence similarities with Mas could represent an independent, second receptor for Ang-(1-7). We firstly transfected COS cells with the receptor with highest homology to Mas within our selection, MrgH (~41%; **Fig. 17**). In these cells only Ang IV had a significant impact on AA release (**Fig. 18a**) compared to pcDNA3.1-transfected cells stimulated with the same peptides. In contrast to Mas, the two receptors from the MrgX-subfamily, MrgX2 (~34% homology to Mas, **Fig. 17**) and MrgX1 (~32% homology), inhibited the Ang-(1-7)-stimulated AA release observed in pcDNA3.1-transfected cells treated with Ang-(1-7) (MrgX2: **Fig. 18b**; MrgX1: **Fig. 18c**; *P*<0.001). Ang II stimulation did not alter the AA release in cells transfected with MrgX1, while it reduced the AA release significantly in MrgX2-transfected cells (*P*<0.05 vs. pcDNA3.1+Ang II). Ang III increased AA release in MrgX2-transfected cells (*P*<0.05 vs. pcDNA3.1+Ang III), whereas it had no effect on MrgX1-over-expressing cells. Both receptors mediated a slight but highly significant increase in AA release after stimulation with Ang IV (*P*<0.001 vs. pcDNA3.1+Ang IV). MrgD (~32% homology to Mas, **Fig. 17**) did not mediate AA release after stimulation with either Ang II or Ang III (**Fig. 18d**). However, MrgD mediated an increase in released AA following Ang IV (*P*<0.001 vs. pcDNA3.1+Ang IV) and Ang-(1-7) (*P*<0.001 vs. pcDNA3.1+ Ang-(1-7)) stimulation that was the highest for both peptides measured for our six Mas-related receptors tested.

Stimulation of MrgF-transfected cells (~31% homology to Mas, **Fig. 17**) with the Ang metabolites led to a pattern similar to MrgX1 with diminished amounts of released AA after Ang-(1-7) stimulation (*P*<0.05 vs. pcDNA3.1+ Ang-(1-7); **Fig. 18e**). Very similar to MrgD-transfection, cells expressing MRG showed an increased AA release after Ang IV and Ang-(1-7) stimulation (Ang IV: *P*<0.01 vs. pcDNA3.1+Ang IV; Ang-(1-7): *P*<0.001 vs. pcDNA3.1+ Ang-(1-7); **Fig. 18f**), although it is the receptor with the lowest homology to Mas (~30%; **Fig. 17**). Ang III stimulation had no influence on the receptor-mediated AA release. Interestingly, MRG was the only receptor in our selection inducing a significant increase in AA release after stimulation with Ang II (*P*<0.05 vs. pcDNA3.1+Ang II).

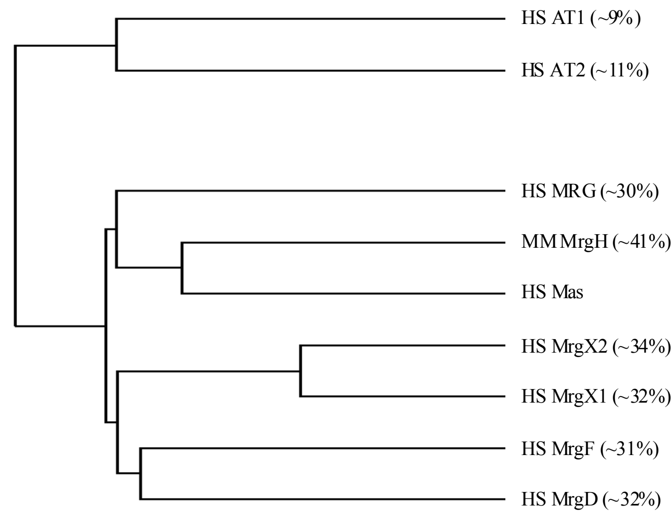

Figure 17 Phylogenetic analysis of receptors belonging to the Mrg family. The percentage in homology to human Mas is parenthesized. The protein sequences were aligned using CLUSTALW.

After stimulation with Ang II, AT_1 activates beside the AA release[254] a variety of other independent intracellular signalling pathways,[255] leading to, e.g., Ca^{2+} release,[256] cytoskeletal rearrangement,[257] cellular proliferation[258] and hypertrophy,[259] and activation of transcription factors.[260] Thus, we measured transcriptional activation using exemplary SRF, a transcription factor mainly regulating proliferation and the cytoskeleton,[261] to investigate whether the identified signalling of the Ang metabolites via the Mrg-family is restricted to AA release.

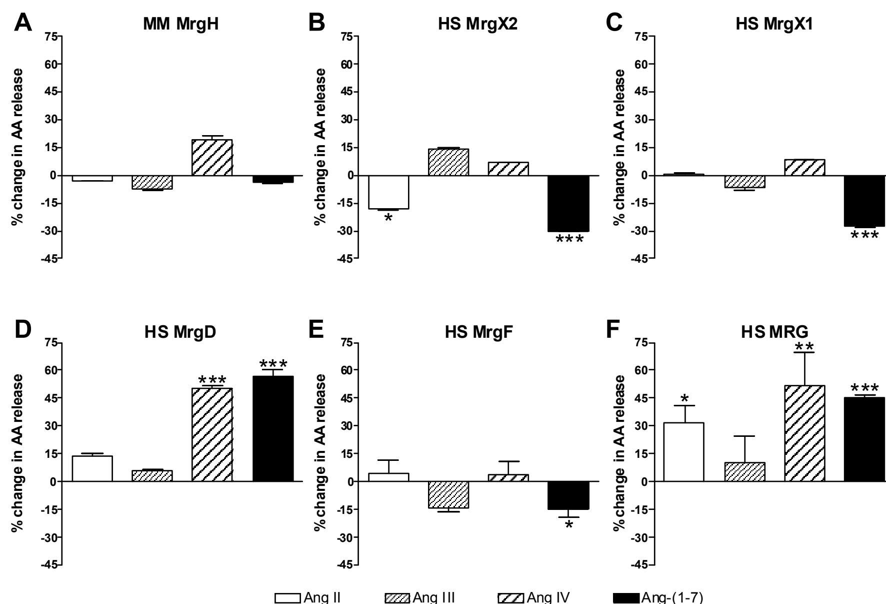

Figure 18 (**A-F**) Changes in [^{3}H]arachidonic acid release after stimulation of COS cells overexpressing receptors of the Mrg family with different Ang metabolites compared to pcDNA3.1-transfected cells stimulated with the corresponding peptide. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* pcDNA3.1-transfected COS cells treated with the same peptide; n≥4.

To show the functionality of our assay conditions, we firstly transfected HEK cells with AT_1 and stimulated these transfected cells with the four Ang peptides. As expected, Ang II and Ang III vigorously activated SRF shown as a significant increase in luciferase activity (Ang II: ~+1116%; Ang III: ~877%, $P<0.001$ vs. saline; **Fig. 19a**). Ang-(1-7) could not stimulate AT_1-mediated activation of SRF. Notably, we found a significant increase in SRF-regulated luciferase expression due to Ang IV stimulation via AT_1 (~+252%, $P<0.001$ vs. saline) by approximately one-fifth of the stimulation seen for Ang II and one-fourth for Ang III, respectively. For further characterization of the Ang IV-induced SRF activation, we generated dose-response curves for Ang II and Ang IV stimulation in AT_1-transfected cells (**Fig. 19b**). The efficacy of Ang II to activate SRF was significant higher than of Ang IV for all tested concentrations (Ang II: EC_{50} ~3.37×10^{-7} M, $E_{max½}$ 6.78 RU; Ang IV: EC50 ~3.08×10^{-6} M, $E_{max½}$ 5.35 RU).

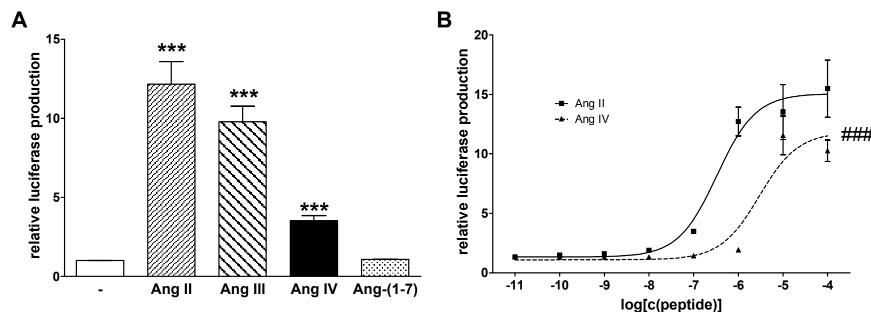

Figure 19 (**A**) Relative luciferase production in AT_1-transfected HEK cells after stimulation with different Ang metabolites. Data are presented as mean±SEM. ***$P<0.001$ *vs.* AT_1-transfected HEK cells treated with saline; n≥8. (**B**) Dose-response curves for AT_1-mediated serum response factor activation by Ang II (squares) and Ang IV (triangles). ###$P<0.001$ *vs.* AT_1-transfected HEK cells treated with Ang II; n≥6

Secondly, as for AA release, we started to test cells transfected with either pcDNA3.1 or the receptor Mas. The Ang peptides tested could not stimulate transcriptional activation by SRF in either pcDNA3.1- (**Fig. 20a**) or *Mas*-transfected cells (**Fig. 20b**). Also for all other tested receptors, no significant increase in SRF activation was detectable (**Fig. 21a-f**).

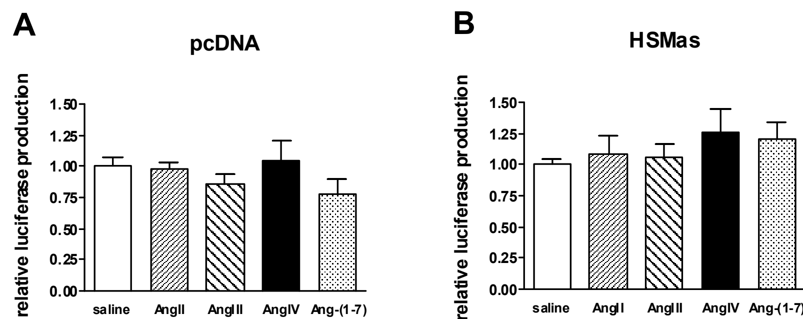

Figure 20 (**A**) Serum response factor (SRF) activation after stimulation with different Ang peptides in control vector-transfected HEK cells. Data are presented as mean±SEM; n≥6. (**B**) SRF activation after stimulation with different Ang peptides in Mas-transfected HEK cells. Data are presented as mean±SEM; n≥6

5.5 Discussion

The RAS plays a major role in the regulation of blood pressure and in the pathogenesis of cardiovascular diseases.[262,263] Long time, Ang II was classified as the only active peptide of the RAS. By now it is known that other Ang metabolites like Ang III, Ang IV, and Ang-(1-7) may also mediate actions of the RAS.[86,88]

Already in 1988 it has been postulated that the GPCR Mas plays a role in the RAS and potentially poses as a receptor for Ang II and Ang III.[145] Nowadays, it is certain that the receptor Mas is associated with Ang-(1-7) signalling[143] and can hetero-oligomerize with the AT_1 and by doing so inhibiting the actions of Ang II.[242]

Since the receptor Mas interacts with Ang-(1-7), we hypothesized that also other Ang metabolites with sequence and structural similarities to the heptapeptide may interact with Mas. In search of potential Ang-(1-7)-independent ligands also stimulating AA release via Mas, we stimulated *Mas*-transfected cells with other Ang peptides, namely, Ang II, Ang III, and Ang IV.

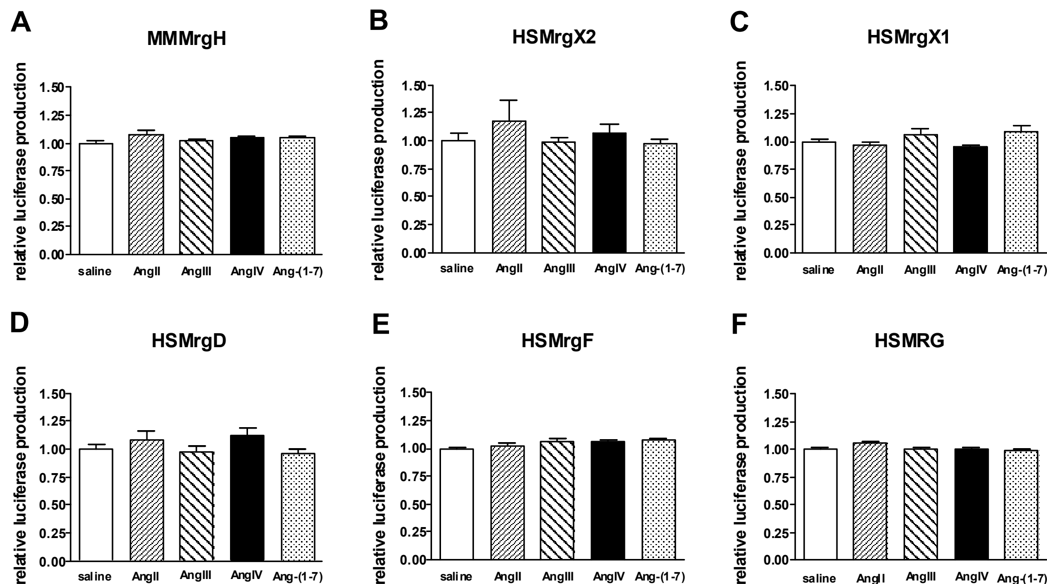

Figure 21 (**A-F**) Serum response factor (SRF) activation after stimulation with different Ang peptides in HEK cells overexpressing receptors of the Mrg family. Data are presented as mean±SEM; n≥6

Our findings clearly demonstrate that beside Ang-(1-7), although less pronounced, also Ang III and Ang IV induce a significant AA release in *Mas*-transfected cells compared to pcDNA3.1-transfected cells (control vector) stimulated with Ang III or Ang IV (**Fig. 16a**), while Ang II does not mediate a significant AA release via Mas. The difference between the Ang II and Ang III effects on Mas is all the more interesting, because it is well accepted that Ang II and Ang III have nearly the same binding affinity to their main receptors, AT_1 and AT_2, and often comparable physiological effects.[86] It could be postulated that the recently described interaction of Mas with AT_1[242] causes the differences in Ang II and Ang III effects due to a conformational change in the endogenous AT_1 when hetero-dimerizing after *Mas* transfection. However, the Ang III-mediated AA release should be an AT_1-independent pathway (**Fig. 16b**), because effects stimulated by Ang III as well as Ang-(1-7) and Ang IV in *Mas*-transfected cells can be blocked by A779, while A779 cannot block AT_1-mediated effects modulated by Mas/AT_1 interaction.
Since Ang II induces an AA release via AT_1 but also activates transcription factors like NF*κ*B, NFAT,[264] and SRF,[265] we stressed the question whether the Ang metabolites releasing AA via Mas can also activate transcription factors via this receptor. Thus, we have chosen exemplary SRF for investigating a different signalling pathway. In contrast to AT_1 (**Fig. 19**), Mas transforms ligand interaction in AA release but is not able to activate SRF (**Fig. 20**). These findings are congruent with the described signalling pathways of Ang-(1-7). While most of its effects are mediated via AA[143] and NO generation,[266] Ca^{2+} release and those related activations by, e.g., the transcription factor NFAT have not been described.
The second hypothesis for our here described work was related to the fact that the two receptors for Ang II and Ang III, AT_1 and AT_2, share more than 30% sequence homology. Thus, we investigated whether Mas-sequence-similar receptors of the Mrg family - they share between 30% and 41% homology with Mas - may also serve as a receptor for Ang-(1-7) or the other investigated Ang metabolites.

As shown before for Mas, the six investigated Mrg receptors do not show any significant activation of SRF after stimulation with the Ang metabolites. Interestingly, the receptor with the highest homology to Mas, MrgH, did not induce a significant alteration in AA release compared to control-vector-transfected cells after stimulation with Ang-(1-7). This finding indicates that highest homology not necessarily leads to most similar agonist specificity. Since Ang-(1-7) was most potent in AA release in *Mas*-transfected cells, it was primarily hypothesized that especially the heptapeptide promotes AA release via one or more of the tested Mas-related receptors. Two of the six investigated receptors (MrgD, MRG) showed a highly significant AA release in response to Ang-(1-7) stimulation ($P<0.001$). Similar to the impact on intracellular Mas signalling, Ang IV, with exception for MrgF, also induces a significant AA release in all tested Mrg receptors which was highest for MrgD. Ang III could only induce a slight, but significant AA release in cells transfected with MrgX2. Ang II failed to induce AA release in cells transfected with one of the other receptors of the Mrg family. Interestingly, the transfection with three of the six receptors reduced significantly the AA release stimulated by at least one of the four peptides when compared to the one in pcDNA-transfected cells. Consequently, further experiments, e.g., binding-assays, dose-response curves, and comparison with already described receptor agonists,[152-154] have to be carried out for a better characterization of receptor activation by Ang metabolites. As shown in **Fig. 16a**, COS cells show already basically AA release after treatment with all four peptides implicating basal expression of AT_1 and Mas. The latter was even proven because A779 could not only block the AA release in *Mas*-transfected cells but also blocked the activation of endogenous Mas in control vector-transfected COS cells (**Fig. 16b**).
Our studies identified Mas to be, beside being a receptor associated with specific Ang-(1-7) signalling, also a receptor mediating robust AA release stimulated by two other Ang metabolites, Ang III and Ang IV. Furthermore, our results indicate that receptors, sharing sequence similarities with Mas, also interact with Ang peptides. Even though the signalling pathway activated by the peptides is identical, the activation pattern differs for each receptor tested. Importantly, this investigation describes for the first time Ang IV-induced effects directly mediated by a GPCR independent of its partial agonistic effects on AT_1. Up to now only a transmembrane enzyme, insulin-regulated membrane aminopeptidase (IRAP), was described as an Ang IV ‘receptor’ mediating indirect Ang IV effects due to inhibition of endogenous IRAP substrate degradation. In addition, we have shown that Ang II and Ang III can initiate signalling via receptors being independent from their receptors AT_1 and AT_2. Since we have found different patterns of receptor activation for Ang II and Ang III, our findings suggest distinct functions for the Ang II metabolite Ang III.
Our data implicate that it would be worthwhile to investigate also the reactivity of the other Mrg family members, approximately 50 orphan receptors are described in rodents and human, toward Ang peptides to identify new target receptors specific for Ang metabolites.

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Chapter 6

The G Protein-coupled receptor Mas is a physiological antagonist of the angiotensin II type 1 receptor

Based on: 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(14):1806-1813

6.1 Abstract

We previously identified the G protein-coupled receptor Mas, encoded by the *Mas* proto-oncogene, as an endogenous receptor for the heptapeptide angiotensin-(1-7); however, the receptor is also suggested to be involved in actions of angiotensin II. We therefore tested whether this could be mediated indirectly through an interaction with the angiotensin II type 1 receptor, AT_1.

In transfected mammalian cells, Mas was not activated by angiotensin II; however, AT_1-mediated, angiotensin II-induced production of inositol phosphates and mobilization of intracellular Ca^{2+} was diminished by 50% after co-expression of *Mas*, despite a concomitant increase in angiotensin II binding capacity. Mas and the AT_1 formed a constitutive hetero-oligomeric complex that was unaffected by the presence of agonists or antagonists of the 2 receptors. *In vivo*, Mas acts as an antagonist of the AT_1; mice lacking the *Mas* gene show enhanced angiotensin II-mediated vasoconstriction in mesenteric microvessels.

These results demonstrate that Mas can hetero-oligomerize with the AT_1 and by so doing inhibit the actions of angiotensin II. This is a novel demonstration that a G protein-coupled receptor acts as a physiological antagonist of a previously characterized receptor. Consequently, the AT_1-Mas complex could be of great importance as a target for pharmacological intervention in cardiovascular diseases.

6.2 Introduction

The *Mas* proto-oncogene was first detected through its tumourigenic activity in *in vivo* tumour assays.[144] In mammals, the gene is expressed predominantly in testis and distinct areas of forebrain, including hippocampus and amygdala, and less strongly but at detectable levels in kidney and heart.[164,267] It encodes a protein with 7 transmembrane domains that contain features characteristic of class I G-protein-coupled receptors (GPCRs), and in early studies, it was suggested to be a receptor for the octapeptide angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system.[145] However, Ambroz *et al.*[146] and Ardaillou[147] later showed that Ang II-induced elevation of intracellular Ca^{2+} in *Mas*-transfected cells was only observed in cells that endogenously expressed the Ang II type 1 receptor (AT_1), one of the 2 receptors now known to represent the natural targets for Ang II. Thus, Mas is not *per se* a receptor for Ang II. Despite this, clear evidence indicates a physiological role for Mas in the function of Ang II. For example, Walther *et al.*[148,234,268] demonstrated an alteration of neuronal AT_1 signalling after Ang II stimulation in mice in which the *Mas* proto-oncogene had been inactivated, and these mice also display gender-specific alterations in heart rate and blood pressure regulation. Furthermore, we have recently identified Ang-(1-7), a peptide metabolite generated mainly from Ang I and with distinct biological functions, as the endogenous agonist for the receptor Mas.[87,143] Taken together, our previous findings provide evidence for an *in vivo* interaction between the Mas and AT_1 GPCRs, although the molecular events that govern this interaction remain unknown.

One mechanism of receptor-receptor interaction that has recently gained much prominence in the field of GPCRs is the phenomenon of receptor dimerization/oligomerization.[269,270] In some cases, oligomerization is a prerequisite for efficient agonist binding and signalling, and in others, it simply occurs constitutively without overt biological consequences.[269-271] Previous studies have shown that the AT_1 can form hetero-oligomeric complexes with certain other GPCRs. For example, dimerization between the AT_1 and the bradykinin B_2 receptor results in enhanced function of Ang II and may underlie much of the hypertension associated with the condition of preeclampsia in pregnant women.[73,272] Interactions between AT_1 and AT_2 have also been demonstrated *in vitro*, but in such circumstances, the AT_2 appears to functionally antagonize the AT_1.[72] To date, however, studies of class I GPCRs have not

established the *in vivo* physiological relevance of hetero-dimerization, the postulated AT_1-B_2 receptor interaction being characteristic of a pathophysiological state. Given our strong evidence for a physiological interaction between Mas and AT_1, we chose to examine the potential for direct interactions between these 2 receptors as the molecular basis underlying their interactions *in vivo*.

6.3 Methods

6.3.1 Cell Culture and Transfection

CHO-K1 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (10,000 IU/ml to 10,000 µg/ml), and 2 mol/l HEPES (radioligand binding assays) or basal ISCOVE medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (10,000 IU/ml to 10,000 µg/ml), and 2 mmol/l L-glutamine (signalling studies). For radioligand binding assays, CHO-K1 cells at 50% to 80% confluence were transfected transiently with the indicated cDNAs (21 µg of plasmid DNA per 150-cm^2 tissue culture flask) with Lipofectamine reagent (GIBCO BRL) according to the manufacturer's instructions. For cell-signalling assays, 2×10^5 CHO-K1 cells were seeded into 35-mm dishes. About 24 h later, cells were transfected transiently at 50% to 80% confluence with the indicated receptor cDNAs (2 or 0.06 µg of total plasmid DNA/35-mm well) with Lipofectamine. In co-transfection experiments, 1 µg of receptor DNA each was used.

6.3.2 Inositol Phosphate Accumulation Assay

Inositol phosphate accumulation assays were performed essentially as described previously.[273]

6.3.3 Fluorometric Imaging Plate Reader Cell Signalling Assay

Six to 18 h after transfection, CHO-K1 cells were seeded into 96-well plates at a density of 50,000 cells per well and cultured for 18 to 24 additional h until used in a fluorometric imaging plate reader (FLIPR; Molecular Devices) assay. CHO-K1 cells were loaded with 95 µL of HBSS containing 20 mmol/l HEPES, 2.5 mmol/l probenecid, 4 µmol/l fluorescent calcium indicator dye Fluo4 (Molecular Devices), and 1% fetal bovine serum for 1 h (37°C, 5% CO_2). Cells were washed 3 times with PBS containing 1 mmol/l $MgCl_2$, 1 mmol/l EDTA, and 2.5 mmol/l probenecid in a Tecan cell washer. After the final wash, 100 µL of residual volume remained on the cells in each 96-well plate. Peptides were dissolved in water or the appropriate solvent as 2-mmol/l stock solutions and diluted at least 1:100 into the washing buffer described above. Peptides were separated into aliquots as 3× solutions into a 96-well plate before the assay. Agonist activity was determined with peak fluorescent counts.

6.3.4 [^{3}H]Ang II Binding Assay

Forty-eight h after transfection, CHO-K1 cells expressing either the AT_1 plus vector, the receptor Mas plus vector, or AT_1 plus Mas were used for saturation binding assays with [tyrosyl-3,5-^{3}H]Ang II ([^{3}H]Ang II; 72 Ci/mmol; Amersham-Pharmacia-Biotech) as the radioligand. CHO cells were harvested by trypsinization followed by centrifugation (300×*g*, 3 min) and re-suspension of the pellet in HEPES buffer (in mmol/l: NaCl 110, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 1, glucose 25, HEPES 50, and sucrose 58; pH 7.4), repeated twice. Subsequently, 10^5 cells/tube were distributed into assay tubes that contained increasing concentrations of [^{3}H]Ang II (0.2 to 50 nmol/l) in a final volume of 1 ml/tube and allowed to equilibrate for 3 h at 4°C. Nonspecific binding was determined with 100 nmol/l unlabeled Ang II. Reactions were terminated by rapid filtration with ice-cold HEPES buffer through Whatman GF/C filters. Filters were washed 3 times with 4 ml aliquots of ice-cold buffer and dried before

being exposed to 5 ml of scintillation cocktail (Ultima Gold LSC-cocktail; Packard Bioscience). Radioactivity was determined via scintillation counting.

6.3.5 Bioluminescence Resonance Energy Transfer

6.3.5.1 Construction of Plasmids

Enhanced yellow fluorescent protein (eYFP) was appended to the C-terminal tail of the AT_1 from which the stop codon was eliminated, and Mas was modified by C-terminal, in-frame addition of *Renilla* luciferase. The production of thyrotropin-releasing hormone (TRH) receptor-1-eYFP and demonstration of the improved resolution of energy transfer signal from the emission spectrum of *Renilla* luciferase that is produced by the use of a variant of the bioluminescence resonance energy transfer ($BRET^2$) have been described elsewhere.[274]

6.3.5.2 Bioluminescence Resonance Energy Transfer

HEK293 cells were grown to ~60% confluence before transfection with Lipofectamine reagent according to the manufacturer's instructions. The majority of traditional BRET experiments (e.g., **Figs. 25a and 25c**) were performed with a Spex fluorolog spectrofluorimeter (SPEX). Cells were harvested 48 h after transfection. Media were removed from cell culture dishes, and cells were washed twice with PBS before they were detached to form a suspension. Approximately 3×10^6 cells in 1.5 ml of PBS buffer were then added to a glass cuvette; an equal volume of PBS containing 10 µmol/l h-coelenterazine was added, and the contents of the cuvette were mixed. The emission spectrum (400 to 600 nm) was acquired immediately with the excitation lamp turned off (slit width 10 nm, 2 s per increment). For comparisons between experiments, emission spectra were normalized with the peak emission from *Renilla* luciferase in the region of 480 nm being defined as an intensity of 1.00. Energy transfer signal was calculated by measuring the area under the curve between 500 and 550 nm. Background was taken as the area of this region of the spectrum when we examined emission of cells that expressed only the energy donor, e.g., Mas-*Renilla* luciferase. In a number of cases, BRET (e.g., **Fig. 25b**) measurements were performed with a Mithras LB 940 (Berthold Technologies Ltd) because its sensitivity to detect BRET is greater than that of the Spex fluorolog spectrofluorimeter. These experiments were performed in 96-well whitewall microtiter plates, with each treatment being performed in triplicate. To 100 µL of cells expressing appropriate constructs, an equal volume of 10 µmol/l coelenterazine was added; this was mixed and immediately placed in the Mithras LB 940. When the cells were treated with drugs, the cells were incubated at 37°C for 5 min before addition of coelenterazine. Each well was counted for 1 second, and the data were collected with emission filters at 480 nm (luciferase emission) and 530 nm (energy transfer). In these examples, data are presented as the ratio (530/480 nm) of these values.

To perform $BRET^2$ assays, cells were washed 3 times in PBS and then harvested in PBS supplemented with magnesium (0.1 g/l) and glucose (1 g/l). They were then counted on a haemocytometer, and _700,000 cells per well were dispensed into a 96-well white-walled culture plate (Packard Biosciences). DeepBlueC (Packard Biosciences) reagent was prepared in accordance with the manufacturer's directions and added to a final concentration of 10 µmol/l. $BRET^2$ signals were then measured immediately in a Mithras LB940 with a 410 nm filter (band pass 80 nm) to measure light emitted from DeepBlueC and a 515 nm filter (band pass 30 nm) to measure light emitted from a modified green fluorescent protein (GFP^2). Energy acceptor to energy donor expression ratios were measured as described previously.[274]

6.3.6 Co-immunoprecipitation of AT_1-eYFP and Mas-$G\alpha_{11}$

HEK293T cells were transfected transiently to express AT_1-eYFP, Mas-$G\alpha_{11}$, or both. Sixteen hours later, cells were lysed with RIPA buffer in the presence of a cocktail of protein

inhibitors (Complete, EDTA free, Roche). The lysates were centrifuged (100,000×*g*, 1 h) then pre-cleared with protein-G sepharose. Protein content was adjusted to 1 mg/ml, and 500 µL was immunoprecipitated with an anti-GFP antiserum. After 3 washes with RIPA, addition of Laemmli buffer and separation by SDS-PAGE were performed. The proteins were transferred to nitrocellulose and immunoblotted with an anti-$G\alpha_q/G\alpha_{11}$ antiserum.

6.3.7 Effects on Vascular Tone of Mesenteric Microvessels
Wild-type and *Mas*-deficient mice were anesthetized with sodium pentobarbital 70 mg/kg i.p. and exsanguinated. The mesentery was removed, and third branch mesenteric arteries were removed and placed on a small-vessel myograph[275] capable of measuring isometric tension and containing a physiological solution, as already reported.[276] Their passive tension and internal circumference were determined, and the mean internal diameter ranged between 150 and 300 µm, with non-significant differences among the different groups of mice. Arteries were then contracted with 125 mmol/l K^+ (equimolar substitution of KCl for NaCl in the physiological solution) for 2 minutes. The bath was then washed several times, and a further resting period of 30 minutes was allowed. Afterward, the arteries were submitted to increasing concentrations of Ang II (10 pmol/l to 1 µmol/l) or endothelin-1 (10 pmol/l to 0.1 µmol/l) at 2 minute intervals, either in the absence or presence of 1 µmol/l losartan, according to procedures described previously.[277]

6.3.8 Statistical Analysis
Radioligand saturation binding data sets were fitted globally via nonlinear regression to mass-action models of both total and nonspecific binding with Prism 4.0 (GraphPad Software) to derive estimates of radioligand affinity (K_d) and maximal receptor density (B_{max}). All concentration-response data were fitted to a 4-parameter Hill equation with Prism. Statistical evaluation of compound effects on the vascular tone of mesenteric microvessels was performed by deviations from the mean with regard to the curves for Ang II and losartan or endothelin-1 with factorial 2-way ANOVA. Student *t*-test was used in other comparisons (mean±SEM). A value of $P<0.05$ was considered statistically significant.

6.4 Results

Initially, cDNAs encoding the human forms of Mas and AT_1 individually and in combination were expressed in CHO-K1 cells, and mobilization of intracellular calcium ($[Ca^{2+}]_i$) on stimulation with varying concentrations of Ang II was recorded. Expression of Mas alone did not confer responsiveness to Ang II (**Fig. 22a**). In contrast, in cells expressing AT_1, Ang II potently mediated elevation of $[Ca^{2+}]_i$ with a log EC_{50} -9.51±0.11 (n=3; **Fig. 22a**, filled squares). Importantly, co-expression of Mas with AT_1 significantly impaired the potency (log EC_{50} -8.90±0.11; n=3; $P<0.05$) and the maximal effectiveness of Ang II to enhance $[Ca^{2+}]_i$ mobilization (**Fig. 22a**, open squares). To test whether the impairment by Mas of Ang II-mediated $[Ca^{2+}]_i$ mobilization via AT_1 is retained at lower expression levels, identical co-expression experiments were performed with 1/30th of the cDNA for transfections. Under these conditions, Ang II mediated elevation of $[Ca^{2+}]_i$ via AT_1 receptor with a log EC_{50} -9.40±0.13; n=3 (**Fig. 22a**, filled circles). Notably, co-expression of Mas impaired Ang II-mediated $[Ca^{2+}]_i$ mobilization to a similar extent (log EC_{50} -8.85±0.29; n=3; **Fig. 22a**, open circles). Increasing concentrations of the AT_1 antagonist losartan induced significant rightward shifts of the Ang II concentration-response curves of cells expressing AT_1 alone (**Fig. 22b**) or AT_1 in combination with Mas (**Fig. 22c**). Schild plots for losartan were linear over a wide concentration range (0.03 to 3.0 µmol/l); the slopes of the plots were not significantly different from unity ($P>0.05$), and antagonist potency estimates were similar in the absence or presence of Mas. Despite the effect of Mas on responsiveness to Ang II, these

results indicate that AT_1 retains its pharmacological characteristics with respect to competitive antagonists, such as losartan. Both Ang II elevation of $[Ca^{2+}]_i$ and its diminution in the presence of Mas were unaffected by the presence of the Ang metabolite Ang-(1-7) (**Fig. 22d**), which has been described recently as an endogenous agonist for Mas.[143]

We next investigated whether impairment by Mas of Ang II-mediated activation of AT_1 is also detectable at the level of inositol phosphate production, for which less signal amplification exists than with Ca^{2+} mobilization (**Fig. 22e**). Evidently, co-expression of Mas impaired the potency and efficiency of Ang II-mediated generation of inositol phosphates in a manner similar to that observed in the Ca^{2+} mobilization assays (**Fig. 22a**).

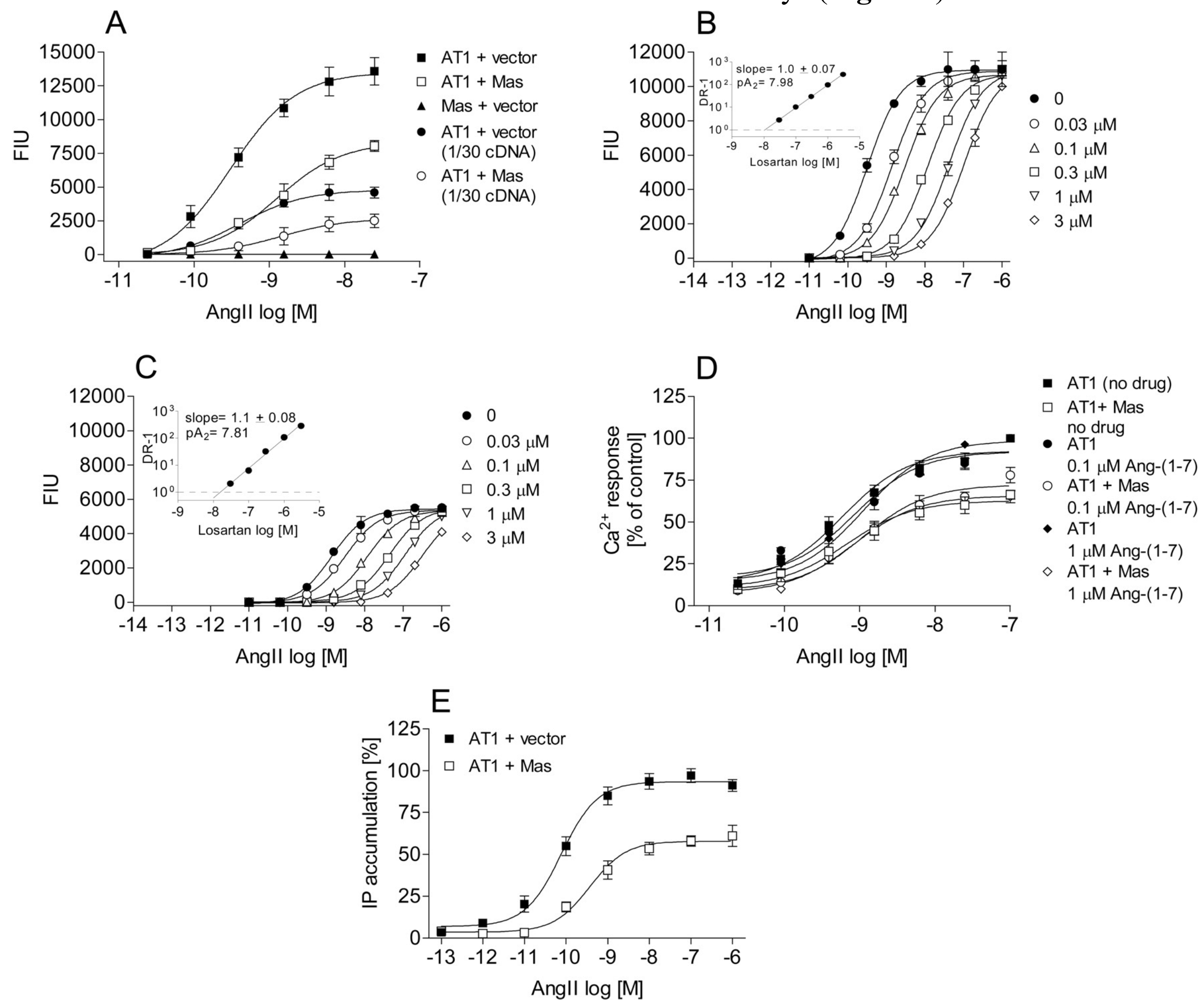

Figure 22 Coexpression with Mas reduces AT_1-mediated elevation of intracellular calcium and inositol phosphate generation. Mas and AT_1 were expressed in CHO-K1 cells either alone or in combination. pcDNA3.1 vector was used to equalize amounts of total DNA used in each transfection. (**A**), Elevation of $[Ca^{2+}]_i$ in response to varying concentrations of Ang II was recorded. Squares represent those curves performed after transfection of 2 µg of total DNA per 35-mm dish, and circles display curves recorded after transfection of 0.06 µg of total DNA per 35-mm dish. (**B**) and (**C**), Ang II concentration-response curve in presence of various concentrations of specific AT_1 antagonist losartan in CHO cells transiently transfected with AT_1 (**B**) or AT_1 and Mas (**C**). Insets in (**B**) and (**C**), Transformation of dose ratios into Schild plots for losartan. D, Concentration-response curve for Ang II in AT_1 versus AT_1/Mas-transfected cells in absence and presence of Mas agonist Ang-(1-7). (**A**), Representative experiment performed in triplicate. (**B**) and (**C**), Representative dose-response curves performed in duplicate; in Schild plots, each point is mean of 4 independent experiments. (**D**), Values are percentage of maximal Ang II-induced stimulation of $[Ca^{2+}]_i$ measured in AT_1-expressing cells. Data are mean±SD of at least 3 independent experiments performed in duplicate. (**E**), Elevation of inositol phosphates (IP) in response to varying concentrations of Ang II in IP accumulation assays. Values are expressed as percentage of maximal Ang II-mediated IP production by AT_1. Data are mean±SD of 3 independent experiments performed in duplicate. Where not shown, error bars lie within dimensions of symbols. FIU indicates fluorescence intensity units; DR, dose ratio.

To explore the specificity of the effect of Mas on AT_1-mediated mobilization of $[Ca^{2+}]_i$, the AT_1 was co-expressed with a series of other class I GPCRs. In contrast to the effects of Mas, Ang II mobilization of $[Ca^{2+}]_i$ was unaffected by the presence of the Gq/11-coupled sphingosine-1-phosphate [S1P3], urotensin II, or muscarinic M3 acetylcholine receptors

(**Fig. 23**). Specific ligands of each receptor stimulated $[Ca^{2+}]_i$ in AT_1-co-transfected cells in a manner identical to that observed with each receptor alone, and Mas did not interfere with $[Ca^{2+}]_i$ mobilization of the bona fide Gq-coupled urotensin II receptor (**data not shown**). Mas thus appeared to act specifically as a functional antagonist of the AT_1, and it can be excluded that AT_1 is, in general, an indiscriminate coupling partner for other GPCRs or that Mas represents an indiscriminate inhibitor.

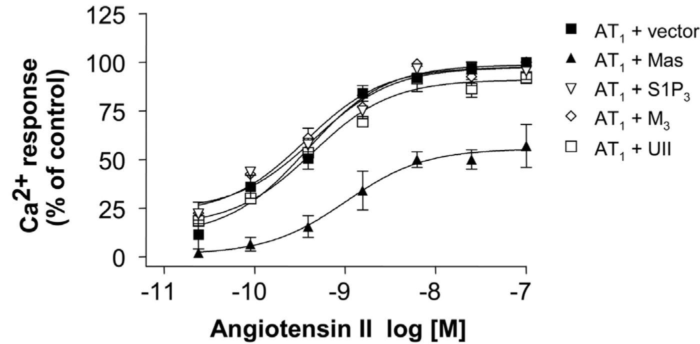

Figure 23 AT_1-mediated elevation of intracellular $[Ca^{2+}]_i$ is not affected by coexpression of range of G protein-coupled receptors (GPCR). AT_1 was coexpressed with pcDNA3.1 vector DNA (control) or series of GPCRs (Mas, sphingosine-1-phosphate [S1P3], muscarinic M3, and urotensin II receptor) in CHO-K1 cells, and Ang II-mediated mobilization of intracellular calcium was recorded in functional FLIPR assay as described in Figure 1. Only coexpression with Mas significantly reduced functional response of Ang II. Values are percentage of maximal Ang II-induced stimulation of $[Ca^{2+}]_i$ measured in AT_1-expressing cells. Data are mean±SD of at least 3 independent experiments performed in duplicate.

Because it was possible that the antagonistic effect of Mas on AT_1 signalling was due to a reduction in AT_1 expression, we performed $[^3H]$Ang II binding experiments to directly determine B_{max} and K_d for the radioligand to AT_1 in the absence and presence of Mas. In contrast to the effects observed on Ang II-mediated signalling, co-expression of Mas resulted in a significant enhancement (235%) of the $[^3H]$Ang II B_{max} value; there was no significant effect on radioligand affinity (**Fig. 24**). The paradoxical effect of *Mas* expression on $[^3H]$Ang II binding capacity was not due to radioligand binding to Mas, because there was no specific binding observed in cells transfected with Mas plus vector alone (**Fig. 24 inset**). One possible explanation for the enhancement in $[^3H]$Ang II B_{max} is an alteration in steady state cell-surface expression due to a change in AT_1 trafficking properties, as has been demonstrated for other GPCR heterodimers.[278]

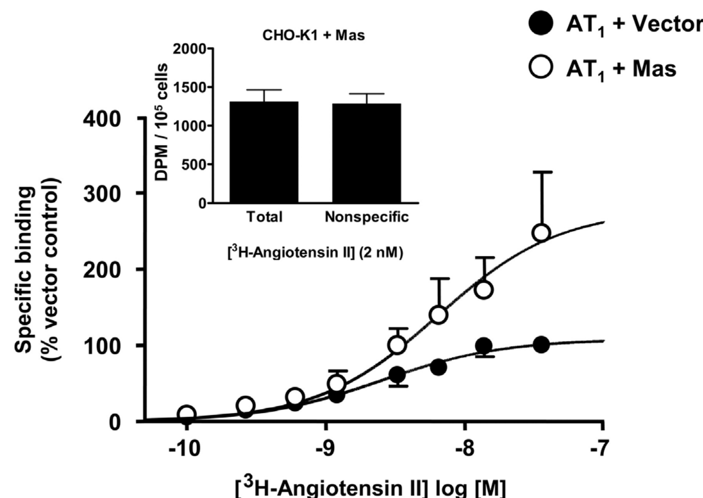

Figure 24 Coexpression with *Mas* does not reduce expression of AT_1. Saturation binding of $[^3H]$Ang II to CHO-K1 cells transiently cotransfected with human AT_1 together with empty pcDNA3.1 vector (●) or with vector containing human *Mas* gene (○). Nonlinear regression analysis yielded negative log dissociation constants of 8.13±0.13 and 8.45±0.08 (n=3), respectively, for control and Mas-transfected cells. Owing to variations in transfection efficiency, data are expressed in terms of percentage of maximal binding (*ca.* 480 fmol per 10^5 cells) observed in absence of *Mas* cotransfection. Inset, Lack of binding of 2 nmol/l $[^3H]$Ang II in CHO cells transfected with *Mas* alone. DPM indicates disintegrations per minute.

The differential effects on AT_1-mediated Ang II binding capacity on the one hand and signalling on the other by the co-expression of Mas suggested that the AT_1 was physically altered by Mas such that the enhanced cell-surface expression of the AT_1 was offset by reduced receptor functionality, most likely due to a conformational constraint on AT_1 signalling within the hetero-oligomer. The potential for such direct interactions between *Mas* and the AT_1 was thus investigated with various forms of BRET.[274,279,280] eYFP was appended to the C-terminal tail of the AT_1 from which the stop codon was eliminated. When this construct was expressed transiently in HEK293 cells, fluorescence microscopy demonstrated it to be present largely at the cell surface (**data not shown**). Such eYFP-tagged constructs act as appropriate energy acceptors of light generated from the oxidation of coelenterazine by the luciferase from *Renilla reniformis* if the luciferase and eYFP are within 50 to 100 Å.[279,280] Mas was modified by the in-frame addition of *Renilla* luciferase to the C-terminal tail of this polypeptide. Expression of Mas-*Renilla* luciferase in HEK293 cells followed by the addition of coelenterazine resulted in the generation of luminescence with a single peak centred at 480 nm (**Fig. 25a**). By contrast, addition of coelenterazine to HEK293 cells transfected to express a BRET-positive control, consisting of a fusion protein between *Renilla* luciferase and eYFP, resulted in both the peak at 480 nm and a second peak at 527 nm that reflects energy transfer (**Fig. 25b**).[275,277] Co-expression of Mas-*Renilla* luciferase and AT1-eYFP followed by the addition of coelenterazine also generated a spectrum consistent with strong energy transfer between the luciferase and eYFP (**Fig. 25a**). Such results are consistent with the hypothesis that these 2 GPCRs are able to form an oligomeric complex.[271,281] This interaction was constitutive, because it was not altered significantly by addition of receptor ligands that act as agonists at the AT_1 but not at Mas (Ang II), at Mas but not at the AT_1 [Ang-(1-7)], or as an antagonist at the AT_1 (losartan; **Figs. 25a and 25b**).[143]
Co-expressed GPCRs frequently display a propensity to interact and can often be co-immunoprecipitated.[276] It was important to examine whether the interaction between Mas-*Renilla* luciferase and AT_1-eYFP was selective. We therefore co-expressed Mas-*Renilla* luciferase with a C-terminal eYFP-tagged form of the TRH receptor-1.[274] We have previously shown this receptor construct to interact with only low affinity with the KOP opioid receptor.[274] TRH receptor-1-eYFP was expressed at similar levels as AT_1-eYFP as monitored directly by the fluorescence that corresponded to eYFP. No significant BRET signal was obtained (**Fig. 25c**), which suggests there was little or no interaction between Mas and TRH receptor-1.
We also wished to explore direct protein-protein interactions between the AT_1 and Mas with co-immunoprecipitation. Because available antisera against the native receptors were unable to immunoprecipitate with high efficiency (**data not shown**), we used the AT_1-eYFP construct in conjunction with a form of Mas tagged at the C-terminus with the G protein $G\alpha_{11}$. Immunoprecipitation with an anti-GFP antiserum of transfected HEK293 cells resulted in co-immunoprecipitation of Mas-$G\alpha_{11}$ when the 2 receptor constructs were co-expressed but, importantly, not when lysates of cells that individually expressed either AT_1-eYFP or Mas-$G\alpha_{11}$ were mixed before immunoprecipitation (**Fig. 25d**). In addition to polypeptides in the region of 75 to 80 kDa that were consistent with the anticipated size of Mas-$G\alpha_{11}$, a band of some 60 kDa was detected in the co-immunoprecipitation studies and may represent a proteolytic fragment.

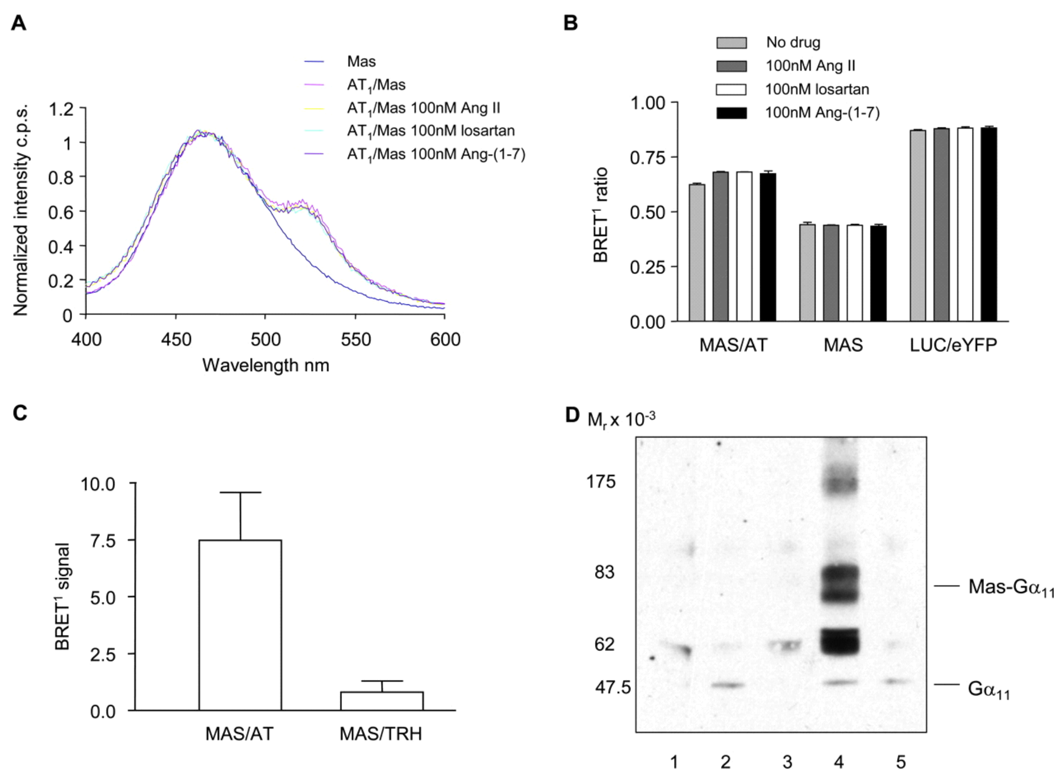

Figure 25 Hetero-oligomeric interactions after coexpression of Mas and AT_1. *Mas-Renilla* luciferase was expressed in isolation in HEK293 cells (**A**, purple; **B**, Mas) or in combination with AT_1-eYFP (**A**, all other colors; **B**, MAS/AT). In certain experiments, fusion protein between *Renilla* luciferase and eYFP was expressed (**B**, LUC/eYFP). Coexpressions of Mas-*Renilla* luciferase with AT_1-eYFP (MAS/AT) and Mas-*Renilla* luciferase with TRH receptor-1–eYFP (MAS/TRH) were also performed (**C**). Coelenterazine and either no ligand (**A**, purple; **B**, light gray), 100 nmol/l Ang II (**A**, yellow; **B**, dark gray), 100 nmol/l losartan (**A**, blue; **B**, white), or 100 nmol/l Ang-(1-7) (**A**, red; **B**, black) were added. Emission spectra were then collected on Spex fluorolog spectrofluorimeter (**A** and **C**), or BRET data (**B**) were measured with Mithras LB 940 as detailed in Methods. (**D**), HEK293T cells were mock-transfected (1) or transfected to express AT_1-eYFP (2), Mas-$G_{\alpha 11}$ (3), or both AT_1-eYFP and Mas- $G_{\alpha 11}$ (4). In (5), cells expressing either AT_1-eYFP or Mas-G $G_{\alpha 11}$ were mixed. Lysates from these cells were immunoprecipitated with anti-eYFP antibody, and after resolution by SDS-PAGE, immunoblot was performed with anti-$G_{\alpha q}$/ $G_{\alpha 11}$ antiserum. c.p.s. indicates counts per second.

In “saturation” BRET assays, the ratio of the energy acceptor to energy donor is varied over a significant range. With increasing ratio of acceptor to donor, it is expected that a maximal BRET signal will be reached when all donor molecules interact with an acceptor.[282] A hyperbolic saturation curve should thus be generated for pairs of interacting proteins in contrast with non-interacting proteins. Recent developments in BRET technology have improved the signal to background noise ratio that can be obtained by introduction of a novel luciferase substrate that, on oxidation, emits light at shorter wavelengths. This results in less spill-over into the region of the spectrum used to measure energy transfer. So-called $BRET^2$ was thus used in saturation BRET assays.[274,282] The ratio of co-expression of Mas-*Renilla* luciferase and the AT_1 linked to the modified fluorescent protein GFP^2 was varied in populations of HEK293 cells and calculated as in Methods. Addition of deep blue coelenterazine (DeepBlueC) as substrate resulted in energy transfer that approached saturation with increasing energy acceptor (AT_1-GFP^2) to energy donor (*Mas-Renilla* luciferase) ratios (**Fig. 26a**). As a control, Mas-*Renilla* luciferase and the isolated GFP^2 were co-expressed in varying ratios. The $BRET^2$ signal was almost undetectable. It was much smaller at equal energy acceptor to energy donor ratios and did not saturate but simply increased in a linear fashion with increasing GFP^2 to Mas-*Renilla* luciferase expression ratios (**Fig. 26a**). Isolated GFP^2 is a soluble polypeptide and thus is not anticipated to be in the same cellular compartment as Mas-*Renilla* luciferase. The lack of energy transfer between co-expressed Mas-*Renilla* luciferase and GFP^2 also confirms that the energy transfer interactions generated by the Mas-AT_1 pairing reflect interactions between the GPCRs and not between the luciferase and GFP^2. To investigate the functional consequences that varying levels of

Mas exert on AT_1 signalling, Ang II-mediated Ca^{2+} elevation was measured in CHO cells transiently co-transfected with a fixed amount of AT_1 and increasing amounts of Mas cDNA (**Fig. 26b**). Notably, impairment by Mas of Ang II-mediated Ca^{2+} release via AT_1 was inversely correlated to the amounts of Mas cDNA used in the co-transfection experiments. These data imply that AT_1 signalling *in vivo* may be regulated in a cell- and tissue-specific manner by the level of Mas expression.

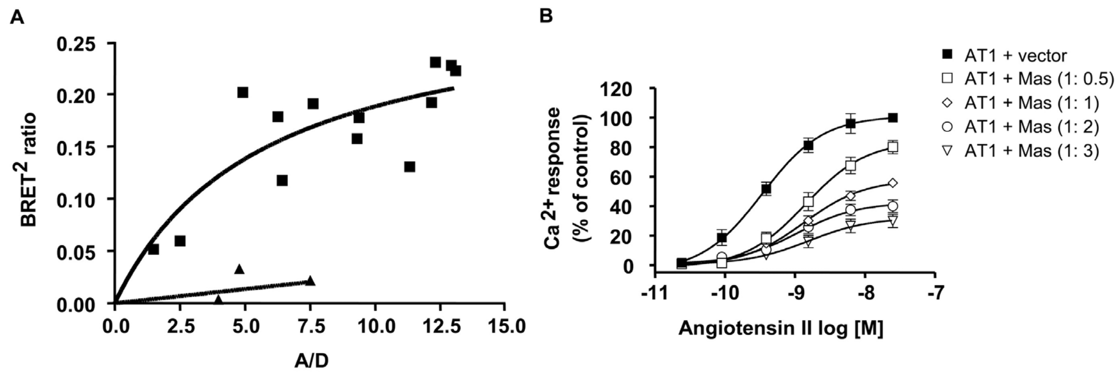

Figure 26 Analysis of interactions between Mas and AT_1 with saturation $BRET^2$ and Ca^{2+} mobilization. (**A**), Varying amounts of Mas-*Renilla* luciferase were coexpressed with either GFP^2 (▲) or AT_1-GFP^2 (■). Energy acceptor (A; GFP^2 or AT_1-GFP^2) to donor (D; *Mas-Renilla* luciferase) ratios were calculated and plotted against $BRET^2$ energy transfer signals obtained after addition of DeepBlueC to cells . Data for Mas/AT_1 were fit by hyperbolic curve with $BRET^2$ max=0.30 and $BRET^2_{50}$ achieved at A/D ratio=5.7. (**B**), Varying amounts of Mas cDNA were coexpressed with fixed amount of AT_1, and Ang II–mediated elevation of Ca^{2+} was recorded. pcDNA3.1 vector DNA was used to equalize total amount of cDNA in transfections. Data are mean±SD of 4 experiments, each performed in duplicate.

The physiological relevance of hetero-oligomerization between the AT_1 and Mas was further investigated with mesenteric microvessels of wild-type mice, which express both receptor mRNAs as demonstrated by reverse transcription-polymerase chain reaction (**data not shown**), in comparison to *Mas*-knockout mice. Specifically, although Ang II produced robust contraction of vessels from wild-type mice in its own right, this effect was markedly enhanced in vessels from the *Mas*-knockout animals at all concentrations of Ang II (**Fig. 27a**). In both types of mice, these contractions were abolished by the AT_1 antagonist losartan (1 µmol/l). The specificity of the effect of genetic ablation of *Mas* on Ang II-mediated contractions was further confirmed by measuring the effect of another vasoconstrictor peptide, endothelin-1, in *Mas*-deficient microvessels. Although the absolute response to endothelin-1 was greater than that observed for Ang II in microvessels of both genotypes, the pattern of the contractile response in the absence versus the presence of Mas was different for one vasoconstrictor peptide relative to the other. *Mas*-deficient microvessels did not show a general over-reactivity but rather a less pronounced contractile response to higher endothelin-1 concentrations compared with wild-type vessels (**Fig. 27b**).

Taken together, our findings demonstrate that the protein encoded by the *Mas* proto-oncogene exhibits direct antagonistic properties on the AT_1 *in vitro* and that this oligomeric interaction may represent a natural state for these receptors *in vivo* in some tissues. Specifically, we have shown that the expression of Mas interferes with functional activity of the AT_1 in transfected mammalian cell lines *in vitro*, even though it enhances maximal cell-surface expression of the AT_1, and that the mechanism is most likely a consequence of the constitutive physical association between the 2 receptors *ex vivo*. Native tissue data strongly support the cell-line findings, because the absence of the receptor Mas in *Mas*-knockout animals significantly enhanced the vasoactive properties of Ang II but showed a different pattern of effect on those induced by endothelin-1. The fact that the increased response to Ang II was abolished by the AT_1 antagonist losartan, with pharmacology comparable to that of wild-type-derived vessels (**Fig. 27a**), clearly indicates an AT_1-specific effect in the native tissues. In conjunction with the oligomerization demonstrated in the BRET studies, the present findings in native tissues suggest that the receptor Mas can act as an *in vivo* functional

antagonist of the AT_1 owing to formation of a hetero-oligomeric complex. Given that recent evidence has also shown that the AT_2 can act as an antagonist of AT_1 function, at least *in vitro*, it is likely that these collective findings are indicative of a novel property of GPCR signalling, namely, the direct regulation of signal transduction via one GPCR by the physical presence of another.[72]

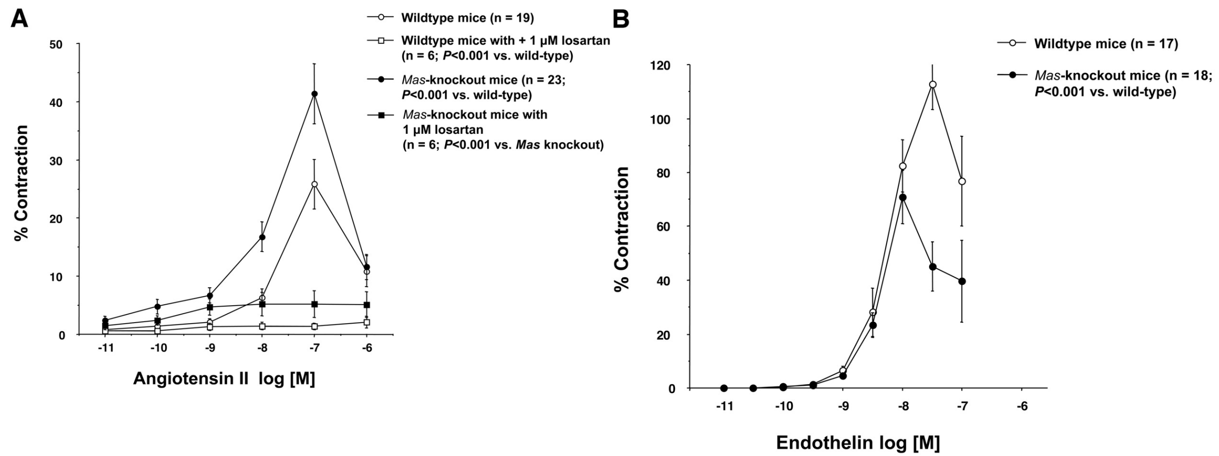

Figure 27 Enhanced vasoconstrictor activity of Ang II in mesenteric microvessels from *Mas*-knockout mice. (**A**), Contractile responses to Ang II in isolated mesenteric microvessels from wild-type and *Mas*-knockout mice, either in absence or presence of 1 µmol/l losartan. Vasoactive responses are expressed as percentage of previous contraction elicited by 125 mmol/l K^+, which yielded values as follows: wild-type, 3.20±0.37 and 3.27±0.54 mN in untreated and losartan-treated segments, respectively; *Mas* knockout, 3.19±0.38 and 3.82±0.38 mN in untreated and losartan-treated segments, respectively (not significant). (**B**), Contractile responses to endothelin-1 in isolated mesenteric microvessels from wild-type and *Mas*-knockout mice. Vasoactive responses are expressed as percentage of previous contraction elicited by 125 mmol/l K^+, which yielded values as follows: wild-type, 4.52±0.34 mN; *Mas* knockout, 4.09±0.32 mN (not significant). Number of segments used and significance between curves are in parentheses. For each curve, mesenteric microvessels from 3 to 8 animals were used.

6.5 Acknowledgment

The study was supported by the Medical Research Council UK (G.M.) and the Sonnenfeld-Stiftung (T.W.).

Chapter 7

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

Based on: Gembardt F, Heringer-Walther S, van Esch JH, Sterner-Kock A, van Veghel R, Le TH, Garrelds IM, Coffman TM, Danser AH, Schultheiss HP, Walther T. Cardiovascular phenotype of mice lacking all three subtypes of angiotensin II receptors. *FASEB J.* **2008**;22(8):3068-3077

7.1 Abstract

Angiotensin II activates two distinct receptors, the angiotensin II receptors type 1 (AT_1) and type 2 (AT_2). In rodents, two AT_1 subtypes were identified (AT_{1a} and AT_{1b}). To determine receptor-specific functions and possible angiotensin II effects independent of its three known receptors we generated mice deficient in either one of the angiotensin II receptors, in two, or in all three (triple knockouts). Triple knockouts were vital and fertile, but survival was impaired. Hypotension and renal histological abnormalities in triple knockouts were comparable to those in mice lacking both AT_1 subtypes. All combinations lacking AT_{1a} were distinguished by reduced heart rate. AT_{1a} deletion impaired the *in vivo* pressor response to angiotensin II bolus injection, whereas deficiency for AT_{1b} and/or AT_2 had no effect. However, the additional lack of AT_{1b} in AT_{1a}-deficient mice further impaired the vasoconstrictive capacity of angiotensin II. Although general vasoconstrictor properties were not changed, angiotensin II failed to alter blood pressure in triple knockouts, indicating that there are no other receptors involved in direct angiotensin II pressor effects. Our data identify mice deficient in all three angiotensin II receptors as an ideal tool to better understand the structure and function of the renin-angiotensin system and to search for angiotensin II effects independent of AT_1 and AT_2.

7.2 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.[16] Ang II mediates its functions through stimulation of two pharmacologically defined receptors, the Ang II receptor type 1 (AT_1) and the Ang II receptor type 2 (AT_2).[16] Molecular cloning of AT_1 and AT_2 has shown that both receptors belong to the family of G protein-coupled receptors (GPCRs).[16] The two Ang II receptor subtypes have a low sequence homology (~35%), with the highest similarity in the seven predicted transmembrane domains.[16] In rodents, two isoforms of 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).[283] The AT_1 subtype is present in almost all tissues, and most of the known actions of Ang II are mediated through its stimulation. The AT_{1a} isoform predominates in most organs, including, e.g., heart, aorta, kidney, lung, and brain,[23] whereas the AT_{1b} isoform is abundantly expressed in pituitary gland and adrenal cortex.[23,284] AT_2 expression in adult tissue is restricted to a few organs, i.e., brain, adrenal, heart, vascular endothelium, kidney, and ovary,[285] whereas it is widely distributed in fetal tissues.[50] The predominant expression of AT_2 in fetal tissues suggests a role of these receptors in differentiation and developmental processes.[286]

It was demonstrated in genetically modified mice, lacking either the AT_{1a} or AT_{1b} isoforms, that blood pressure control is dominated by the AT_{1a} isoform. Baseline blood pressure was significantly decreased in mice lacking AT_{1a},[30] whereas AT_{1b}-deficient animals were normotensive.[31] Interestingly, AT_2 knockout mice showed an increase in blood pressure under basal conditions,[32] supporting the hypothesis of its AT_1-counteracting effects in blood pressure regulation.[287]

The vasoconstrictor effect of exogenous Ang II was almost completely blunted in AT_{1a} knockout mice,[30] whereas animals lacking AT_{1b} showed normal responsiveness to Ang II.[31] In AT_2 knockout mice, Ang II stimulation led to a significant pronounced vasoconstriction, a further indicator for the AT_1-counteracting effects of AT_2 on blood pressure regulation.[32] The kidney abnormalities observed in mice lacking angiotensinogen (Agt)[288] or angiotensin-converting enzyme (ACE),[12] such as cortical thinning, focal areas of atrophy, and thickened

walls of renal vessels, were less pronounced in AT_{1a} knockout mice[30] and absent in AT_{1b} and AT_2 knockout mice,[32] whereas mice lacking AT_{1a} and AT_{1b} showed a phenotype similar to that of Agt and ACE knockout mice.[31]
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 and normal renal development. Furthermore, we wanted to clarify whether there are Ang II effects on blood pressure control not related to one of the known Ang II receptor subtypes.

7.3 Materials and methods

7.3.1 Animals

AT_{1a} (-/-//+/+//+/+),[30] AT_{1b} (+/+//-/-//+/+),[31] and AT_2 (+/+//+/+//-/-)[32] single-knockout mice were used to generate the recently described AT_{1a}/AT_{1b} double-knockout mice (-/-//-/-//+/+),[31] with 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 (WT) (+/+//+/+//+/+), double-knockout, and triple-knockout mice, the heterozygous offspring were intercrossed and bred on a 129×C57BL/6 background at the animal facilities of Charité Berlin, Campus Benjamin Franklin, Berlin, Germany, whereby experimental homozygous animals were generated by cross-breeding of heterozygous mice to ensure the most similar genetic background. For basal cardiac phenotyping and Ang II infusion, a set of 7-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 end point of 12 months. In addition, age-matched animals lacking Agt[27] were used for histological comparison. Experiments were performed according to the regulations of the Animal Care Committee of the Erasmus Medical Centre, in accordance with the “APS Guiding Principles for the Care and Use of Animals in Research” (American Physiological Society) or according to the guidelines of the federal law on the use of experimental animals in Germany (Animal Welfare Act) and were approved by the local authorities.

7.3.2 Polymerase chain reaction (PCR) (genotyping)

Genotyping was performed by PCR with specific primers for each genotype. PCRs for AT_{1a} and AT_2 were described earlier.[289] For AT_{1b}-specific PCRs, two pairs of primers that amplified products specific for the AT_{1b} WT (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.[290]

7.3.3 RNA isolation and RNase protection assay

RNA of frozen organs was isolated using TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) following the manufacturer’s protocol as described previously.[291]
AT_{1a}, AT_{1b}, and AT_2 expressions were analyzed by RNase protection assays using the commercially available RPA II kit [Ambion (Europe) Ltd., Huntingdon, UK], according to the manufacturer’s protocol. Thirty micrograms of total RNA was hybridized with one of the following probes:
Probe MMAT1A: A recently described vector[292] was used to transcribe a radioactive probe complementary to a 352-bp fragment specific for AT_{1a} mRNA.
Probe MMAT1B: 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 sub-cloned 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 (due to sequence homology between AT_{1a} and AT_{1b} between bp 1 and 170) and a 255-bp fragment specific for AT_{1b} mRNA (whole sequence protected).
Probe MMAT2: A 468-bp fragment from genomic mouse DNA, amplified by PCR using the genotyping primers,[289] was sub-cloned 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 (BD PharMingen International, San Diego, CA, USA), complementary to a 127-bp fragment of rL32 mRNA, was used as a housekeeping control as described earlier.[210]
RNA samples were hybridized with 40,000 cpm of the radiolabelled probe for MMAT1A, MMAT1B, or MMAT2 and 20,000 cpm of the radiolabelled 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% polyacrylamide, 8 mol/l urea) and analyzed using a FUJIX BAS 2000 PhosphoImager system (Raytest GmbH, Straubenhardt, Germany). Quantitative analysis was performed by measuring the intensity of the AT_{1a} bands normalized by the intensity of rL32.

7.3.4 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 in inside diameter × 0.025 in outside diameter) 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 to 15 min, 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). The hemodynamic response to Ang II (0.5 µM 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 min), the response to 0.5 mM phenylephrine (PE) (100 µl) was determined, and this protocol was repeated for 2 µM 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 (KW)/body weight (BW) and heart weight (HW)/BW were used as indices of organ hypertrophy.

7.3.5 Ang 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.[293] Both were stored at -80°C until further processing. Ang II was determined by radioimmunoassay after SepPak extraction and high-performance liquid chromatography separation as described before .[294]

7.3.6 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 or periodic acid-Schiff (PAS).

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

7.4 Results

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

Cross-breeding 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 28a** shows PCR results identifying a WT (+/+//+/+//+/+) mouse (top panel) and a triple-knockout mouse (bottom panel).

All eight receptor combinations were vital and fertile, although survival in AT_{1a}/AT_{1b} double- and triple-knockout mice was significantly impaired until weaning (**Fig. 28b**). However, mortality was not increased in these mice from weaning to the defined end point of 12 months (**data not shown**).

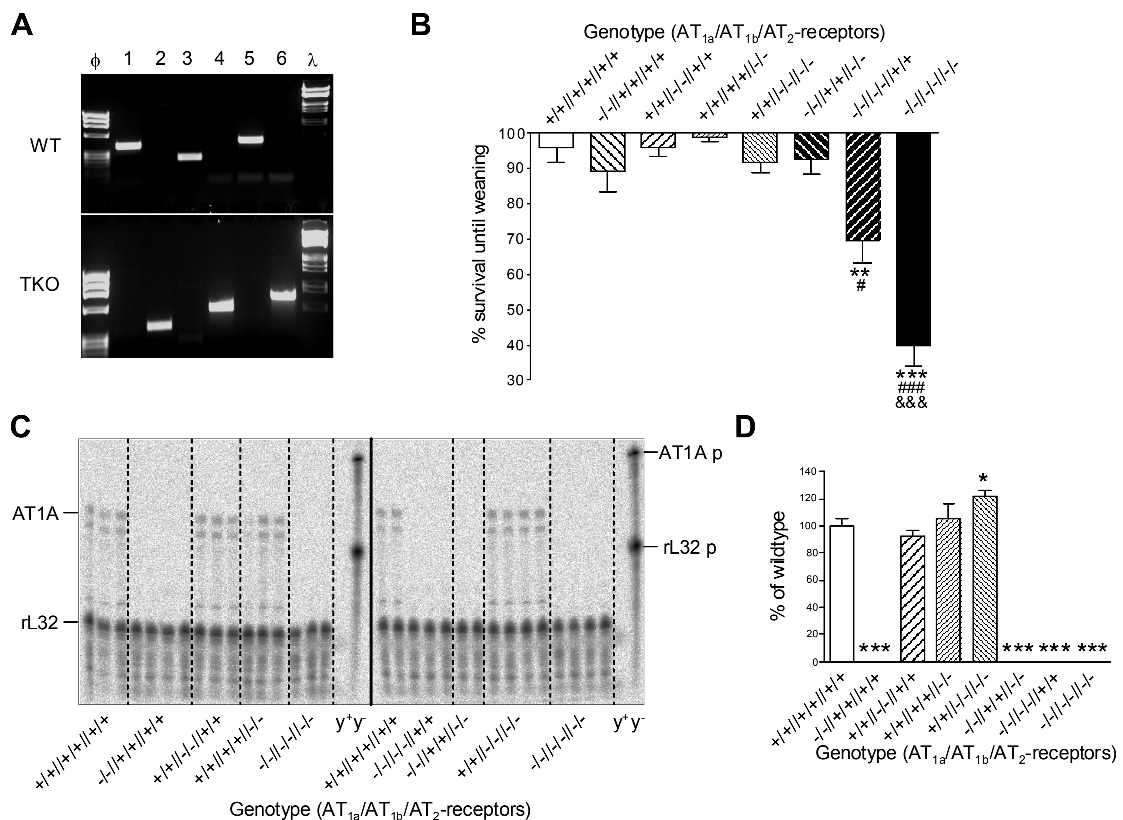

Figure 28 (**A**) The genotyping PCRs for AT_{1a} (lanes 1 and 2), AT_{1b} (lanes 3 and 4), and AT_2 (lanes 5 and 6) produced distinct bands for WT (top row) and knockout (bottom row) alleles, depending on the appearance or absence of the Ang II receptors. TKO, triple-knockout; Φ, ΦX174 DNA (*Bsu*RI digested) size marker; λ, λ DNA (*Eco*RI+*Hind*III digested) size marker. (**B**) Survival of pups until weaning for all eight genotypes. Data are shown as percentage of surviving mice per litter. $^{**}P<0.01$, $^{***}P<0.001$ *vs.* +/+//+/+//+/+; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ *vs.* -/-//+/+//+/+; $^{\&\&\&}P<0.001$ *vs.* -/-//-/-//+/+ ($n\geq8$). (**C**) Representative RNase-protection assays showing AT_{1a} receptor (352 bp) and rL32 (127 bp) expression in the heart. The undigested AT_{1a} and rL32 probes are shown in y^- (control lane). y^+, yeast RNA with RNase A; y^-, yeast RNA without RNase A. (**D**) 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 mice were set to 100%. $^{*}P<0.05$; $^{***}P<0.001$ *vs.* +/+//+/+//+/+ ($n\geq6$).

7.4.2 Regulation of Ang II receptors in different knockout variants

To clarify the effects of the genetic modifications on the expression level of the three Ang II receptors, we measured receptor-specific mRNA in the heart. **Figure 28c** shows representative RPA blots of heart RNA using an AT1a-specific probe (MMAT1A). All 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} (+/+//-/-//-/-)

(**Fig. 28d**). Investigation of the samples with a probe discriminating between AT_{1a} and AT_{1b} (MMAT1B) revealed the same pattern of regulation for AT_{1a} mRNA. However, AT_{1b}- and AT_2-specific mRNA expression was below the RPA detection level, independent of the receptor combination used (**data not shown**) as proven with this probe and the specific probe for AT_2 mRNA (MMAT2).

7.4.3 Basal phenotyping

BW recording confirmed previous findings that the lack of just one Ang II receptor did not influence body weight (**Tab. 3**).[30-32] 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.[31] Interestingly, this BW reduction was partially compensated for in triple knockouts. Notably, the observed reduction in BW was unrelated to the size of the animals as tibia length was unchanged (+/+//+/+//+/+: 2.16±0.03 cm; -/-//-/-//-/-: 2.17±0.06 cm).
In all variants deficient for AT_{1a}, the HW/BW was decreased (**Tab. 3**), with the lowest HW in AT_{1a}/AT_{1b} double-knockouts. A less pronounced HW reduction was seen in AT_2-deficient animals.

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 *** ##

Table 3 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; * P<0.05; ** P<0.01; *** P<0.001 vs. +/+//+/+//+/+; # P<0.05; ## P<0.01 vs. -/-//+/+//+/+; & P<0.05 vs. -/-//-/-//+/+; n≥6).

KW/BW ratio was unaltered in AT_{1a} and AT_{1b} single-knockout mice (**Tab. 3**). In contrast, animals deficient for AT_2 showed a reduced KW/BW ratio. KW 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, the KW/BW ratio was significantly reduced.[31] However, we observed a further, profound reduction of the 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 (**Fig. 29**). In mice expressing only AT_{1a}, the Ang II plasma level was unchanged (98.8±34.0 fmol/ml), compared with that in WT mice (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 II plasma concentration (885.8±217.2 fmol/ml). Notably, Ang II levels in triple knockouts (315.8±104.7 fmol/ml) ranged between those for WT and AT_{1a}/AT_{1b} double-knockouts without significant differences compared with any other group.

7.4.4 Renal histopathological examination

As described earlier, kidneys of AT_2-deficient animals showed morphology comparable to that of WT animals (**Fig. 30**, top panel, left) and were characterized by an inconspicuous histological appearance (**Fig. 30**, middle panel, left).[295] 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 (**Fig. 30**, bottom panel, left), and media hyperplasia of small- and medium-sized vessels.[31]

Furthermore, kidneys of these AT_{1a}/AT_{1b}-deficient mice showed prominent renal mononuclear infiltrates, partially with a blastic appearance and a high rate of partial mitosis, 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 performed. The additional deficiency in AT_2 did not affect 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 either AT_{1a} or AT_{1b} exclusively to investigate whether one of the AT_1 subtypes alone is capable of restoring 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 (**Fig. 30**, middle panel, middle). In contrast, kidneys of mice harbouring only the AT_{1b} showed multifocal glomeruli atrophy, partially with a cystic dilated Bowman's space (**Fig. 30**, middle panel, right). Nevertheless, compared with the severely changed phenotype observed in kidneys of AT_{1a}/AT_{1b} double-knockouts and triple-knockouts, kidneys harbouring only AT_{1b} had less damaged glomeruli, and the majority were 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 Agt gene coding for the angiotensin precursor. Morphohistological investigations revealed that kidneys of Agt-deficient mice showed the same malformations (**Fig. 30**, bottom panel, right) as mice lacking both AT_1 subtypes and as the triple-knockouts.

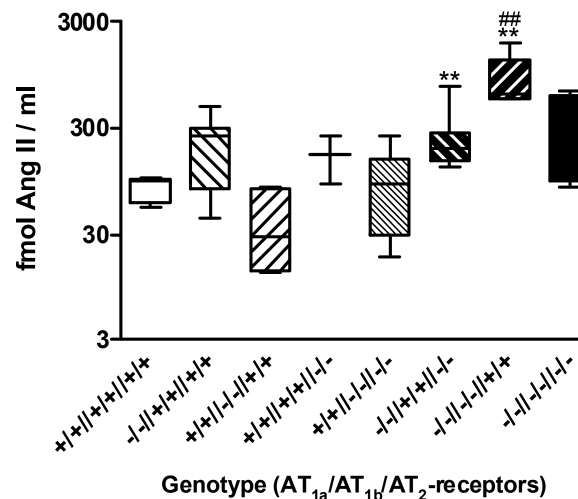

Figure 29 Ang II levels in blood plasma of mice of all eight genotypes. Boxes show median with interquartile range. ***P*<0.01 *vs.* +/+//+/+//+/+; ##*P*<0.01 *vs.* -/-//+/+//+/+ (*n*=2 for +/+//+/+//-/- and *n*≥5 for all other groups).

7.4.5 Effects of Ang II receptors on basal hemodynamic parameters

Baseline MAP in AT_{1a}-deficient mice was markedly reduced (**Tab. 3**) compared with that in WT animals. The lack of only 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.[31] We also confirmed the rise in blood pressure in AT_2 knockout mice.[32] However, in contrast to AT_{1b}, the lack of AT_2 on top of AT_{1a} deficiency did not modify the hypotensive phenotype. Interestingly, the AT_{1b} deficiency also 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 (**Tab. 3**). Deficiencies of 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.

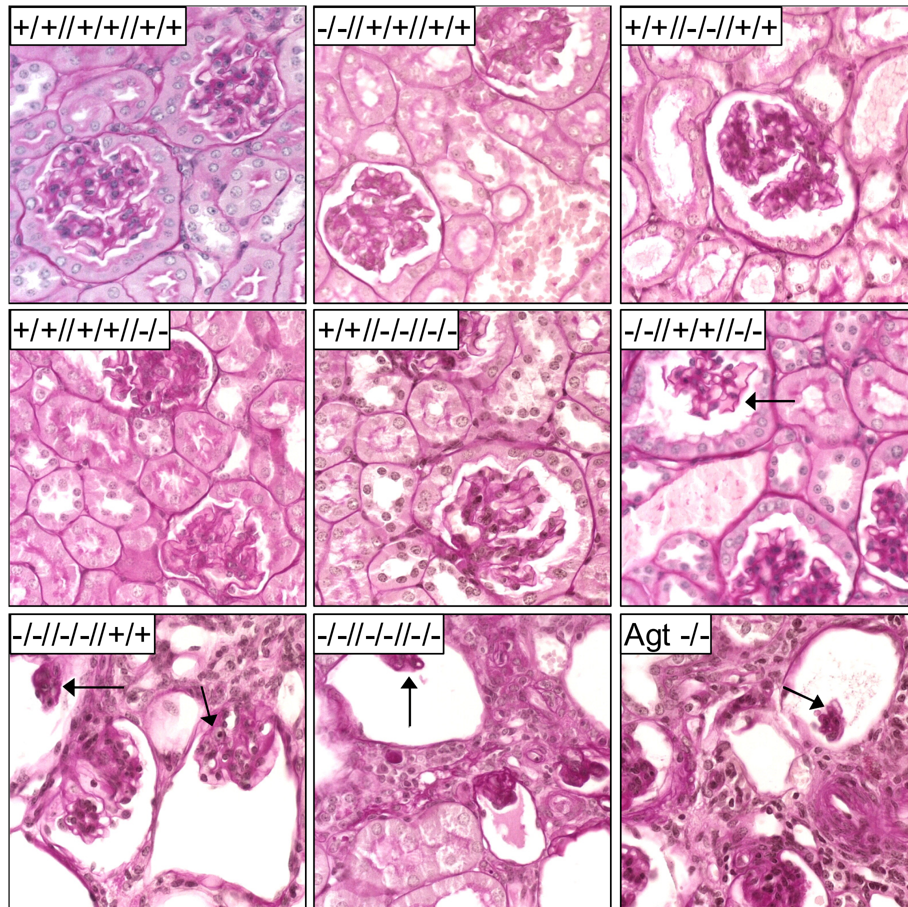

Figure 30 PAS-stained sections of kidneys of WT (+/+//+/+//+/+), AT_{1a} (-/-//+/+//+/+), AT_{1b} (+/+//-/-//+/+), and AT_2 (+/+//-/-//+/+) single-knockout mice, mice exclusively expressing AT_{1a} (+/+//-/-//-/-), AT_{1b} (-/-//+/+//-/-), or AT_2 (-/-//-/-//+/+), triple knockouts (-/-//-/-//-/-), and Agt-deficient (Agt -/-) mice. Atrophic glomeruli are indicated by an arrow.

7.4.6 Effects of Ang II infusion

To clarify the effects of the three Ang II receptors on blood pressure and heart rate control, we administered a single bolus injection of Ang II. In WT mice, Ang II injection caused a fulminant increase in MAP and heart rate (**Fig. 31a-b**). 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 with AT_{1a} single-knockouts.

The time needed for normalization of MAP after Ang II infusion was significantly reduced in AT_{1a}/AT_{1b} double-knockout and triple-knockout mice (-/-//-/-//+/+: 151.0±33.7 s; -/-//-/-//-/-: 122.2±43.0 s) compared with WT mice (+/+//+/+//+/+: 286.9±20.6 s; $P<0.01$). Interestingly, the time until the peak occurred was unchanged in AT_{1a}/AT_{1b} double-knockouts compared with WT animals(+/+//+/+//+/+: 29.8±3.5 s; -/-//-/-//+/+: 39.9±6.5 s), whereas the peaking time was significantly shortened in triple-knockouts (-/-//-/-//-/-: 11.6±1.9 s; $P<0.01$ *vs.* +/+//+/+//+/+).

7.4.7 Effects of infusion of other vasoactive substances

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. First, 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 (**Fig. 32a**). Interestingly, the negative chronotropic effect of ET-1 in WT animals (**Fig. 32b**) 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 that in WT controls. Nevertheless, ET-1 infusion again mediated a positive chronotropic effect in animals deficient for all three Ang II receptor subtypes.
Second, we used the non-peptidic sympathicomimetic compound PE. In contrast with ET-1, the PE-stimulated α_1-adrenoreceptor-mediated increase in MAP was comparably reduced in AT_{1a}/AT_{1b} double-knockout (**Fig. 33a**) and triple-knockout animals compared with controls. In WT mice, administration of PE reduced heart rate by 12% (**Fig. 33b**). The PE effect on heart rate was inverted in AT_{1a} single-knockout mice to a positive chronotropic response, whereas deletion of AT_{1b} and AT_2 alone did not influence changes in heart rate observed in WT animals. Notably, additional AT_{1b} deficiency reversed the positive chronotropic effects of PE infusion seen in AT_{1a} single-knockouts. In contrast with all other genotype variants, PE infusion did not mediate any chronotropic effect in animals lacking all three receptor subtypes.

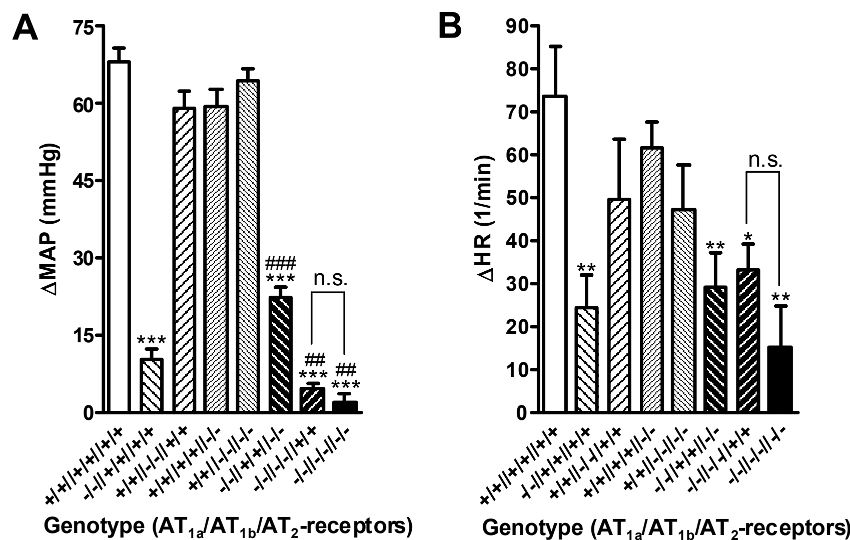

Figure 31 Changes in mean arterial pressure (MAP) (**A**) and heart rate (HR) (**B**) after a bolus injection of Ang II. ΔMAP, change in MAP after injection; ΔHR, change in HR after injection. *P<0.05, **P<0.01, ***P<0.001 vs. +/+//+/+//+/+; #P<0.05, ##P<0.01, ###P<0.001 vs. -/-//+/+//+/+ (n≥6).

7.5 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.[296] Inhibition of Ang II actions is achieved clinically by either blocking with antagonists against its type 1 receptors or preventing Ang II generation with ACE inhibitors.[297,298] Both pharmacological interventions are used primarily for blood pressure control, but research during the last decade has demonstrated that they also provide cardiovascular protective effects that are independent of blood pressure lowering, as, e.g., evidence was found that the anti-inflammatory component of both drugs is part of their success.[299]
The generation of AT_1- and AT_2-specific antagonists gave a first tool that allowed identification of 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 over-expressing Ang II type 1 receptors lacking intracellular signalling via heterotrimeric G proteins has shown that AT_1 can mediate effects, e.g., induction of cardiac hypertrophy and bradycardia, independent of Ang II.[300] 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 stimulation by Ang II. However, none of the single-receptor knockouts is capable of reproducing 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 specificity, because it could not be discriminated whether the observed phenotype was related to a lack of receptor stimulation due to the deficiency or due to an overstimulation of the residual two receptors. Therefore, we first generated new transgenic mice exclusively expressing either AT_{1a} or AT_{1b} for verification of Ang II-mediated AT_1 subtype-specific effects. Using these animals and the previously described mice deficient for both AT_1 subtypes[31] exclusively expressing AT_2, we could demonstrate that basal blood pressure and heart rate are mainly affected by AT_{1a}, whereas the role of AT_{1b} differs between both parameters. In mice exclusively expressing AT_{1a}, basal blood pressure is slightly increased (~118% of WT), probably due to higher expression of AT_{1a} (**Fig. 28** and **Tab. 3**) and/or the missing inhibitory properties of AT_2 on AT_{1a} signalling.[72,301] Mice expressing AT_{1b} solely show a less pronounced decrease in blood pressure (~70% of wild type) than the mice expressing only AT_2 (~55% of WT), indicating that AT_{1b} is partially capable of compensating for the lack of AT_{1a}. Although the reduced blood pressure in all groups lacking AT_{1a} may account for the reduced HW/BW ratio, 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 with that in WT mice, whereas in mice lacking AT_{1a} and AT_{1b} it is reduced (~-23%). Mice uniquely expressing AT_{1b} show a reduction in heart rate (~-19%) similar to that of animals lacking only AT_{1a} (~-15%) or both AT_1 subtypes, suggesting that in regard to heart rate, the lack of AT_{1a} cannot be compensated for by AT_{1b} in contrast to partial normalization of blood pressure. This difference may be best explained by the lack of a valuable level of AT_{1b} in the heart but a significant expression of AT_{1b} in vessels.[23]

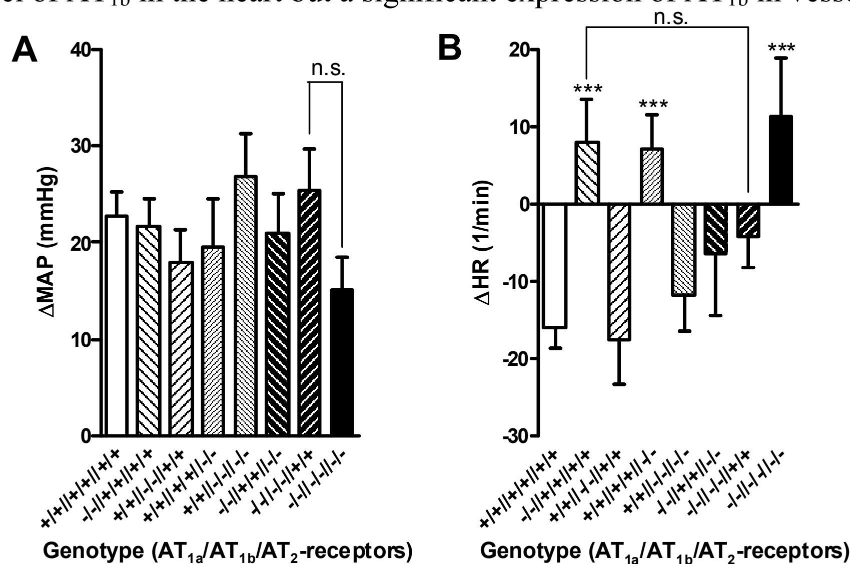

Figure 32 Changes in mean arterial pressure (MAP) (**A**) and heart rate (HR) (**B**) after a bolus injection of ET-1. ΔMAP, change in MAP after injection; ΔHR, change in HR after injection. ***$P<0.001$ *vs.* +/+//+/+//+/+ ($n \geq 6$).

We also determined that the formerly described blood pressure raising[302] and positive chronotropic[303] effects of exogenous Ang II were mediated primarily 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 WT), whereas the responsiveness to Ang II is partially restored in animals exclusively expressing AT_{1b} (~30% of WT). The Ang II-induced increase in heart rate in WT mice is not altered in animals harbouring only AT_{1a} compared with that in WT animals. In mice exclusively expressing either AT_{1b} or AT_2 and thus lacking AT_{1a}, the positive chronotropic effect of Ang II is substantially reduced (to ~40 and ~44% of that in WT mice, respectively). The inability of AT_{1b} to compensate for 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.
In recent investigations it was shown that chronic infusion of Ang II[304] and the lack of both AT_1 subtypes[305] are regulators of the endothelin system, e.g., affection of expression levels of ET-1 and its receptors. However, to our knowledge we are the first to administer 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}[305] 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. Thus, these distinct regulatory effects of the Ang II receptor subtypes on the endothelin system and their clinical relevance open new avenues in the research of peptide systems interactions.

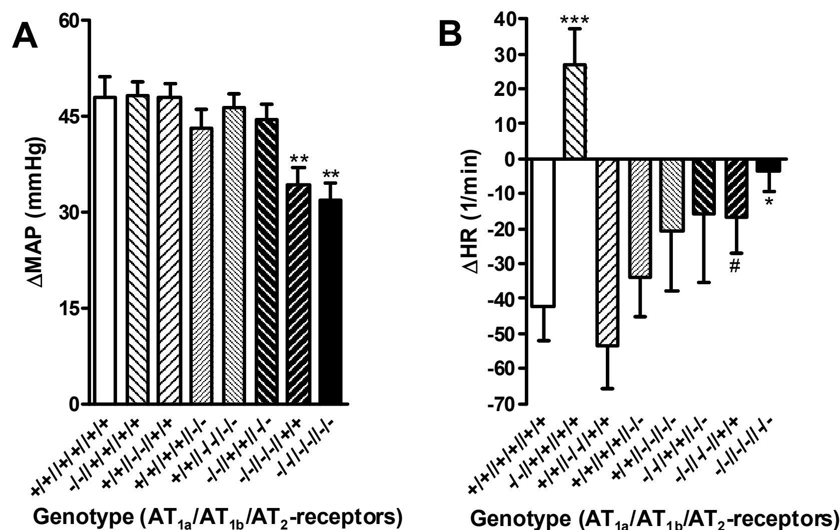

Figure 33 Changes in mean arterial pressure (MAP) (**A**) and heart rate (HR) (**B**) after a bolus injection of PE. ΔMAP, change in MAP after injection; ΔHR, change in HR after injection. *P<0.05, **P<0.01, ***P<0.001 vs. +/+//+/+//+/+ #P<0.05 vs. -/-//+/+//+/+ (n≥6).

As was shown earlier, Ang II-mediated stimulation of AT_1 leads to increased release of norepinephrine and epinephrine.[306] Ang II and the catecholamines increase vascular tone by using the same intracellular pathway. Previous investigations have shown that blockade of AT_1 receptors reduces the effects of α-sympathomimetics on vascular resistance and MAP.[307] Batenburg *et al.*[306] have demonstrated that AT_1 and α-adrenergic receptors are able to form hetero-oligomers and therefore could influence each other not only through enhanced second messenger release but also by physical interaction. The reduced rise in blood pressure in mice lacking both AT_1 subtypes or all three Ang II receptors, but not in any genotype expressing at least one AT_1 subtype, indicates that the cross-talk between the two systems requires either AT_{1a} or AT_{1b}. Our data illustrate the importance of AT_1 receptors on the regulation of the sympathetic system. However, the effects of the different genotype combinations on the regulation of the sympathetic system and the relevance of this regulation need further investigations.

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[31] or Agt[288] is caused primarily by the lack of Ang II stimulation of AT_{1a}. This is best illustrated by the fact that 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 partial atrophy of glomeruli. Of importance, it was shown earlier that those histological alterations are paralleled by reduced urinary osmolarity due to an increase in urine volume.[31,308] Notably, even without structural changes of the kidneys, AT_2-deficient mice (**Fig. 30**) demonstrate significantly reduced KW (**Tab. 3**). Because AT_2 deficiency likewise reduces HW, further investigations have to be undertaken to clarify whether the reduced kidney and heart weights are the results of less cell proliferation, smaller cell size, increased apoptosis, or even increased necrosis.
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 those 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 kidney morphology comparable to that 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 with AT_{1a}/AT_{1b} double-deficient animals may suggest a kidney-protective role for AT_2. This hypothesis may also be supported by findings that AT_2 knockouts mimic the morphological changes seen in common forms of human congenital anomalies of the kidney and urinary tract (CAKUT) with an incidence of 2-3%.[309] Further support is given by genetic observations. In patients with CAKUT, the occurrence rate of a loss-of-function mutation in the gene encoding AT_2 (*Agtr2*) is significantly higher than that in control subjects unaffected by CAKUT.[310]
In addition to their AT_1-dependent 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.[311] 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, as they can be used to visualize the effects of Ang II independent of its known receptors and identify effects of other Ang metabolites dependent or independent of AT_1 and AT_2.

7.6 Acknowledgements

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Chapter 8

Cardiac phenotype and angiotensin II levels in AT_{1a}, AT_{1b}, and AT_2 receptor single, double, and triple knockouts

Based on: van Esch JH, Gembardt F, Sterner-Kock A, Heringer-Walther S, Le TH, Laßner D, Stijnen T, Coffman TM, Schultheiss HP, Danser AH, Walther T. Cardiac phenotype and angiotensin II levels in AT_{1a}, AT_{1b}, and AT_2 receptor single, double, and triple knockouts. *Cardiovasc Res*. **2010**; 86(3):401-409.

8.1 Abstract

Our aim was to determine the contribution of the three angiotensin (Ang) II receptor subtypes (AT_{1a}, AT_{1b}, AT_2) to coronary responsiveness, cardiac histopathology, and tissue Ang II levels using mice deficient for one, two, or all three Ang II receptors.
Hearts of knockout mice and their wild-type controls were collected for histochemistry or perfused according to Langendorff, and kidneys were removed to measure tissue Ang II. Ang II dose-dependently decreased coronary flow (CF) and left ventricular systolic pressure (LVSP), and these effects were absent in all genotypes deficient for AT_{1a}, independently of AT_{1b} and AT_2. The deletion of Ang II receptors had an effect neither on the morphology of medium-sized vessels in the heart nor on the development of fibrosis. However, the lack of both AT_1 subtypes was associated with atrophic changes in the myocardium, a reduced CF and a reduced LVSP. AT_{1a} deletion alone, independently of the presence or absence of AT_{1b} and/or AT_2, reduced renal Ang II by 50% despite a five-fold rise of plasma Ang II. AT_{1b} deletion, on top of AT_{1a} deletion (but not alone), further decreased tissue Ang II, while increasing plasma Ang II. In mice deficient for all three Ang II receptors, renal Ang II was located only extracellularly.
The lack of both AT_1 subtypes led to a baseline reduction of CF and LVSP, and the effects of Ang II on CF and LVSP were found to be exclusively mediated via AT_{1a}. The lack of AT_{1a} or AT_{1b} does not influence the development or maintenance of normal cardiac morphology, whereas deficiency for both receptors led to atrophic changes in the heart. Renal Ang II levels largely depend on AT_1 binding of extracellularly generated Ang II, and in the absence of all three Ang II receptors, renal Ang II is only located extracellularly.

8.2 Introduction

The biological actions of angiotensin (Ang) II are mediated via Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. In rodents, two sub types of AT_1 have been identified: AT_{1a} and AT_{1b}[312] which share 94% sequence homology[283] and have similar ligand binding affinities and signal transduction properties. To date, there are no pharmacological antagonists that discriminate between AT_{1a} and AT_{1b}, and the function of AT_2 is still only partly understood.
To get more insight in the function of the three Ang II receptors, we generated mice which are deficient for either one, two, or all three Ang II receptors.[313] AT_2 deletion increased baseline mean arterial pressure (MAP), whereas mice lacking AT_{1a} were hypotensive and displayed a reduced heart weight/body weight (HW/BW) ratio. Blood pressure and HW/BW ratio dropped further in mice lacking both AT_1 subtypes. AT_{1a} deletion impaired the *in vivo* pressor response to Ang II bolus injections, 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. Ang II failed to alter MAP in mice lacking all three Ang II receptors (triple knockouts), indicating that no other receptors than the AT_{1a}, AT_{1b}, and/or AT_2 mediate the pressor effects of Ang II.
In the present study, we set out to quantify, in the above-described knockout mice, the contribution of the three Ang II receptors to cardiac hemodynamic *in vitro*, using the Langendorff heart preparation. Given the reduced heart size in AT_{1a} knockout mice, we also quantified brain natriuretic peptide (BNP) expression and studied morphological changes such as fibrosis and remodelling. Finally, we measured tissue Ang II levels in these mice to verify earlier findings by us and others showing that tissue Ang II is AT_1-bound.[314-318]

8.3 Methods

8.3.1 Animals

Males (aged ~5 months) of all eight possible genotypes ($AT_{1a}/AT_{1b}/AT_2$: +/+//+/+//+/+, -/-//+/+//+/+, +/+//-/-//+/+, +/+//+/+//-/-, +/+//-/-//-/-, -/-//+/+//-/-, -/-//-/-//+/+, -/-//-/-//-/-) of the recently described Ang II receptor deficient mice[313] were used in this study. In addition, age-matched animals lacking angiotensinogen (Agt)[27] were used for histological comparison. Experiments on adult mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), the regulations of the Animal Care Committee of the Erasmus MC, and the Federal Law on the Use of Experimental Animals in Germany and were approved by the local authorities (Landesamt für Gesundheit und Soziales des Landes Berlin).

8.3.2 Langendorff heart preparation

Mice were killed by cervical dislocation. 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% O_2 and 5% CO_2.[287,319,320] 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.[321] Two needle electrodes were placed at the right atrium and the hearts were paced at ~600 b.p.m. (10 Hz, 4 ms duration, 4 V) using a Grass stimulator (Grass Instruments Co., Quincy, MA, 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.[321,322] 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, NY, USA). After a stabilization period of 10–15 min, 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 to 0.1 mmol/l) were applied.

8.3.3 RNA isolation and RNase protection assay

Total RNA was isolated from heart using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) as described earlier.[323] Mouse BNP mRNA expression was identified by RPA using the Ambion RPA II kit (Ambion Europe Ltd, Huntingdon, UK) as described elsewhere.[323] In brief, SP6-RNA polymerase transcribed a radioactive antisense probe complementary to BNP mRNA (290-bp).[324] RNA complementary to 127 nucleotides of rL32 mRNA was used as a positive control.[210,291] Thirty microgram of each RNA sample was hybridized with of the radiolabelled antisense probes in the same reaction. The hybridized fragments protected from RNase A + T1 digestion were separated by electrophoresis and analyzed using a FUJIX BAS 2000 Phospho-Imager system (Raytest GmbH, Straubenhardt, Germany). Quantitative analyses were performed by measuring the intensity of the target bands normalized by the intensity of rL32.

8.3.4 Histology

Hearts were isolated and fixed in 4% buffered formalin and processed according to standard protocols. In brief, the hearts were embedded in paraffin and sectioned at 2 mm. Slides were deparaffinised and hydrated. Sections of hearts were stained for histology with haematoxylin-eosin (HE) solution, periodic acid-Schiff (PAS) solution, or van Gieson solution as described

previously.[291,325] Tissue sections (n≥4) were evaluated in four planes for morphological changes and connective tissue production.

8.3.5 Ang II levels in kidney
Kidneys were removed from the abdomen, rapidly frozen in liquid nitrogen, and stored at -80°C until processing. Ang II was determined by radioimmunoassay following SepPak extraction and high-performance liquid chromatography separation as described before.[294]

8.3.6 Data analysis
CF and LVSP data were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments, Akron, OH, USA). After a manual selection of the desired signals pre- and post-injection, data were analysed using Matlab (Mathworks, Inc., Natick, MA, USA). Six consecutive beats were selected for the determination of CF and LVSP. Results are represented as mean±SEM or geometric mean and range. Concentration–response curves were analysed as described,[326] using Graph Pad Prism 3.01 (Graph Pad Software, Inc., San Diego, CA, USA), to obtain pEC50 ($-^{10}\log EC_{50}$) 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 was performed using the SPSS 11.0 statistical package (SPSS, Inc., Chicago, IL, USA). Multiple regression analysis was conducted to determine the contribution of the three receptors as independent variables and, in case one of the receptors did not exert an independent effect, their interaction. $P<0.05$ was considered significant.

8.4 Results

8.4.1 Langendorff heart studies
Table 4 shows the baseline CF and LVSP values in the eight genotypes. Regression analysis revealed that the deletion of individual Ang II receptor subtypes did not affect baseline CF. However, combined deletion of AT_{1a} and AT_{1b}, independently of the presence or absence of AT_2, lowered baseline CF (P=0.020; **Fig. 34**, left panel). Although baseline LVSP was unaffected by individual AT_{1a} or AT_2 deletion, it was lower in hearts lacking AT_{1b}, independently of the presence of AT_{1a} or AT_2 (P=0.005; **Fig. 34**, middle panel). In addition, AT_{1a} deletion on top of AT_{1b} deletion further decreased baseline LVSP (P=0.003; **Fig. 34**, right panel).

Genotype	+/+//+/+//+/+	+/+//-/-//+/+	+/+//+/+//-/-	+/+//-/-//-/-	-/-//+/+//+/+	-/-//+/+//-/-	-/-//-/-//+/+	-/-//-/-//-/-
n	8	4	6	7	5	7	4	4
CF (mL/min)	2.5±0.2	3.0±0.3	2.8±0.1	3.1±0.1	2.7±0.1	3.0±0.2	2.4±0.3	2.5±0.3
LVSP (mmHg)	98.5±3.7	98.2±6.3	96.5±3.5	98.9±8.3	114.3±6.8	112.6±7.1	86.0±6.2	72.3±6.7

Table 4 Baseline coronary flow (CF) and left ventricular pressure (LVSP) according to genotype. Data is presented as mean±SEM

In hearts of wild-type mice, bolus injections of Ang II dose dependently decreased CF and LVSP (**Fig. 35**) by maximally 56±5% and 39±4%, respectively (pEC_{50} 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.[327-329]
Bolus injections of Ang II exerted no effects in hearts of mice deficient for AT_{1a} receptors. Interestingly, no differences were found between hearts of mice lacking AT_{1a} alone and hearts of mice deficient for AT_{1b} and/or AT_2 on top of AT_{1a} deficiency. The effects of Ang II bolus injections in hearts of mice lacking AT_{1b} and/or AT_2 were identical to those in wild-type mice. This suggests that the cardiac effects of Ang II are exclusively AT_{1a} mediated.

8.4.2 Cardiac histopathological examination and expression of BNP

We investigated the impact of the Ang II receptors on morphological changes in medium-sized vessels in the heart using PAS-stained sections. Deficiency for one, two, or all three Ang II receptor subtypes did not influence the morphology of medium-sized arteries in the heart (**Fig. 36**). To investigate the impact of lack in Ang II receptor expression on fibroblast quantities, we determined the basal production of connective tissue by van Gieson staining. In none of the eight possible receptor combinations, a change in basal connective tissue production was detected (**Fig. 37**).

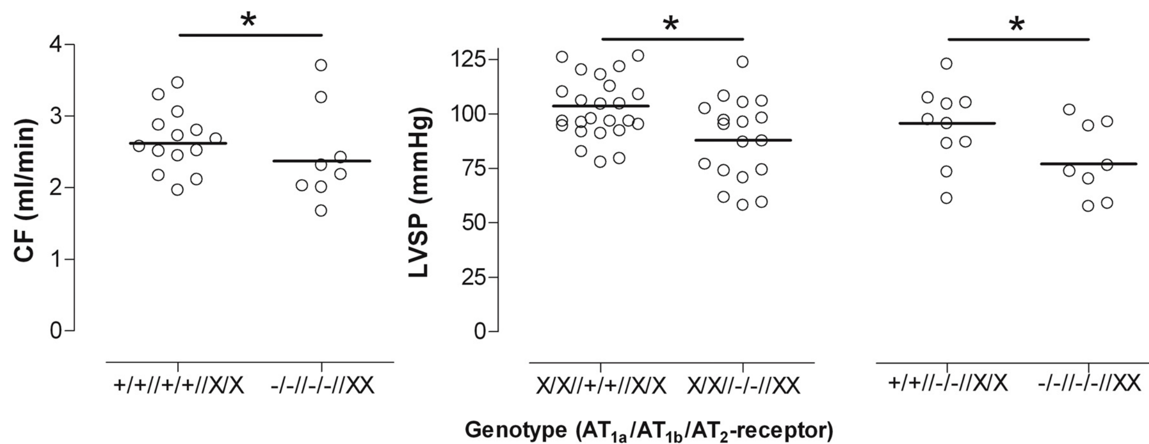

Figure 34 Baseline coronary flow (CF) following deletion of AT_{1a} and AT_{1b}, independently of the presence or absence of AT_2 (left panel), baseline left ventricular systolic pressure (LVSP) following deletion of AT_{1b}, independently of the presence of AT_{1a} or AT_2 (middle panel), and baseline LVSP following deletion of AT_{1a} on top of AT_{1b}, independently of the presence of AT_2 (right panel). **P*< 0.05; data (n=8–26) are represented as scatter dot plot. The horizontal bar represents the mean. The use of 'X' implies that for that specific receptor, both +/+ and -/- animals were included, i.e. that the comparison occurred independently of the presence or absence of that receptor.

As we have reported earlier, all genotypes lacking the AT_{1a} receptor showed a significantly reduced HW/BW ratio.[313] Interestingly, there was oligo- to multi-focal atrophy of cardiac muscle evident in animals lacking both AT_1 subtypes (-/-//-/-//+/+ and -/-//-/-//-/-) and Agt-deficient mice, whereas animals deficient for none or only one of the AT_1 subtypes showed no structural changes within the heart (**Fig. 38**). Atrophic muscle fibres revealed a more pronounced, dense, reticular network, with partial collapse of the reticulin fibres. Atrophic muscle fibres had an angular outline, and widened partially enlarged interstitial spaces.

BNP is a marker for heart failure. Thus, we tested whether the deficiency of one of the receptors and/or the structural changes have an impact on BNP expression in all eight groups. None of the eight genotypes showed increased BNP expression (**data not shown**).

8.4.3 Ang II levels in plasma and kidney

As the hearts had already been used for *ex vivo* hemodynamic studies, these measurements were performed in kidneys. Kidneys have much higher Ang II levels than the heart,[330] and changes in renal Ang II content exactly parallel changes in the Ang II content of other tissues (including the heart).[330-332] In other words, the renal Ang II levels can be used as a reflection of what happens at the tissue level in general. Renal Ang II levels were compared with the plasma Ang II levels (in the same animals) reported previously by our group.[313] For the sake of clarity, **Fig. 39** shows the geometric mean and range of the Ang II levels in both kidney (A) and plasma (B). Regression analysis revealed that AT_{1a} deletion alone, independently of the presence or absence of the AT_{1b} and/or AT_2, diminished renal Ang II by ~50% (*P*=0.002; **Fig. 40a**, left panel) and increased plasma Ang II ~5-fold (*P*<0.0001; **Fig. 40b**, left panel).

AT_{1b} deletion alone did not affect plasma and renal Ang II (**Fig. 39**). However, on top of AT_{1a} deletion, independently of the presence or absence of the AT_2, AT_{1b} deletion further diminished renal Ang II by a factor of 4 (*P*<0.0001; **Fig. 40a**, middle panel) and doubled plasma Ang II (*P*=0.003; **Fig. 40b**, middle panel).

AT_2 deletion alone did not affect plasma and renal Ang II. However, on top of AT_{1a} deletion, independently of the presence or absence of the AT_{1b}, AT_2 deletion reduced plasma (but not renal) Ang II (*P*=0.018; **Fig. 40a and b**, right panels).
We have previously shown that tissue Ang II is protected against metabolism by binding to AT1 and subsequent internalization.[316] To verify this concept, kidneys of two mice lacking all three Ang II receptors were kept at room temperature for 30 min before freezing them in liquid nitrogen. Ang II levels in these kidneys were 2 and 8 fmol/g, whereas the (geometric) mean Ang II level in the kidneys of AT_{1a}/AT_{1b}/AT_2 triple knockout mice that had been frozen immediately was 35 fmol/g.

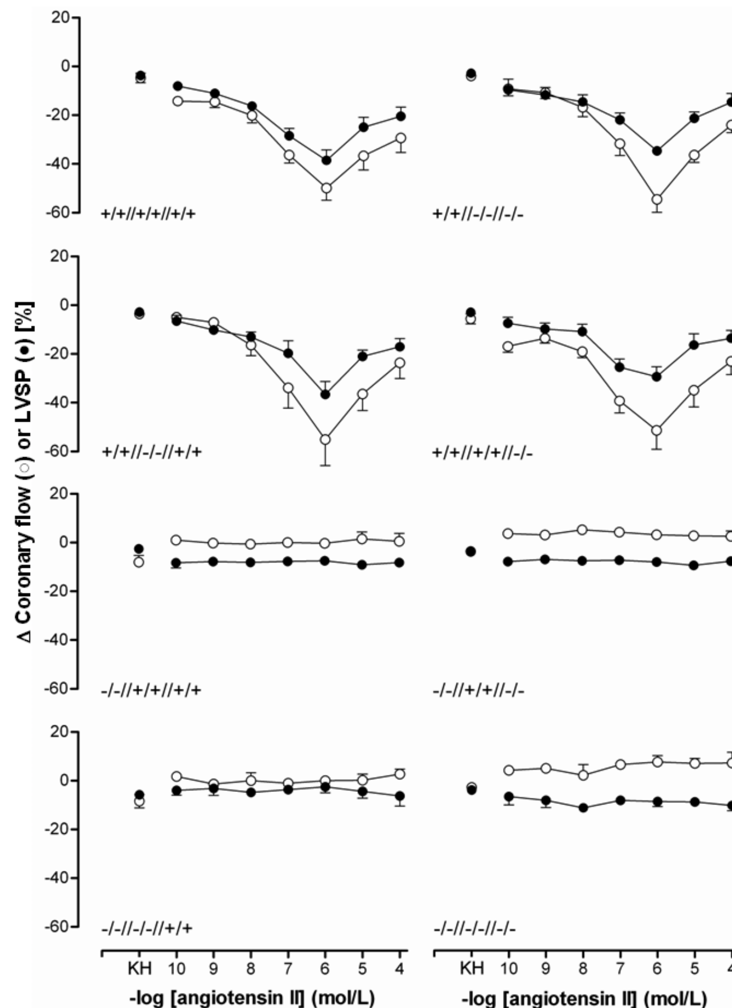

Figure 35 Changes in coronary flow (CF; open symbols) and left ventricular systolic pressure (LVSP; closed symbols) after a bolus injection of Ang II in the Langendorff heart according to genotype. Data (mean±SEM of 4–8 experiments) represent percentage change from baseline. KH, bolus injection of Krebs-Henseleit buffer. The x-axis displays the Ang II concentration in the injection fluid.

8.5 Discussion

We have previously shown that AT_{1a} deletion impaired the *in vivo* pressor response to Ang II bolus injections, whereas deficiency for AT_{1b} and/or AT_2 had impact only on top of AT_{1a} deletion.[313] The importance of AT_{1a} is also apparent in our current studies using the mouse heart Langendorff model. Deletion of one, two, or all three Ang II 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 AT_{1a} independently of the presence or absence of AT_{1b} and/or AT_2. These effects were completely abolished in all genotypes deficient for the AT_{1a}. Thus, the AT_{1a} exclusively mediates the Ang II-induced

negative inotropy and vasoconstriction in the mouse heart, and AT_{1b} does not exert such effects, possibly because it is not expressed in the heart in sufficient amounts. Whether the negative inotropic and vasoconstrictor effects of Ang II occurred independently of each other could not be determined in the present study. Negative inotropic effects of Ang II in the mouse heart have been described before,[333,334] and contrast with the positive inotropic effects of Ang II in human trabeculae.[335] Yet, it is also possible that the decrease in LVSP simply is the consequence of the Ang II-induced decrease in CF, i.e. that Ang II has no direct effect on the mouse cardiomyocyte.

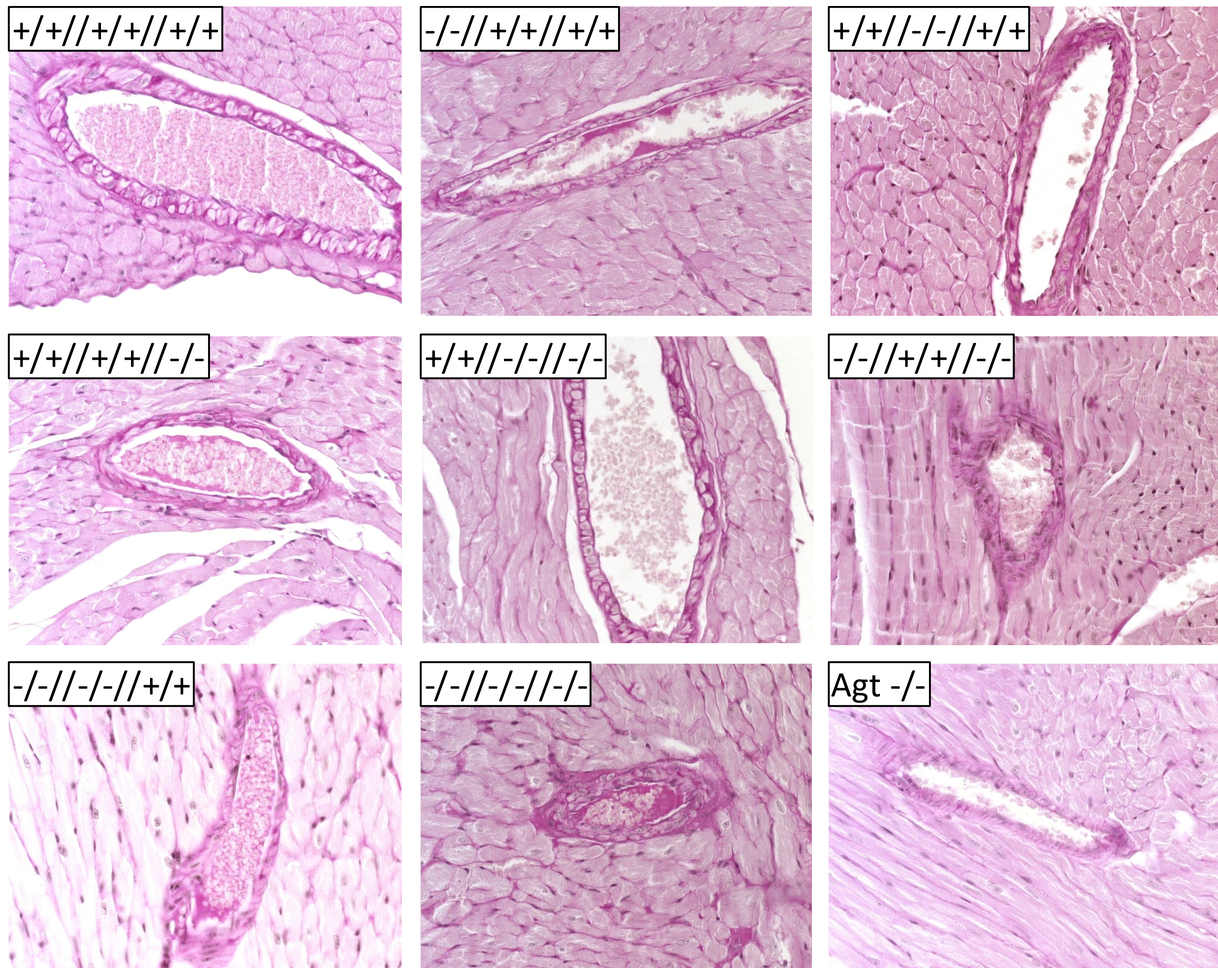

Figure 36 Medium-sized arteries in hearts of wild-type (+/+//+/+//+/+), AT_{1a}- (-/-//+/+//+/+), AT_{1b}- (+/+//-/-//+/+), and AT_2- (+/+//+/+//-/-) single-knockout mice, mice exclusively expressing AT_{1a} (+/+//-/-//-/-), AT_{1b} (-/-//+/+//-/-), or AT_2 (-/-//-/-//+/+), triple-knockout mice (-/-//-/-//-/-), and angiotensinogen-deficient (Agt -/-) mice do not show genotype-related morphological alterations (PAS-stained; magnification: 400-fold; representative sections for all groups from n≥4 sections).

Unexpectedly, absence of the AT_2 did not enhance the AT1-mediated effects in the heart. This contrasts with other studies in the heart where AT_2 blockade potentiated the effects of Ang II.[287,336-338] On the basis of these enhanced effects, it has been concluded that AT_2 counteract the AT_1-mediated effects. An explanation for the absence of such enhanced cardiac effects in the AT_2 knockout mice in the present study could be that chronic absence (unlike acute blockade) of AT_2 affects AT_{1a} signalling modifying pathways. Alternatively, life-long absence of AT_2 may have resulted in structural changes. However, histological examination did not reveal such alterations, and baseline CF and LVSP were identical in all genotypes. Moreover, the vascular and cardiac effects of other agonists (phenylephrine and endothelin-1) in these mice are unaltered.[313] Thus, the discrepancy between pharmacological blockade and the AT_2 knockout related to specific parameters could be also a result of ectopic effects of the AT_2 blocker as significant ectopic effects have been described for a variety of

AT_1 blockers.[339,340] The AT_2 blocker used in these studies, PD123,319, has been originally described as an AT_2 agonist.[16] Taken this in consideration, the genetically receptor-deficient mice are a superior experimental model compared with the pharmacological blockade of AT_2. Furthermore, we also did not observe direct AT_2-mediated effects in AT_1 knockout mice. This is in line with our previous inability to observe cardiac AT_2-mediated effects during AT_1 blockade in wild-type mice.[287] Apparently therefore, AT_2-mediated effects in the mouse heart can only be demonstrated during simultaneous AT_1 stimulation, i.e. they occur in conjunction with AT_{1a} activation.

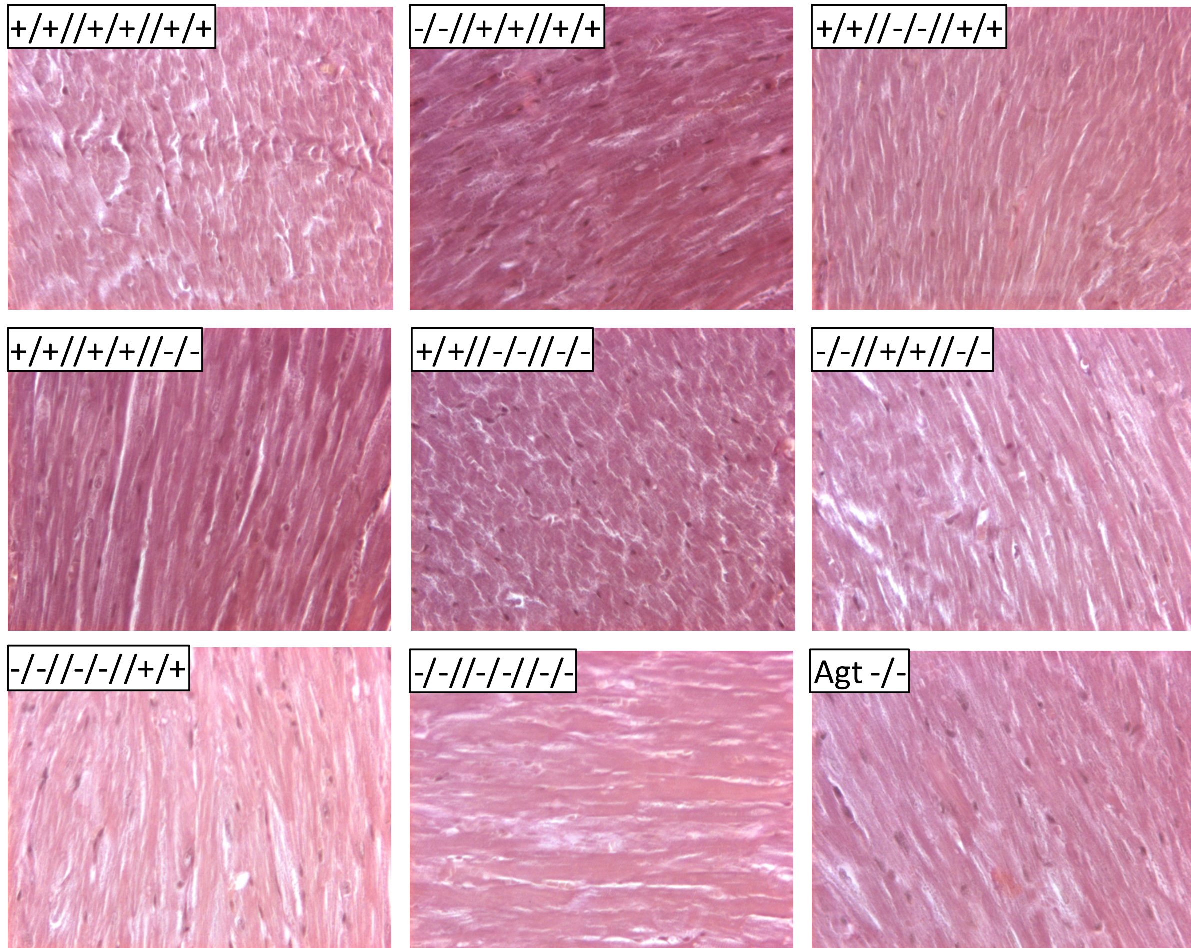

Figure 37 Sections of hearts from wild-type (+/+//+/+//+/+), AT_{1a}- (-/-//+/+//+/+), AT_{1b}- (+/+//-/-//+/+), and AT_2- (+/+//+/+//-/-) single-knockout mice, mice exclusively expressing AT_{1a} (+/+//-/-//-/-), AT_{1b} (-/-//+/+//-/-), or AT_2 (-/-//-/-//+/+), triple-knockout mice (-/-//-/-//-/-), and angiotensinogen-deficient (Agt -/-) mice do not show changes in basal connective tissue production (van Gieson-stained; magnification: 400-fold; representative sections for all groups from n≥4 sections).

However, our findings also show that this concept does not account for all tissues or Ang II-mediated effects. Our data on plasma Ang II levels implicate AT_2-mediated effects independent of AT_1, because AT_2 deficiency led to lower Ang II concentrations in plasma of AT_1-deficient animals.

Recently, we reported reduced heart weights for mice lacking AT_{1a} in all combinations, in AT_2-deficient mice, and mice lacking Agt.[313] In this study, we found a similar, genotype-specific atrophy in the myocardium of AT_{1a}/AT_{1b} double-, AT_{1a}/AT_{1b}/AT_2 triple-, and Agt-deficient mice. Interestingly, mice only deficient for AT_{1a} and not for AT_{1b} (-/-//+/+//+/+ and -/-//+/+//-/-) did not show the described atrophic changes. Despite the low AT_{1b} expression in the heart, our data suggest a rescue of AT_{1a} function by AT_{1b} in the maintenance of normal cardiac morphology, whereas AT_{1b} cannot compensate for the lack of growth stimulation in the heart due to the absence of AT_{1a}. The reduction in baseline CF and LVSP in mice deficient for both AT_{1a} and AT_{1b} may be the consequence of these atrophic changes. Thus,

atrophy alone cannot explain the reduced heart weight in the five genotypes where lower organ weight has been observed, because mice deficient for AT_{1a} and/or AT_2 (-/-//+/+//+/+, +/+//+/+//-/-, and -/-//+/+//-/-) do not show the atrophic changes seen in mice lacking both AT_1 subtypes. Therefore, further experiments have to investigate both phenomena; the reduced heart weight and the atrophy in the heart of mice with lacking AT_{1a}/AT_{1b} stimulation. Finally, the present study yielded important information on the regulation of tissue Ang II levels. Normally, circulating Ang II is sequestered by multiple tissues via AT1-mediated endocytosis.[316] Yet, the majority of tissue Ang II is formed at tissue sites.[341,342] Studies with ^{125}I-labelled Ang I have revealed that this production occurs extracellularly and is followed by rapid AT_1-mediated endocytosis.[314-318] Therefore, despite its extracellular synthesis, e.g. in the interstitial fluid compartment or on the cell surface, the majority of tissue Ang II is located intracellularly.[318] Its levels are particularly high in endosomes.[343] The intracellular accumulation of Ang II may either facilitate its activation of nuclear receptors[344,345] and/or results in its destruction.[316] The half-life of AT_1-bound tissue Ang II is 20–30 times longer than that of extracellular Ang II,[316] thereby explaining, at least in part, why tissue Ang II levels far exceed plasma Ang II levels. Clearly, receptor-binding protects Ang II against rapid metabolism by angiotensinases.

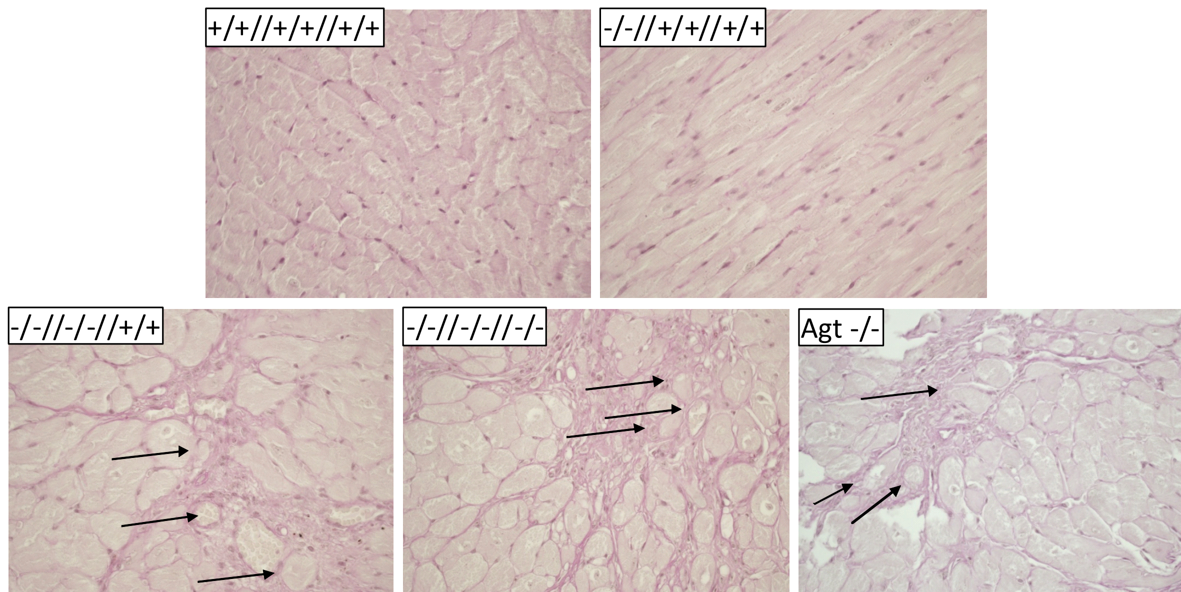

Figure 38 Oligo- to multi-focal atrophy of cardiac muscle is evident in animals lacking both AT1 subtypes (-/-//-/-//+/+ and -/-//-/-//-/-) and angiotensinogen-deficient (Agt -/-) mice, whereas animals lacking none or only one AT1-subtype (exemplarily shown +/+//+/+//+/+ and -/-//+/+//+/+). Arrows indicate dense reticular network, partially collapsed, with individualized muscle fibres, and occasional angular outline, as well as loss of muscle fibres (HE-stained; magnification: 400-fold representative sections for all groups from n≥4 sections).

As AT_2 does not internalize after ligand binding,[346,347] the intracellular accumulation of Ang II exclusively depends on AT_1. The current study now shows that both AT_1 subtypes contribute to this accumulation and that the contribution of the AT_{1a} exceeds that of the AT_{1b}. Making use of single AT_{1a} knockout mice, a similar conclusion was already drawn by Li and Zhuo.[348]

Not having AT_{1a} caused a five-fold rise in plasma Ang II. Despite this rise in extracellular Ang II, the renal tissue level of Ang II decreased by 50%. Not having AT_{1b} does not have an effect by itself on either plasma or tissue Ang II, thereby demonstrating that AT_{1a} can make up for the consequences of the deletion of AT_{1b}. However, missing AT_{1b} on top of AT_{1a} deletion further increased plasma Ang II (by a factor of 2) and lowered tissue Ang II by a factor of 4. Therefore, in mice not expressing AT_1, the tissue/ plasma Ang II concentration ratio is far below that in wild-type mice. Additional AT_2 deletion did not alter renal Ang II, in agreement with the above concept of non-internalizing AT_2.

Renal Ang II levels in the absence of its three receptors are not zero. To verify whether the measured Ang II is putatively extracellular in $AT_{1a}/AT_{1b}/AT2$-triple knockout mice, we determined the Ang II content of kidneys that were kept at room temperature for 30 min. Normally, over this time period, the level of AT_1-bound Ang II does not change,[316] whereas the level of extracellular Ang II rapidly decreases.[317] Indeed, tissue Ang II levels in mice deficient for all three Ang II receptors following this procedure decreased by more than 70%, confirming the rapid metabolism of non-receptor bound, extracellular Ang II by angiotensinases. Furthermore, given that the extracellular fluid content of renal tissue consists of blood plasma and interstitial fluid (accounting for ~5% and ~10% of tissue weight, respectively), and assuming that the renal interstitial Ang II levels resemble those in blood (~320 fmol/ml in triple knockout mice), it can be estimated that the renal tissue Ang II levels in triple knockout mice should be in the order of ~50 fmol/g, if Ang II is limited to the extracellular fluid compartment. This is within the range of the tissue levels that were found (35 fmol/g), thereby again confirming that tissue Ang II in triple knockout mice is extracellular. Consequently, the experimental data in our triple knockout mice clearly argue against the formerly expressed hypothesis of intracellular Ang II generation.[82]

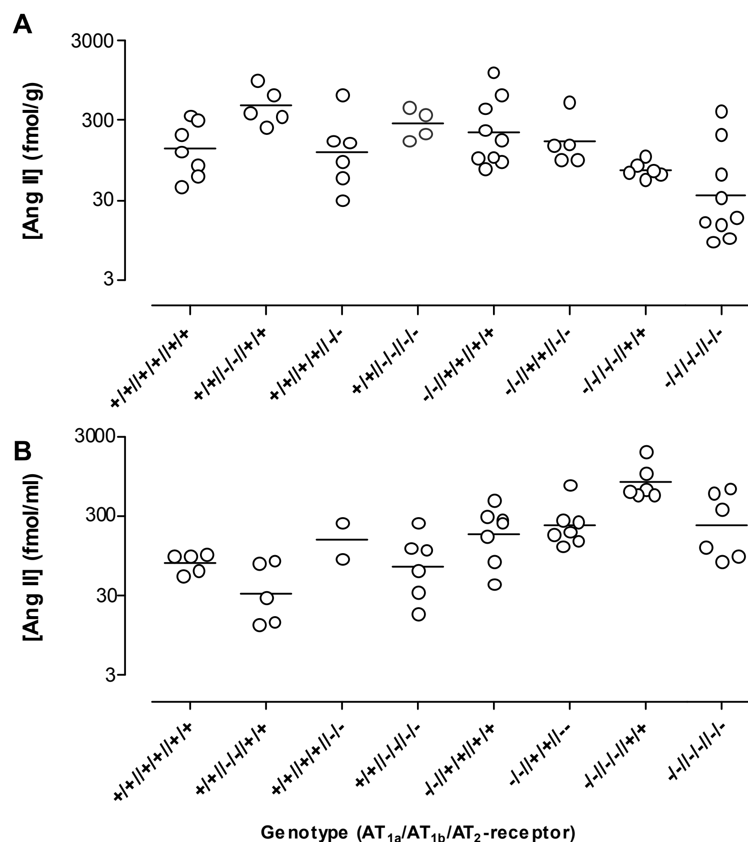

Figure 39 Ang II levels in kidney (**A**) and blood plasma (**B**) of wild-type (+/+//+/+//+/+), AT_{1a}- (-/-//+/+//+/+), AT_{1b}- (+/+//-/-//+/+), and AT_2- (+/+//+/+//-/-) single-knockout mice, mice exclusively expressing AT_{1a} (+/+//-/-//-/-), AT_{1b} (-/-//+/+//-/-), or AT_2 (-/-//-/-//+/+), and triple-knockout mice (-/-//-/-//-/-). Data (n=2–9) are represented as scatter dot plot. The horizontal bar represents the geometric mean. Plasma levels were redrawn from Gembardt *et al.*[309]

On the basis of our new findings using animals with different combinations of Ang II receptor deficiencies, we can draw the following conclusions: (i) the lack of both AT_1-subtypes led to reduced baseline CF and LVSP, (ii) the coronary constrictor and negative inotropic effects of Ang II are exclusively mediated via AT_{1a}, (iii) deficiency of only one AT_1 subtype has no effect on development or maintenance of normal cardiac morphology, whereas the lack of both receptors led to atrophic changes in the heart, (iv) tissue Ang II

levels largely depend on AT_1-mediated endocytosis of extracellular Ang II; (v) in the absence of the three Ang II receptors, tissue Ang II is only located extracellularly.

Figure 40 Ang II levels in kidney (**A**) and blood plasma (**B**) following deletion of AT_{1a}, independently of the presence or absence of AT_{1b} or AT_2 (left panels), deletion of AT_{1b} on top of AT_{1a} deletion, independently of the presence of AT_2 (middle panels), or deletion of AT_2 on top of AT_{1a} deletion, independently of the presence of AT_{1b} (right panels). *P<0.05; Data (n=12-29) are represented as scatter dot plot. The horizontal bar represents the geometric mean. The use of 'X' implies that for that specific receptor, both +/+ and -/- animals were included, i.e. that the comparison occurred independently of the presence or absence of that receptor. Plasma levels were obtained from Gembardt *et al.*[309]

8.6 Conflict of interest

None declared.

8.7 Funding

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Chapter 9

Summary and general discussion

9.1 Summary

9.1.1 Peptidases involved in the regulation of RAS activation (Chapters 2 and 3)
After the generation of Ang I by renin from Agt, Ang I is further hydrolyzed by ACE to Ang II. By stimulating its receptors, Ang II mediates vasopressor effects. Peripheral Ang II plays a critical role in the regulation of vascular tone, central Ang II regulates water intake and is crucial in alcohol-consuming behaviour. Beside Ang II also some of its metabolites, e.g. Ang-(1-7) and Ang III, have biological activity. Ang-(1-7), which mediates distinct effects through the receptor Mas, can be directly generated from Ang II by ACE2. We analysed ACE2 expression in different tissues of mice and rats on mRNA and protein levels. Corresponding to mRNA, ACE2 activity was highest in the ileum of both species. Interestingly, tissue distribution differs between the two rodent species we investigated, best illustrated by the high ACE2 activity in mouse kidney but weak in rat.
Ang III, which is discussed to mediate Ang II-related central effects, is produced by conversion of Ang II by APA. The genetic deficiency for APA led to an increased ACE2 activity in the kidney, whereas the activity of ACE2 was unchanged in lungs and different brain regions in these animals. The inhibition of Ang III production in APA-deficient mice led to an increased water intake. The reduction in water consumption by social stress was blunted in mice lacking APA. However, neither basal voluntary alcohol consumption, nor changes in alcohol-drinking behaviour were altered in the knockout model.
Our data on ACE2 expression and our findings demonstrating the impact of a shift in the ratio of bioactive Ang peptides point to the fact that the RAS becomes increasingly complex.

9.1.2 Mas and the receptor family of Mas-related genes in the RAS (Chapters 4 to 6)
The *Mas* proto-oncogene encodes a G protein-coupled receptor. Early investigations have suggested a potential role for Mas as an Ang II receptor. More recent investigations have demonstrated that Mas is not a receptor for Ang II, but is associated with intracellular signalling induced by Ang-(1-7). Interestingly, we have shown that vessels of Mas-deficient animals showed altered responses to vasorelaxant compounds, showing all characteristics of an endothelial dysfunction. In *in vitro* experiments, we have shown that Mas is not mediating Ang II stimuli, but diminishes AT_1-mediated intracellular effects. Furthermore, we could demonstrate that the impact of Mas on AT_1 signalling was due to a physical interaction of both receptors. In an *ex vivo* setting, we demonstrated that the hetero-dimerization of the 2 receptors *in vitro* was not an artefact due to the artificial cell/receptor system, but also influences vasoreactivity. Our results clearly demonstrate the importance of the AT_1-Mas complex as a target for pharmacological intervention in cardiovascular diseases.
Recently, the Mrg-receptor family was identified. The Mrg-receptors share high sequence homology with the Ang-(1-7)-associated receptor Mas. Most of these receptors have no known endogenous ligand. Our data shows that Ang peptides can stimulate transcriptional activation and/or AA release mediated by Mrg-receptors in peptide-specific patterns. Furthermore, our results show AT_1- and AT_2-independent effects induced by Ang II and Ang III and describe an AT_1-independent direct Ang IV signalling.

9.1.3 New insights in Ang II receptor function and regulation (Chapters 7 and 8)
Ang II can stimulate two distinct GPCRs, the AT_1 and AT_2. In rodents, two AT_1 subtypes were identified (AT_{1a} and AT_{1b}), which are not distinguishable by pharmacological treatment. In our work using mice deficient for one, two, or all three Ang II receptors, we could demonstrate that AT_{1b} can partly compensate lacking AT_{1a} stimulation in regard to Ang II-induced vasoconstriction, but not in regard to heart rate alterations and to the reduction of CF and LVSP. Furthermore, our data clearly demonstrates that Ang II-induced pressor effects

exclusively depend on AT_1 and AT_2, and that there is no intracellular Ang II in the absence of its known 3 receptors. While deficiency for single Ang II receptors had no impact on morphology of medium-sized vessels and the basal production of connective tissue, the lack of both AT_1 subtypes was associated with atrophic changes in the myocardium. We could identify the mice deficient for all three Ang II receptors as an ideal tool to improve the knowledge on the non-AT_1-, AT_2-mediated effects of the RAS.

9.2 General Discussion

The RAS plays a major role in the regulation of blood pressure and in the pathogenesis of cardiovascular diseases.[262,263] Not only the finding that beside Ang II some of its metabolites exhibit biological activity,[83-85,125] but also the discovery of new enzymes[105,107,108] and receptors[143,152,153] regulating the activity of the RAS have drawn a more complex picture of the RAS.
Investigations of peptidases involved in the regulation of RAS activity, revealed new functions for these enzymes. In *in vitro* experiments, ACE2 was identified to be a receptor for the SARS virus.[123] Another group confirmed this finding *in vivo,*[349] showing that the interaction of the virus with ACE2 induced a downregulation of the enzyme leading to a decreased ACE2 activity. Furthermore, it was demonstrated that ACE2 can interact with integrin β1, a critical fibronectin receptor, in the failing heart.[350] Integrin β1 is involved in many regulatory signalling cascades[351] and is therefore discussed to be involved in a variety of diseases. The modulation of integrin-mediated signalling by ACE2 has to be better characterised to identify new targets for the treatment of diseases strongly influenced by integrins. Besides the intracellular signalling mediated by ACE2, also ACE-mediated outside-in signalling was described.[352] Binding of inhibitors to ACE induce phosphorylation of the intracellular domain of the enzyme. ACE phosphorylation triggers c-Jun NH_2-terminal kinase and casein kinase-2 activation, subsequently stimulating transcription of ACE itself[353] and other proteins.[354,355] Beside the described intracellular pathway activated by ACE, the phosphorylation also induces shedding of the ectodomain. Shedding is a common mechanism for the regulation of biological functions mediated by cell surface proteins. Commonly shedding is an active process in which the cell surface protein is released by proteinases of the MMP (matrix metalloproteinases) or ADAM (a disintegrin and metalloproteinase) families. While the proteinase responsible for ACE2 shedding was identified as ADAM17 (tumour necrosis factor-α converting enzyme),[356] the sheddase for ACE still remains unknown, therefore further investigations have to reveal its identity. Recent investigations have shown that exogenous ACE upregulates the expression of both bradykinin receptor subtypes, B_1 and B_2, by pathways involving NFκB and AP-1.[357] ACE also activates focal adhesion kinase and SHP-2, independently of the modulated transcriptional activity.[358] The proteins responsible for ACE binding and signal transduction have to be identified. Furthermore, it has to be investigated if exogenous or shedded ACE2 also mediates intracellular signalling unrelated to its enzymatic activity or if the beneficial effects seen by the administration of ACE2 in acute lung injury[122] are only mediated by the reduction in Ang II or the increase in Ang-(1-7). For other cell surface proteins it is known that after shedding the remaining intracellular domain can have signalling functions, e.g. the Alzheimer's amyloid precursor protein.[359] Thus it has to be determined if similar pathways exist for ACE and/or ACE2.
Not only new functions for known enzymes were identified, but also a new homologue of ACE, named ACE3, was recently identified.[360] This protein lacks the essential HE*XX*H zinc-binding domain and therefore does not possess catalytic activity. ACE3 is expressed in several mammalian species, but it seems to be a pseudogene in humans. A first investigation using ACE3-deficient mice could not reveal physiological functions for this new

homologue,[361] therefore further studies are needed to better characterise the importance of the protein.

In the 'new' RAS, two different arms can be activated, the ACE/Ang II/AT_1-axis and the ACE2/Ang-(1-7)/Mas-axis. Beside the classical role in cardiovascular physiology and pathology, both axes of the RAS play critical roles in the (patho)physiology of other biological actions, e.g. inflammation,[299,362] haematopoiesis,[142,363,364], or acute lung injury.[122]

The newly established ACE2/Ang-(1-7)/Mas-axis can oppose many of the detrimental Ang II-induced effects. While AT_1 stimulation by Ang II produces cardiac remodelling through a complex mechanism resulting in reduction of cardiac performance and increased susceptibility to cardiac events,[365] Ang-(1-7) can reduce cardiac remodelling by decreasing hypertrophy and fibrosis induced by Ang II.[366] Furthermore, it was shown that Ang-(1-7)-stimulation of cardiac fibroblasts diminishes the Ang II-induced production of endothelin-1 and leukaemia inhibitory factor.[245] Moreover, opposite effects of both peptides were described for the regulation of cell growth.[367] Ang-(1-7) also inhibits endothelin-1- and serum-induced hypertrophic growth of neonatal rat cardiomyocytes.[136] In recent investigations, antiproliferative effects of Ang-(1-7) were demonstrated in stent-induced neointima proliferation[368] and also in lung cancer cells.[139] Taken together with older findings demonstrating preserved cardiac function, coronary perfusion, and aortic endothelial function by Ang-(1-7)-treatment of rats after myocardial infarction,[134] the ACE2/Ang-(1-7)/Mas-axis counteracts the detrimental effects of the ACE/Ang II/AT_1-axis. These findings suggest a beneficial effect for the stimulation of the ACE2/Ang-(1-7)/Mas-axis under pathological conditions. Interestingly, recent investigations have demonstrated beneficial effects for the disruption of the ACE2/Ang-(1-7)/Mas-axis at least in the kidney. In mice lacking the receptor Mas, the inflammatory response to ureter obstruction and renal ischemia/reperfusion was blunted.[362] In the Mas-deficient mice, the activation of the pro-inflammatory transcription factor NFκB was significantly diminished, whereas Ang-(1-7)-infusion into wild-type mice lead to an augmented activation of NFκB. Furthermore, Ang-(1-7) can stimulate growth of mesengial cells.[369] The signalling cascades activated by Ang-(1-7) leading to cell growth are normally associated with Ang II-induced signalling, e.g. TGF-β1 synthesis. Recently it was shown that Ang-(1-7) can stimulate the proliferation of early hematopoietic progenitor cells.[142] While the increased production of blood cells can be beneficial after chemo- and radiotherapy, the increased proliferation of white blood cells can lead to boosted inflammation or even leukaemia.

Mas is involved in mediating Ang-(1-7)-induced signalling, interacts with AT_1, and shows constitutive activity. Recent data implicate that Mas is not *per se* the Ang-(1-7) receptor,[238,370] but Mas-associated signalling requires interaction with or dissociation of an intracellular co-factor, or the generation of a heterodimeric complex with another receptor to induce a conformational change on Mas, or the interacting receptor(s), or the receptor complex to be accessible to Ang-(1-7) and thus allowing the induction of its intracellular signalling. Therefore, further experiments have to identify the co-factor(s) and/or receptor(s) interacting with Mas to build the functional receptor for the cardiovascular protective Ang-(1-7). Beside the association of Mas with Ang-(1-7)-stimulated effects[143] and the ability of Mas to inhibit AT_1-mediated Ang II signalling,[242] it was shown that the receptor Mas is constitutively active.[150,151]

This complex scheme drawn for the possible signalling properties of Mas, has to be better characterised by further investigations to identify the mechanisms diverting the actions of the ACE2/Ang-(1-7)/Mas-axis between beneficial and detrimental. Only if the pathways activated in the development and/or progression of diseases by the ACE2/Ang-(1-7)/Mas-axis are better understood, the pharmacological manipulation of the axis can yield new options for the treatment and prevention of diseases.

Nederlandse samenvatting en discussie

Peptidases die betrokken zijn bij de regulatie van RAS activatie (Hoofdstuk 2 en 3)

Na de vorming van Ang I door renine uit Agt, wordt Ang I door ACE omgezet in Ang II. Door stimulatie van AT receptoren medieert Ang II bloeddrukverhogende effecten. Perifeer Ang II speelt een belangrijke rol bij de regulatie van de vaattonus, centraal Ang II reguleert de waterinname en bepaalt ook ons drankinnamegedrag wanneer het alcoholische dranken betreft. Ang II heeft ook een aantal actieve metabolieten, zoals Ang-(1-7) en Ang III. Ang-(1-7), dat specifieke effecten veroorzaakt via de Mas receptor, kan direct uit Ang II gevormd worden door ACE2. We analyseerden de ACE2 expressie (mRNA en eiwit) in verschillende weefsels van muizen en ratten. Op grond van mRNA expressie was de ACE2 activiteit het hoogst in het ileum van zowel muizen als ratten. Een interessant punt is dat de weefseldistributie verschillend is in de twee knaagdiersoorten die we onderzocht hebben, wat het best te zien is aan de hoge ACE2 activiteit in de nieren van de muis, terwijl deze juist laag was in de rat.
Ang III, waarvan gemeend wordt dat het de Ang II-gerelateerde centrale effecten medieert, wordt geproduceerd door de afbraak van Ang II door APA. APA knockout leidde tot verhoogde ACE2 activiteit in de nieren, terwijl de activiteit van ACE2 onveranderd bleef in de longen en hersenen van deze dieren. De remming van Ang III productie in muizen zonder APA leidde ertoe dat de dieren meer gingen drinken. De gebruikelijke afname in waterconsumptie onder invloed van sociale stress was afwezig in muizen zonder APA. Echter zowel de basale alcoholconsumptie als het alcoholdrinkgedrag waren ongewijzigd in het knockout model.
Onze data over ACE2 expressie en onze bevindingen die laten zien dat er verschillende angiotensine metabolieten kunnen ontstaan laten zien dat het RAS steeds complexer wordt.

Mas en de receptor familie van Mas-gerelateerde genen in het RAS (Hoofdstuk 4 t/m 6)

Het *Mas* proto-oncogen codeert voor een G-eiwit gekoppelde receptor. Eerder onderzoek suggereerde dat er een mogelijke rol is voor Mas als een Ang II receptor. Recenter onderzoek wees uit dat Mas geen receptor voor Ang II is, maar een receptor waarvan de intracellulaire signaaltransductie op gang komt onder invloed van Ang-(1-7). We hebben aangetoond dat bloedvaten van dieren met een verminderde Mas functie anders reageerden op bloedvatverwijdende middelen - er was sprake van een verstoorde endotheelfunctie. In *in vitro* experimenten hebben we aangetoond dat Mas niet betrokken is bij de Ang II stimulatie, maar juist de door AT_1 veroorzaakte intracellulaire effecten tegengaat. Bovendien konden we laten zien dat het effect van Mas op de AT_1 signaaltransductie te wijten is aan een fysieke interactie tussen beide receptoren. In een *ex vivo* opstelling lieten we zien dat de hetero-dimerisatie van de 2 receptoren *in vitro* niet een artefact van het gebruikte cel/receptor systeem was, maar dat het ook invloed heeft op de reactiviteit van de bloedvaten. Onze resultaten laten duidelijk zien dat het AT_1-Mas complex belangrijk is als doel voor farmacologische interventie in cardiovasculaire ziekten.
Recent is de Mrg receptor familie geïdentificeerd. De Mrg-receptoren delen een hoge sequentie-homologie met de Mas receptor. De meesten van deze receptoren hebben een onbekend endogeen ligand. Onze data laat zien dat Ang peptiden transcriptie activatie en/of de afgifte van AA door middel van Mrg receptoren op een peptide-specifieke manier kunnen stimuleren. Bovendien tonen onze resultaten aan dat AT_1- en AT_2-onafhankelijke effecten veroorzaakt kunnen worden door Ang II en Ang III, en ze ondersteunen AT_1-onafhankelijke Ang IV signalling.

Nieuwe inzichten in Ang II receptor functie en regulatie (Hoofdstuk 7 en 8)

Ang II kan twee specifieke GPCRs stimuleren: de AT_1 en AT_2. In knaagdieren zijn er twee subtypes van AT_1 gespecificeerd (AT_{1a} en AT_{1b}) die niet onderscheiden kunnen worden met behulp van selectieve blokkers. In ons werk met knockout muizen die een, twee of drie Ang II receptoren missen, konden we aantonen dat AT_{1b} deels het gebrek aan AT_{1a} stimulatie kan compenseren wat de Ang II-geïnduceerde vaatwandconstrictie betreft, maar niet wat betreft de veranderingen in hartfrequentie en de afname in CF en LVSP. Bovendien laten onze data duidelijk zien dat de bloeddrukeffecten die veroorzaakt worden door Ang II volledig afhankelijk zijn van AT_1 en AT_2 en dat er geen intracellulair Ang II is bij de afwezigheid van de 3 receptoren die nu bekend zijn. Terwijl de afwezigheid van één Ang II receptor geen invloed had op de samenstelling van de vaatwand, leidde de afwezigheid van beide AT_1 subtypes tot atrofische veranderingen in het hart. We kunnen de muis die de drie bekende Ang II receptoren mist betitelen als het meest geschikte model om de kennis over effecten van het RAS die niet via AT_1 en AT_2 optreden te vergroten.

Algemene Discussie

Het RAS speelt een grote rol in de regulatie van de bloeddruk en het ontstaan van cardiovasculaire ziekten.[262,263] Niet alleen de bevinding dat naast Ang II sommige van de metabolieten biologische activiteit vertonen,[83-85,125] maar ook de ontdekking van nieuwe enzymen[105,107,108] en receptoren[143,152,153] die de RAS activiteit reguleren hebben het beeld van het RAS complexer gemaakt.

Onderzoek naar de peptidases die betrokken zijn bij de regulatie van RAS activiteit heeft nieuwe functies van deze enzymen aangetoond. In *in vitro* experimenten is ACE2 geïdentificeerd als een receptor voor het SARS virus.[123] Een andere groep bevestigde deze bevinding *in vivo*,[349] en liet zien dat de interactie van het virus met ACE2 een vermindering van de enzymproductie veroorzaakte wat leidt tot een verminderde ACE2 activiteit. Bovendien is aangetoond dat ACE2 kan inwerken op integrine $\beta 1$, een belangrijke fibronectine receptor, bij hartfalen.[350] Integrine $\beta 1$ is betrokken bij vele signaaltransductiecascades[351] en is daarom in verband gebracht met betrokkenheid bij verscheidene ziekten. De regeling van door integrine-gemedieerde signaaltransductie door ACE2 moet beter gekarakteriseerd worden om nieuwe doelen voor de behandeling van ziekten die sterk beïnvloed worden door integrines te kunnen identificeren. Naast de intracellulaire signaaltransductie die door ACE2 gemedieerd wordt, is ook door ACE gemedieerde outside-in signaaltransductie beschreven.[352] Binding van remmers aan ACE veroorzaakt fosforylering van het intracellulaire domein van het enzym. ACE fosforylering triggert de activering van c-Jun NH_2-terminal kinases en caseïne kinase-2, wat vervolgens de transcriptie van ACE zelf[353] en andere eiwitten stimuleert.[354,355] Naast de beschreven intracellulaire cascade die geactiveerd wordt door ACE veroorzaakt de fosforylering ook zgn. shedding van het ectodomein. Een dergelijke afscheiding is een veel voorkomend mechanisme dat de biologische functie van eiwitten aan het celoppervlak bepaalt. Meestal is het afscheiden een actief proces waarbij het eiwit op het celoppervlak vrijgemaakt wordt door proteinases van de MMP (matrix metalloproteinases) of ADAM (a disintegrin and metalloproteinase) families. Terwijl ADAM17 (tumor necrosis factor-α converterend enzym)[356] geïdentificeerd is als het proteinase dat verantwoordelijk is voor de afscheiding van ACE2, blijft de stof die verantwoordelijk is voor de afscheiding van ACE onbekend, dus er zou meer onderzoek gedaan moeten worden om hier achter te komen. Onlangs hebben onderzoekers aangetoond dat exogeen ACE de expressie van beide bradykinine receptor subtypen,B_1 en B_2, verhoogt via wegen waarbij NFκB en AP-1 betrokken zijn.[357] ACE activeert ook focal adhesion kinase en SHP-2 onafhankelijk van effecten op

transcriptieniveau.[358] De eiwitten die verantwoordelijk zijn voor de binding en signaaltransductie van ACE moeten nog geïdentificeerd worden. Bovendien dient nog onderzocht te worden of exogeen of gesecreteerd ACE2 een intracellulaire signaaltransductie op gang kan brengen (los van de enzymatische activiteit van dit enzym) en of de gunstige effecten die gezien worden bij de toediening van ACE2 bij acute longschade[122] alleen tot stand komen door de afname van Ang II of de toename van Ang-(1-7). Van andere celoppervlak eiwitten is bekend dat na afscheiding de achtergebleven intracellulaire gedeeltes signaalfuncties kunnen hebben, bijvoorbeeld het amyloïde precursor eiwit bij Alzheimer.[359] Het dient dus nagegaan te worden of gelijksoortige activeiten bestaan voor ACE en/of ACE2.
Er zijn niet alleen nieuwe functies van reeds bekende enzymen ontrafeld, maar recent is ook een nieuwe homoloog van ACE, genaamd ACE3, ontdekt.[360] Dit eiwit heeft niet het kenmerkende HEXXH zink-bindende domein en het vertoont daardoor ook geen catalytische activiteit. ACE3 komt voor in verscheidene zoogdiersoorten maar lijkt een pseudogen te zijn in de mens. Het eerste onderzoek bij knockout muizen zonder ACE3 leverde geen fysiologische functies voor dit nieuwe homoloog op,[361] en dus zijn verdere studies nodig om het belang van het eiwit beter te karakteriseren.
In het ‘nieuwe’ RAS kunnen twee verschillende assen geactiveerd worden: de ACE/Ang II/AT_1 as en de ACE2/Ang-(1-7)Mas as. Naast de klassieke rol in cardiovasculaire fysiologie en pathologie spelen beide assen een belangrijke rol in de (patho)fysiologie van andere aandoeningen, zoals inflammatie,[299,362] haematopoiese,[142,363,364] en acute longschade.[122]
De pas ontdekte ACE2/Ang-(1-7)/Mas as kan veel van de door Ang II veroorzaakte nadelige effecten tegenwerken. Terwijl AT_1 stimulatie door Ang II cardiale remodeling teweegbrengt middels een complex mechanisme dat resulteert in de reductie van hartfunctie en een verhoogde kans op cardiale stoornissen,[365] kan Ang-(1-7) cardiale remodeling juist verminderen door de hypertrofie en fibrose die onder invloed van Ang II ontstaan af te doen nemen.[366] Tevens is aangetoond dat Ang-(1-7) in cardiale fibroblasten de door Ang II aangezwengelde productie van endotheline-1 en de leukaemia inhibiting factor vermindert.[245] Bovendien werden tegengestelde effecten van beide peptiden voor de regulatie van celgroei beschreven.[367] Ang-(1-7) remt ook de door endotheline-1 of serum veroorzaakte hypertrofie van neonatale ratte cardiomyocyten.[136] In recente onderzoeken werden antiproliferatieve effecten van Ang-(1-7) aangetoond in een model waar neointima proliferatie optreedt na stent implantatie,[368] maar ook in longkankercellen.[139] In combinatie met oudere bevindingen die het behoud aantoonden van hartfunctie, coronaire perfusie, en aorta endotheelfunctie bij behandeling van ratten met Ang-(1-7) na een myocardiaal infarct,[134] lijkt het er dus op dat de ACE2/Ang-(1-7)/Mas as de slechte effecten van de ACE/Ang II/AT_1 as afremt. Deze bevindingen wijzen er op dat er een positief effect is na de stimulatie van de ACE2/Ang-(1-7)/Mas as onder pathologische omstandigheden. Interessant is dat recent onderzoek heeft uitgewezen dat er ook een positief effect is van het onderbreken van de ACE2/Ang-(1-7)/Mas as, tenminste in de nieren. In muizen zonder de Mas receptor was de ontstekingsreactie bij ureter obstructie en ischaemie/reperfusie in de nieren sterk verminderd.[362] In muizen zonder Mas was de activatie van de pro-ontstekings transcriptie factor NFκB duidelijk afgenomen, terwijl Ang-(1-7) infusie in wildtype muizen leidde tot een verhoogde activatie van NFκB. Bovendien kan Ang-(1-7) de groei van mesangiale cellen bevorderen.[369] De door Ang-(1-7) geactiveerde signaaltransductie keten die tot celgroei leidt wordt normaliter geassocieerd met door Ang-II veroorzaakte signaaltransductie, bijvoorbeeld wat betreft TGF-β1 synthese. Onlangs is aangetoond dat Ang-(1-7) de proliferatie van vroege hematopoietische progenitor cellen kan stimuleren.[142] Terwijl de toegenomen productie van bloedcellen voordelig kan zijn na chemo- en radiotherapie, kan de toename van proliferatie van witte bloedcellen leiden tot een uitbraak van ontstekingen of zelfs leukemie.

Mas medieert de door Ang-(1-7) veroorzaakte signaaltransductie, werkt in op AT_1, en vertoont constitutieve activiteit. Nieuwe data suggereren dat Mas niet *per se* de Ang-(1-7) receptor is,[238,370] maar dat Mas-gerelateerde signaaltransductieroutes interacteren met/dissociëren van een intracellulaire co-factor, en/of dat de receptor een heterodimeer vormt met een andere receptor. Toekomstig onderzoek is nodig om deze co-factor(en) en/of de receptor(en) die samenwerken met Mas te identificeren zodat een compleet beeld ontstaat van de cardioprotectieve effecten van Ang-(1-7). Naast de associatie van Mas met door Ang-(1-7) gestimuleerde effecten[143] en de mogelijkheid van Mas om AT_1-gemedieerde Ang II signaaltransductie af te remmen,[242] is ook belangrijk dat de Mas receptor constitutieve activiteit vertoont.[150,151]
Dit complexe schema dat geschetst wordt voor de mogelijke signaaltransductiefuncties van Mas moet nu beter gekarakteriseerd worden door middel van verder onderzoek om de mechanismen te vinden die bepalen of de effecten van de ACE2/Ang-(1-7)/Mas as gunstig of schadelijk zijn. Alleen met dergelijke kennis, kan de farmacologische manipulatie van deze as nieuwe opties bieden voor de behandeling en preventie van ziekten.

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