THE ALTERNATIVE RENIN-ANGIOTENSIN SYSTEM: **EXPLORATION OF ITS THERAPEUTIC POTENTIAL**

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The alternative renin-angiotensin system: exploration of its therapeutic potential

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THE ALTERNATIVE RENIN-ANGIOTENSIN SYSTEM:

EXPLORATION OF ITS THERAPEUTIC POTENTIAL

Het alternatieve renine-angiotensine systeem:

verkenning van de therapeutische mogelijkheden

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To my wife and children

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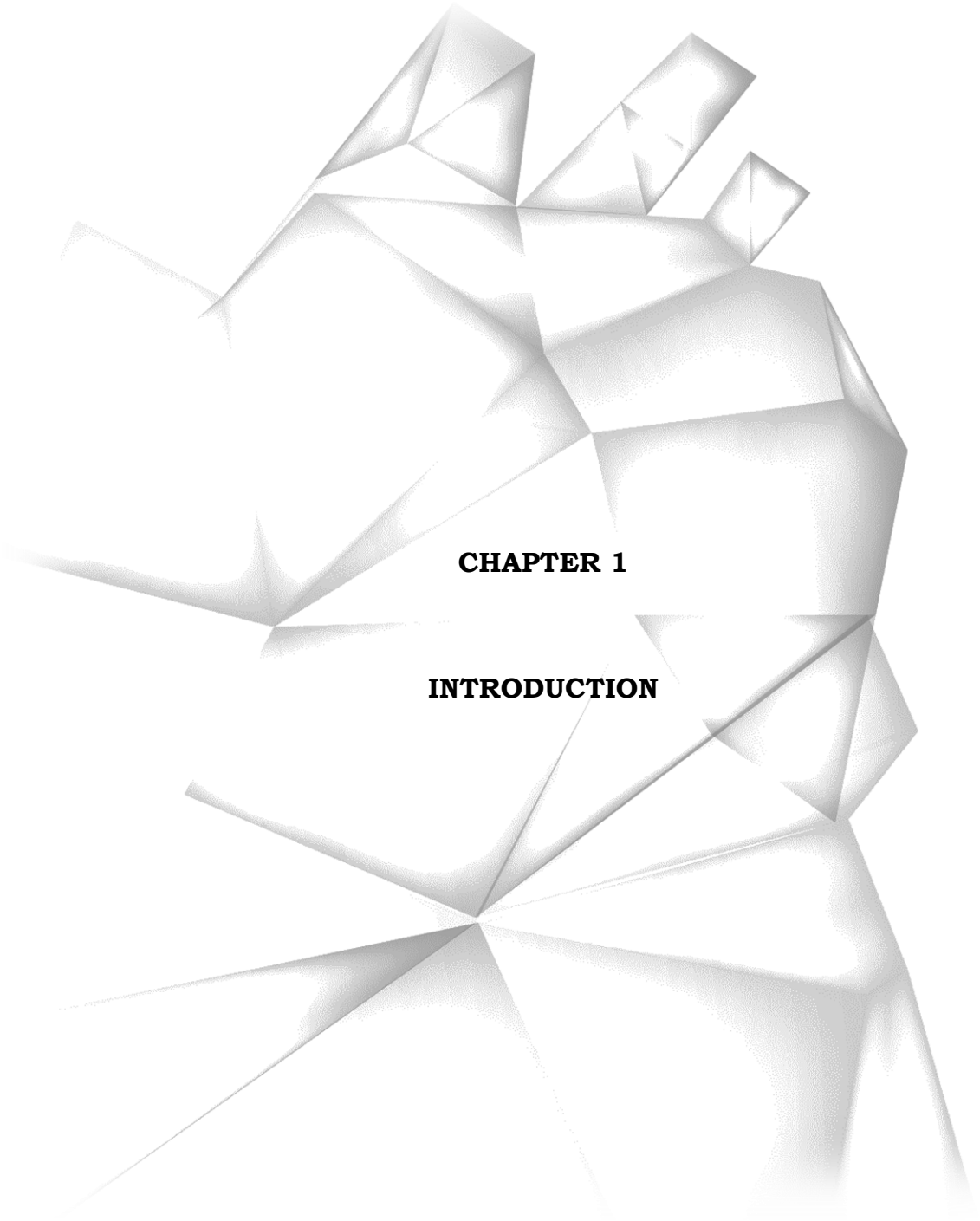

CHAPTER 1

INTRODUCTION

Based on: *Key developments in renin-angiotensin-aldosterone system inhibition.* Sevá Pessôa B, van der Lubbe N, Verdonk K, Roks AJM, Hoorn EJ, Danser AHJ. Nat Rev Nephrol. 2013;9:26-36.

ABSTRACT

The renin–angiotensin system (RAS) was initially thought to be fairly simple. However, this idea has been challenged following the development of RAS blockers, including renin inhibitors, angiotensin-converting-enzyme (ACE) inhibitors, type 1 angiotensin II (AT_1)-receptor blockers and mineralocorticoid-receptor antagonists. Consequently, new RAS components and pathways that might contribute to the effectiveness of these drugs and/or their adverse effects have been identified. For example, an increase in renin levels during RAS blockade might result in harmful effects via stimulation of the prorenin receptor (PRR), and prorenin—the inactive precursor of renin—might gain enzymatic activity on PRR binding. The increase in angiotensin II levels that occurs during AT_1-receptor blockade might result in beneficial effects via stimulation of type 2 angiotensin II receptors. Moreover, angiotensin 1–7 levels increase during ACE inhibition and AT_1-receptor blockade, resulting in Mas receptor activation and the induction of cardioprotective and renoprotective effects, including stimulation of tissue repair by stem cells. Finally, a role of angiotensin II in sodium and potassium handling in the distal nephron has been identified. This finding is likely to have important implications for understanding the effects of RAS inhibition on whole body sodium and potassium balance.

INTRODUCTION

In the past few decades our understanding of the complexity of the renin–angiotensin system (RAS) has increased considerably, largely as a result of knowledge obtained following the introduction of RAS blockers, such as renin inhibitors, angiotensin-converting-enzyme (ACE) inhibitors, type 1 angiotensin II (AT_1) receptor blockers (ARBs) and mineralocorticoid receptor antagonists (MRAs). Although these agents do not always uniformly suppress angiotensin II and aldosterone levels or activity—breakthroughs, during which angiotensin II and aldosterone levels return to, or even increase above, their pretreatment levels often occur[1, 2]—they have been used successfully to treat cardiovascular and renal diseases.[3] The efficacy of RAS blockers despite the occurrence of angiotensin II and aldosterone breakthroughs suggests that their beneficial effects are not solely attributable to blockade of the angiotensin II–AT_1 receptor–aldosterone–mineralocorticoid receptor axis. For example, high angiotensin II levels, particularly during AT_1 receptor blockade, might stimulate activation of type 2 angiotensin II (AT_2) receptors, which are thought to oppose the effects of AT_1 receptor activation. The latter include vasoconstriction, stimulation of growth and remodelling, sympathetic nervous system activation, and Na^+ and water retention.[4, 5] Similarly, treatment with ACE inhibitors and ARBs results in the production of high levels of angiotensin II metabolites (Figure 1), most importantly angiotensin 1-7 (Ang-(1-7)), which exerts cardioprotective and renoprotective effects via the Mas receptor.[6] Ang-(1-7)–Mas receptor interactions might also contribute to the success of RAS blockade.

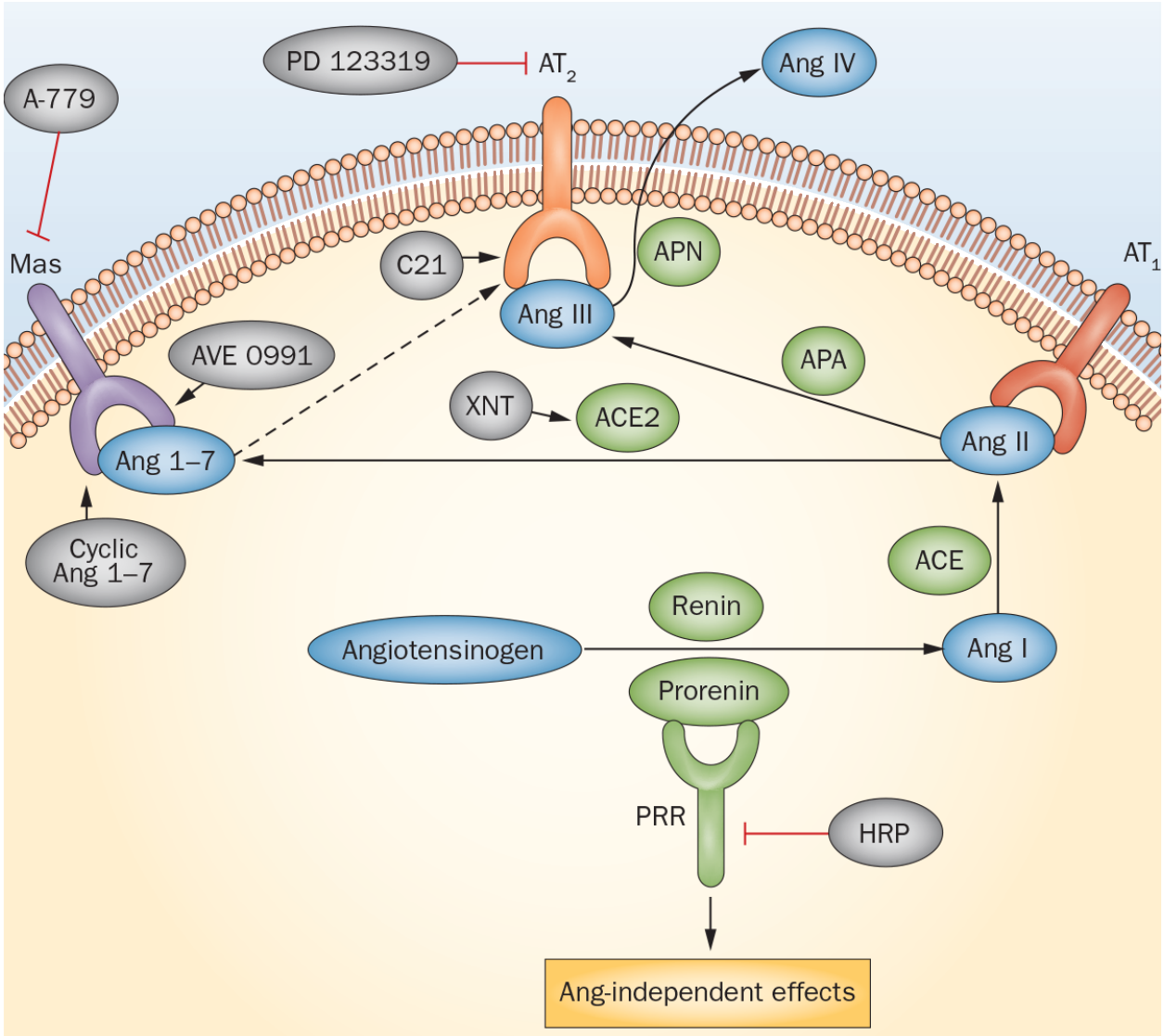

Figure 1: New developments in the renin–angiotensin-system cascade. Binding of the renin precursor, prorenin, to the PRR activates the protein, enabling prorenin to cleave angiotensinogen and generate angiotensin 1. Renin and prorenin also act as agonists of the PRR and induce angiotensin-independent effects, such as ERK1/2 activation. APA generates angiotensin III from angiotensin II, and APN subsequently degrades angiotensin III to angiotensin IV. Binding of angiotensin II to the AT1 receptor induces vasoconstriction, stimulation of growth and remodelling, sympathetic nervous system activation, and sodium and water retention. Angiotensin III is thought to be the preferred endogenous agonist of the AT2 receptor, which mediates effects that either resemble or oppose those of AT1- receptor activation. ACE2 generates angiotensin 1–7 from angiotensin II, and this metabolite activates the Mas receptor (and possibly AT2 receptors), thereby inducing beneficial effects (such as improvement in endothelial function, reduced fibrosis and enhanced tissue repair by stem cells) that oppose those of AT1-receptor activation. New drugs in development include antagonists of the PRR (HRP), AT2 receptor (PD123319) and Mas receptor (A779), and activators of the AT2 receptor (C21), Mas receptor (AVE 0991, cyclic angiotensin 1–7) and ACE2 (XNT). Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting-enzyme 2; Ang, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; AT1, type 1 angiotensin II; AT2, type 2 angiotensin II; ERK1/2, extracellular signal-regulated kinase 1/2; HRP, handle region peptide; PRR, prorenin receptor.

RAS blockade causes an increase in renin levels[7] as a result of interference with the negative feedback loop between angiotensin II and renin release. This effect explains, at least in part, why angiotensin II and aldosterone breakthroughs occur. In addition to renin, the levels of its precursor, prorenin, might also increase. Since the discovery of the prorenin receptor (PRR),[8] which *in vitro* induces profibrotic effects following renin or prorenin binding, it has been proposed that increases in renin and prorenin levels will not only result in diminished RAS suppression, but also in unwanted effects via PRR stimulation.[9]

Aldosterone interacts with angiotensin II in a synergistic manner and might exert nongenomic, acute effects via a mineralocorticoid-receptor-independent mechanism.[10-12] Unravelling this interaction at the level of the kidney is of importance to obtain a full understanding of the different effects of aldosterone during hypovolemia (sodium retention, potassium conservation) and hyperkalemia (potassium secretion), that is the aldosterone paradox. A better understanding of this paradox and of the specific roles of angiotensin II and aldosterone in sodium and potassium handling in the kidney is likely to be important to fully appreciate the effects of RAS inhibition on sodium and potassium balance.

In this Review, we describe the available evidence regarding the roles of the PRR, AT_2 receptor, Ang-(1–7) and aldosterone-angiotensin II interactions in the RAS cascade. Focusing mainly on the kidney, we discuss the potential of agents such as PRR blockers, AT_2-receptor agonists and Mas-receptor agonists to further enhance the degree of RAS blockade with potentially beneficial effects in patients with cardiovascular and renal diseases.

THE PRORENIN RECEPTOR

Activation of prorenin

The PRR is an almost ubiquitously expressed 350-amino-acid protein that can bind both renin and prorenin.[8, 13, 14] Binding to the PRR induces prorenin to undergo a conformational change, which allows the prosegment to move out of the catalytic cleft, resulting in full, nonproteolytic activation of the protein.[8, 13] This mechanism differs from the proteolytic activation of prorenin in the kidney, during which the prosegment is cleaved off by an as yet unidentified enzyme.[15] Given the much higher levels of prorenin (which is largely derived from the kidney, but also from extrarenal sources)[15] than renin in blood plasma, and the fact that prorenin–renin converting enzymes are only expressed in the kidney, the hypothesis that PRR binding enables the normally inactive prorenin to gain angiotensin-generating activity at tissue sites is attractive.[16] However, a study showed that following renin or prorenin exposure, the PRR directly activated the extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway independently of the formation of angiotensin II.[8, 17] This activation was not blocked by renin inhibitors, suggesting that binding of these inhibitors to prorenin does not prevent binding of prorenin to the PRR, although inhibitor binding did prevent the generation of angiotensin I.[13, 17]

As the affinity of prorenin for the human PRR is 3–4-fold higher than that of renin,[14] the assumption that the prosegment facilitates binding to the PRR seems reasonable. The prosegment contains a 'handle region' (peptides 10–19), which is thought to bind to the PRR and enable catalytic activation of prorenin.[18] Peptidic antagonists, known as handle region peptides (HRPs), bind competitively to the PRR, thereby preventing PRR-mediated prorenin activation and reducing RAAS activity in tissues.[19, 20] The structure of prorenin is highly species-specific so different HRPs have been designed for use in humans, rats and mice.

RAS-dependent function

In rats, ubiquitous overexpression of the human PRR resulted in proteinuria, glomerulosclerosis[21] and renal cyclooxygenase-2 (COX-2) upregulation.[22] The human PRR binds, but does not activate rat prorenin,[21] therefore, it was not surprising that overexpression of the human PRR did not alter angiotensin levels in the plasma and tissues of transgenic rats.[22] However, these animals showed increased levels of aldosterone in their blood plasma.[23] This increase was unlikely to be the result of prorenin-induced PRR stimulation because neither renin nor prorenin affected aldosterone synthesis in the human adrenocortical cell lines H295R and HAC15.[23] An infusion of HRP prevented the development of glomerulosclerosis in transgenic rats that expressed the human PRR.[21]

In diabetic rats, HRP therapy normalized high renal angiotensin levels without affecting blood pressure, and prevented the development of nephropathy.[19] HRP therapy also prevented the development of nephropathy in diabetic type 1a angiotensin II (AT_{1A}) receptor-deficient mice,[20] suggesting that this effect was not dependent on the suppression of local angiotensin generation. Interestingly, extracellular signal-regulated kinase 1 and 2 (ERK1/2) as well as phospho-p38 mitogen-activated protein kinase (MAPK) and phospho-Jun N-terminal kinase (MAPK) were upregulated in the diabetic kidneys of both wild-type and AT_{1A} receptor-deficient mice, and HRP (but not ACE inhibitor) therapy fully normalized this increased phosphorylation.[20]

Evidence supporting a renin–PRR interaction came from a study that showed that renin activated ERK1/2 in mesangial cells, even in the presence of renin inhibitors, ACE inhibitors or ARBs.[24] This activation resulted in production of transforming growth factor-β1 (TGF-β1) and the subsequent upregulation of genes encoding profibrotic molecules, such as plasminogen-activator inhibitor-1 (PAI-1), fibronectin and collagens.[8, 24] ERK1/2 activation occurred in response to <1 nmol/l of renin, and could be fully prevented by

PRR small interfering RNA (siRNA).[24] Similar profibrotic effects with both renin and prorenin were observed in human embryonic kidney cells, although concentrations of 100 nmol/L were required to induce gene upregulation.[25] Remarkably, in podocytes, 2 nmol/l human prorenin increased ERK1/2 activation without affecting TGF-β1 or PAI-1 levels.[26] These data suggest that the PRR–ERK1/2-mediated activation of profibrotic pathways does not occur in all cell types. Interestingly, in cultured nephritic glomeruli from rats with glomerulonephritis, concomitant PRR inhibition (using siRNA) and angiotensin II blockade (using an ACE inhibitor) had a greater suppressive effect on TGF-β1 and PAI-1 expression than either inhibitor alone.[27] The authors attributed this effect to the fact that ACE inhibition increases renin mRNA levels, thereby counteracting the consequences of angiotensin II suppression, since renin upregulates TGF-β1 and PAI-1 by stimulating the PRR.[27]

In a gene expression profiling study, treatment of human mesangial cells with renin (20 nmol/l) and prorenin (50 nmol/L) activated pathways implicated in organ damage.[28] For example, upregulation of COX-2 and activation of the TGF-β1–PAI-1 pathway were observed. Although the effects of renin were greater than the effects of prorenin, there was extensive overlap between the gene signatures, indicating that renin and prorenin act through similar pathways. In podocytes, COX-2 overexpression exacerbated diabetic nephropathy by increasing PRR expression.[29] Thus, not only does PRR stimulation result in COX-2 upregulation,[22] but the reverse is also true, possibly because COX-2 metabolites promote PRR activation.[29] Indeed, the presence of this positive feedback loop was confirmed in a study that showed that high glucose levels upregulated expression of the PRR and prorenin in rat mesangial cells, thereby facilitating generation of angiotensin II, which subsequently induced expression of COX-2 and interleukin-1β.[30] In addition, COX-2 inhibition reduced glucose-induced PRR upregulation, suggesting that products generated by COX-2 upregulate the PRR.[29, 30] Expression of the PRR

in glomeruli and renal tubules was increased in diabetic rats, possibly as a result of enhanced AT_1 receptor and NADPH oxidase activity.[31] In diabetic rats, HRP treatment did not seem to affect PRR expression *in vivo*, whereas ARB therapy reduced PRR expression.[32]

Effects of RAS blockade

Consistent with the stimulatory effects of AT_1 receptors on PRR expression,[31] RAAS blockade reduced PRR expression in the kidneys of diabetic rats.[33] However, in a Goldblatt rat (high angiotensin II) model of hypertension, parallel increases in PRR expression and renin levels were observed.[34] PRR upregulation also occurred in the remnant kidneys (particularly in the tubular cells) of five-sixths nephrectomized rats,[35] and in the kidneys (mainly in the tubular cells and collecting ducts) of patients with end-stage renal disease caused by diabetic nephropathy.[36] These contrasting findings suggest that PRR expression might also be regulated in a RAS-independent manner, for example via the NADPH oxidase-dependent generation of reactive oxygen species.[25, 31]

Despite the above data, we still do not know for certain whether prorenin–PRR interactions occur *in vivo*, and whether the therapeutic effects of HRPs are attributable to interference with such interactions. Importantly, the affinity of the PRR for renin and prorenin is in the high nanomolar range (possibly even 20 nmol/l for renin),[14] which is difficult to reconcile with the picomolar levels of renin (~0.5 pmol/l) and prorenin (~5 pmol/l) in extracellular fluid. Which is difficult to reconcile with the picomolar levels of renin (~0.5 pmol/l) and prorenin (~5 pmol/l) in extracellular fluid.[37] However, this affinity is consistent with the findings that PRR overexpression did not alter the levels of RAS components in rats,[22, 38] and that in most *in vitro* studies in which prorenin–PRR interaction were investigated, high nanomolar prorenin concentrations (≤100 nmol/L) were required for activation of ERK1/2 and upregulation of profibrotic genes.[6, 25, 28, 39] Moreover, in rodents, prorenin overexpression (which resulted in an up to 400-fold increase in plasma

prorenin levels), increased blood pressure in an angiotensin-dependent manner[40-42] but did not cause the fibrosis or glomerulosclerosis that was expected based upon the findings of *in vitro* studies with renin and prorenin. [17, 25, 28, 29] Even larger increases in plasma prorenin levels might be required to produce such effects. However, under pathological conditions and/or during RAS blockade, prorenin and renin levels do not normally increase more than three orders of magnitude above baseline. Whether such high levels occur in prorenin-synthesizing tissues, such as in renal interstitial fluid, is not known. The maximum increases in plasma prorenin levels that have been shown in humans—for example during severe heart failure or RAS blockade—are ~50–100-fold for renin (although the increase is normally well below 10-fold), and twofold to threefold for prorenin.[1, 37, 43]

Not surprisingly, as a result of the negative feedback loop between angiotensin II and renin, the largest increases in renin levels occur during the highest levels of RAS blockade. Consequently, dual or even triple RAS blockade (using a combination of RAS blockers) resulted in a greater increase in renin levels than did RAS blockade using a single RAS blockade approach.[44] Studies of spontaneously hypertensive rats kept on a low-sodium diet, showed that dual RAS blockade resulted in massive increases (up to several-hundred-fold) in both plasma and renal renin levels, and depletion of angiotensinogen.[44] As a consequence, a large decrease in blood pressure and severe renal failure occurred. These findings are reminiscent of those of the ALTITUDE study, in which the efficacy of the renin inhibitor aliskiren was evaluated versus placebo in high-risk patients with type 2 diabetes mellitus and renal dysfunction, whose blood pressure was normalized using one or more antihypertensive drugs, including ACE inhibitors and ARBs.[45] The trial was stopped prematurely because of a lack of beneficial effects and an increased incidence of serious adverse events, including hypotension, hyperkalaemia and renal complications, in the patients who received aliskiren. The latter adverse-effect profile is entirely consistent with the consequences of angiotensin II and/or aldosterone

depletion, and thus deleterious PRR stimulation in response to increased renin and/or prorenin levels during combined aliskiren and ACE inhibitor or aliskiren and ARB treatment does not need to be invoked to explain these adverse effects. Moreover, although the levels of prorenin and renin in the patients who received aliskiren in the ALTITUDE trial have not yet been reported, it is highly unlikely that they were ≥10,000 times greater than the levels in untreated patients and, therefore, within the range required to stimulate the human PRR *in vivo*.[39] In addition, as discussed above, RAS blockade in rodents induced a decrease, rather than an increase, in PRR expression.[31-33]

RAS-independent functions

If prorenin levels *in vivo* are too low to induce PRR stimulation, even during RAS blockade or under pathological conditions, the phenotype that develops in response to PRR overexpression *per se* must be the result of RAS-independent effects of PRR stimulation. Colocalization of the PRR with V-type proton ATPase (V-ATPase) in the kidney has been reported.[46] This colocalization might occur because the 8.9 kDa accessory protein ATP6AP2 of V-ATPase is a post-translationally truncated version of the PRR, which resembles the C-terminal domain of the receptor.[14] V-ATPases have an important role in the acidification of subcellular compartments and the PRR is indispensable for V-ATPase integrity; in mice with podocyte-specific PRR knockout, the abundance of several V-ATPase subunits in podocytes was reduced, resulting in defective autophagy, severe endoplasmatic stress, and podocyte necrosis.[47] As a consequence, the mice died at 2–4 weeks of age due to renal failure. The PRR also functions as an adaptor between V-ATPase and receptors for members of the Wnt family of signalling molecules.[48] These findings clearly indicate the importance of the PRR beyond renin/prorenin binding, and raise doubt that the PRR binds to and activates prorenin *in vivo*. However, they do not rule out the possibility that the PRR has an important role in tissue damage, or that the

beneficial effects of RAS blockade occur as a result of a reduction in PRR expression.

The available data on the effects of HRPs are inconclusive, mainly because no study has convincingly shown that these drugs block PRR–prorenin interactions *in vivo*. A possibility exists that HRPs are partial PRR agonists that exert their effects independently of renin and prorenin,[49] thus explaining some of the conflicting results. However, given the interference with V-ATPase, PRR blockade *per se* would not necessarily have beneficial effects.

AT_2 RECEPTORS

AT_2 receptors are generally thought to oppose classical AT_1-receptor-mediated effects, such as vasoconstriction, sodium retention and inflammation. In the kidney, AT_2 receptors are expressed in the proximal tubules, collecting duct and renal resistance arteries,[4] suggesting a role in renal haemodynamics and tubular function. Pathological conditions, such as diabetes and kidney injury, usually increase renal AT_2-receptor expression, and concomitant ARB treatment further enhances this upregulation.[50-52] AT_2-receptor-knockout mice had either increased blood pressure at baseline or an increased hypertensive response to angiotensin II compared with wildtype mice, supporting a vasodilatory role of AT_2 receptors.[53, 54] The knockout mice also showed a rightward shift of the pressure-natriuresis curve and a more pronounced antinatriuretic response to angiotensin II than wildtype mice, again supporting a counter-regulatory role of AT_2 receptors.[55] Moreover, unilateral urethral obstruction resulted in increased renal interstitial fibrosis and apoptosis in AT_2 receptor knockout mice compared with wildtype mice.[56, 57] However, an important consideration is that AT_2-receptor-null mice showed increased expression of the AT_1 receptor, which might explain these findings.[58]

Effects of stimulation

ARB pretreatment is usually required to allow angiotensin II-induced, AT_2-receptor-mediated hypotensive and natriuretic effects to be observed in wild-type animals,[4, 59] suggesting that the opposing actions of the AT_1 receptor usually predominate. Interestingly, in rats angiotensin III (angiotensin 2–8) rather than angiotensin II seems to be the preferred agonist of the AT_2 receptor (Figure 1);[60] blockers of aminopeptidase type A, which converts angiotensin II into angiotensin III, inhibited AT_2-receptor-mediated natriuresis, whereas blockers of the degradation of angiotensin III (by aminopeptidase N) enhanced this effect.[61, 62]

Development of the selective AT_2 receptor agonist C21 has made investigation of the effects of AT_2 receptor stimulation without concomitant ARB treatment possible. In obese Zucker rats, C21 induced natriuresis in a NO/cGMP-dependent manner.[63, 64] This effect was prevented by preinfusion of the AT_2 receptor antagonist PD123319.[63, 64] C21 infusion also increased the fractional excretion of lithium, suggesting involvement of the proximal tubules in AT_2 receptor-mediated natriuresis.[63] Although AT_2 receptor stimulation has been reported to increase renal blood flow in Sprague-Dawley rats, AT_2 receptor stimulation did not affect glomerular filtration rate, and thus altered renal hemodynamics are unlikely to underlie the increased natriuresis.[65] AT_2 receptor-mediated anti-inflammatory effects have been observed in the rat 2-kidney, 1-clip model of hypertension; C21 decreased the renal interstitial levels of TNF-α, interleukin-6, and TGF-β1, and activated the NO/cGMP cascade in a blood-pressure-independent manner.[64]

Based upon findings in AT_2 knockout animals, AT_2 receptor-mediated natriuresis, and C21-induced activation of the NO/cGMP cascade, one would expect that C21 infusion lowers blood pressure. Remarkably, however, the majority of studies have shown no effect of C21 on blood pressure, whereas other studies showed hypertension or hypotension in response to C21

treatment.[66] Increases in blood pressure in response to C21 treatment might be a result of nonselective AT_1 receptor stimulation occurring at high C21 doses.[4] C21-induced decreases in blood pressure could not be blocked by PD123319, and might be the result of a C21-dependent blockade of calcium transport into the cell.[66]

Effects of blockade

Studies of the effects of AT_2 receptor blockade using PD123319 have also yielded conflicting results. In subtotally nephrectomized rats, PD123319, alone or in combination with ARB treatment, decreased proteinuria as well as monocyte and macrophage infiltration into the remnant kidney.[67] Similarly, dual AT_1-receptor blockade and AT_2-receptor blockade, using PD123319 and the AT_1 antagonist losartan, prevented activation of the NFκB pathway in the mouse unilateral ureteral obstruction model of renal injury,[68] and in rats, the glomerular infiltration of monocytes and macrophages following angiotensin II infusion depended on AT_2-receptor-mediated chemokine induction.[69] In a rat renal wrap hypertension model, angiotensin II–AT_2 receptor interactions increased renal interstitial bradykinin, thus activating the protective NO/cGMP pathway.[70] In rats, however, PD123319 treatment following renal ablation increased renal damage and blood pressure, most likely because AT_2-receptor-mediated vasodilation was prevented, and, therefore, ischaemic damage was increased in the remnant tissue.[50] Finally, AT_2-receptor stimulation in stroke-prone, spontaneously hypertensive rats reduced monocyte and macrophage infiltration in the aorta and the kidney.[71]

Detrimental effects

Although data from a wide range of studies in healthy and diseased animal models support a beneficial role of AT_2-receptor stimulation, resulting in anti-inflammatory, hypotensive and natriuretic effects,[50, 53-65, 71] the opposite has also been reported.[66-69] One explanation for these contrasting data is that the phenotype of AT_2 receptors is altered under pathological conditions and/or

with age. Indeed, AT_2-receptor-mediated vasoconstriction has been observed in spontaneously hypertensive rats,[72] and only lowering blood pressure enabled the return of bradykinin/NO-mediated relaxant responses.[73] The change of phenotype might relate to the location of the receptor (endothelium or smooth muscle cell), as well as its capacity to heterodimerize with AT_1 receptors.[4] At this stage, the clinical application of an AT_2-receptor agonist seems premature, particularly because a comparison of the effects of ACE inhibitors and ARBs in humans has not revealed major outcome differences.[74] Nevertheless, the availability of a selective AT_2-receptor agonist will help obtain a better understanding of the location, age and disease-dependent roles of the AT_2 receptor and determine in which diseases such drugs might be applied.

ACE2–ANGIOTENSIN 1-7–MAS RECEPTOR AXIS

The ACE homologue ACE2 cleaves angiotensin II to generate Ang-(1–7), which opposes AT_1-receptor-mediated effects via stimulation of the Mas receptor (Figure 1).[6] Ang-(1–7) can also be generated from angiotensin I, via angiotensin 1–9, again with the help of ACE2.[75] According to some studies, Ang-(1–7) binds to AT_2 receptors, and high levels of Ang-(1–7) might stimulate AT_1 receptors.[76] ACE2 and Mas receptors are expressed in kidney, heart and vascular tissue, suggesting that Ang-(1–7) might have a physiological, protective role in these organs.[77] Indeed, in rodent models of cardiac ischemia and heart failure, Ang-(1–7) infusion or ACE2 overexpression preserved cardiac and endothelial function, and in models of hypertension and diabetes, such treatment prevented renal and cardiovascular anomalies.[77, 78] In spontaneously hypertensive rats, the Mas receptor antagonist A779 abrogated the antihypertensive and antiproteinuric effects of ACE inhibitors, suggesting that these effects depend, at least in part, on activation of the Mas receptor.[79, 80] Mas-receptor activation increased endothelial NO release and reduced oxidative stress, thereby eliminating the pro-oxidant properties of angiotensin II, and thus resulting in antihypertrophic, antifibrotic and renoprotective effects.[81-83] Somewhat confusingly, transfection of the Mas receptor into murine renal

proximal tubular cells attenuated angiotensin II-stimulated TGF-β1 expression, whereas in human mesangial cells, Ang-(1–7) stimulated the TGF-β1-dependent profibrotic pathway—an effect that was inhibited by A779.[84, 85] These conflicting data, which are reminiscent of the contrasting data regarding AT_2-receptor-mediated effects, might relate to the existence of Mas–AT1 receptor heterodimers.[86]

Role in stem cell regulation

In the bone marrow, haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) differentiate into cardiovascular and renal progenitor cells, which have a major role in tissue repair. Importantly, clinical studies have shown that cardiac grafting of autologous bone marrow cells improved cardiac performance in patients with ischaemic heart disease, an effect that appeared to be mainly due to improved angiogenesis.[87] Similarly, MSC infusion improved kidney function in patients with chronic kidney disease.[88, 89] Given that all of the major components of the RAAS are present in bone marrow,[90] this system is now believed to have an important role in HSC and MSC regulation. In mice, treatment with Ang-(1–7) after irradiation or chemotherapy stimulated regeneration of bone marrow.[91, 92] Another rodent study showed that activation of the Ang-(1–7)–Mas-receptor pathway stimulated bone marrow cells to form early endothelial progenitor cells,[93] although, paradoxically, antiangiogenic properties of Ang-(1–7) have also been reported.[94] Nevertheless, stimulation of Mas receptors in bone marrow might be a strategy to improve tissue repair by stem cells.

Novel therapies

Given the rapid breakdown of the peptide Ang-(1–7) *in vivo*, novel therapies are now being developed to stimulate the ACE2–angiotensin 1–7–Mas receptor axis. Ang-(1–7) encapsulation in an oligosaccharide (hydroxypropyl β-cyclodextrin) offers protection during gastrointestinal passage, and oral application of encapsulated Ang-(1–7) was cardioprotective in infarcted rats.[95] Cyclic

angiotensin 1–7 (cAng-(1–7)), an Ang-(1–7) analogue with a thioether bridge between amino acid residues 4 and 7, is resistant to metabolism, and can be delivered via oral and pulmonary routes.[96] Interestingly, cAng-(1–7) selectively activated Mas receptors without binding to AT_1 or AT_2 receptors[97] and improved cardiac remodelling and endothelial function after myocardial infarction.[98] Furthermore, the nonpeptide Ang-(1–7) analogue AVE 0991 prevented endorgan damage in spontaneously hypertensive rats treated with a NO synthesis inhibitor[80] and also enhanced acetylcholine-induced vasodilation in Wistar rats.[99] Finally, the ACE2 activator XNT improved cardiac function, and reduced cardiac, renal and pulmonary fibrosis in diabetic rats and in spontaneously hypertensive rats.[100, 101]

In summary, Mas-receptor stimulation is a promising new approach to improve tissue repair by stem cells. Current strategies are directed either at generating high endogenous levels of Ang-(1–7) using ACE2 activators, administering stable Ang-(1–7) analogues or encapsulating Ang-(1–7) in oligosaccharides to slow down its breakdown following oral administration.

ANGIOTENSIN–ALDOSTERONE INTERACTIONS

The classical paradigm

Physiologically, the RAS operates in the kidney to increase renal sodium retention during hypovolaemia and potassium secretion during hyperkalaemia. The classical paradigm states that angiotensin II primarily increases sodium reabsorption by the proximal tubule, whereas aldosterone stimulates sodium uptake and potassium secretion in the more distal parts of the nephron—the distal convoluted tubule, connecting tubule, and collecting duct.[102]

Sodium potassium transporters

In the past two decades, the major sodium and potassium transport proteins in the nephron have been cloned and characterized. In the proximal tubule, angiotensin II mediates sodium bicarbonate reabsorption through

sodium/hydrogen exchanger 3,[103] and in the distal nephron, the sodium chloride cotransporter (NCC),[104] epithelial sodium channel (ENaC),[105] and the renal outer medullary potassium channel (ROMK)[106, 107] have all been shown to be sensitive to aldosterone. The NCC is located in the 'early' distal convoluted tubule, whereas the ENaC is located in the 'late' distal convoluted tubule, connecting tubule and collecting duct, and the ROMK is expressed along the entire distal nephron.[108] These aldosterone-sensitive transporters are important clinically because they are direct or indirect targets of several antihypertensive drugs, including ACE inhibitors, ARBs, thiazide diuretics, amiloride and MRAs.

Angiotensin II actions in the distal nephron

The paradigm that angiotensin II acts proximally in the kidney has now been challenged. Several studies, including ours, have shown that angiotensin II also regulates sodium and potassium handling in the distal nephron.[109-115] Although abundantly located in the proximal tubule, ^{125}I– angiotensin II binding studies have shown that AT receptors are also present in the thick ascending limb, distal convoluted tubule and collecting duct.[116] Wang and Giebisch were some of the first to investigate the effects of angiotensin II on sodium and potassium transport in the distal tubule.[117] Using isolated perfused tubules, they showed that administration of angiotensin II increased sodium transport and decreased potassium transport in the early and late distal convoluted tubule.[117] Treatment with either an ARB or the ENaC blocker, amiloride, abolished these effects.[117] These data suggest that angiotensin II activates the ENaC and NCC, although the effect of NCC blockade using thiazides was not investigated. More compelling evidence that angiotensin II activates the NCC came from a rat study, which showed that ACE inhibition stimulated acute trafficking of the NCC from the apical plasma membrane to subapical cytoplasmic vesicles.[109] This effect was reversed by angiotensin II infusion. However, because angiotensin II also induces aldosterone secretion

and aldosterone also activates NCC,[104] the effect of angiotensin II on NCC was still not proven unequivocally.

To more clearly determine the effect of angiotensin II and aldosterone on the NCC, we treated adrenalectomized rats with a chronic infusion of either aldosterone or angiotensin II.[110] The latter therapy increased the levels of both total NCC and phosphorylated NCC in plasma membrane fractions independently of aldosterone. We also showed that angiotensin II caused sodium retention and that this was reversed by thiazides.[110] In a subsequent study, we investigated whether aldosterone required angiotensin II to activate the NCC.[111] We showed that aldosterone treatment increased the expression of NCC and ENaC in adrenalectomized rats, even in the presence of losartan; therefore, aldosterone alone is sufficient for these effects. However, the addition of losartan to the infusion of aldosterone reduced the levels of phosphorylated, and, therefore, active, NCC. These data suggest that angiotensin II might have an additive effect on aldosterone-induced NCC activation.

In our studies, the effects of angiotensin II on ENaC expression were less pronounced in terms of plasma membrane abundance than the effects on NCC expression; angiotensin II increased the expression of the β-subunit of the ENaC only.[110] However, more functional studies have shown that angiotensin II increases the open probability of the ENaC.[112, 113] In freshly isolated, split-opened murine distal nephrons, angiotensin II acutely increased the open probability of the ENaC, whereas more prolonged exposure to angiotensin II induced a translocation of α-ENaC to the apical plasma membrane.[112] Interestingly, aldosterone did not acutely increase the open probability of the ENaC and the effect of angiotensin II on the ENaC persisted when mineralocorticoid receptors were saturated during the infusion of high doses of aldosterone. Thus, angiotensin II seems to have an additive effect to that of aldosterone in the activation of both the NCC and the ENaC.[111, 112] The effects of angiotensin II on the ROMK have also been investigated using patch clamp recordings in split-opened collecting ducts.[114] Angiotensin II dose-dependently

inhibited ROMK channel activity in rats on a low potassium diet, but not in rats on a normal potassium diet.[114] Conversely, a high potassium diet inhibited NCC and increased ROMK activity.[114, 118] In summary, the observations of Wang and Giebisch[117] can now be explained by a stimulatory effect of angiotensin II on the NCC and the ENaC, and an inhibitory effect on the ROMK.

Regulation of sodium and potassium transporters

In addition to receptor–transporter interactions, the intracellular signalling pathways that regulate sodium and potassium transporters have also been a matter of intense research. This interest was stimulated by the discovery that mutations in with-no-lysine (WNK) kinases directly affected transporter activity and caused hypertension.[119] The current model of kidney transporter regulation by kinases has evolved to a complex kinase network that also includes the serine/threonine-protein kinase Sgk1 (SGK1) and STE20/SPS1-related proline-alanine-rich protein kinase (SPAK). This kinase network regulates the NCC, ENaC, and ROMK.[120] For example, in oocytes, cells[115] and WNK4-knockout mice,[121] angiotensin II has been shown to activate the NCC through a SPAK-WNK4 dependent pathway. In rodents, a low sodium diet or an infusion of aldosterone or angiotensin II increased the SPAK-mediated phosphorylation of NCC.[110, 122] The response of NCC to dietary sodium and potassium is mediated by SGK1,[123] whereas the inhibition of ROMK activity by angiotensin II is mediated by both SGK1 and WNK4.[124] These kinases are, therefore, obvious drug target candidates.[125]

The aldosterone paradox

The findings discussed above increase our understanding of the aldosterone paradox; the question as to how the same hormone can cause sodium retention during hypovolaemia and potassium secretion during hyperkalemia.[126, 127] During hypovolaemia, levels of both angiotensin II and aldosterone are increased, causing a synergistic activation of both NCCs and ENaCs, which

promotes maximal sodium reabsorption (Figure 2). At the same time, potassium is conserved because sodium reabsorption through the NCCs is electroneutral and does not require potassium secretion through the ROMK, which is inhibited by angiotensin II.[114, 124] Conversely, during hyperkalaemia, aldosterone levels are maximally increased, whereas angiotensin II levels are suppressed, resulting in NCC inhibition and increased sodium delivery to ENaCs. Maximum ENaC activity facilitates electrochemical sodium reabsorption that promotes kaliuresis through the ROMK. Hyperkalaemia or a high potassium diet might also directly inhibit NCC activity by increasing expression of WNK4.[128]

A better understanding of the aldosterone paradox is not only important physiologically, but also to understand the effects of RAS inhibition on sodium and potassium homeostasis. For example, the synergistic activation of NCCs and ENaCs by angiotensin II and aldosterone could provide a rationale for dual blockade, that is, a combination of an ARB with an MRA to inhibit the combined effects of angiotensin II and aldosterone on NCCs and ENaCs. Notably, high doses (>25–50 mg per day) of the MRAs spironolactone and eplerenone might be required to establish a diuretic effect.[129, 130] However, any combination of RAS inhibitors confers a risk of hyperkalaemia and acute or chronic kidney injury, as discussed above.[45] Whether more selective inhibition of NCCs or ENaCs using drugs that interfere with WNK and SPAK kinases is a promising strategy for the treatment of hypertension remains to be determined. Finally, it should be noted that in addition to their natriuretic and antihypertensive effects, RAS inhibitors also exert renoprotective effects by suppressing renal fibrosis and improving glomerular and podocyte function.[130]

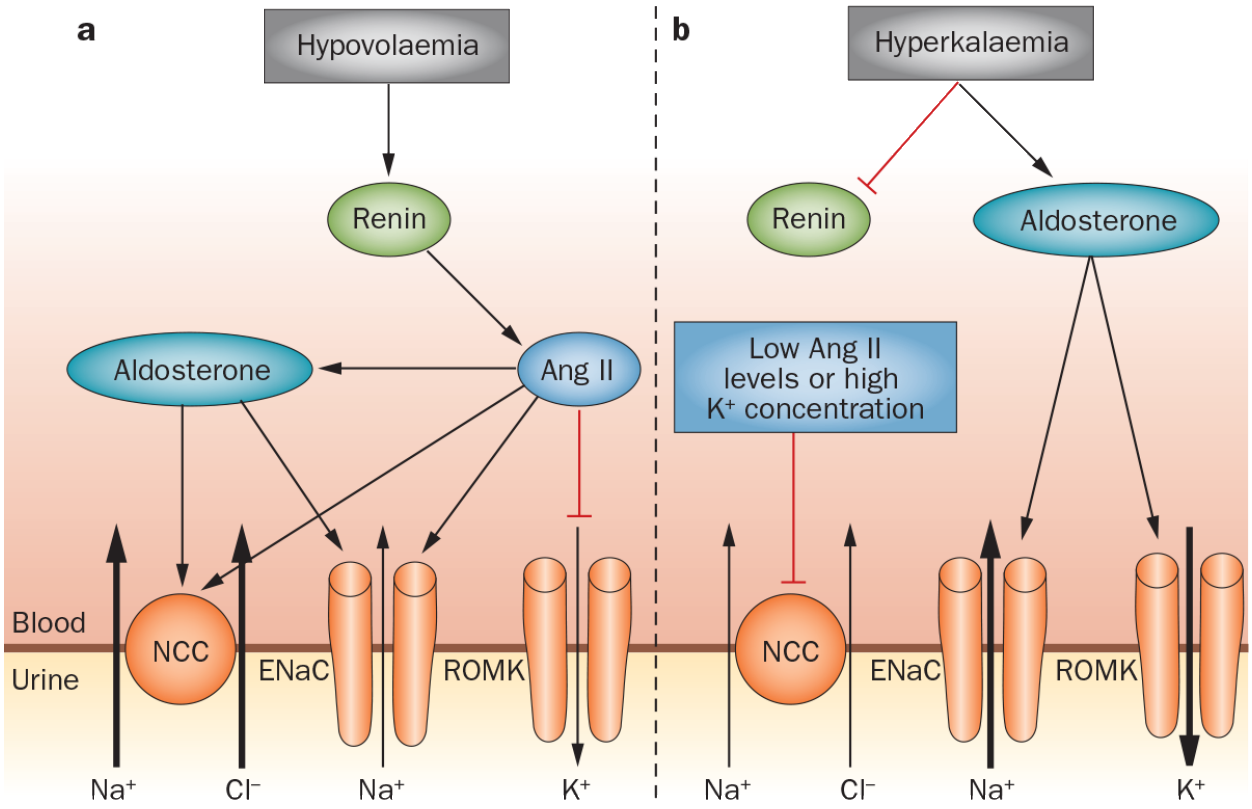

Figure 2: The aldosterone paradox. Aldosterone exerts differential effects during hypovolaemia and hyperkalaemia. a | During hypovolaemia, angiotensin II and aldosterone act synergistically to increase the activity of the NCC and the ENaC. Angiotensin II also inhibits the activity of the ROMK. These actions results in maximal sodium reabsorption to correct hypovolaemia and conserve potassium. b | Hyperkalaemia directly stimulates aldosterone secretion independently of renin and angiotensin II. In the absence of high angiotensin II levels the ROMK is not inhibited and hyperkalaemia reduces the activity of the NCC. However, the ENaC and the ROMK are maximally activated by aldosterone. These combined effects result in maximal sodium delivery to the ENaC, which facilitates the coupled secretion of potassium through the ROMK. Abbreviations: Ang, angiotensin; ENaC, epithelial sodium channel; NCC, sodium chloride cotransporter; ROMK, renal outer medullary potassium channel.

CONCLUSIONS

The PRR was initially thought to be the key to prorenin activation at tissue sites. However, recent findings suggest that the PRR has other, perhaps more important, RAS-independent functions. Contradictory data regarding the effects of HRPs—the only PRR blockers that currently exist—are therefore, perhaps unsurprising. Given the lethal consequences of PRR knockout, whether PRR blockade should be a therapeutic target is questionable. Similarly, the contrasting data regarding AT_2-receptor function suggest that a complete understanding of why the actions of this receptor differ depending on age, disease and location is required before AT_2-receptor agonists can be utilized in the clinic. Compelling evidence suggests a beneficial effect of Mas-receptor stimulation on stem-cell-mediated tissue repair. Thus, stable agonists are required to either stimulate the receptor or activate ACE2, the enzyme that generates the endogenous Mas-receptor agonist, Ang-(1–7). If acting locally, such ACE2 activators might overcome the rapid metabolism of Ang-(1–7) *in vivo*. Finally, the emerging role of angiotensin II in sodium and potassium handling in the distal nephron is likely to improve our understanding of the effects of RAS inhibition on total body sodium and potassium balance. For example, the synergistic actions of angiotensin II and aldosterone on sodium and potassium transport in the distal nephron could be targeted to better treat diseases characterized by avid sodium reabsorption, including salt-sensitive hypertension and the cardiorenal syndrome, while at the same time preventing hyperkalaemia.

AIM OF THE THESIS

As described above, the RAS is a major physiological regulator of blood pressure and volume homeostasis and, consequently, it is involved in the pathogenesis of renal and cardiovascular diseases. Therefore, this system plays a central role on many of the cardiovascular and renal pathologies that are not inborn or non-communicable. The classical treatment of heart failure and hypertension is directed at inhibition of the Ang II-producing enzymes renin and ACE, and of the AT_1 receptor. ACE inhibition and AT_1 receptor blockade have been developed and explored exhaustively and are widely applied in the clinic. However, although being explored for over 3 decades as an interventional target, renin inhibition is relatively less developed as a therapy. Optimal use of renin inhibition, as compared to ACE inhibition and AT_1 receptor blockade, does not seem to have been accomplished yet. Perhaps this is partly due to the lack of diversity in available compounds, aliskiren being the only clinically used renin inhibitor until now. Since most drugs have unique properties, the exploration of novel renin-inhibiting compounds might offer opportunities to improve therapy. To this end we compared the pharmacodynamic and pharmacokinetic profile of the new renin inhibitor VTP-27999 versus placebo and aliskiren in salt-depleted healthy volunteers in Chapter 2.

It is widely believed that optimization of RAS modulation as a therapy can also be achieved in other ways. As discussed here, in the past 15 years new components of the RAS have been identified, including ACE2,[131] Mas and AT_2 receptor signaling,[6] and the PRR[13], and with them new possible treatments for cardiovascular and renal diseases have emerged. In addition, progenitor cells have been identified as potential treatment targets in the cardiovascular system. Their interaction with the RAS may particular occur at the level of these new RAS components.[132] Moreover, there is emerging evidence that regulation of at least some of the components of the RAS might be sex-dependent. This is already known in the case of the AT_2 receptor which is located on the X chromosome.[133]

In this thesis, we focused on the 'alternative RAS', and its additional link to sex-differences with respect to AT_2 signaling, to explore new ways of modulating RAS activity. In Chapter 3 we investigated the sexual dimorphism in response to Ang II. Using C57bl/6 mice and the genetically modified "four core genotype" mouse model (on a C57bl/6 background), in which the sex chromosome complement (XX or XY) is unrelated to the animal's gonadal sex, we investigated the dependency of the constrictor effects of Ang II on sex hormones and sex chromosomes, making use of both intact and gonadectomized animals. Moreover, in Chapter 4 we review the role of Ang II and Ang-(1-7) and their respective receptors in hematopoietic and mesenchymal stem cells, and discuss possible therapeutical implications.

Subsequently, we investigated alternative intervention strategies based on stimulation of the Ang-(1-7)-Mas receptor axis. In Chapter 5 we present the data of a metabolically stable Ang-(1-7) analogue, cyclic Ang-(1-7), on progenitor cell recruitment and on the heart post-myocardial infarction. Moreover, in Chapter 6, we explored the relationship between angiotensin receptor signalling and the differentiation process of stem / progenitor cells.

Finally, in Chapter 7 we summarize the main findings of this thesis. We also discuss the limitations of the studies and the implications of our findings.

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CHAPTER 2

MULTIPLE ASCENDING DOSE STUDY WITH THE NEW RENIN INHIBITOR VTP-27999: NEPHROCENTRIC CONSEQUENCES OF TOO MUCH RENIN INHIBITION

Based on: *Multiple ascending dose study with the new renin inhibitor VTP-27999: nephrocentric consequences of too much renin inhibition*. Balcarek J, Sevá Pessôa B, Bryson C, Azizi M, Ménard J, Garrelds IM, McGeehan G, Reeves RA, Griffith SG, Danser AHJ, Gregg R. Hypertension. 2014;63:942-50.

ABSTRACT

This study compared the pharmacodynamic/pharmacokinetic profile of the new renin inhibitor VTP-27999 in salt-depleted healthy volunteers, administered once daily (75, 150, 300, and 600 mg) for 10 days, versus placebo and 300 mg aliskiren. VTP-27999 was well tolerated with no significant safety issues. It was rapidly absorbed, attaining maximum plasma concentrations at 1 to 4 hours after dosing, with a terminal half-life of 24 to 30 hours. Plasma renin activity remained suppressed during the 24-hour dosing interval at all doses. VTP-27999 administration resulted in a dose-dependent induction of renin, increasing the concentration of plasma renin maximally 350-fold. This induction was greater than with aliskiren, indicating greater intrarenal renin inhibition. VTP-27999 decreased plasma angiotensin II and aldosterone. At 24 hours and later time points after dosing on day 10 in the 600-mg group, angiotensin II and aldosterone levels were increased, and plasma renin activity was also increased at 48 and 72 hours, compared with baseline. VTP-27999 decreased urinary aldosterone excretion versus placebo on day 1. On day 10, urinary aldosterone excretion was higher in the 300- and 600-mg VTP-27999 dose groups compared with baseline. VTP-27999 decreased blood pressure to the same degree as aliskiren. In conclusion, excessive intrarenal renin inhibition, obtained at VTP-27999 doses of 300 mg and higher, is accompanied by plasma renin rises, that after stopping drug intake, exceed the capacity of extrarenal VTP-27999 to block fully the enzymatic reaction. This results in significant rises of angiotensin II and aldosterone. Therefore, renin inhibition has an upper limit.

INTRODUCTION

The renin–angiotensin–aldosterone system (RAAS) is a hormone system that regulates blood pressure, plasma sodium and potassium levels, and extracellular fluid volume in the body. The RAAS sequentially processes angiotensinogen to angiotensin II (Ang II), a peptide hormone that is a potent vasoconstrictor. Renin catalyzes the first and rate-limiting step of the RAAS cascade and is a specific protease for angiotensinogen. Direct renin inhibition leads to a decrease in plasma renin activity (PRA) and is expected to decrease Ang II and aldosterone levels.[1, 2] Plasma renin concentration (PRC) and prorenin levels are expected to increase through a compensatory feedback induction mechanism. The only direct renin inhibitor currently available for clinical use is aliskiren, marketed at 150 and 300 mg/d. Its use at doses >300 mg/d is hampered by the occurrence of gastrointestinal side effects including cramping and diarrhea.

VTP-27999 is a highly potent direct renin inhibitor being developed for the treatment of chronic renal disease and end-organ protection.[3, 4] Its oral bioavailability is ≈10-fold higher than that of aliskiren. This study examines the safety and tolerability, pharmacokinetics, and pharmacodynamics of VTP-27999 compared with a placebo and to the full marketed dose of aliskiren, 300 mg/d, after 10 days of administration to salt-depleted healthy volunteers.

MATERIALS AND METHODS

The Methods section is available in the Data Supplement.

RESULTS

Subject Demography and Disposition

A total of 37 subjects participated in the study (35 men, 2 women: 3 Asian, 8 black, and 26 white; age, 18–45 years; mean age, 27 years; weight, 60–111 kg; and mean weight, 80 kg). Two subjects were discontinued from the study: 1 subject because of an adverse event (AE) unrelated to study medication and 1 subject who was discontinued by the investigator for having a prestudy measurement that did not meet entry criteria.

Pharmacokinetics

After oral administration in the fasted state, VTP-27999 was rapidly absorbed, attaining C_{max} at 1 to 4 hours (Figure 1; Table S1 in the Data Supplement), after which plasma concentrations decreased slowly in a multi-exponential manner. Administration of VTP-27999 exhibited dose-proportional pharmacokinetics, based on area under the curve $(AUC)_{0–24}$ and C_{max} (Table S1). The $t_{1/2}$ for VTP-27999 ranged from 24 to 30 hours across all doses and steady-state levels were reached by day 7 (data not shown). Plasma VTP-27999 exposures (AUC) on day 10 were ≈2-fold higher than on day 1, also consistent with a $t_{1/2}$ of 24 to 30 hours. Aliskiren pharmacokinetic parameters were in agreement with those reported previously, its $t_{1/2}$ being ≈30 to 40 hours.[5] Urine was collected on days 1 and 10, and at steady state on day 10, between 5% and 10% of the daily dose was excreted unchanged into the urine (Table S2).

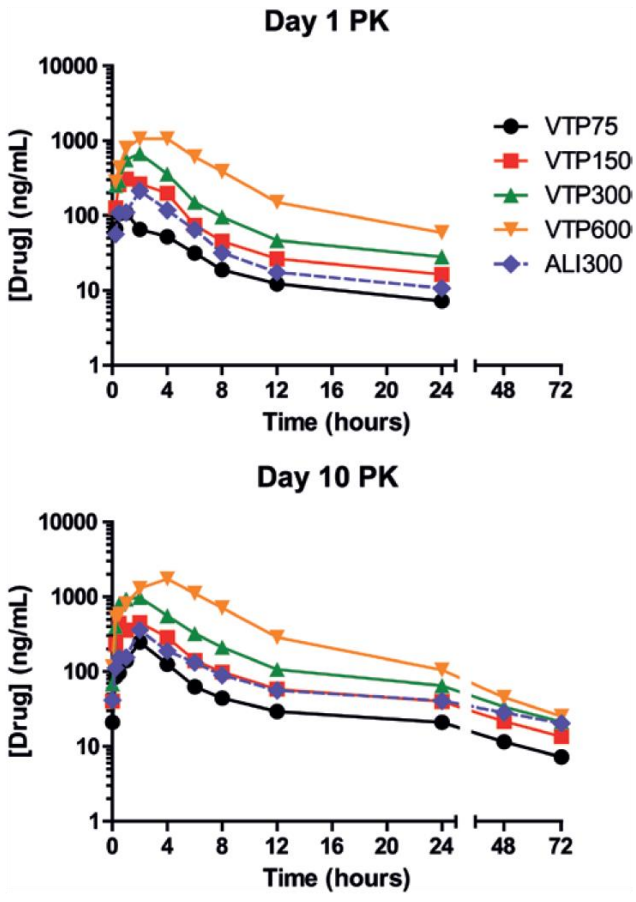

Figure 1: Drug levels (pharmacokinetics [PK] on treatment days 1(top) and 10 (bottom), and after stopping treatment. Data are mean±SEM of n=4 to 6.

Changes in Plasma RAAS Components

As expected with the low-salt diet, baseline PRA, PRC, plasma Ang II, and aldosterone levels were increased when compared with reference values from healthy volunteers on a normal sodium diet.[6] PRA declined rapidly and dose dependently after dosing on day 1, reaching a blockade of >90% within 15 minutes at all doses of VTP-27999 (Figures 2 and 3). Although PRA started to rise again after 4 to 6 hours, suppression was maintained for 24 hours post dose on day 1 at all doses of VTP-27999 and with aliskiren, with little difference in the PRA values between the various VTP-27999 doses and aliskiren during the 24 hours. There was little change in PRA during the 24 hours with placebo. On day 10, predose PRA was below baseline (and similar to the 24-hour value on day 1) in all active dose groups, and there was an acute drop in PRA after dosing. The PRA returned to day 10 predose levels by 12 hours and remained stable during the subsequent 60 hours (through 72 hours post dose) except for the 600-mg group, in which the PRA continued to rise through 48 hours post dose, ending at 1.5 to 2× the day 10 predose level. There was a minor decrease in day 10, predose PRA in the placebo group (perhaps reflecting temporal changes during clinic confinement) with no intraday reduction.

Plasma Ang II levels largely followed PRA (Figures 2 and 3; Figure S1) on day 1 except that recovery to baseline levels occurred by 24 hours. On day 10, the predose Ang II levels were similar to baseline in all dose groups. After dosing on day 10, Ang II again decreased after VTP-27999 or aliskiren administration and returned to day 10, predose levels by 6 hours. At 72 hours in the 600-mg group, and similar to the PRA values, the mean Ang II value was higher than baseline by 2- to 3-fold.

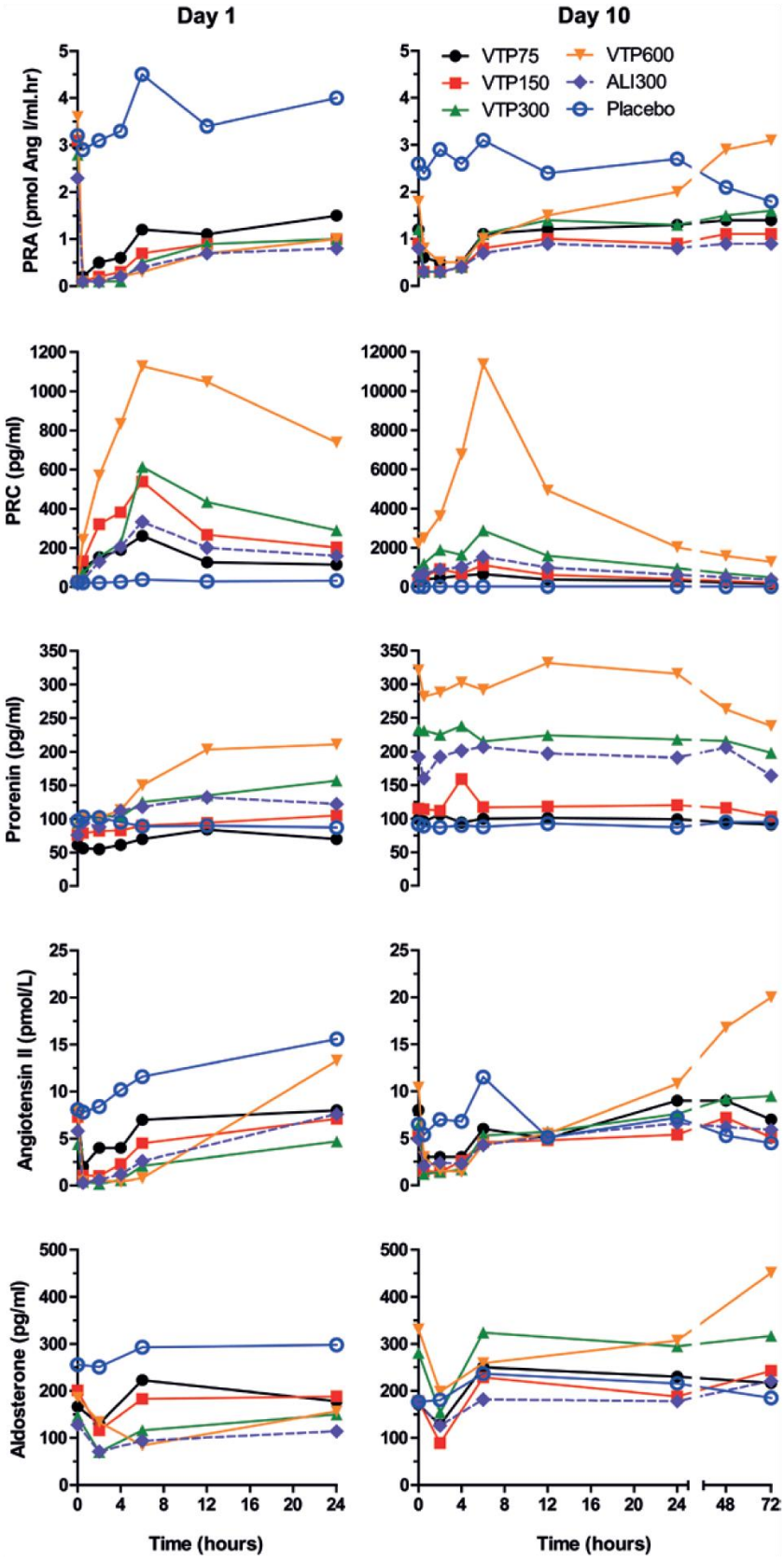

Figure 2: Plasma renin activity (PRA) and the plasma concentration of renin (PRC) and prorenin, angiotensin II, and aldosterone, respectively, on treatment days 1 (left) and 10 (right), and after stopping treatment. Data are mean±SEM of n=4 to 6. Note that the PRC scale is 10-fold greater for day 10 compared with day 1.

For plasma aldosterone, the degree of suppression on day 1 was less than that for PRA or Ang II, and the levels generally recovered by 24 hours (Figures 2 and 3). The day-1 plasma aldosterone levels were higher in the placebo group than the drug-treated groups at all time points, including the baseline time point, for unknown reasons, but the values were stable during the 24 hours. On day 10, aldosterone levels were lower in the placebo group than on day 1, and similar to the day-1 baseline values for the other groups. In the VTP-27999 75-and 150-mg groups and the aliskiren group, baseline aldosterone levels on day 10 were similar to those on day 1. In the 300- and 600-mg dose groups, levels tended to be somewhat higher at predose on day 10. The aldosterone levels for all groups returned to day 10, predose levels by 6 hours and remained stable through 72 hours post dose except for the 600-mg group. In that group, and similar to the PRA and Ang II values, the aldosterone levels continued to rise through the 72-hour final measurement.

VTP-27999 dose dependently increased PRC and prorenin on day 1, with up to a 35-fold increase over baseline for the C_{max} of PRC in the 600-mg VTP-27999 group (Figures 2 and 4). Peak levels were reached after ≈6 and >12 hours, respectively, for PRC and prorenin, with persistent elevations through hour 24. Substantial further rises in PRC (≤350-fold for the C_{max} of PRC at 600 mg VTP-27999) occurred during treatment on day 10, when the placebo group showed a minor reduction in PRC and no change in prorenin levels. Note that in Figure 2, the PRC scale for day 10 is 10-fold greater than the day-1 scale. After stopping treatment, PRC decreased roughly in parallel with plasma VTP-27999 concentrations, albeit somewhat more slowly (see terminal slopes in Figure 4). However, at 72 hours after the last dose of drug, the PRC levels were still ≈10-fold higher for aliskiren and the lower VTP-27999 dose groups, and to ≤50-fold higher for the 600-mg dose group compared with the baseline levels on day 1. Prorenin levels increased in a dose-dependent manner, and this was particularly apparent after 10 days of dosing (Figures 2 and 4). The prorenin levels remained high after stopping treatment and started to decrease only after 48 hours consistent with the known longer half-life of prorenin.

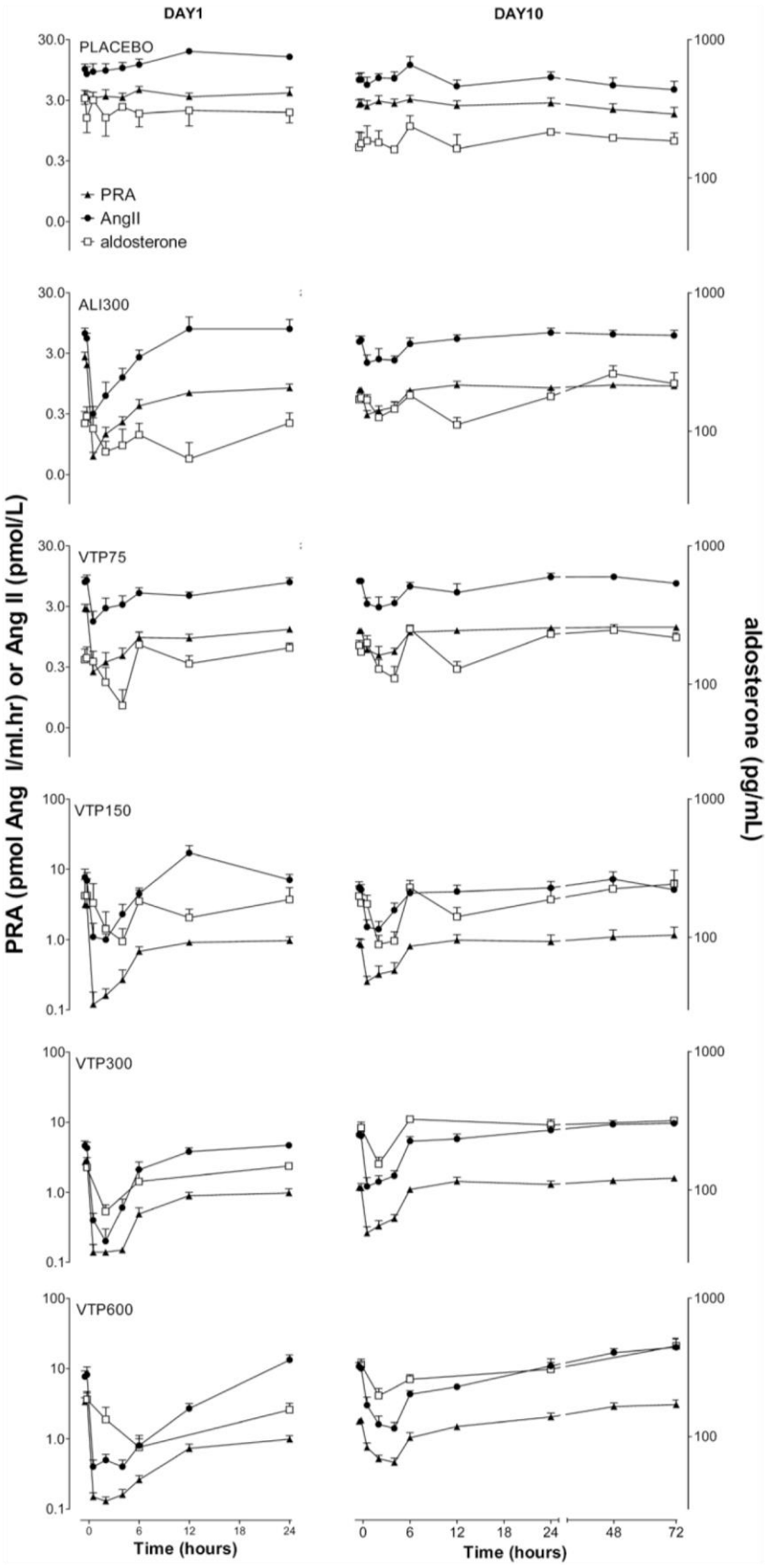

Figure 3: Plasma renin activity (PRA), plasma angiotensin II concentration, and plasma aldosterone concentration on treatment days 1 and 10, and after stopping treatment. Each panel represents a different dose of drug. Data are mean±SEM of n=4 to 6.

Drug levels correlated positively with PRC (Figure 5). PRC variation versus drug levels diminished when comparing $AUC_{0–24}$ for both drug and PRC (Figure 5). To measure the relative level of inhibition of renin in the circulation, the PRA values were divided by the PRC values, and the PRA/PRC ratio was then normalized such that 100% was set to the predose ratio in the placebo group on day 1 (Figure 6). Renin was inhibited between 90% and 99.8% on day 1 in a dose- and exposure-dependent fashion and between 95% and 99.9% on day 10. Even at 72 hours after the last dose, renin was inhibited by 90% to 98%, with the greatest inhibition being in the 600-mg group.

Changes in Urinary RAAS Components

The urinary renin excretion rate increased dose dependently during VTP-27999 treatment on day 1, and the increases were even more prominent on day 10 (Figure 7). Note the different scales used on the graphs for the 2 different days. These changes generally reflected the changes seen in PRC and were greatest in the VTP-27999 600-mg dose group. Changes in urinary renin resulting from aliskiren administration were comparable with those observed with 75 and 150 mg VTP-27999. Urinary angiotensinogen and prorenin excretion rates were unaffected by drug treatment (data not shown).

The urinary aldosterone excretion rate was decreased during all treatments on day 1 versus placebo, particularly in the 0- to 6-hour interval (Figure 7). On day 10, urinary aldosterone excretion rates for the 2 lower doses of VTP-27999 and aliskiren were again below the placebo group for 0 to 6 hours and were near placebo for the remainder of the day. Conversely, aldosterone excretion rates on day 10 were above placebo for the 300- and 600-mg doses of VTP-27999. The aldosterone urinary excretion rates reflected the elevated plasma aldosterone levels for these 2 groups. On day 1, 24-hour urinary sodium excretion rates (normalized for creatinine excretion) were, as expected, higher at all doses of VTP-27999 and aliskiren than with placebo (Table S3). On day 10, these rates had decreased and were similar to that observed in the placebo group.

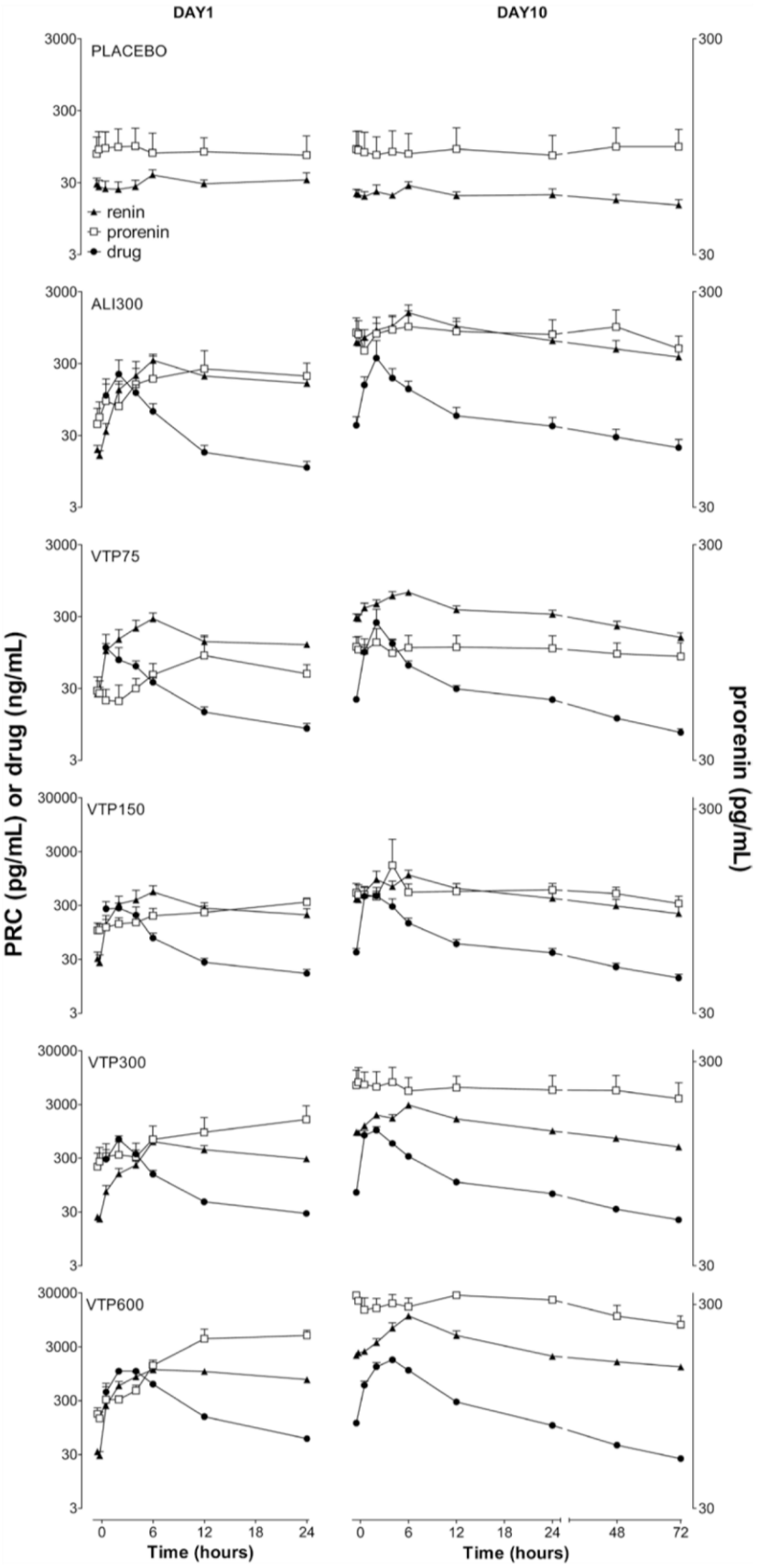

Figure 4: Plasma prorenin, renin, and drug concentrations on treatment days 1 and 10, and after stopping treatment. Each panel represents a different dose of drug. Data are mean±SEM of n=4 to 6. PRC indicates plasma renin concentration.

Safety Profile and Hemodynamic Effects

Two of the 37 subjects who received study medication were discontinued from the study. One subject was withdrawn for an AE of sinus tachycardia that was also present pretreatment; the investigator considered this AE to be because of anxiety and unrelated to study medication (VTP-27999 75 mg). One subject was discontinued by the investigator after 1 dose of placebo when it was noticed that the subject's predose ECG did not meet entry criteria. There were no serious AEs. Eleven of the 25 subjects who received VTP-27999 reported ≥1 AE (Table S4). AEs were mild to moderate in intensity and all were reported as resolved by the follow-up visit on day 17. The greatest number of AEs was reported at the 600-mg dose of VTP-27999; the majority of which were nausea and vomiting. There were no important findings on physical examinations, ECGs, or laboratory tests; serum potassium levels were not elevated (Table S5).

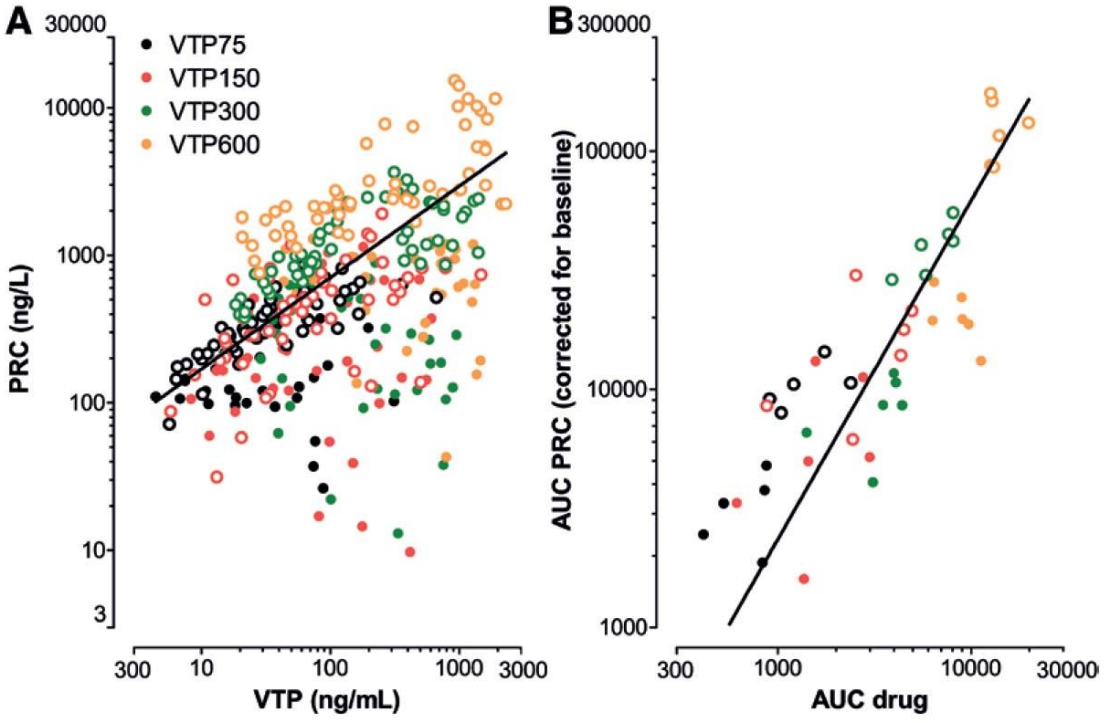

Figure 5: Correlation between the plasma concentrations of VTP-27999 and renin on treatment days 1 and 10, either expressed as individual data points (**A**) or as area under the curve $(AUC)_{0-24}$ for the plasma concentrations of VTP-27999 and renin (ng/mL×hours and pg/mL×hours, respectively; **B**). Day 1 values are represented by closed symbols and day 10 values by open symbols. PRC indicates plasma renin concentration.

In these normotensive but sodium-depleted subjects, VTP-27999 and aliskiren decreased blood pressure on days 1 and 10 compared with placebo (Figure 8). However, because of the small sample size and the inherent variability in blood pressure within and between days, the relative effects on blood pressure between the different doses of VTP-27999 and aliskiren could not be differentiated. Heart rates tended to be somewhat elevated in all dose groups on day 10 versus placebo, with a more substantial increase in the heart rate in the 600-mg dose group.

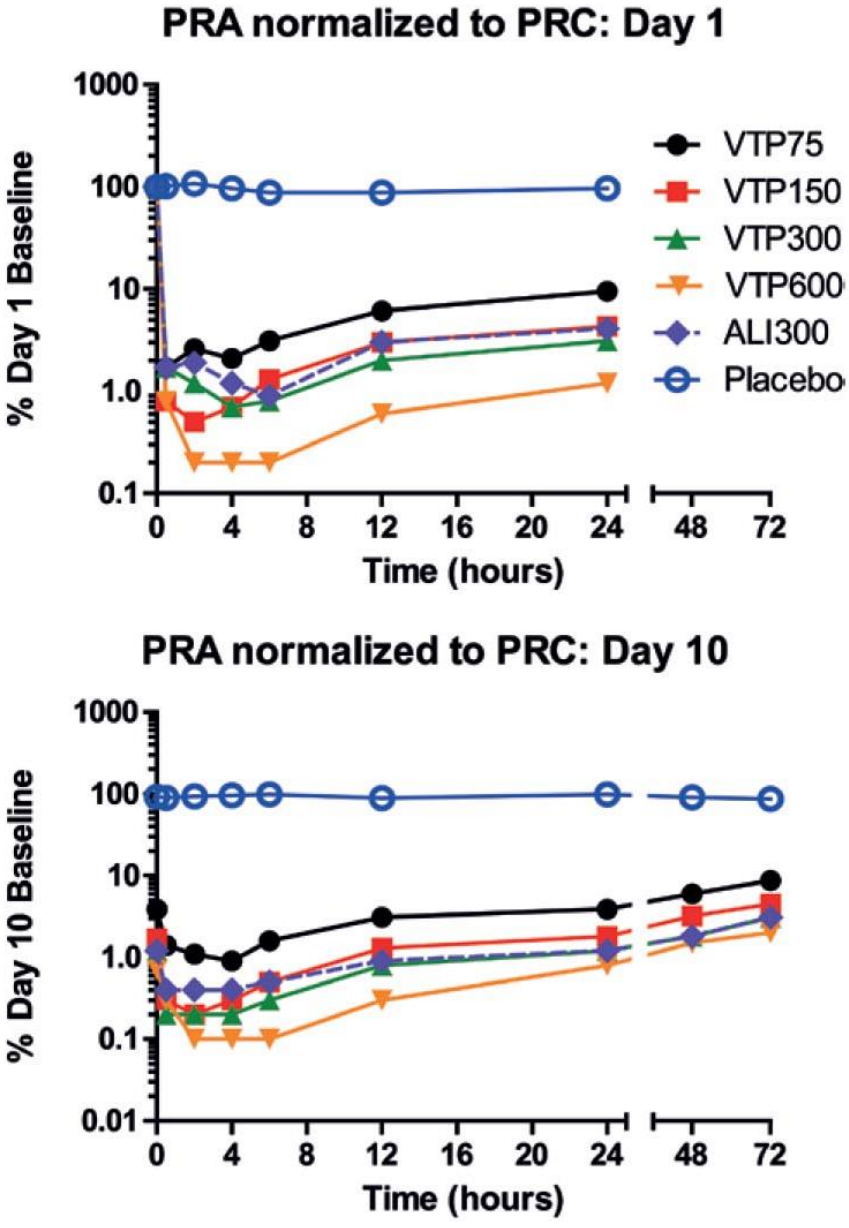

Figure 6: Plasma renin activity (PRA)/plasma renin concentration (PRC) ratios on treatment days 1 and 10, and after stopping treatment. Data are mean±SEM of n=4 to 6.

DISCUSSION

This is the first description of the pharmacokinetic and pharmacodynamic characteristics of a new renin inhibitor, VTP-27999, in human subjects. VTP-27999 is rapidly absorbed, reaching C_{max} between 1 and 4 hours after oral dosing, and it had a multi-exponential decay curve from plasma. There was a rapid distribution phase followed by a terminal removal phase with a $t_{1/2}$ of 24 to 30 hours. The pharmacokinetics were dose-proportional, and the day-10 pharmacokinetic parameters were as predicted from the day 1 kinetics; day 10 C_{max} values were ≈50% greater than the day 1 values, and the AUC was approximately doubled. Relatively little of the drug was excreted in the urine (<10%), and thus the liver and metabolism are apparently the major routes of clearance. With the long terminal $t_{1/2}$ and dose proportionate exposure, the pharmacokinetics supports once a day dosing for VTP-27999.

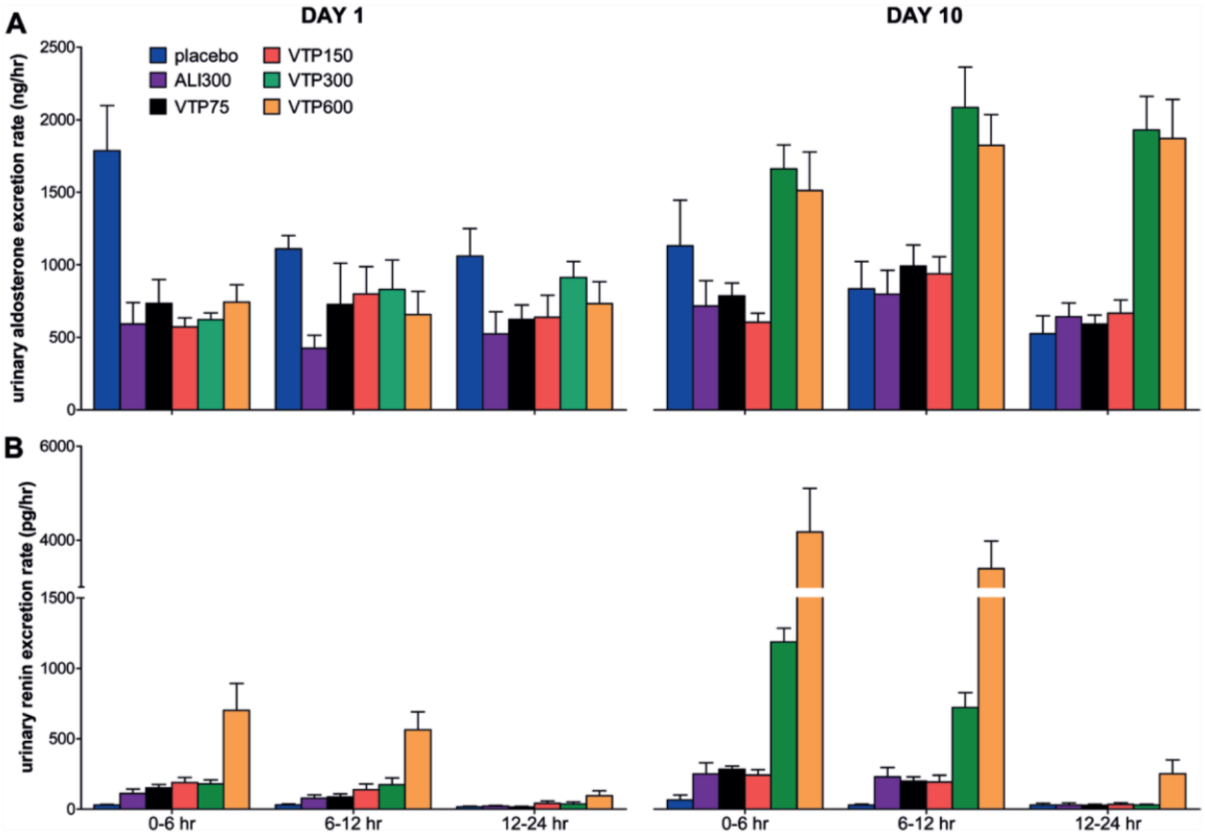

Figure 7: Urinary renin (**A**) and aldosterone (**B**) excretion for 0 to 6, 6 to 12, and 12 to 24 hours on treatment days 1 and 10. Data are mean±SEM of n=4 to 6.

VTP-27999 was generally safe and well tolerated in these salt-restricted, healthy, normal volunteers. Nausea and some vomiting were observed in the 600-mg dose group, but this was not observed at the lower doses of VTP-27999, and no subject discontinued drug because of tolerability issues. There were no clinically significant changes in laboratory values or ECGs, and more specifically, no significant changes in serum potassium levels. Changes in blood pressure are not easily seen in normotensive volunteers, even when following a low-salt diet, and differences in blood pressure in drug-treated versus placebo only could be observed. There were no drug-related discontinuations or significant AEs.

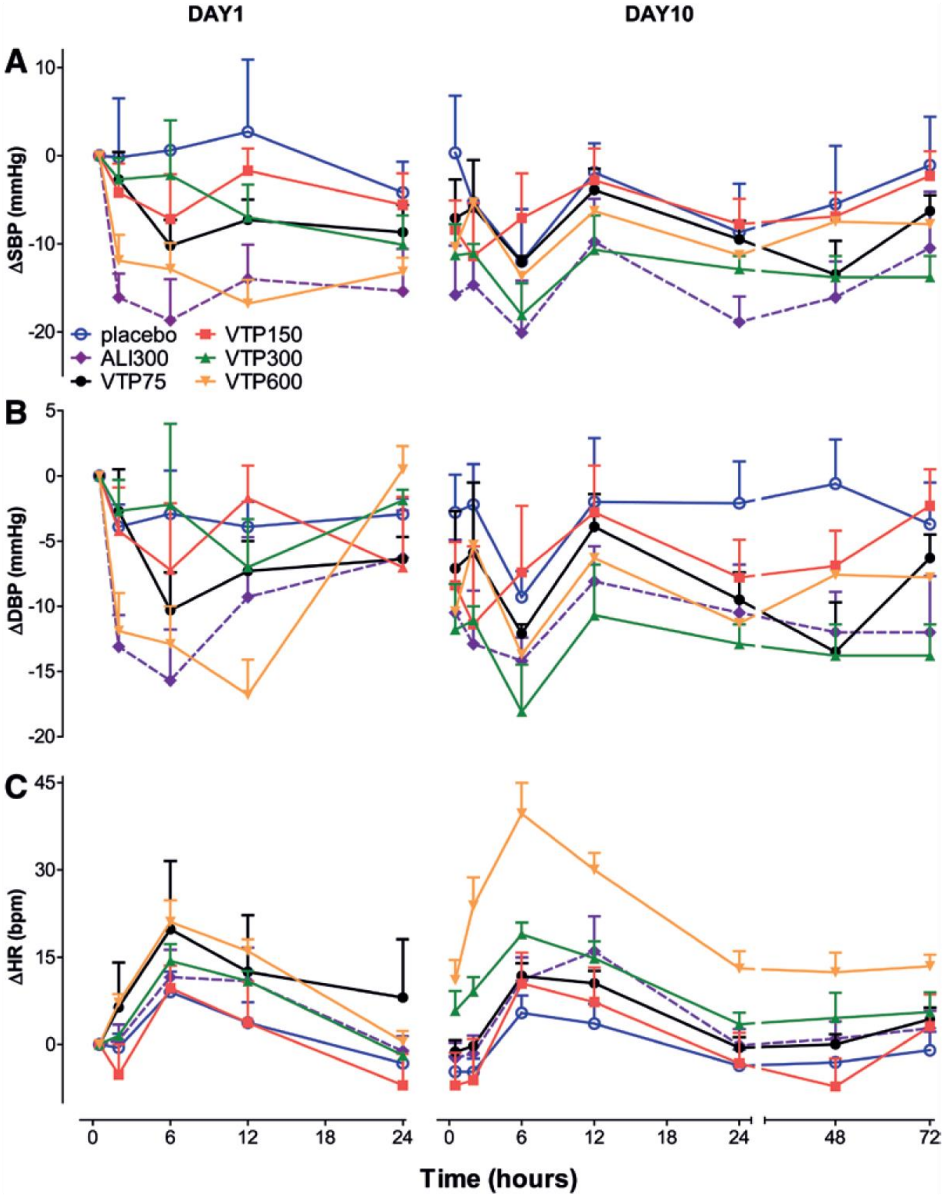

Figure 8. Change from baseline in systolic blood pressure (SBP; **A**), diastolic blood pressure DBP; **B**), and heart rate (HR; **C**) on days 1 and 10, and after stopping treatment. Data are mean±SEM of n=4 to 6.

The pharmacodynamics of VTP-27999 demonstrated a rapid onset and almost complete inhibition of PRA; >90% at 15 minutes after dosing on day 1 at all dose levels, and significant inhibition of PRA was maintained for 24 hours on day 1. There was also substantial inhibition of PRA at all dose levels on day 10, supporting once a day dosing, consistent with the pharmacokinetics. There was a clear dose–response for PRC and a significant induction between days 1 and 10, with a 2- to 10-fold higher C_{max} on day 10 compared with day 1 for the different doses of drug. With the PRC providing the clearest dose–response, one can compare the relative pharmacodynamics potency of orally administered VTP-27999 with aliskiren. With the day-1 PRC measurements, on an oral dose basis, VTP-27999 was ≈2- to 3-fold more potent than aliskiren, and on day 10, it was ≈2-fold more potent. Given the comparable IC_{50} of both inhibitors,[3, 4] it can be concluded that the greater oral potency of VTP-27999 is related to its greater bioavailability.

The full pharmacodynamic data set demonstrates an interesting and surprising relationship between the dose of VTP-27999 administered, the intrarenal inhibition of renin, and the blockade of the systemic RAAS. Because the majority of renin is synthesized and secreted from juxtaglomerular cells in the renal glomeruli,[7] and because renin secretion by the kidney is downregulated by Ang II, plasma levels of renin are inversely related to, and are a reflection of, inhibition of renin in the kidney.[8]

Biochemical data obtained from day 10 and out to 72 hours after the day 10 dose are consistent with the 600-mg dose of VTP-27999 being less effective than 300 mg on decreasing PRA, Ang II production, and aldosterone production at 48 and 72 hours after dosing. After the 600-mg dose, the rise in the PRC C_{max} on day 10 was ≤350-fold compared with the day 1 baseline values. PRA remained below baseline (ie, predose level) during the full 24 hours after the last dose, and thereafter started to rise. Ang II and aldosterone also started to rise 24 hours after the last dose in the 600-mg cohort, even reaching plasma concentrations that were several fold above baseline. These rises were not seen for the other doses of VTP-27999 or for aliskiren, except for a 50% rise in

plasma aldosterone in the VTP-27999 300 mg cohort at 72 hours after the day 10 dose.

Thus, a dissociation occurs in the 600-mg dose group at the later time points (>24 hours after dosing) between the intrarenal blockade of renin, which increases the synthesis and secretion of renin, and the systemic blockade of renin. This dissociation occurs at times when there are declining plasma levels of the renin inhibitor. After the last dose of drug on day 10, the decrease in PRC generally paralleled the decrease in renin inhibitor concentration (Figure 3). However, in the case of 600-mg VTP-27999 cohort, PRC decreased significantly more slowly than VTP-27999 (≈1/3 as fast) after stopping treatment, and this is the likely cause of the PRA and plasma Ang II and aldosterone overshoot. Renin is still inhibited 98% to 99% at these times (Figure 6), but because the renin levels are so elevated, the absolute PRA values are increased 1.5- to 2-fold.

When high doses of VTP-27999, aliskiren, and other renin inhibitors, such as ciprokiren and remikiren, are administered, all of which are known to accumulate in the kidney,[9, 10] the degree of renal/juxtaglomerular RAAS blockade may be greater than that outside the kidney. Under such conditions, there is a sustained stimulation of renal renin release secondary to the intrarenal inhibition of renin and subsequent decrease of Ang II at the level of the juxtaglomerular cells, even when extrarenal renin inhibitor levels are no longer sufficient to fully block renin systemically. However, once released into the circulation, these VTP-27999–bound renin molecules face declining VTP-27999 concentrations in plasma, and thus dissociation of the renin–VTP-27999 complex is likely to occur.

The capacity of the human body to counteract RAAS blockade through the synthesis and secretion of renin did not reach its limit in the present study, and interestingly, the renin levels observed at 600 mg VTP-27999 were comparable with those in patients with renin-producing tumors.[11] If a stimulation for renin secretion persists during a long period of time, the increased secretory capacity involves increased synthesis of prorenin through

hypertrophy of juxtaglomerular cells and metaplastic transformation of preglomerular vascular smooth muscle cells into renin-producing cells.[12, 13] On day 10, the PRC AUC values were 2 to 10× higher than the AUC values on day 1. Thus, there is a large increase in renin secretion with drug treatment and also with drug treatment between days 1 and 10. This is almost certainly because of both strong inhibition of intrarenal renin and an increase in the overall secretory capacity, particularly at the higher doses of VTP-27999. This hypertrophy and metaplastic transformation may also contribute to the overshoot or escape of the systemic RAAS. For the same level of decrease in intrarenal Ang II, there will be a greater capacity for renin production and release by the increased mass of the juxtaglomerular cells in the glomeruli.

When the degree of renal RAAS blockade exceeds that outside the kidney, plasma renin will increase as will the PRA, and this rise in PRA will lead to a counter-regulatory increase in the concentrations of Ang II and aldosterone (escape). It is likely that this would occur with aliskiren too, had it been able to be tested at higher doses. Our findings are reminiscent of the nephrocentric view of angiotensin-converting enzyme inhibition noted 25 years ago in patients with congestive heart failure.[14] It was asked why the kidneys continue to release renin in such patients; the answer being that they do everything possible to preserve renal function and glomerular filtration, apparently at the expense of the hemodynamic burden on the heart. This situation mimics our findings, although obtained in healthy volunteers, at the highest dose of VTP-27999 where the kidneys respond to renal RAAS suppression by releasing large quantities of renin, resulting in elevations of PRA, Ang II, and aldosterone. Such elevated Ang II levels might also, either directly or indirectly (via the facilitation of norepinephrine release), be responsible for the rise in heart rate that was observed at the highest VTP-27999 dose.

The PRC correlated with the plasma levels VTP-27999, and if assessments are limited to the PRC-VTP-27999 concentration relationship on day 1 (ie, before the induction of renin synthesis) or day 10 (ie, after the induction of renin synthesis), the variation in PRC levels for a given drug

concentration varies between 30- and 100-fold. There are several potential reasons for this variation. First, there are biological interindividual variations in renin levels. Second, peak levels for renin occurred 2 to 4 hours after peak levels of VTP-27999. When the time/drug level/PRC relationship was plotted, there was clear hysteresis for the relationship between drug levels and the PRCs (plots not shown). To compensate for this hysteresis, we compared the $AUC_{0–24}$ for VTP-27999 versus the $AUC_{0–24}$ for PRC, and did this for both on days 1 and 10. By doing this, the variation in the PRC AUC for a given VTP-27999 AUC was reduced to <10. PRA also correlated strongly with Ang II and less with aldosterone (Figure S1).

Prorenin, like renin, increased dose dependently, the only difference being that this rise required >12 hours on day 1, because of the fact that it depends on the actual induction of prorenin synthesis, because prorenin is not stored in the kidney. On day 10, prorenin was elevated compared with day 1 predose values, and there was little change in prorenin for 24 hours, reflecting the longer half-life of prorenin in the circulation. This was followed by a slow decline in concentration during the subsequent 2 days. Changes in urinary renin and aldosterone levels generally reflected the alterations in their levels in plasma at the various doses and various times of collection, suggesting that urinary renin originates largely, if not completely, from filtration from the systemic circulation into the urine and not from direct secretion from the kidney into the urine.

PERSPECTIVES

The data obtained in the present study support that VTP-27999 is a potent and generally well-tolerated, renin inhibitor, capable of effectively suppressing the RAAS with once a day dosing, and on an oral dose-basis, VTP-27999 is approximately twice as potent as aliskiren. VTP-27999, which is known to be sequestered in the kidney, has a temporal asynergism, dependent on the tissue and plasma levels of the inhibitor, between intrarenal renin inhibition, with its consequent stimulation of renin synthesis and release, and extrarenal inhibition with its consequent decrease in PRA, Ang II, and aldosterone. With VTP-2799 at doses >300 mg/d, the high level of intrarenal renin inhibition, with subsequent dramatic increases in renin release, results in systemic renin levels that are greater than the systemic levels of VTP-27999 can fully inhibit at 24 to 72 hours after the last dose. This raises the interesting possibility of VTP-27999 at the highest dose having superior renal renin inhibition and protection while having increased systemic PRA, Ang II, and aldosterone levels. This study also demonstrates once more the variability of local renin–angiotensin systems when stressed by a renin inhibitor, and the need for more studies investigating, with an appropriate and complex technology, the pharmacodynamics of the RAAS, within and outside the kidneys. The dose of VTP-27999 to optimally balance the antiproteinuric, nephroprotective, antihypertensive, and cardioprotective effects remains to be determined.

SOURCES OF FUNDING

This study was funded by Vitae Pharmaceuticals.

DISCLOSURES

Dr Danser received research support from Vitae Pharmaceuticals. J. Balcarek, C. Bryson, G. McGeehan, and R. Gregg are employees of Vitae Pharmaceuticals. The other authors report no conflicts.

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SUPPLEMENTAL INFORMATION

MATERIAL AND METHODS

Study Protocol

This was a randomized, double-blind, placebo- and active-controlled study in which once daily doses of 75, 150, 300, or 600 mg VTP-27999 were administered orally as 75 mg tablets for 10 days to healthy subjects (males and females not of childbearing potential, aged between 18-45 years, and with a BMI ranging from 18-32 kg/m^2) in 4 sequential ascending dose cohorts. The study was approved by the PRACS Institute, Ltd. Institutional Review Board. All subjects provided written, informed consent and the study was conducted in accordance with accepted standards of Good Clinical Practice (GCP), as defined in the International Conference on Harmonization (ICH) E6 Guideline for Good Clinical Practice, in agreement with the latest revision of the Declaration of Helsinki.

Subjects visited the clinic on Day -5 to receive dietary instructions and low salt meals (10 mEq/day) for outpatient consumption. Subjects were admitted to the clinic on Day -1 to undergo baseline assessments and were verified to have a spot urinary Na+/creatinine ratio of <30 mEq/g creatinine before they could continue in the study. Subjects were randomized within each dose cohort to receive VTP-27999, placebo or aliskiren 300 mg, all administered orally once daily in the fasted state at approximately 08:00 hours. VTP-27999 75 mg tablets and a matching placebo were supplied to the clinical site by Vitae Pharmaceuticals, Inc. Aliskiren 300 mg tablets were purchased by the site pharmacy from a commercial source. The 75 mg and 150 mg cohorts each included 8 subjects (6 on VTP-27999, 1 on aliskiren and 1 on placebo matching VTP-27999). The 300 mg and 600 mg cohorts each included 10 subjects (6 on VTP-27999, 2 on aliskiren and 2 on placebo). Dosing began on the morning of Day 1 and continued through Day 10. Subjects were released from the clinic on Day 13 and returned for a safety visit on Day 17±2 days, after which they were discharged from the study.

Tolerability and Safety Assessments

Routine safety assessments included vital signs, physical examination, standard laboratory tests, and 12-lead ECGs. Adverse events (AE) were monitored throughout the study.

Pharmacokinetic and Pharmacodynamic Assessments

Blood samples for the determination of plasma VTP-27999 and aliskiren concentrations were collected at 0 hour (pre-dose), at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours after dosing on Days 1 and 10, predose on Days 5 and 7, and at 48 and 72 hours after dosing on Day 10. Samples were processed on ice in the presence of a serine protease inhibitor and stored at -70°C until shipment to the bioanalytical laboratory.

Blood samples for determination of plasma renin activity (PRA), plasma renin concentration (PRC), plasma prorenin concentration, Ang II, and aldosterone were obtained as described previously[1] at -30 and -15 minutes (pre-dose) and at 0.5, 2, 4, 6, 12, and 24 hours post-dose on Day 1 and Day 10, pre-dose on Day 5 and Day 7, and at 48 and 72 h after the last dose on Day 10. PRA, PRC, prorenin, and aldosterone samples were processed at room temperature and stored at -70°C until analysis. Ang II samples were placed on ice, processed at 4°C and stored at -70°C until analysis.

Urine was collected for the measurement of VTP-27999, Na+, creatinine, renin, angiotensinogen, aldosterone, Ang II, and prorenin over the intervals of 0-6, 6-12, and 12-24 hours post-dose on Days 1 and 10. Urine samples were stored at -20°C until analysis. Blood pressure and heart rate were measured in triplicate, after quiet semi-supine rest before each blood sample collection, using an automated device.

Bioanalytical Methods

Plasma VTP-27999 concentrations were measured using a solid phase extraction method and LC-MS/MS analysis developed and validated by Cetero Research, Houston, TX, USA. The standard curve ranged from 1-200 ng/mL. The analytes and internal standard peak areas were exported to Watson Laboratory Information Management System (LIMS) to create calibration curves

using weighted ($1/x^2$) least squares regression fit to a linear model. The concentrations of the standards, QCs, and samples were calculated using Watson LIMS. Plasma aliskiren concentrations were determined using a of solid phase extraction method and LC-MS/MS analysis developed at Vitae Pharmaceuticals. The standard curve ranged from 1-500 ng/mL. The analyte and internal standard peak areas were calculated by Analyst 1.4.2® Software to create a calibration curve using weighted (1/x2) least squares regression fit to a linear model. The concentrations of the standards, QCs, and samples were calculated using Analyst 1.4.2® Software.

PRA was determined by measuring Ang I generation during incubation of plasma at 37°C and pH 7.4. PRC was measured with an immunoradiometric kit (Renin III, Cisbio, Gifsur-Yvette, France).[2] Urinary renin was measured with the same renin kit after concentrating the samples as described before.[3] Plasma Ang II was measured by radioimmunoassay after SepPak extraction as described before. Plasma aldosterone and free urine aldosterone were measured by solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA).[4] Plasma prorenin was measured with a direct prorenin enzyme-linked immunosorbent assay (Molecular Innovations, Novi, MI, USA).[5] Urinary angiotensinogen was measured as the maximum quantity of Ang I that was generated during incubation with excess recombinant renin.

Pharmacokinetic Parameters

Peak concentration (C_{max}), concentration at trough (C_{min}), time to C_{max} (t_{max}), terminal half–life ($t_{1/2}$), renal clearance (Cl_r), and area under the concentration-time curve up to 24 h postdose (AUC_{0-24}) for each individual plasma concentration-time profile were determined by a non-compartmental method, using WinNonlin® (Pharsight® Corporation, Mountain View, CA, USA), Version 5.0.1. Values below the lower limit of quantitation (BLQ) were presented as zero in the plasma concentration tables and were treated as zero when calculating the descriptive summary of the concentrations by time point. When calculating PK parameters, BLQ values were treated as zero when occurring at the

beginning of the profile and missing when occurring at the end of the profile or between two values that were above lower limit of quantitation (LLOQ).

Statistical Analysis

All subjects who received at least one dose of study drug (VTP-27999, aliskiren or placebo) are included in the safety analyses. Concentration data from all subjects who received VTP-27999 were used in the calculation of PK parameters and no subjects were excluded from the final VTP-27999 plasma and urine PK analysis. One subject was excluded from the aliskiren PK analysis dataset because this subject inadvertently received placebo on Day 1. Only subjects with samples from Day 1 and Day 10 were included in the pharmacodynamic (PD) dataset. Two subjects (one each for the aliskiren and placebo cohorts) were excluded from the PD analysis because they inadvertently had their doses switched on Day 1.

Safety data from all subjects who received at least one dose of study medication were reported and summarized. Plasma VTP-27999 PK parameters included Cmax, Cmin, tmax, AUC_{0-24}, and $t_{1/2}$. All available plasma VTP-27999 concentration and PK results were summarized using appropriate descriptive statistics. Mean and individual concentration versus time curves were plotted for each analyte. Descriptive statistics, including changes from baseline were calculated for all plasma PD analytes and BP (mean of triplicate readings at each time point), by dose and time point. Percent change from baseline was also calculated for plasma PD analytes. Descriptive statistics for urine excretion amount and rates for aldosterone and renin were calculated by dose and collection interval.

Table S1. Plasma Pharmacokinetic parameters for VTP-27999 and Aliskiren. Data are mean±SD or median and range. VTP=VTP-27999, ALI=aliskiren.

Drug	N	AUC_{0-24} (ng·hr/mL)	C_{max} (ng/mL)	t_{max} (hr)	C_{min} (ng/mL)	$t_{1/2}$ (hr)
Day 1						
75 mg VTP	5	602 ± 291	202 ± 96	0.8 (0.3 - 2.1)		--
150 mg VTP	6	1754 ± 937	441 ± 267	1.0 (0.6 - 4.0)		--
300 mg VTP	6	3453 ± 979	786 ± 266	1.5 (1.0 - 2.0)		--
600 mg VTP	6	8466 ± 2031	1207 ± 264	3.0 (1.0 - 4.0)		--
300 mg ALI	5	1123 ± 543	269 ± 209	2.5 (0.5 – 4.0)		
Day 10						
75 mg VTP	5	1408 ± 489	320 ± 216	2.0 (1.0 – 4.0)	21 ± 3.4	30.2 ± 4.02
150 mg VTP	6	3035 ± 1485	676 ± 468	2.0 (0.5 – 4.0)	40 ± 18	29.4 ± 2.28
300 mg VTP	6	6210 ± 1481	1126 ± 283	2.0 (0.5 – 2.0)	65 ± 11	29.1 ± 3.49
600 mg VTP	6	13663 ± 3111	1736 ± 320	4.0 (4.0 – 4.1)	105 ± 23	23.8 ± 2.24
300 mg ALI	5	2348 ± 1813	411 ± 520	2.5 (0.5 – 4.0)	40 ± 25	33.6 ± 1.00

Table S2. Summary of urinary excretion ($A_{e(0-24)}$) and renal clearance (Cl_r) for VTP-27999.

VTP-27999		$A_{e\ (0-24)}$ (µg)	Cl_r (L/hr)
Day 1	**N**	**Mean ± SD (%CV)**	
75 mg	6	1504 ± 651.4 (43.3)	2.8 ± 0.90 (32.6)
150 mg	6	4503 ± 848.9 (18.9)	3.3 ± 1.91 (59.0)
300 mg	6	13938 ± 5073 (36.4)	3.9 ± 0.68 (17.3)
600 mg	6	34062 ± 14422 (42.3)	3.9 ± 1.58 (40.3)
Day 10			
75 mg	5	3169 ± 893.6 (27.0)	2.3 ± 0.46 (19.6)
150 mg	6	8498 ± 2543 (29.9)	3.3 ± 1.55 (46.6)
300 mg	6	22259 ± 5969 (22.2)	3.6 ± 0.74 (20.5)
600 mg	6	57794 ± 18932 (32.8)	4.1 ± 0.62 (14.8)

Table S3. Renal excretion of sodium and creatinine. Data are mean±SD.

	Na^+ (mmol/24 hours)		Creatinine (mmol/24 hours)	
Treatment	**Day 1**	**Day 10**	**Day 1**	**Day 10**
75 mg VTP-27999	51±14	29±2	15.7±0.9	15.6±1.0
150 mg VTP-27999	54±3	36±2	15.0±1.2	16.3±1.2
300 mg VTP-27999	98±13	32±1	17.0±0.6	17.0±1.2
600 mg VTP-27999	46±9	26±2	17.7±1.1	18.0±1.0
300 mg Aliskiren	42±3	22±4	19.3±2.9	17.5±2.3
Placebo	29±2	23±3	19.2±1.6	16.7±2.0

Table S4. Summary of number of subjects with treatment-emergent adverse events (AE), presented in order of frequency.

	VTP-27999 Doses				Aliskiren	Placebo
Preferred Term	**75 mg N=6**	**150 mg N=6**	**300 mg N=7**	**600 mg N=6**	**Pooled N=7**	**Pooled N=5**
AEs (N)	**2**	**5**	**3**	**31**	**3**	**4**
Subjects with AEs (N)	**2 (33%)**	**2 (33%)**	**3 (42%)**	**4 (67%)**	**1 (14%)**	**1 (20%)**
Nausea				4 (67%)		
Vomiting				2 (33%)		
Dizziness				2 (33%)		
Cold sweat				2 (33%)		
Abdominal pain upper		2 (33%)				1 (20%)
Lymphadenopathy				1 (17%)		
Procedural dizziness				1 (17%)		
Dyspnoea				1 (17%)		1 (20%)
Pallor				1 (17%)		
Palpitations			1 (14%)	1 (17%)		1 (20%)
Asthenia			1 (14%)			
Musculoskeletal chest pain			1 (14%)			
Headache		1 (17%)			1 (14%)	1 (20%)
Dry eye		1 (17%)				
Oral pain		1 (17%)				
Sinus tachycardia	1 (17%)					
Cough	1 (17%)					

N = number of subjects dosed with each treatment. Blank cell = no reported AE. One subject, randomized to placebo, received one dose of aliskiren on Day 1. Another subject, randomized to aliskiren, received placebo on Day 1. Both subjects were counted in the aliskiren group for the safety analysis, but were excluded from the PK/PD analysis.

Table S5. Serum potassium levels. Data are mean±SD.

Treatment	K^+ (mmol/L)	
	Day -1	Day 9
75 mg VTP-27999	4.7±0.5	4.3±0.2
150 mg VTP-27999	4.4±0.3	4.1±0.2
300 mg VTP-27999	4.4±0.3	4.5±0.3
600 mg VTP-27999	4.3±0.2	4.5±0.3
300 mg Aliskiren	4.6±0.3	4.4±0.4
Placebo	4.7±0.3	4.2±0.6

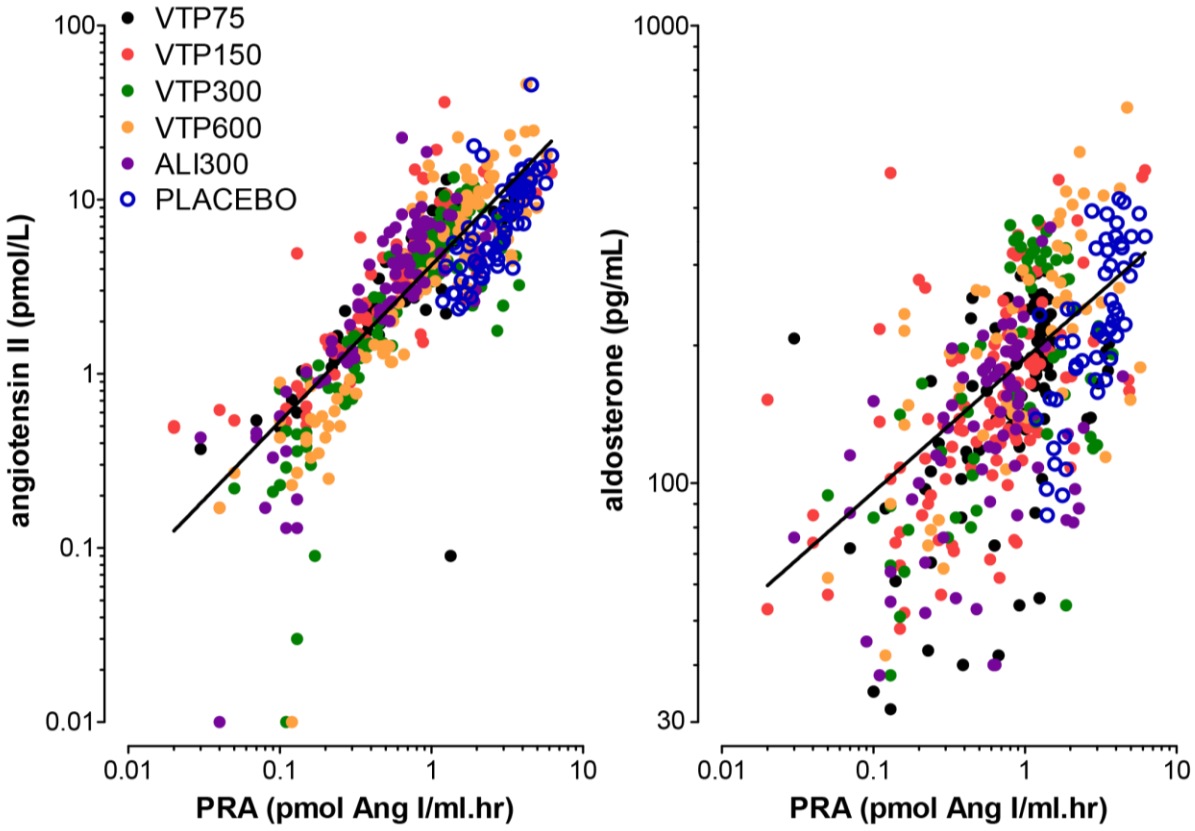

Figure S1. Plasma renin activity (PRA) versus plasma angiotensin (Ang) II and aldosterone in subjects treated with placebo, VTP-27999 (75, 150, 300 or 600 mg) or 300 mg aliskiren.

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CHAPTER 3

ANGIOTENSIN II TYPE 2 RECEPTOR- AND ACETYLCHOLINE-MEDIATED RELAXATION: THE ESSENTIAL CONTRIBUTION OF FEMALE SEX HORMONES AND CHROMOSOMES

Based on: *Angiotensin II type 2 receptor- and acetylcholine-mediated relaxation: the essential contribution of female sex hormones and chromosomes*. Sevá Pessôa B, Slump DE, Grefhorst A, van Veghel R, Garrelds IM, Roks AJM, Kushner SA, Danser AHJ, van Esch JHM. *Manuscript in preparation.*

ABSTRACT

Angiotensin II induces vasoconstriction via its type 1 receptors (AT_1R), while type 2 (AT_2) R are believed to be vasodilator. The latter is not a universal finding and may be limited to women. AT_2R-induced vasodilation, if occurring, is mediated via nitric oxide (generated by endothelial NO synthase, eNOS) and/or endothelium-derived hyperpolarizing factors (EDHFs). Studies in eNOS knockout mice suggest that EDHF predominate in women. To distinguish the contribution of female sex hormones and chromosomes to AT_2R function and EDHF-mediated vasodilation, we made use of the four core genotype (FCG) model, where the testis-determining *Sry* gene has been deleted (Y^-) from the Y chromosome, allowing XY^- mice to develop a female gonadal phenotype. Simultaneously, by incorporating the *Sry* gene onto an autosome, XY^-*Sry* and XX*Sry* transgenic mice develop into gonadal males. FCG underwent a sham or gonadectomy (GDX) operation, and after 8 weeks, animals were sacrificed and iliac arteries were collected to assess vascular function. Vascular function was also studied in C57bl/6 males treated with estrogen after GDX. XY^-*Sry* males responded more strongly to Ang II than XX females, and the AT_2R antagonist PD123319 revealed that this was due to a dilator AT_2R-mediated effect occurring exclusively in XX females. The latter could not be demonstrated in XX*Sry* males and XY^- females, nor in XX females after GDX, suggesting that it depends on both sex hormones and chromosomes. Indeed, treating C57bl/6 GDX males with estrogen could not restore Ang II-mediated, AT_2R-dependent relaxation. To block acetylcholine-induced relaxation of iliac arteries obtained from FCG XX mice, both eNOS - and EDHF inhibition were required, while in FCG XY animals eNOS inhibition alone was sufficient. These findings were independent of gonadal sex, and unaltered after GDX. AT_2 receptor-induced relaxation requires both estrogen and the XX chromosome sex complement, while only the latter is required for EDHF. Estrogen treatment of male mice confirms that this approach is insufficient to re-introduce AT_2 receptor-induced relaxation.

INTRODUCTION

It is generally accepted that angiotensin (Ang) II type 2 (AT_2) receptors oppose Ang II type 1 (AT_1)-mediated responses by evoking vasorelaxation, natriuresis, antigrowth and anti-inflammatory effects.[1] According to some studies, these effects occur exclusively in women,[2] possibly due to the fact that estrogen upregulates AT_2 receptors.[3] As a consequence, low-dose Ang II even decreases blood pressure in young adult female rats, at levels that increase pressure in age-matched males.[4] Moreover, under certain conditions, e.g. in the spontaneously hypertensive rat (SHR), AT_2 receptors become AT_1 receptor-like,[5, 6] and induce constriction. The mechanism behind this phenotypic change is unclear, but might be due to a difference in location of endothelial cell versus vascular smooth muscle cell and/or heterodimerization with AT_1 receptors.[1]

AT_2 receptor-mediated vasodilation involves bradykinin type 2 (B_2) receptor activation, which results in the generation of NO by endothelial NO synthase (eNOS) and endothelium-derived hyperpolarizing factors (EDHFs), respectively.[7, 8] Although the identity of the latter varies according to vessel size, species and vascular bed, EDHF-induced relaxation always depends on the activation of intermediate-conductance and small-conductance Ca^{2+}-activated K^+-channels (IK_{Ca}, SK_{Ca}). Of interest, studies in eNOS KO mice revealed that EDHF is the predominant relaxant pathway in females, and as such might contribute to the lower incidence of cardiovascular diseases in premenopausal women.[9]

Sex differences arise from direct effects of gonadal hormones and sex chromosome genes. An ideal model to investigate the sex chromosome complement (XX versus XY) independently of gonadal sex is the four core genotype (FCG) mouse.[10] In this model, the *Sry* gene, which is the dominant testis-determining gene, is deleted from the Y chromosome through a natural mutation (Y^-). As a consequence, the XY^- mouse develops ovaries and expresses a female gonadal hormone phenotype. Simultaneously, by incorporating the *Sry* gene onto an autosome, XY^-*Sry* and XX*Sry* transgenic mice develop into

gonadal males. Unexpectedly, after gonadectomy, XX FCG mice displayed a larger blood pressure response upon Ang II infusion than XY mice, suggestive for adverse sex chromosome effects encoded within the XX sex chromosome complement that contribute to hypertension.[11] In the present study, we made use of the FCG model to dissect the contribution of gonadal hormones and sex chromosome genes to AT_2 receptor-mediated vasodilation and the EDHF component of acetylcholine-induced relaxation.

METHODS

Animals

C57bl/6J mice (19 males, 8 females; age 8-12 weeks) were purchased from Charles River. Four core genotype (FCG) mice (19 XY$^-$*Sry* and 22 XX*Sry* gonadal males, 23 XX and 23 XY$^-$ gonadal females) were bred at the department of Neuroscience using XY$^-$*Sry* males on a C57Bl/6J background (Jackson Laboratories) and female C57Bl/6J mice (Harlan). PCR genotyping for the presence or absence of the Y chromosome was done as described before using DNA isolated from toes (10 days old), and again confirmed after sacrifice (ear).[12] At 10-14 weeks of age, mice were randomized to undergo gonadectomy or sham operation via an incision in the midline, followed by removal of the ovaries or testes. To study the effect of estrogens, 1-week gonadectomized C57bl/6J males (Charles River Laboratories, Maastricht, The Netherlands) were randomized to receive a daily subcutaneous injection of 100 µg/kg of diethylstilbesterol (DES) (Steraloids Inc., Newport, RI) dissolved in olive oil or vehicle (olive oil) during one week. Intact C57bl/6J and FCG mice were sacrificed (60 mg/kg i.p. pentobarbital) at 18-22 weeks of age. Treated gonadectomized C57bl/6 males were sacrificed at 12-14 weeks of age by cardiac puncture under isoflurane anesthesia. Blood from FCG mice was obtained immediately prior to sacrifice by cannulation of the hepatic portal vein. Uteri were rapidly excised, weighed and stored at -80^{o}C. Tibias were collected and cleaned using hydrogen peroxide. Iliac arteries and were removed and

either used directly or after overnight storage in cold, oxygenated Krebs-Henseleit solution.

Mulvany myographs

Iliac arteries (diameter ~350 μm) were cut into 6 ring segments of approximately 1.5-2 mm length and mounted in a Mulvany myograph with separated 6-mL organ baths containing gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure. Following a 30-min stabilization period, the maximal contractile response was determined by exposing the vessels to 100 mmol/L KCl. Segments were then incubated for 30 min in fresh buffer in the absence or presence of 100 μmol/L L-NAME, 1 μmol/L irbesartan (not in FCG mice), or 1 μmol/L PD123319. Next, concentration-response curves (CRCs) were constructed to Ang II, endothelin-1 (ET-1) and phenylephrine. Finally, after a washout period of 30-minutes, segments were again incubated for 30 min in fresh buffer with or without 100 μmol/L L-NAME, the SK_{Ca} inhibitor apamin (100 nmol/L), and/or the IK_{Ca} inhibitor TRAM34 (10 μmol/L), and preconstricted with U46619 (100-300 nmol/L). Thereafter, CRCs to acetylcholine (ACh) were constructed. CRCs for Ang II and ACh in presence and absence of L-NAME were performed in the same segments, whereas ACh CRCs in presence of TRAM34+apamin and/or L-NAME were randomized across the remaining segments.

Data analysis

Data are expressed as mean±SEM. Statistical analysis between groups for basal characteristics was performed by one-way ANOVA, followed by post-hoc evaluation according to Bonferroni. Relaxant responses to ACh are expressed as a percentage of the contraction to U46619. Contractile responses are expressed as a percentage of the contraction to 100 mmol/L KCl. CRCs were analyzed as described to obtain E_{max} and pEC_{50} ($-^{10}\log EC_{50}$) values. Differences

in CRCs within and between groups were tested by general linear model ANOVA for repeated measures. $P<0.05$ was considered significant.

RESULTS

Baseline characteristics

As expected, males had a higher BW than females (Table 1), and gonadectomy diminished this difference. Tibia length was similar in all animals. Gonadectomy reduced the uterus weight/TL ratio from 5.1±0.6 to 2.0±0.5 mg/mm in XX females, and from 4.1±0.6 to 1.2±0.2 mg/mm in XY^- females ($P<0.05$ for both), thus confirming that gonadectomy was successful.

Studies in healthy C57bl/6 mice

Ang II responses in iliac arteries from healthy C57bl/6 males were higher than those in arteries from healthy females ($P<0.0001$; Figure 1). Irbesartan abolished all Ang II responses ($P<0.001$), while PD123319 partially reduced the response in males ($P=0.013$), and enhanced it in females ($P=0.003$). L-NAME enhanced the response to Ang II in females and males, but the increase was significant in females only ($P<0.001$). After L-NAME, Ang II responses in males and females were identical. In females, the response after L-NAME was identical to that after PD123319, whereas in males, the response after L-NAME was increased versus that after PD123319 ($P=0.001$).

Table 1. Body weight (BW) and tibia length (TL) in C57bl/6 and four core genotype mice.

Strain	**C57bl/6 mice**		**Four Core Genotype mice**							
SCC	**XY**	**XX**	**XY$^-$**		**XX**		**XX**		**XY$^-$**	
Sry	**Yes**	**No**	**Yes**		**No**		**Yes**		**No**	
Gonadal	**Male**	**Female**	**Male**		**Female**		**Male**		**Female**	
Gonadal status	**Intact**	**Intact**	**Intact**	**GDX**	**Intact**	**GDX**	**Intact**	**GDX**	**Intact**	**GDX**
N	7	8	9	10	9	14	10	12	10	13
BW (g)	28.0±1.1	22.7±0.2#	26.6±0.8	24.4±0.4	23.1±0.7#	23.9±0.5	29.8±1.2	25.5±0.8	21.0±0.5#	21.9±0.4
TL (mm)	17.1±0.1	16.9±0.1	17.4±0.1	17.4±0.1	17.1±0.1	17.3±0.1	17.6±0.1	17.4±0.1	16.9±0.1	17.2±0.1

#P<0.05 vs. corresponding male.

Studies in FCG mice

Ang II responses in iliac arteries (Figures 2A & 2B) obtained from FCG XY$^-$*Sry* males (E_{max} 53±11%) and XX females (E_{max} 40±10%) were comparable to those in healthy C57bl/6 males (E_{max} 59±9%; Figure 1A) and females (E_{max} 51±7%; Figure 1B). Moreover, like in C57bl/6 females, PD123319 (P=0.054) and L-NAME (P=0.007) increased the Ang II response identically in XX females, so that the response after L-NAME was not different from that after PD123319. Like in C57bl/6 males, L-NAME increased the Ang II response in XY$^-$*Sry* males versus PD123319 (P=0.032), while the difference versus control was of borderline significance (P=0.057). PD123319 alone marginally decreased the response to Ang II (P=NS). Irbesartan was not evaluated in these studies. Importantly, all responses in XX*Sry* males and XY$^-$ females (Figures 2C & 2D) were identical to those in XY$^-$*Sry* males. Gonadectomy did not change these responses (Figures 2E, 2G & 2H), except in XX females (Figure 2F), where PD123319 now decreased the response to Ang II instead of enhancing it (P=0.001 for difference versus intact XX females; Figure 2B). These data indicate that the AT_2 receptor-induced relaxant effect requires both estrogen and the XX chromosome sex complement. Responses to ET-1 or phenylephrine did not display sex hormone or chromosome-dependent differences (supplemental Figure).

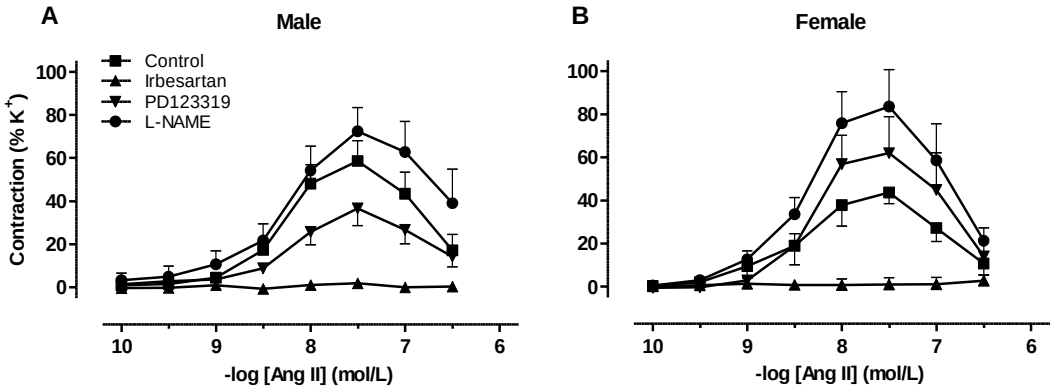

Figure 1. Contractions of iliac arteries, obtained from healthy male and female C57bl/6 mice to angiotensin II in the absence (control) or presence of irbesartan, PD123319, or L-NAME. Contractions (mean±SEM of n=5-16) have been expressed as a percentage of the response to 100 mmol/L K^+.

ACh relaxed iliac arteries of FCG XX females more strongly than iliac arteries of XY$^-$*Sry* males (P=0.020; Figures 3A & 3B). TRAM34+apamin prevented this stronger response in XX females, indicating that it was due to EDHF. Near complete blockade was obtained with L-NAME, both in XY$^-$*Sry* males and XX females, and TRAM34+apamin did not exert significant additional effects on top of L-NAME. Findings in XX*Sry* males (Figure 3C) mimicked those in XX females (i.e., TRAM34+apamin exerting an inhibitory effect, P<0.001), while findings in XY$^-$ females (Figure 3D) were identical to those in XY$^-$*Sry* males (i.e., TRAM34+apamin having no significant effect). Taken together, these findings indicate that ACh-induced EDHF release occurs exclusively in XX mice.

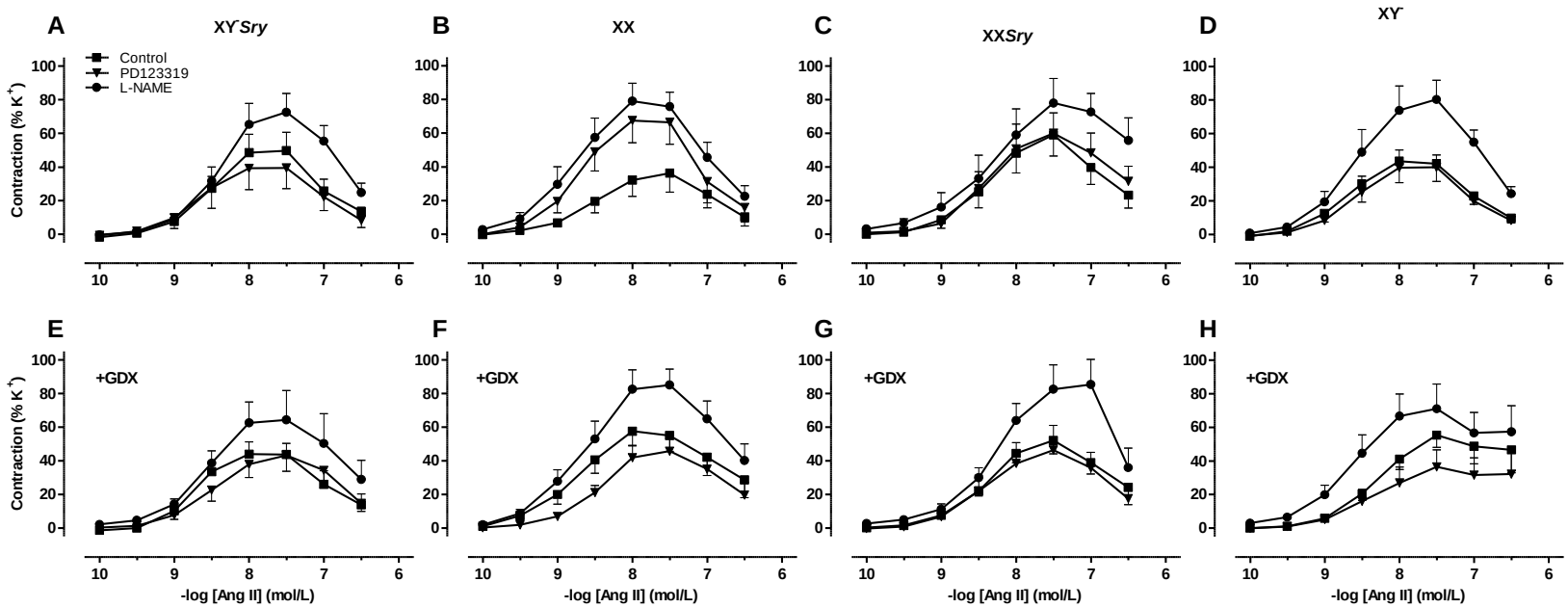

Figure 2. Contractions of iliac arteries, obtained from FCG mice (XY-*Sry* males, XX females, XX*Sry* males, and XY- females) before (top panels) or after (bottom panels) gonadectomy, to angiotensin II in the absence (control) or presence of PD123319 or L-NAME. Contractions (mean±SEM of n=7-13) have been expressed as a percentage of the response to 100 mmol/L K^+.

Observations after gonadectomy (Figures 3E-3H) were essentially the same, the only difference being that ACh responses in XX females (Figure 3F) versus XY-*Sry* males (Figure 3E) now were not only larger under control conditions (P=0.039), but also in the presence of L-NAME (P=0.022). Moreover, TRAM34+apamin exerted an inhibitory effect (P=0.015) on top of L-NAME in XX females (Figure 3F), but not in XY-*Sry* males. A similar tendency (P=0.075) was observed for the effect of TRAM34+apamin in the absence of L-NAME in XX females. In other words, gonadectomy allowed a better distinction of the EDHF response in XX animals, independently of the NO response. Findings in XX*Sry* male mice after gonadectomy were identical to those in gonadectomized XX females, while findings in gonadectomized XY- females were identical to those in gonadectomized XY-*Sry* males. This is further illustrated in Figure 4, which compares all data in gonadectomized animals pooled according to sex chromosome complement (i.e., independent of gender). Indeed, Figure 4A shows that TRAM34+apamin exert an inhibitory effect both at baseline (P<0.0001) and on top of L-NAME (P=0.017) in XX mice, but not XY mice (Figure 4B). Moreover, the response to ACh in XX mice (panel 4A) is larger than that in XY mice (panel 4B), both at baseline (P=0.022) and after L-NAME (P=0.004).

Studies in gonadectomized male C57bl/6 mice

Neither PD123319, nor L-NAME, enhanced the Ang II-induced contractions in iliac arteries of gonadectomized male C57bl/6 mice (Figure 5). DES treatment upregulated the response to L-NAME (P<0.05), but not to PD123319. This indicates that estrogen treatment upregulates the NO pathway, but cannot induce AT_2 receptor-mediated vasodilation. The latter would be in agreement with the observation that such vasodilation additionally requires the XX chromosome sex complement.

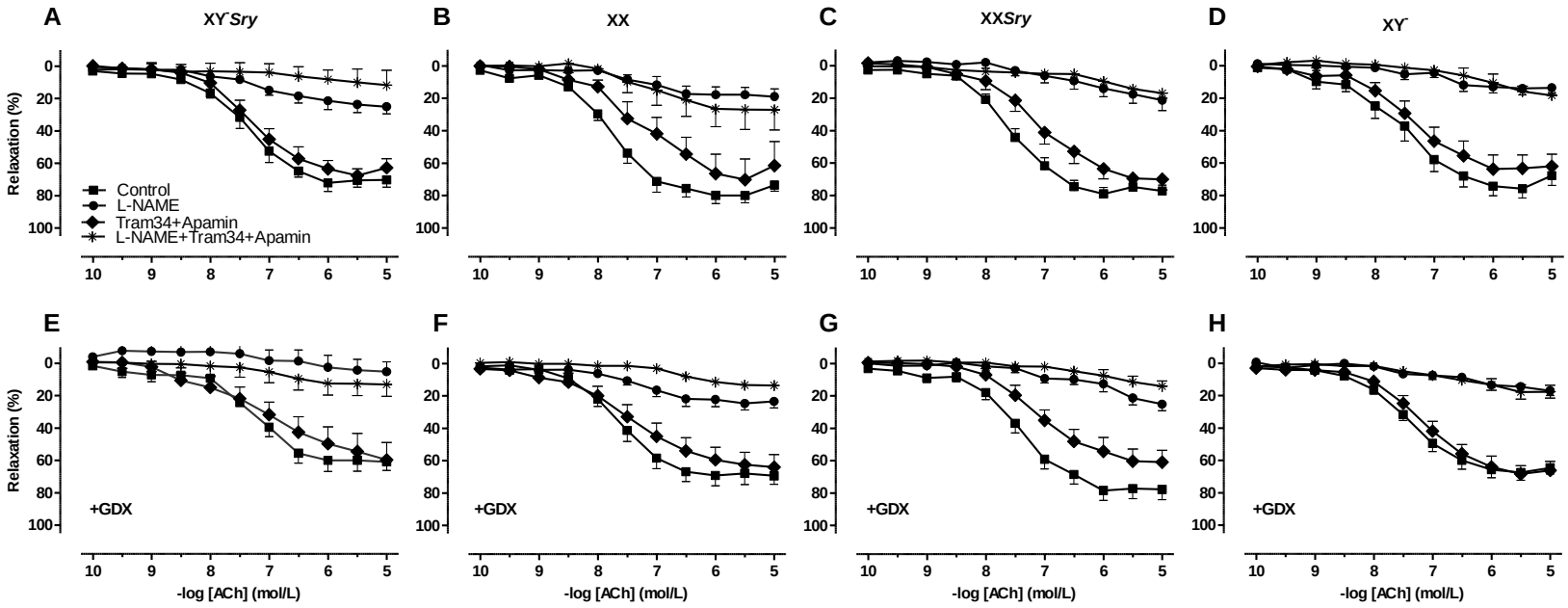

Figure 3. Relaxations of iliac arteries, obtained from FCG mice (XY$^-$*Sry* males, XX females, XX*Sry* males, and XY$^-$ females) before (top panels) or after (bottom panels) gonadectomy, to acetylcholine in the absence (control) or presence of L-NAME, TRAM34+apamin, or L-NAME+TRAM+apamin. Effects (mean±SEM, n=6-13) have been expressed as a percentage of the response to U46619.

DISCUSSION

This study is the first to show that AT_2 receptor-mediated relaxation requires the presence of both female sex hormones and the XX chromosome sex complement. The EDHF component of ACh-induced relaxation depends on the latter only. In contrast, ET-1 and phenylephrine-induced constrictions were sex hormone- and sex chromosome-independent. These data imply that the protective cardiovascular profile of premenopausal women is not due to estrogen only. Moreover, estrogen treatment of men will be insufficient to introduce a relaxant AT_2 receptor phenotype, as demonstrated in our DES-treated gonadectomized male mice.

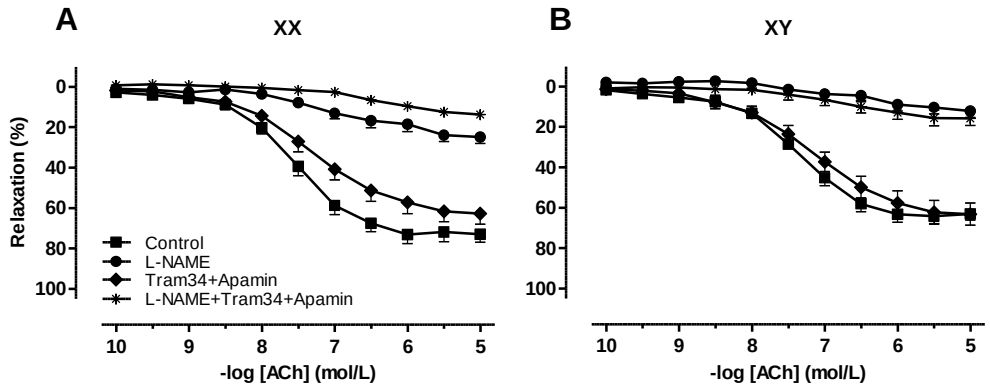

Figure 4. Relaxations of iliac arteries, obtained from gonadectomized FCG mice pooled according sex chromosome complement (XX vs. XY), to acetylcholine in the absence (control) or presence of L-NAME, TRAM34+apamin, or L-NAME+TRAM+apamin. Effects (mean±SEM, n=16-24) have been expressed as a percentage of the response to U46619.

A logical consequence of AT_2 receptor-mediated relaxation occurring exclusively in women is that women will display more modest hypertensive responses to Ang II. Animal models fully support this view: not only is the pressor response to chronic Ang II infusion much larger in male than in female mice,[11] but also was the increase in renal blood flow in Sprague-Dawley rats greater in females than in males,[13] while in SHR Ang II-induced renal vasodilation was limited to female rats only.[14] The underlying explanation might be that that the density of AT_2 receptors is larger in women, resulting in

a rise in the AT_2/AT_1 receptor ratio.[15, 16] However, although estrogen is known to induce AT_2 receptor expression,[3] this view may be too simplistic. Recent studies have shown that moderate cardiac AT_2 receptor overexpression in transgenic mice protected against maladaptive remodeling and dysfunction, whereas a massive, 9-fold overexpression did not yield such positive effects.[17] Thus, more AT_2 receptors do not necessarily translate into enhanced protective effects. Moreover, under pathological conditions AT_2 receptors sometimes become AT_1 receptor-like,[5, 6] and induce constriction. To what degree this involves a difference in location (e.g., endothelial cell versus vascular smooth muscle cell) and/or heterodimerization with AT_1 receptors remains to be determined.[1, 18] Importantly, in the absence of AT_1 receptors, direct AT_2 receptor-mediated dilation does not occur.[19] Thus, whether AT_2 receptors induce dilation depends on the presence of the AT_1 receptor, the AT_2/AT_1 receptor ratio (and most likely the degree of heterodimerization) and their location. Our current study now adds the XX chromosome sex complement to this list.

Of interest is that AT_2 receptor-induced dilation is mediated via B_2 receptors, which act through NO and EDHF,[20, 21] and that the latter now also appears to be determined by the XX chromosome sex complement. Thus, our findings on AT_2 receptor-induced vasodilation and EDHF may reflect the same phenomenon. Unfortunately, investigating the EDHF contribution to AT_2 receptor-mediated dilation in FCG mice is technically challenging, as such dilation was observed indirectly only, as an enhanced response to Ang II in the presence of PD123319. Studying enhanced responses on top of inhibitors of EDHF and NO synthase would have required large additional numbers of animals. Instead, we therefore choose to study the contribution of EDHF to ACh-induced relaxation.

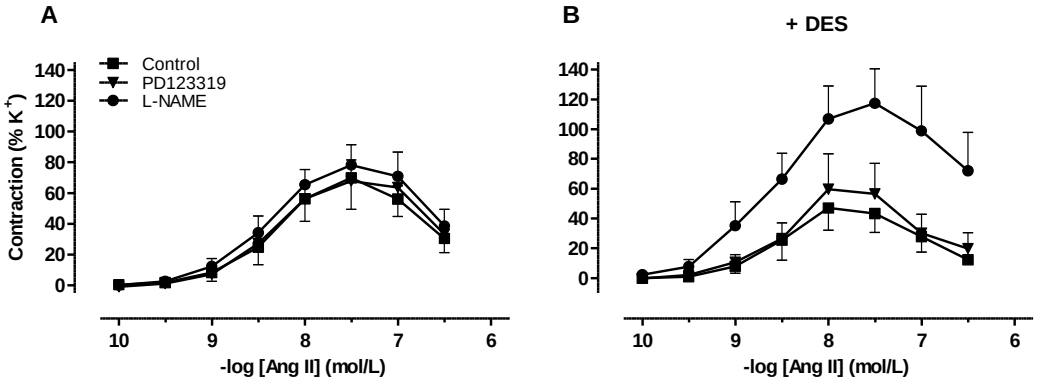

Figure 5. Contractions of iliac arteries, obtained from gonadectomized male C57bl/6 mice before (left panel) or after (right panel) treatment with DES, to angiotensin II in the absence (control) or presence of PD123319 or L-NAME. Contractions (mean±SEM of n=5-6) have been expressed as a percentage of the response to 100 mmol/L K^+.

There is not one single EDHF. Multiple EDHF candidates have been proposed, ranging from K^+, cytochrome-P450 products from arachidonic acid (epoxyeicosatrienoic acids) and H_2O_2 to *S*-nitrosothiols, and the underlying pathways involve IK_{Ca} and SK_{Ca} in endothelial cells, and inwardly rectifying K^+ channels, Na^+-K^+-ATPase and/or large-conductance Ca^{2+}-activated K^+-channels in vascular smooth muscle cells.[22-24] Concomitant blockade of IK_{Ca} and SK_{Ca} with TRAM34 + apamin is the gold standard to distinguish the contribution of EDHF, irrespective of what its exact identity is. Studies in eNOS KO mice have already revealed that EDHFs are of particular importance in females,[9] in full agreement with our findings. Obviously, given the multiple EDHF candidates that exist, and the fact that their identity varies according to vessel size, species and vascular bed, our conclusions at this stage are valid for the mouse iliac artery only, and do not necessarily imply that there is no EDHF in men.

How does the sex chromosome complement relate to AT_2 receptor function and EDHF? Although the AT_2 receptor gene is located on the X chromosome, none of the well-known EDHF components are. Yet, the NADPH oxidase subunits Nox1 and Nox2 do map to the X chromosome, and as such may determine the generation of superoxide-derived H_2O_2 in a sex

chromosome-dependent manner. Future studies should investigate which EDHFs/EDHF pathways are sex chromosome-dependent. Sex hormones play an additional, sex chromosome-independent role, at least with regard to AT_2 receptor function. Indeed, estrogens are known to increase angiotensinogen, AT_2 receptor density and eNOS, while they decrease renin, ACE, AT_1 receptor density, Nox1 and Nox2.[16, 25] These alterations are suggestive for reduced ROS formation combined with an upregulation of AT_2 receptor stimulation and NO release. Our data after DES treatment, showing that NOS inhibition greatly increased the response to Ang II, confirm the latter. Simultaneously, they did not reveal the re-introduction of an AT_2 receptor-mediated relaxant effect in male mice after DES exposure. Thus, unlike eNOS upregulation, AT_2 receptor-induced vasorelaxation requires more than estrogen treatment alone. Furthermore, testosterone increases renin, ACE and AT_1 receptors, and downregulates AT_2 receptors, thereby favouring Ang II-induced constriction. In postmenopausal women the balance will shift towards vasoconstriction, unless they receive hormone replacement therapy.[25]

In apparent contrast with our data, Ji et al. observed an enhanced response to Ang II in gonadectomized FCG XX mice as compared to XY mice, despite their earlier observation that females display more modest Ang II pressor responses than males.[11] They attributed this unexpected finding to adverse sex chromosome effects within the XX sex chromosome complement. There are no follow-up data that support this concept. Clearly, in view of our data, these effects, if occurring, are non-adrenergic, non-ET-1 and non-Ang II-mediated.

What are the clinical implications of our findings? It is well-accepted that premenopausal women are protected from the development of cardiovascular disease in comparison to age-matched men.[26] The current study, combined with previous data, supports a major role for the renin-angiotensin system (RAS) in sex-related differences. Yet, there are no sex-specific recommendations for antihypertensive therapy, nor is there currently any evidence that men and

women respond differently to RAS blockers. One retrospective study in patients with heart failure claimed a higher efficacy of ACE inhibitors in males, and of AT_1 receptor blockers in females.[27] Although this potentially supports the importance of AT_2 receptor stimulation in women, large prospective studies are warranted to confirm such claims.

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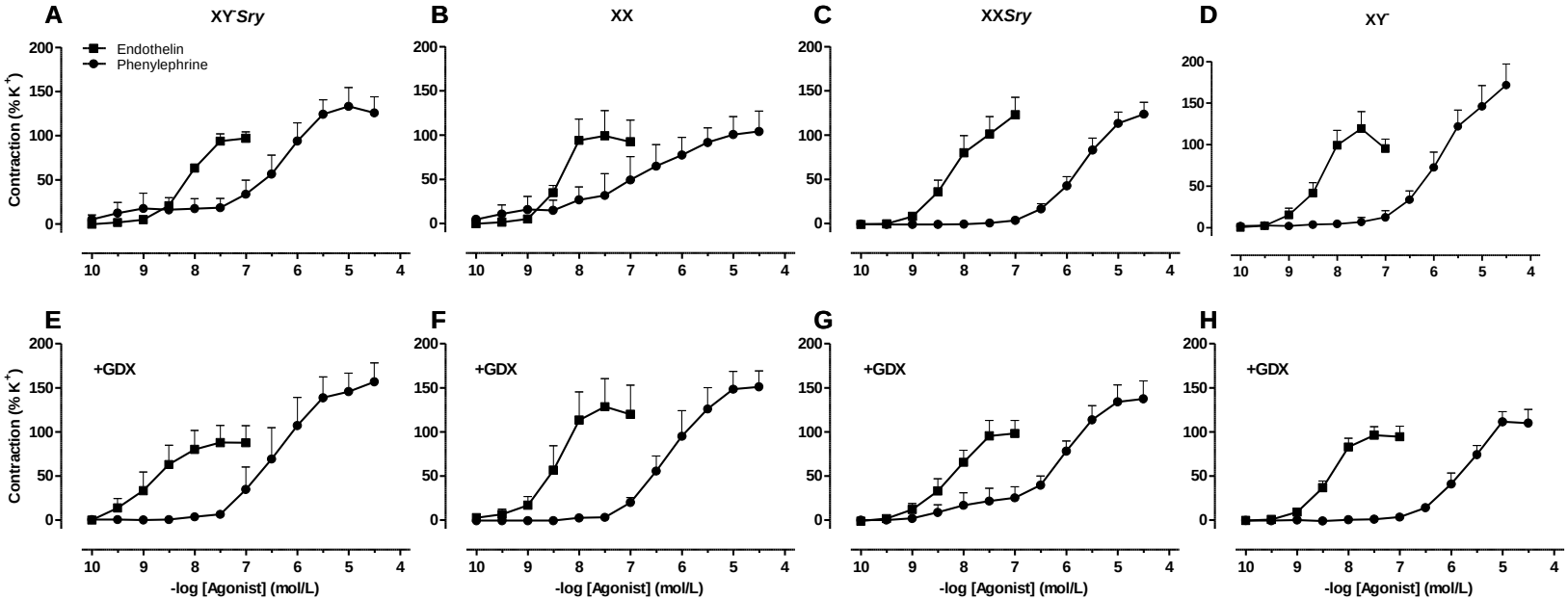
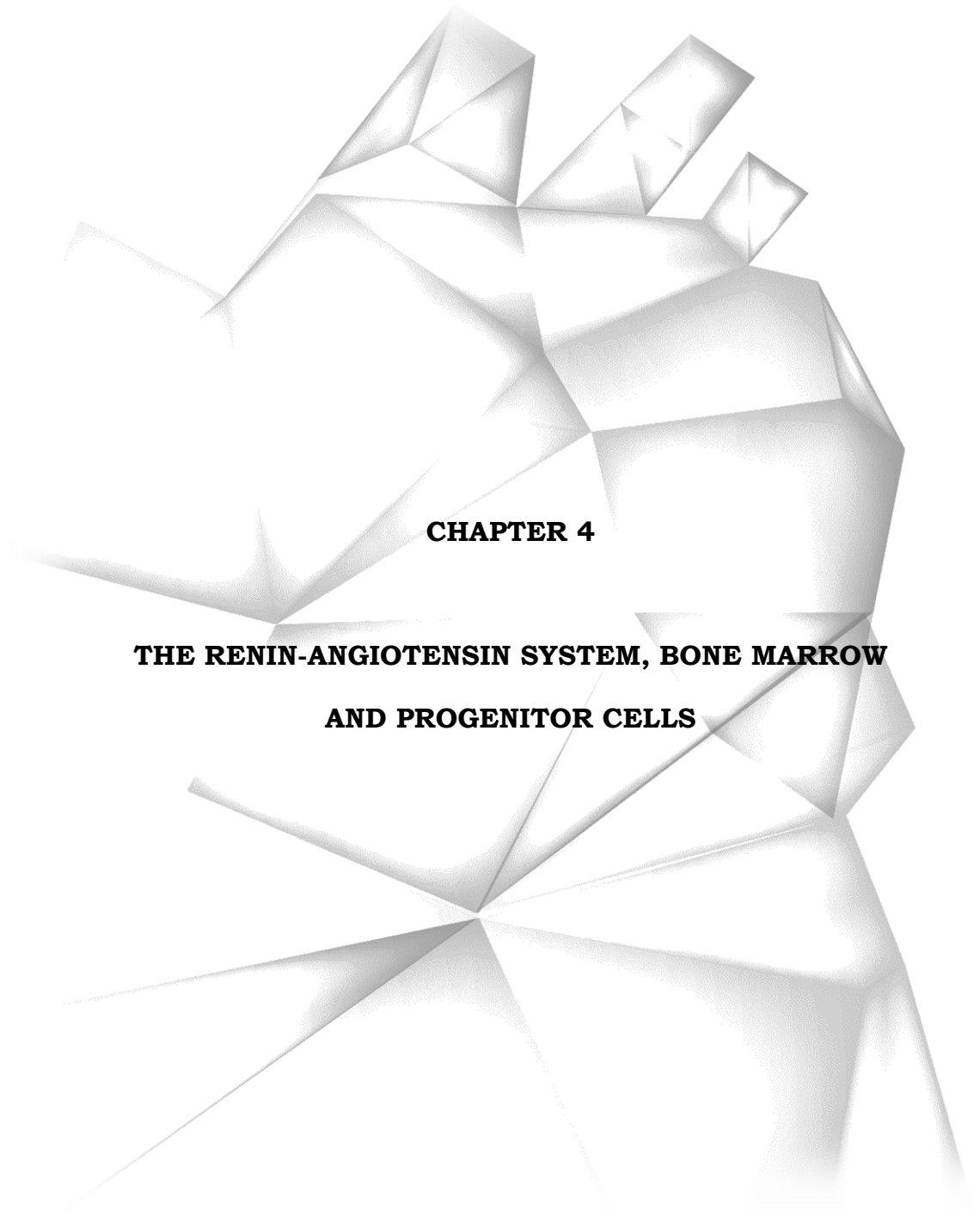
SUPPLEMENTAL MATERIAL



Supplemental Figure. Contractions of iliac arteries, obtained from FCG mice (XY$^-$*Sry* males, XX females, XX*Sry* males, and XY$^-$ females) before (top panels) or after (bottom panels) gonadectomy, to endothelin-1 or phenylephrine. Contractions (mean±SEM of n=3-10) have been expressed as a percentage of the response to 100 mmol/L K^+.



CHAPTER 4

THE RENIN-ANGIOTENSIN SYSTEM, BONE MARROW AND PROGENITOR CELLS

Based on: *The renin-angiotensin system, bone marrow and progenitor cells*. Durik M, Sevá Pessôa B, Roks AJM. Clin Sci (Lond). 2012;123:205-23.

ABSTRACT

Modulation of the renin-angiotensin system, in particular of the function of the hormones angiotensin II and angiotensine-(1-7), is an important target for pharmacotherapy in the cardiovascular system. In the classical view, such modulation affects cardiovascular cells to decrease hypertrophy, fibrosis, and endothelial dysfunction, and improves diuresis. In this view, excessive stimulation of angiotensin II type 1 receptors fulfills a detrimental role, as it promotes cardiovascular pathogenesis, and this is opposed by stimulation of angiotensin II type 2 and the angiotensin-(1-7) receptor coded by the Mas proto-oncogene. In recent years, this view has been broadened with the observation that the renin-angiotensin system regulates bone marrow stromal cells and stem cells, thus involving hematopoiesis and tissue regeneration by progenitor cells. This change of paradigm has enlarged the field of perspectives for therapeutic application of existing as well as newly developed medicines that alter angiotensin signaling, and now stretches beyond cardiovascular therapy. In this article we review the role of angiotensin II and angiotensin-(1-7) and their respective receptors in hematopoietic and mesenchymal stem cells, and discuss possible pharmacotherapeutical implications.

GENERAL INTRODUCTION

Angiotensin II versus angiotensin-(1-7)

The renin-angiotensin system (RAS) is a major regulator of renal and cardiovascular function and plays a central role in the homeostasis of the cardiovascular system and of the hydro-electrolyte balance.[1] For a long period, research was focussed on production and signaling of angiotensin II (Ang II), highlighting angiotensin-converting enzyme (ACE) and renin with respect to production, and the angiotensin II type 1 receptor (AT_1 receptor) with respect to signaling of Ang II. Since Ang II is an important player in unfavourable remodeling of cardiovascular tissue, ACE inhibitors (ACEi), AT_1 receptor blockers (ARB) and renin inhibitors are successfully used in treatment of cardiovascular disease, and novel tools to further optimize intervention in the RAS are being developed.[1]

In the past two decades these optimization efforts resulted in the identification of novel therapeutic targets within the RAS, including the Ang II type 2 receptor (AT_2 receptor),[2] angiotensin-(1-7) (Ang-(1-7)) and its G-coupled receptor Mas,[3] and angiotensin-converting enzyme 2 (ACE2),[4,5] which is important for Ang-(1-7) formation. An increasing amount of evidence shows that Ang-(1-7) is one of the most important hormones of the RAS.[6] In the cardiovascular system, Ang-(1-7) mainly has opposite actions as compared to Ang II, leading to a dichotomy in the RAS, namely the proliferative, prothrombotic and vasoconstrictor actions of Ang II versus the anti-proliferative, anti-thrombotic and vasodilator actions of Ang-(1-7).[7] Ang-(1-7) can be formed directly from Ang II by ACE2, but also from Ang I. As ACE metabolizes Ang-(1-7), ACEi treatment increases Ang-(1-7) levels, and this is believed to play an important role in beneficial cardiovascular effects of ACEi.[7,8] As an alternative, improvement of Ang-(1-7) formation by ACE2 is now under consideration as a novel cardiovascular therapy.[9] Similarly, stimulation of AT_2 receptor, a receptor for both Ang II and Ang-(1-7), often opposes AT_1 receptor signaling, and this principle is being explored for cardiovascular therapy with specific AT_2 receptor agonists.[10]

Stem cells: general features and clinical use

At the same time that novel pharmacological tools within the RAS are being found for cardiovascular therapy, a novel target tissue for RAS intervention has been identified, namely the bone marrow (BM) and other sources of stem cells and progenitor cells. These cells can either be hematopoietic stem cells (HSC) or hematopoietic progenitor cells (HPC), or can be mesenchymal stem cells (MSC), also called multipotent stromal cells. Many articles use the term HSC for cells that are in a very early stage of phenotypic development, still allowing differentiation towards diverse lineages, and that may have a potential for self-renewal. HPC is then used for cells that are further differentiated, and have lost the potential for self-renewal. HSC and HPC form cells of the erythroid, myeloid, or lymphoid lines (Fig. 1). Since the boundaries between these two hematopoietic populations are not always clear, we will here use the term HSC for both populations. Endothelial progenitor cells (EPC) and fibrocytes are special HPC types that are involved in angiogenesis and fibrosis respectively. MSC have been identified as cells that can form a plethora of non-hematopoietic cells, including cardiomyocytes, smooth muscle-like cells, renal cell types, connective tissue cells, neural cells etc. (Fig. 2). MSC are most abundantly present in BM, fat tissue and dental pulp, but can also be present in tissues such as the myocardium and the subintima or adventitia of large arteries. Accordingly, these cells are under investigation as a tool for tissue regeneration of any imaginable kind. Since both HSC, MSC and isolated whole bone marrow are interesting for tissue repair, all three sources have been used for cardiac and vascular repair. Other precursor cells that may potentially be used for this purpose are embryonal stem cells or induced pluripotent stem cells.[11] These cells can either serve as sources for novel cardiomyocytes or angiogenic cells, or act as paracrine cells that favourably affect cardiac remodelling or vascular repair. Several clinical studies have been performed with autologous BM-derived stem cells showing moderately improved cardiac performance in cardiac ischemic diseases.[12,13] As to yet, it is not clear which stem cell type and which preparation and infusion method gives the best

results, and this is an important research goal. A second important goal is upgrading of the stem cells' abilities to perform its reparative function. For this purpose, diverse strategies are being studied,[11] and, as will become clear from the present review, pharmacological intervention in the RAS may be one of them.

RAS and stem cells: general implications

The notion that the RAS is involved in regulation of bone marrow and stem cells, and in particular hematopoiesis and erythropoiesis, was generated already shortly after the introduction of ACE inhibitors in the clinic when in 1982 two independent groups observed that a high dose of ACE inhibition causes anemia and leucopenia.[14,15] Later, the presence of all major RAS components in BM cells, including stromal cells, HSC / HPC and MSC, was confirmed. This led to the concept of a potential autocrine-paracrine mechanism for local RAS-mediated regulation of hematopoiesis.[16] Another important reason to study effects of angiotensins and RAS-modulating medicines on BM-derived cells is that in the case of cardiovascular disease where RAS modulation is indicated as a treatment, stem or progenitor cells are under investigation for application in tissue regeneration, in particular of the vascular bed and the myocardium.[17-27] Furthermore, inflammation and fibrosis have been identified as important targets for RAS modulation, and these processes may be related to regulation of HSC and MSC, as will be explained later in the article. Dedicated studies have now provided clear evidence that ACE-, Ang II / AT_1 receptor and Ang-(1-7) / Mas receptor activity is involved in hematopoiesis as well as in the formation of cardiovascular cells and other somatic cell types from progenitor cells.[28]

In the present article we will review the role of Ang II, Ang-(1-7) and their respective receptor / signaling pathways in propagation and differentiation of HSC and MSC, and discuss possible clinical implications. Furthermore, for more extensive discussions regarding ACE substrates and metabolites other than Ang II and Ang-(1-7) we refer to previous reviews.[29,30] The present review contains two summarizing figures: Fig. 1 depicts effects

of Ang II and Ang-(1-7) on hematopoietic stem cells and derived progenitors; Fig. 2 summarizes effects on mesenchymal stem cells. In addition, we provide a table (Table 1) that summarizes beneficial vs. detrimental effects of the main angiotensin receptors with respect to cardiovascular disease.

THE ROLE OF ANG II AND ANG-(1-7) IN HEMATOPOIESIS

Erythropoiesis

Soon after the initial studies that uncovered that manipulation of ACE activity interfered with erythropoiesis, it became clear that the role of the RAS in erythropoiesis is very complex, being involved in virtually every step between the hematopoietic stem cell and the fully differentiated erythrocyte. When looked at in further detail it was shown that the stimulation of AT_1 receptor increased formation of early erythroid progenitors; an effect that requires the presence of erythropoietin.[31] Also, genetic manipulation leading to overactivity of AT1a receptors in mice results in an increase of hematocrit.[32] Conversely, AT_1 receptor knockout mice show a decrease of hematocrit values when compared with wild-type animals.[33] The stimulatory role of AT_1 receptors in erythropoiesis has clinical implications: like ACEi, ARB treatment was reported to reduced erythropoiesis in healthy persons, and also in patients undergoing hemodialysis.[34]

The mechanism of Ang II-mediated regulation of erythropoesis is largely unclear. Most of its effects are exerted in the early phases of erythropoiesis.[31,34] Some authors imply that Ang II acts indirectly via its influence on erythropoietin levels,[35] or erythropoietin sensitivity,[36] while others do not observe a link between erythropoietin and Ang II in erythropoiesis.[37,38] A possible second messenger system via which Ang II could be affecting erythropoiesis is the Jak-STAT pathway, which is known to be stimulated by Ang II,[39] and to be vital in the erythrocyte-stimulating action of erythropoietin.[40]

Since Ang-(1-7) is degraded by ACE, the anemic effect of ACEi might be due to changed Ang-(1-7) levels. However, it was shown that Ang-(1-7)

stimulated erythroid burst-forming units (BFU-E) cultured from mice that were treated with the myelosuppressive agent 5-fluorouracil, and reduced anemia in breast cancer patients after chemotherapy.[41,42] These findings suggest that Ang-(1-7) stimulate erythropoiesis, and should counteract the anemic effect of ACEi. Apparently, this does not happen, and the question remains how important Ang-(1-7) is for erythropoiesis.

General effects of Ang II on hematopoiesis of white blood cells

Leucopenia induced by a high dosage of ACE inhibitors as observed early after introduction of ACEi[15] was an indication that the RAS may be involved in the formation of white blood cells (WBC) by HSC. In agreement, genetic ablation of ACE in mice leads to perturbations in myelopoiesis, which can be reliably recapitulated with ACE inhibition.[43] When focusing the attention on Ang II, it has been shown that Ang II induces the proliferation of mouse bone marrow and human cord blood HSC *in vitro*. This Ang II effect is elicited in part through stimulation of Lin^- BM stromal cells and partly mediated by the direct stimulation of HSC in the presence of colony stimulating factor (CSF).[44] AT_1 receptors mediate this Ang II effect because losartan blocks it, and the presence of AT_1 receptor in both HSC and stromal cells is compatible with the dual pathway.[44] Furthermore, Ang II/AT_1 receptor signaling promotes the monocyte colony stimulating factor (M-CSF)–mediated differentiation/proliferation of BM monocyte-lineage cells.

Through these general mechanisms Ang II can contribute to regulation of WBC hematopoiesis. Strictly spoken, however, AT_1R signaling doesn't seem to be of great importance for hematopoiesis under normal physiological conditions: ACE-KO mice show a block in terminal granulopoiesis which leads to a reduction of segmented neutrophils. Monocytes and macrophages are at normal levels in ACE-KO and AT_1-KO mice, although they are to be functionally immature.[30,43,45] This would correspond to the fact that ARB treatment does not reduce WBC levels. However, as observed recently,[30] under hematopoietic stress Ang II / AT_1 receptor signaling has readily visible effects. This is noticed for instance after chemotherapy or irradiation, where

Ang II infusion improves the repopulation of bone marrow with HSC cells, and thus accelerates restoration of WBC counts. It is with these circumstances in mind that the next section, in which we deliberate specific HSC subtypes, has to be read.

Role of Ang II in specific subtypes of white blood cell precursors

As already indicated, important data on the role of Ang II in regulation of HSC comes from studies that explored the restoration of WBC populations after irradiation or chemotherapy in animal models. Under such circumstances, *in vivo* Ang II infusion leads to accelerated restoration of total blood leucocytes, lymphocytes and platelets, and to increased granulocyte macrophage colony forming units (GM-CFU), granulocyte erythroid megakaryocyte macrophage colony forming units (GEMM-CFU) and BFU-E in *in vitro* expanded bone marrow cells.[46] Therefore, Ang II has a rather broad spectrum when it comes to stimulation of HSC. This is in accordance with the observation that in the early development of HSC to WBC, i.e. in CFU cultured under pan-lineage conditions, Ang II has a proliferative effect.[30]

This proliferative effect is lost when cells are further differentiated due to culturing in lineage-specific media, containing granulocyte-macrophage colony-stimulating factor (GM-CSF) with macrophage colony-stimulating factor (M-CSF) or granulocyte colony-stimulating factor (G-CSF).[43] When the HSC has passed the stage of myeloblast, pharmacological or genetically-induced interruption of AT_1 receptor or ACE activity delays myeloid differentiation rather than proliferation, as witnessed by an increase in the myeloblast / early myelocyte marker CD11b and a decreased development of macrophage and neutrophil markers.[43] This was observed *in vivo* in the absence of hematopoietic stress. Furthermore, AT_1 receptor blockade also leads to the decreased differentiation towards dendritic cells of human monocyte or murine BM cell cultures.[47] AT_2 receptor stimulation counteracts this AT_1 receptor effect. Accordingly, the summed effect of these Ang II functions is an accelerated general increase in leucocytes during

hematopoietic stress, and more subtle qualitative changes in populations of mature leucocytes, which can be seen in the absence of hematopoietic stress.

Similar to erythropoiesis, Ang II does not seem to have a stand-alone effect in development of WBC, but rather plays a facilitating role, as observed during CFU-GM and CFU-GEMM formation. In absence of colony stimulation factors (SCF, GM-CSF, IL-3 and EPO) no growth effect of Ang II on these colonies was observed, but in their presence, Ang II dose-dependently increased CFU-GM and CFU-GEMM formation.[44] The factors that are needed to allow an Ang II effect are released by Lin^- BM stromal cells.[44] Also, in the absence of M-CSF, cultured BM cells of AT_1 receptor KO and WT mice showed no difference in myeloid progenitors and promonocytes.[45] However, the increase of these cell types that finds place upon stimulation with M-CSF was attenuated in AT_1 receptor KO vs. WT. The facilitating role of Ang II on M-CSF was shown to be due to TNF-alpha release from stromal cells which increases expression of C-Fms, the receptor for M-CSF, in HSC.[45]

Apart from stimulating the myeloid path of hematopoiesis Ang II affects the lymphoid development. AT_1 receptor stimulation appears to promote inflammatory activation of lymphocytes. In addition, Ang II infusion leads to a shift of CD4+ T cells (T helper) from Th2 to Th1, which leads to increased production of pro inflammatory cytokines (IFN-gamma, IL-2, and tumor necrosis factor-beta). Blockade of AT_1 receptors decreased this shift and led to reduced infiltration of tissues by activated macrophages and T-cells.[48]

Based on the mild effects of ACE/AngII/AT_1 receptor on WBC population in the absence of hematopoietic stress, it seems that the major part of the effect of RAS modulation on inflammation is mainly caused by its effects that do not directly relate to hematopoiesis. Ang II is known to induce monocyte recruitment to the vascular wall and to stimulate these monocytes to release various inflammatory cytokines. It also increases production of reactive oxygen species (ROS) which in turn stimulate NF-kappaB signaling

leading to a proinflammatory phenotypes in various cell types. These processes are reviewed in detail in other publications.[49,50]

The role of Ang-(1-7) in HSC regulation

The effects of Ang-(1-7) on hematopoiesis are documented in several articles and are, in short terms, similar to the effect of Ang II. Ang-(1-7) stimulates recovery from irradiation and chemotherapy by increasing proliferation of HSC and multi-lineage hematopoietic progenitors.[42,46,51] In NOD/SCID mice the engraftment and proliferation of human mononuclear cells was increased in mice receiving Ang-(1-7) treatment, which also increased numbers of differentiated cells of myelomonocytic and B cell lineages.[52] Like for Ang II, Ang-(1-7) effects are readily visible during hematopoietic stress, but not so evidently under normal physiological circumstances. This is reflected in a recent study in which toxicological studies with Ang-(1-7) infusion did not show apparent effects on blood variables.[53]

The role of angiotensin receptor subtypes for Ang-(1-7) effects on hematopoiesis has not been investigated. However, our own studies in which we used isolated BM mononuclear cells (BM-MNC) from rat and mice, suggest that Mas receptors are mediating the proliferative effect of Ang-(1-7) on HSC.[29,54] Since these tests were done under conditions that promote the development of endothelial progenitor cells, they do not provide conclusive evidence.

Endothelial progenitor cells

Whereas a modest number of studies have addressed the relationship between HSC and RAS, some more intensive research has been done on endothelial progenitor cells (EPC).[28,29] EPC are a special type of HSC or MSC-derived progenitor cells that develop endothelial-like features in specific culturing conditions, and that have been implicated in endothelial repair and vasculogenesis.[55,56] Vasculogenesis is the formation of new blood vessels from progenitor cells such as hemangioblasts, as opposed to angiogenesis, in which new vessels originate from sprouting of pre-existing ones.

Vasculogenesis takes place during embryogenesis, but it has been proposed that it can also occur during adulthood, which would involve EPC.[57]

EPC starting as HSC in the bone marrow are being intensively studied. Their recruitment as HSC from the bone marrow to the circulation involves stress-induced stromal-derived factor-1 (SDF1), which activates proteases that degrade the adherence proteins that bind HSC to endosteal cells. Subsequently, HSC can migrate to the circulation. Once arrived in the vascular lumen they can home as angiogenic monocytes and macrophages that pre-process the tissue that requires neovascularization or endothelial repair, or as EPC that will form the new endothelium.[58] Thus, the process of vasculogenesis involves various hematopoietic cell types with varying progenitor, myeloid and endothelial cell markers, complicating the identification of "true EPC".[28] This identification can either be based on immunohistological staining for stem cell and endothelial membrane markers, followed by flow cytometry, or by culturing of BM- or blood-derived MNC in specialized endothelial culture medium and subsequent colony observation or (immuno)histochemical staining. A simple histochemical staining for cultured angiogenic cells is combined acetylated low density lipoprotein uptake (Ac-LDL uptake; a feature of phagocytotic monocytes, macrophages and endothelial cells) with binding to lectin from *Bandeiraea* (*Griffonia*) *simplicifolia*-1 (BS1) or *Ulex europaeus* (*Ulex europaeus* agglutinin-I: UEA-1).[55] More specific EPC markers, for cultured as well as freshly obtained cells processed for flow cytometry, combine stem cell surface markers c-Kit, Sca-1 and CD133 with an endothelial cell (EC) surface marker (e.g. CD34 or Flk-1 (mouse equivalent for human kinase domain receptor (KDR) or VEFG receptor 2 (VEGFR-2)). With respect to culturing methods, in vitro propagation of blood-derived MNC distinguishes early EPC and colony-forming unit endothelial cells (CFU-EC/CFU-Hill) on the one hand from late EPC (comprising outgrowth endothelial cells (OEC) and endothelial colony forming cells (ECFC)) on the other hand.[57,59-63] Early EPC and CFU-Hill are found from 2 days until 3 weeks of culture and might be more related to angiogenic monocytes and macrophage since they show low proliferation and

tube formation capacity. Late EPC, appearing from 3 weeks up until 12 weeks of culture could represent true EPC, displaying highly proliferative and tube formation capacity. For further critical reviewing of EPC selection criteria we refer to previous publications.[28,64,65]

Although the identity of true EPC and their permanent incorporation into repaired and newly formed vessels is still a matter of debate, it is evident that the various "EPC" contribute to the vascular regeneration and repair processes, and thus bear clinical relevance.[28,29] In addition, EPC plasma levels have been used as a biologic marker for vascular function and cumulative cardiovascular risk. It has been shown that the number of CFU-Hill correlate with brachial endothelial function, measured as reactive hyperemia, arterial calcification, with Framingham risk score, and with several cardiovascular risk factors.[61,66,67] Quantification of cultured EPC might be a rather laborious method for prognostic purposes. Instead, circulating EPC can be measured with flow cytometry, using CD34 alone or in combination with a marker expressed by endothelial cells, often KDR. As such, CD34+KDR+ cell levels were shown to be associated with coronary artery disease, outcome after angioplasty, and traumatic brain injury, although peripheral arterial calcification only correlated with colony number of cultured EPC.[67-70] In a direct comparison between cultured EPC and quantitation with flow cytometry, both methods show association of EPC levels with coronary artery disease, but only cultured EPC are predictive for progression of the disease.[71] Also, an increase of these markers is associated with beneficial effects of RAS intervention, as reviewed earlier.[28] The use of EPC as a standard risk marker in cardiovascular disease is however still remote for the daily practice. Hence, EPC remain under investigation for regenerative, angiogenic therapy in organs after ischemic events and as a prognostic marker during pharmacotherapy directed against vascular disease. Since RAS intervention is often used in those cases it is important to characterize the effects of angiotensins on EPC.

Role of Ang II, AT_1 receptor *and* AT_2 receptor *in EPC*

Although the identity of true EPC is still a question[28] many studies have confirmed the role of angiogenesis-associated progenitor cells, whether they are mobilized from BM to peripheral blood or isolated, cultured and re-injected, in endothelial regeneration, and neovascularization.[72] The fundamental parts of these processes bear relationship with Ang II signaling through AT_1 and AT_2 receptors. AT_1 receptor stimulation can lead to pro-angiogenic effects and recruitment of EPC, but on the other hand the stimulation can reduce EPC proliferation and function. These paradoxical effects can be explained from acute as compared to chronic AT_1 receptor signaling. Acute Ang II signaling is pro-angiogenic. In EPC, this pro-angiogenic effect depends on NADPH oxidase activation and enhanced VEGF anti-apoptotic function through upregulation of VEGF type 2 receptors and improved NO release, as well as on PI3/Akt signaling.[73-75] The deleterious effects of Ang II arises from chronic stimulation, and consists of two consecutive phases.[76] In the first phase, taking place between day 2 and 5 of stimulation of EPC with Ang II, AT_1 receptor-mediated increase of NADPH oxidase activity leads to ASK-1 – JNK/p38 MAPK – Bax/Bcl2 signaling-induced apoptosis involving caspase 3.[77] The second phase, which is noticed from day 5 and onward, involves production of cytotoxic levels of ROS leading to cellular senescence.[78,79] As a result, chronic treatment with Ang II decreases human and mouse EPC numbers and function.[68,77-80] Employing AT_1 receptor KO MNC and BM transplant in wild type and ApoE KO mice, it was shown that AT_1 receptor signaling affects vascular repair function and thus promotes atherogenesis.[77] As commented in detail earlier, it is still a question if these *in vivo* vascular effects solely depend on EPC, or involve an interplay with inflammatory cells or even BM stromal cells.[76]

The role of Ang-(1-7) / Mas receptor signaling in EPC

Ang-(1-7) was shown to improve endothelial vasodilator and eNOS function in various studies.[81-94] Although some of these effects depend on the acute activation of eNOS or inhibition of NADPH oxidase,[95-97] others may point to a

potential role of Ang-(1-7) in endothelial regeneration. Such a role was suggested by the observation that Ang-(1-7) improved the recovery of HSC,[41,42,46] from which EPC are derived. Therefore, dedicated studies to explore Ang-(1-7) effects on EPC were performed.[54] In adherent rat or mice BM-MNC cultures, which most likely resemble early EPC, 7 days of treatment with Ang-(1-7) increased AcLDL/lectin-positive cells, which were found to be positive also for VEGF type 2 receptors. Mas receptor signaling is an important mediator of the stimulatory effect of Ang-(1-7) on BM-MNC and EPC because the effect disappeared when Mas receptor signaling was prohibited by genetic ablation of Mas or treatment with A779. AT_2 receptors did not seem to play a role.[29] The *in vitro* stimulatory effect of Ang-(1-7) on EPC can explain why we found that *in vivo* infusion of the peptide in mice with myocardial infarction led to an increase in VEGF+ and c-kit+ cells in the heart.[54] Since local cardiac overexpression of Ang-(1-7) peptide did not lead to such an effect, we hypothesized that BM-derived, angiogenesis-related progenitor cells were recruited to the heart. This potential mechanism as well as the consequences for myocardial angiogenesis remains to be characterized.

Several other questions regarding angiogenesis need to be clarified as well. Firstly, Ang-(1-7) can also inhibit angiogenesis *in vivo* [98-101] and tends to inhibit *in vitro* tube formation by human umbilical cord vene endothelial cells (HUVEC).[102] This principle is now under investigation in the context of application of Ang-(1-7) as an anti-cancer therapy.[100,103] There can be several explanations for such a discrepancy with our findings on EPC. First, it can relate to differences in cell types, i.e. EPC versus adult endothelial cells. Second, the inhibitory Ang-(1-7) effect on tube formation by HUVEC was not dose-dependent, and involved Mas receptors as wells as AT_1 receptors.[102] Since these were acute responses, the inhibitory effect of Mas receptors on AT_1 receptor signaling[104] might have inhibited angiogenesis. However, this explanation only works if one presumes the presence of spontaneous AT_1 receptor signaling or a paracrine / autocrine RAS. Third, in the sponge model for angiogenesis and after tumor implantation the anti-angiogenic

response might involve effects on surrounding cells that produce angiogenic factors. The myocardium in our experiments may respond differently to Ang-(1-7) with respect to the release of angiogenic factors as compared to the tissue that surrounds sponges or tumours. In fact, there is evidence that tumour angiogenesis markedly differs from that in normal tissues, as extensively reviewed elsewhere.[105] Fourth, the Ang-(1-7) concentration and its relation to the duration of the stimulus may be important. In acute studies, such the mentioned study in HUVEC,[102] Ang-(1-7) might simply display the usual opposite effect Ang II. Instead, our studies in cultured MNC and EPC employed chronic stimulation, and again Ang-(1-7) appear to be opposite of the Ang II effect, i.e. stimulatory vs. inhibitory. Looking in better detail, we found a bell-shaped concentration-dependent effect of a 7-day Ang-(1-7) treatment, given every 2 days, with a maximal response between 10^{-9} and 10^{-8} mol/L.[54] Similarly, pretreatment with 10^{-8} mol/L Ang-(1-7) stimulated porcine BM-MNC to increase tube formation, whilst higher concentrations seem to reduce this ability.[106] This observation prompts another explanation. Recently, it was discovered that Ang-(1-7) leads to Mas receptor internalization.[107] A continuous presence of Ang-(1-7) concentration of 1 nM and higher might induce permanent Mas receptor internalization, thus abolishing Mas receptor signaling. This could in turn even promote chronic AT_1 receptor signaling, thus reducing EPC. Alternatively, Ang-(1-7) might be diverted to AT_2 receptors, which would suppress angiogenesis.[108] Since Ang-(1-7) is rapidly degraded in the presence of serum,[109] the presence of Mas receptors at the cell membrane might be warranted when intermittent administration of the peptide is used, even at concentrations that are slightly higher than 1 nmol/L.

Regardless of the here mentioned reasons for the paradoxical effects of Ang-(1-7) on EPC as compared to angiogenesis, it is clear that in the development of pharmacotherapy based on EPC stimulation by Ang-(1-7) it will be important to dissect the diverse signaling pathways. This concerns both the exploration of the differential function of these pathways as well as the development of optimal pharmacotherapy.

Improvement of EPC recruitment with ARB and ACEi.

The *in vivo* effects of ARB on EPC generally correspond well with the effects that would be predicted from the functions of Ang II and Ang-(1-7) as observed in animal or cell culture studies. ARB treatment in animal studies raises EPC levels during hypertension,[110-112] after nephrectomy[113] or myocardial infarction,[114] in atherosclerosis models,[115] and in the ischemic hindlimb model.[116,117] Although the situation in animal models may quite differ from the clinical presentation of patients, the effects of ARB on EPC from animals and patients correlate rather well. In patients with type II diabetes, ARB treatment alone or as a part of multiple drug therapy was proposed to exerts its beneficial cardiovascular effects by increasing the number of regenerative EPCs.[118,119] In accordance with this idea, ARB treatment increased EPC counts, angiogenic factors and endothelial vasodilator function in normotensive patients with coronary artery disease.[120] Similarly, ARB increase EPC in patients with acute coronary syndrome.[121] The ARB treatment also promotes an increase in EPC levels in kidney transplant patients.[122] A special role might be performed by telmisartan, a combined ARB / PPAR-γ agonist. The PPAR-γ might have additional value when stimulation of EPC is concerned,[123,124] as was discussed in detail in previous reviews.[28,29] Together, the data suggest that in animal models as well as in patients ARB treatment increases EPC levels, thus preserving the endothelium. It seems logical to ascribe these beneficial effects to a decrease of EPC apoptosis and senescence caused by excessive Ang II / AT_1 receptor signaling.

ACEi have been shown to have beneficial effects in cardiovascular disease that are believed to be related to EPC function.[61,68,125-130] However, the actual number of patient studies that explored effects of ACEi on EPC is very limited. ACEi increased the levels, proliferation, migration, adhesiveness, and tube formation of EPC that were cultured from blood of patients with coronary artery disease.[80] In patients with acute coronary syndrome ramipril increased circulating EPC as measured by flow cytometry, and its effect on EPC was nearly identical to that of ARB telmisartan.[121] Very recently, two

studies appeared that claim a stimulatory effect of ACEi on EPC of patients with hypertension and acute coronary syndrome, although the characterisation of EPC in these studies is limited.[131,132] In agreement with clinical studies, ACEi increase EPC in several animal models.[114,116,117,133,134] These effects may involve various factors other than angiotensin metabolism, as amply discussed elsewhere.[28,29]

Differentiation of HSC into fibrocytes

Fibrocytes are CD34+, CD45+, or CD133+ bone marrow-derived circulating cells that co-express collagen I or smooth muscle actin.[135] They are derived from hematopoietic cells and related to monocytes, and infiltrate organs to establish fibrosis. In models of renal fibrosis AT_1 receptor mediates increased bone marrow fibrocytes, increased renal infiltration and stimulates collagen I production in cultured fibrocytes. AT_2 receptor counteracts these effects. Recruitment of fibrocytes to the kidney involves CXCL16, a ligand for the receptor CXCR6.[136] However, it is not certain if this relates to Ang II signaling.

With respect to cardiac remodeling, it was shown that Ang II infusion promotes cardiac fibrosis through the recruitment of fibrocytes in mice.[137,138] Since chemokine (C-C motif) receptor 2 (CCR2) KO abrogated the Ang II effect, the chemokine (C-C motif) ligand (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), is apparently required for Ang II-induced fibrocyte recruitment. Blood pressure is not involved, since CCR2 knockout did not change this variable. MCP-1 is an important stimulator of monocytes, dendritic cells and T cells, and therefore these data show an important relationship between inflammatory response and Ang II-induced fibrosis. It should be noted, however, that the recruitment of fibrocytes by Ang II to the myocardium is not necessarily detrimental. CCR2 knockout, which ablates this Ang II effect, leads to less fibrosis, but also to an exaggerated ventricular dilatation and a worsened systolic function. The fibrotic response seems to provide the necessary matrix to support the hypertrophied myocardium.[137] Therefore, to obtain clinical benefit from suppression of Ang II-induced

fibrosis one should also prevent myocardial hypertrophy to avoid myocardial dilation, which is detrimental.

Mesenchymal stem cells from bone marrow

Mesenchymal stem cells are non-hematopoietic progenitor cells that are able to form many diverse cell types, amongst which cells with features of adipocytes, cardiomyocytes, fibroblasts, endothelial cells vascular smooth muscle cells and renal cells, which all bear relevance for cardiovascular disease and therapy. They are found in many different tissues, including the bone marrow. Given the pluripotency of MSC these cells may play a role in diverse physiological and therapeutical processes. We here outline the role of RAS in various of these processes.

Cardiomyocyte formation from MSC

BM-MSC were shown to be able to develop cardiomyocyte features through reprogramming of the genetic program by *in vitro* treatment with 5-azacytidine, a DNA-demethylating agent.[139] This finding was an important step towards the principle that autologous MSC transplantation could be used for cardiac repair, and led to clinical trials. Meta-analyses have shown that the small trials conducted thus far show only limited benefit, which has prompted novel research on optimization of stem cell therapy.[140,141] Optimization studies include issues like cell type that is used, number of grafted cells, moment of injection, but also the effect of pharmacological modulation, including RAS components. Ang II was shown to promote differentiation of rat BM-MSC to cardiomyocytes *in vitro*, and have an additive effects to 5-azacytidine.[142] Thus, Ang II treatment might reduce the culture time of BM-MSC allowing earlier grafting of the differentiated cultures.

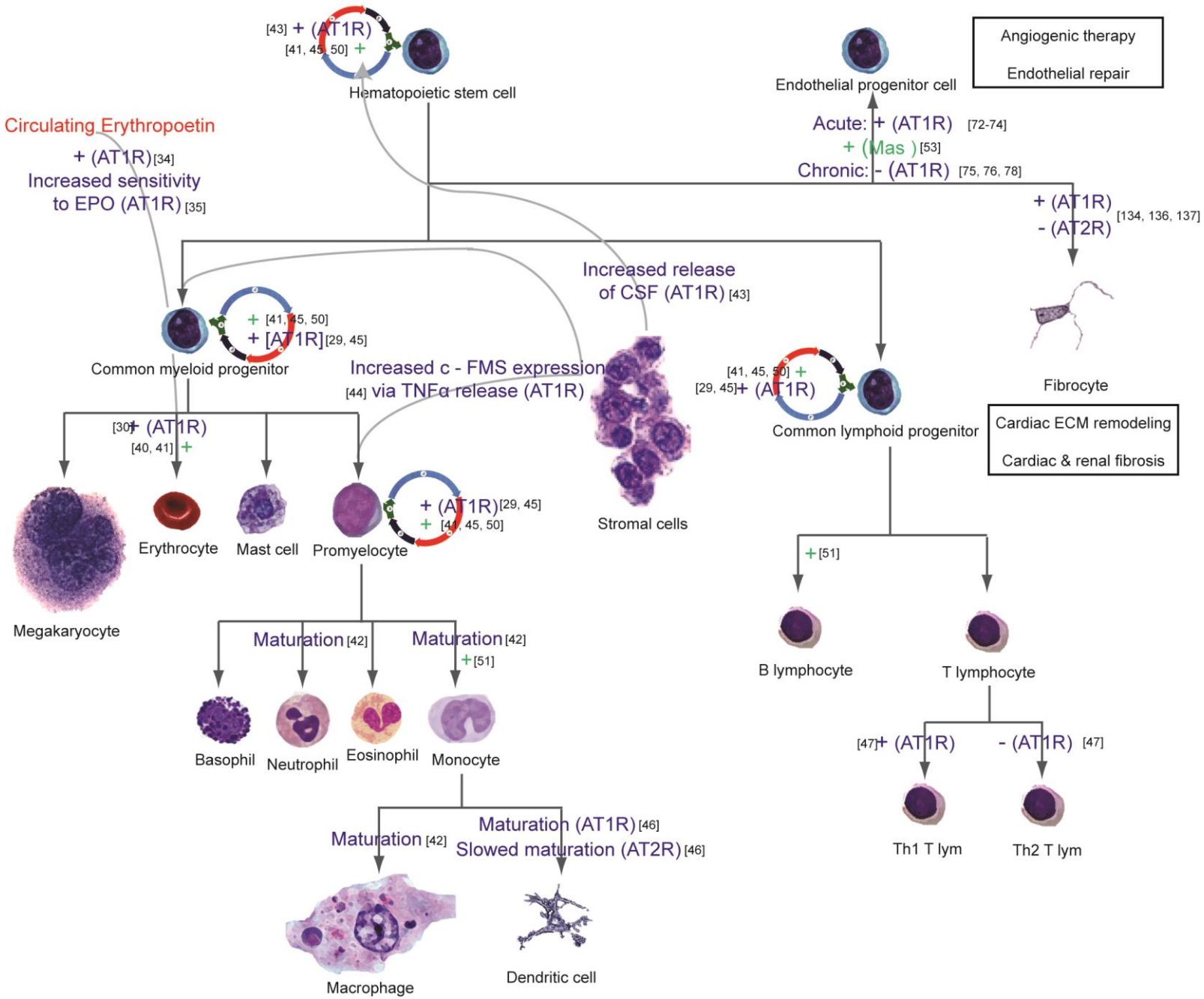


Figure 1: Diagrammatical representation of the effects of Ang II (blue lettering) and Ang-(1-7) (green lettering) on hematopoietic cells. The direction of the effect of Ang II, Ang-(1-7) or angiotensin receptor stimulation is indicated with "+", being stimulatory, or "-", being inhibitory. The involved receptor, if known, is mentioned between curved brackets: (AT1R) = AT_1 receptor signaling; (AT2R) = AT_2 receptor signaling. Straight black arrows indicate effects on differentiation or, when specifically indicated, on maturation. Cell cycle diagrams indicate a proliferative effect. Curved arrows originating from stromal cells represent paracrine effects. Digits

Further characterisation of the involved receptor subtypes came from a study in which human BM-MSC (Yub623) were stimulated to differentiate to cardiomyocytes by co-culturing on murine cardiomyocytes for 2 weeks.[143] Various ARB's were shown to equally improve BM-MSC differentiation, apart from telmisartan that had additional effectiveness because of its pleiotropic actions as a PPAR-γ agonist. The effect was independent from the co-cultured murine cells. AT_2 receptor antagonist PD123319 alone was without effect, but it reduced the effect of candesartan. Since also Ang II alone did not stimulate differentiation in this study, unless given together with candesartan, it was concluded that the differentiation of BM-MSC to cardiomyocytes was mediated through AT_2 receptors. Because ACE inhibition mimicked ARB effects a local RAS in BM-MSC was proposed, as was to be expected from previous PCR studies on rat BM-MSC.[16] However, the fact that renin inhibitor aliskiren was without effect in human BM-MSC suggests that not all RAS components may be present in relevant amounts in those cells. Therefore, it cannot be excluded that ARB's, when given in the absence of exogenously applied Ang II, might have unknown pleiotropic effects, especially when applying the high concentration (3 μmol/L) used in this particular study.

The Ang II-induced differentiation of BM-MSC to cardiomyocytes theoretically is in accordance with the observation that Ang II stimulates cardiac extracellular matrix remodelling through recruitment of fibrocytes.[137] In the context of physiological remodelling these effects of Ang II would be complementary to each other if there is a need for increased myocardial mass, for instance to improve physical performance, because they would provide a balanced increase of myocytes and extracellular matrix. Such a remodeling would beneficially influence cardiac performance. Whether Ang II-induced BM-MNC differentiation into cardiomyocytes occurs, as well as the question if this is in balance with fibrocyte recruitment, remains to be investigated in models for physiological cardiac remodelling. An exaggerated AT_1 receptor-mediated response may, however, cause stiffening of the myocardium and relative ischemia, a concept that fits in the general

paradigm of pathological remodelling due to high blood pressure or ischemia. Blockade of AT_1 receptor binding of Ang II and concurrent redirection of Ang II towards AT_2 receptors would inhibit this detrimental response, because it restores the equilibrium between differentiation of MSC into fibrocytes and cardiomyocytes.

ARB treatment can also be combined with BM-MSC grafting. Indeed, in the rat myocardial infarction model it was shown that pretreatment of BM-MSC with ARB improved the therapeutic effect of BM-MSC grafting on systolic heart function.[143] This was further improved by oral administration of ARB. However, oral ARB intake combined with non-pretreated BM-MSC grafting did not have an additive effect compared to the single ARB treatment. Therefore, pretreatment of BM-MSC with ARB or AT_2 receptor agonists may be the most optimal condition for cardiac repair. It should be noted however that BM-MSC that are injected for cell therapy in the myocardium are only transiently present, as observed in mice, and the fusion with resident cardiomyocytes might be poor.[144] This has favoured the concept that BM-MSC are therapeutic primarily due to a paracrine function.[145] Therefore, it is important to explore these paracrine factors and the impact of RAS modulation thereupon.

Neural repair

Similar to cardiac ischemia, BM-MSC grafting has been tested for brain ischemia.[146] In the mouse mid-cerebral artery occlusion (MCA) model, BM-MSC grafting improved survival, ischemic lesion volume, neurological score and cerebral edema. Cerebral blood flow and TNFα measurements suggest that reduction of inflammation rather than a pro-angiogenic effect is responsible for the effect of BM-MSC grafting. BM-MSC from AT_2 receptor KO mice, however, did not improve neurological variables, and even showed a trend to worsen survival. Pretreatment with the ARB valsartan restored the survival effect of BM-MSC grafting. These results imply that AT_1 receptor signaling, when not counteracted by AT_2 receptor, is detrimental for BM-MSC repair function. ROS-mediated pro-inflammatory AT_1 receptor signaling

could be the provocative stimulus in the case where AT_2 receptor KO cells seem to worsen the outcome. Since AT_2 receptor signaling results in NF-kappaB-mediated neural repair and neural differentiation of embryonic stem cells through stimulation of methyl methanesulfonate-sensitive 2 release,[147,148] it would be worthwhile to study if these mechanisms are also involved in BM-MSC grafting.

BM-MSC grafting is believed to represent a new intervention technique for patient with stroke,[149,150] and AT_1 receptor blockade might provide further improvement. Pretreatment of mice with valsartan in a dose without a blood pressure effect led to comparable improvement of neurological score and better blood flow than BM-MSC grafting.[146,151] Other ARB show comparable results to valsartan.[152] Further neuroprotective effects of ARB were shown in hypertensive patients, who show less stroke-related events when ARB treatment was applied, leading to a better prognosis.[153] In acute stroke, animal models also show improved recovery with ARB treatment.[154-156] However, in patient studies of acute stroke ARB treatment was not at all effective, showing even a tendency towards adverse effects, which might relate to a hypersensitivity of the patients towards blood pressure lowering.[156,157] Therefore, optimization of RAS treatment in acute ischemia might benefit from (pre)clinical research to interaction with endogenous as wells as grafted BM-MSC.

Adipogenesis

Adipogenesis, the formation of fat tissue, is importantly implicated in metabolic syndrome and cardiovascular risk. As extensively reviewed elsewhere.[158,159] Adipose tissue contains a complete RAS needed for Ang II production and signaling. The physiological importance of this local RAS extends to the entire organism, contributing to both local and systemic regulation of RAS. This is believed to contribute to obesity, diabetes, metabolic syndrome and cardiovascular disease. The adipose tissue can be roughly divided in two compartments, the visceral and the subcutaneous adipose tissue. Adipose tissue can form part of normal physiology as an

energy storage compartment. This healthy fat tissue contains small insulin-sensitive adipocytes that store fat until use as an energy source. These cells produce anti-inflammatory effectors, such as adiponectin. Adipose tissue can also be related to diabetes and metabolic syndrome, and in this case is featured by the occurrence of large insulin-resistant adipocytes.[160] These large adipocytes release pro-inflammatory factors amongst which TNF-α, a key player in insulin resistance, diabetes and metabolic syndrome. Obesity is associated with large adipocytes and an activated adipose tissue RAS, and it is believed that obesity thus contributes to cardiovascular disease.[158]

Adult adipocytes may arise from two sources: pre-adipocytes in the fat tissue and MSC from various sources, including the bone marrow. In pre-adipocytes the role of Ang II and its respective receptors is matter for a complicated debate that involves culture conditions, type of adipogenic stimulus, phase of differentiation, etcetera.[161,162] A feasible concept of Ang II-mediated dysregulation of adipogenesis in diabetes and metabolic syndrome is that AT_1 receptor stimulation prevents insulin-induced differentiation of pre-adipocytes into adult small adipocytes, and that as a consequence the pool of large adipocytes increases.[163] Compared to research in pre-adipocytes, research in MSC differentiation to adipocytes is relatively young. It was shown that human BM-MSC cultured under adipocyte-inducing conditions, all key Ang II-related RAS components are expressed. Thus, Ang II seems to enhance differentiation into adipocytes through AT_1 receptors in an autocrine fashion.[161] This effect is inhibited by AT_2 receptor. Since expression of AT_2 receptor is rather high in MSC that are cultured under the given conditions, the blockade of adipogenesis by AT_2 receptors was readily visible when the antagonist PD123319 was added. However, since exogenously given Ang II mimicked the AT_2 receptor-mediated blockade of differentiation, AT_1 receptor signaling is dominant when only the locally produced Ang II is present.

Given the complicated manner in which AT_1 receptor and AT_2 receptor signaling is involved in differentiation of MSC and pre-adipocytes, as well as the question what fate will fall upon MSC with respect to formation of small

vs. large adipocytes, it is in this moment hard to establish the importance of BM-MSC differentiation in therapeutic effects of RAS modulation. Further studies will require BM-MSC-specific RAS component knockout cell culture or mouse models, as was previously suggested also for adipose tissue.[158] Culturing conditions and metabolic status will be important issues in such studies.

The importance of Ang-(1-7) / Mas axis in adipose tissue is only beginning to be uncovered. Ang-(1-7) and ACE2 are present in adipose tissue,[164,165] and ACE2 is regulated by dietary fat.[165] Also, Ang-(1-7) and the Mas receptor were reported to have an impact on fat and glucose metabolism, adiponectin levels and on insulin sensitivity of adipocytes.[166,167] Moreover, the peri-aortic fat may play a role in Ang-(1-7)-induced vasodilations.[168] Therefore, research to the role of Ang-(1-7) in BM-MSC differentiation into adipocytes is warranted.

MSC with vascular smooth muscle cell traits

Vascular smooth muscle-like cells can be derived from both BM stem cell pools as well as MSC populations present in the adipose tissue. In mouse studies it was shown that Ang II stimulates the expression of typical smooth muscle cell markers in cultured adherent BM-MNC and MSC from adipose tissue through AT1 receptors.[169,170] Similar results were obtained with MNC derived from peripheral blood of rabbits.[171] In adipose tissue MSC it was shown that this effect is mediated by TGF-β receptor stimulation and subsequent Smad2 and ERK activation.[170] Replacement of wildtype with GFP-labeled BM in mice showed that GFP-positive α-smooth muscle cell actin (α-SMA) -positive cells incorporated in the neointima of damaged femoral arteries of wild-type C57bl/6 mice.[169] The recruitment of such cells was stimulated by Ang II infusion, and inhibited by AT_1 receptor blockade. Since neointima formation followed a similar pattern it was concluded that Ang II-induced differentiation of BM progenitors to vascular smooth muscle cells (VSMC) contributes to neointima formation. Indeed, BM-derived cells that express typical VSMC markers had been implicated in neointima

formation before.[172,173] In ApoE KO mice with LacZ-positive BM it was shown that local vascular production of SDF1α, a ligand for chemokine (C-X-C motif) receptor type 4 (CXCR4) that is important for chemoattraction of various BM-derived cells, plays a central role in homing of the VSMC-like cells to the neointima. Recently, a study that explored the effect of replacement of normal with AT_1 receptor KO BM in wild-type mice showed a connection between Ang II and SDF1α signaling.[174] AT_1 receptor KO led to diminished plasma levels and neointimal incorporation of Lin^- BM-derived progenitor cells, and decreased neointima formation. Although no specific VSMC markers were used in this study it was proposed that lack of AT_1 receptor signaling leads to decreased VSMC progenitor recruitment. This seemed to depend on decreased SDF1α release by local platelets at the site of injury due to the absence of AT_1 receptor signaling in those platelets. Therefore, AT_1 receptor signaling may affect neointimal homing of VSMC-like cells from BM in two ways: through increased differentiation of progenitor cells into VSMC-like cells and attraction of progenitor cells through stimulation of platelet-derived SDF1α.

It should be noted, however, that VSMC-like progenitor cells may not undergo complete differentiation into adult vascular smooth muscle cells. Some studies show that, when injected in the ischemic myocardium, BM stem cells / BM-MSC with VSMC traits are not incorporated in vessels.[175,176] Indeed, in earlier studies it was suggested that mesenchymal stem cells develop a smooth muscle cell-like contractile apparatus to form myoid cells that use this apparatus in their function to support hematopoiesis of inflammatory cells.[177] Interestingly, it was recently shown that BM-derived cells that incorporate into neointima and atherosclerotic lesions and express α-SMA are also expressing monocyte/macrophage markers.[178] Therefore, VSMC-like cells originating from the BM, and perhaps also from adipose tissue, may play a pro-inflammatory role, rather than that they represent a pool of progenitor cells that will permanently differentiate into adult VSMC. This might explain why we found that all BM-derived cells in neointima after stenting expressed inflammatory cell markers.[179] The importance of such

cells may of course be significant: the effect of Ang II on stimulation of the development of the VSMC-like cells that support or possess traits of inflammatory cells may be another example of the versatility of this peptide as a pro-inflammatory factor. Exploration of the role of AT_2 and Mas receptors on these cells would be of great interest. Also of importance is further exploration of the link with SDF1α-CXCR4 signaling in pro-atherogenic environments, since blockade of this pathway in inflammatory cells leads to increased plaque formation in ApoE KO mice,[180] which is in sharp contrast with the earlier discussed decrease of neointima formation in injured arteries of wild-type mice.[174] It is essential to discover how these contrasting findings would translate to AT_1 receptor blockade effects in a clinical setting. Finally, α-SMA expression in MSC might be part of a transition towards development of connective tissue.[181]

Renal progenitor cells

BM-derived progenitor cells may play a role in the remodeling of renal tissue. About a decade ago it was shown, with the help of replacement of wild-type BM with GFP+ BM in mice, that BM-derived cells incorporate into the glomerular mesangium of the kidney.[182] GFP+ mesangial cells increased over time after BM replacement, suggesting a long lasting, if not permanent residence of these cells. Although some of these cells express macrophage and lymphocyte markers, a large part of these cells do not appear to be leucocytes. Like MSC these cells express α-SMA, and therefore might represent pools of mesangial cells that contribute to glomerular vasomotor control. In accordance with this idea, isolated and cultured GFP+ cells contracted upon exposure to Ang II.[182]

Another α-SMA-expressing cell type that is associated with the glomerulus is the juxtaglomerular cell, present at the intersection of the distal convoluted tubule and the afferent arteriole of the glomerulus. These cells contain renin granules that can be release in reaction to low blood pressure or low filtrate osmolarity. The resulting increase in Ang II production causes normalization of blood pressure and filtrate osmolarity. It

was recently shown that BM-MSC can develop into renin-expressing granular cells that resemble juxtaglomerular cells, and that subsequently start to increase α-SMA-expression.[183] This process is under the control of liver x receptor α (LXRα) stimulation. This observation has shifted the paradigm of the origin of juxtaglomerular cells from being a VSMC-derived cell towards a MSC-derived cell that actually develops VSMC-like features in a later phase of differentiation. Perhaps even, a part of the BM-derived GFP+ glomerular cells that were observed in the fate-tracking experiment in mice might have developed in juxtaglomerular cells, but this was not explicitly explored.[182] Since the VSMC-like features develop relatively late during MSC differentiation it is tempting to speculate that this phenotype is a result of autocrine Ang II signaling following renin upregulation. Thus, Ang II might contribute to the development of cell types that release renin, control glomerular blood flow or both. This in turn would implicate an involvement in hypertension and renal disease.

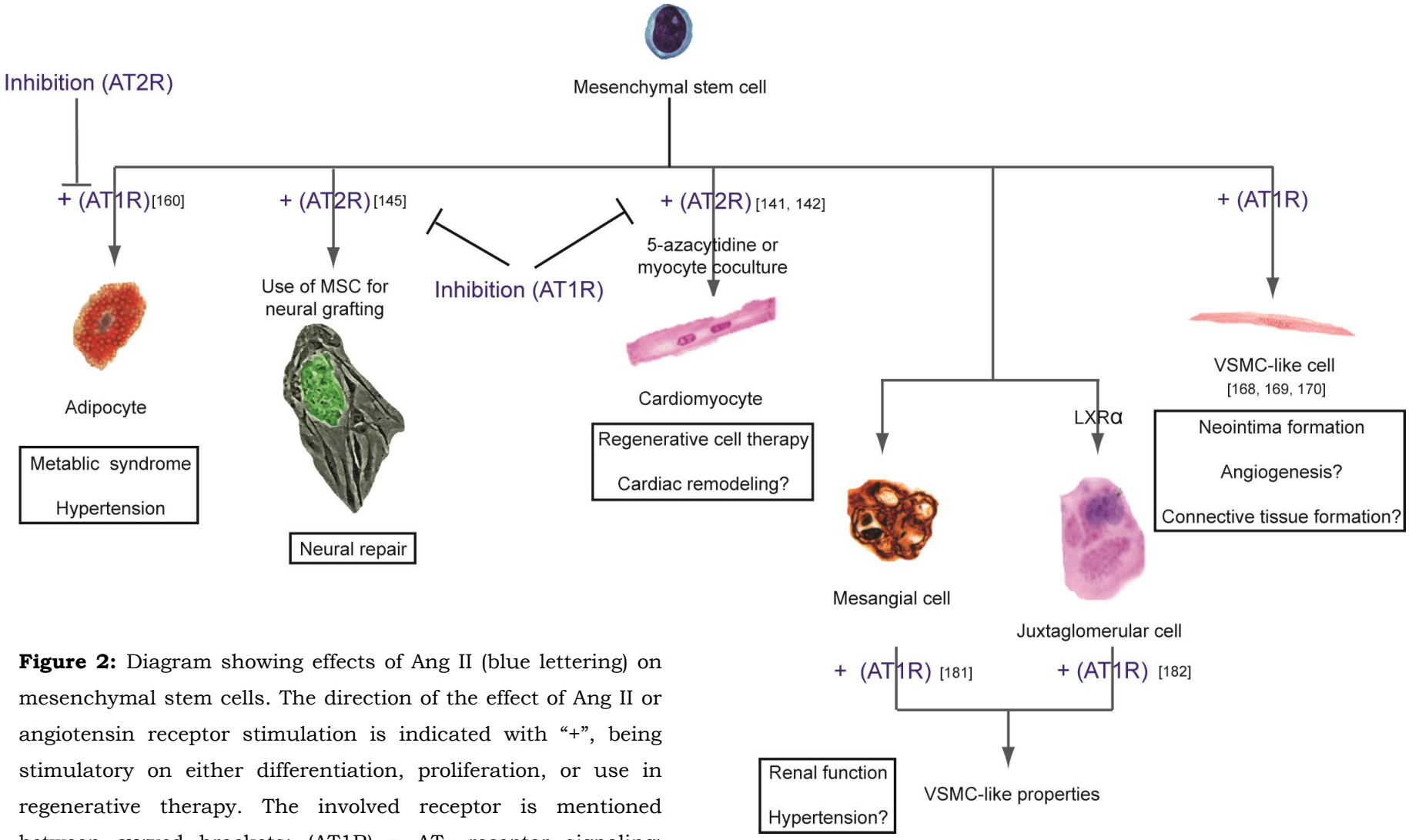


Figure 2: Diagram showing effects of Ang II (blue lettering) on mesenchymal stem cells. The direction of the effect of Ang II or angiotensin receptor stimulation is indicated with "+", being stimulatory on either differentiation, proliferation, or use in regenerative therapy. The involved receptor is mentioned between curved brackets: (AT1R) = AT_1 receptor signaling; (AT2R) = AT_2 receptor signaling. Square brackets indicate references that directly show the Ang II (receptor) effects. Clinical implications or possible applications are mentioned in

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

There is a growing body of evidence that Ang II and Ang-(1-7) can affect proliferation and differentiation of BM-HSC and BM-MSC. The observed effects imply that RAS modulation in these cells can be used to inhibit cardiac, vascular and renal fibrosis as well as neointima formation, to improve renal function, to improve blood pressure control and organ perfusion, and to induces cardiac and renal repair. Beyond the cardiovascular system, RAS modulation in these cells can beneficially influence adipogenesis, neural repair, connective tissue formation, and wound healing, but foremost, stimulation of the Ang-(1-7) / Mas signaling axis is now under evaluation as an anti-cancer therapy with the combined benefit of improving hematological recovery after chemotherapy or irradiation. For a large part, signaling mechanisms remain to be investigated, especially in relation to the stage of differentiation of HSC and MSC, to the culture conditions (*in vitro*) or surrounding tissue (*in vivo*), and to the pathophysiological context. Such information might lead to more refined pharmacotherapy, while novel drugs for stimulation of specific angiotensin receptor subtypes are already being developed.

With respect to these novel drugs, the emerging ACE2/Ang-(1-7)/Mas receptor axis-oriented drugs are of particular interest. Since Ang-(1-7) promotes post-chemotherapy or –irradiation hematopoiesis in the absence of a pressor effect, the peptide is now under evaluation for hematological recovery of cancer patients. This already involves clinical trials with TXA127, an Ang-(1-7)-containing drug formulation.[184] The paradoxical effect of Ang-(1-7) on angiogenesis and its potential dependency on a delicate balance in stimulation of specific receptor subtypes was discussed. Several attempts have been made to specifically improve Ang-(1-7) signaling, for example through infusion of cyclodextrin-enveloped Ang-(1-7)[185] or increased ACE2 expression.[186] Even more specific are the non-peptide analogue CGEN-856S [187] or the thioether-bridged Ang-(1-7) analogue called cyclic Ang-(1-7),[188,189] which are specific Mas receptor agonists. Infusion of cyclic Ang-(1-7) has shown promise in rat models as an intervention for acute respiratory

syndrome or after myocardial infarction.[190,191] It will be of major interest to perform comparative studies on native Ang-(1-7) versus these specific Mas receptor agonists, and to unravel their effects on EPC and other progenitor cells.

As discussed, the AT_2 receptor is importantly involved in Ang II effects on progenitor cells. Through its effect on HSC and MSC, AT_2 receptor stimulation may have beneficial effects on fibrosis by inhibiting fibrocyte development, prevention of heart failure by promoting myocardial regeneration, and improvement of neural repair. Therefore, the design of AT_2 receptor agonists may be an important development. Currently, two agonists are being explored, the non-peptide drug Compound 21 (C21) and Ang II with single β-amino-acid substitutions.[10,192] C21 has shown promise in the rat model of myocardial infarction by improving systolic and diastolic function, and by anti-apoptotic and anti-inflammatory effects.[193] Also it was found that C21 alone or in combination with losartan may improve endothelial function and vascular composition; by reducing oxidative stress, collagen content, fibronectin, and inflammatory cell infiltration in stroke-prone spontaneously hypertensive rats.[194] The development of β-amino-acid Ang II analogues awaits further *in vivo* characterisation, with respect to pharmacokinetic aspects as well as therapeutic effects. Interaction with stem cell therapy is certainly one of the most relevant issues that can be addressed in further studies with these AT_2 receptor agonists.

Another relevant goal for future research is further characterisation of α-SMA-expressing cells types formed by MSC in response to Ang II. It will be important to study the relation between tissue localization and culture conditions and the final phenotype and function that such cells will assume, which comprises VSMC, connective tissue and neointimal cell types, pro-inflammatory cell types, mesangial cells or juxtaglomerular cells. Without any doubt this feature of Ang II-AT_1 receptor signaling will play a role in clinical effects of RAS modulation on arterial and renal remodelling, and perhaps on blood pressure control. Futhermore, the formation of connective tissue cells from MSC is being explored for application in tissue engineering

related to wound healing and repair of cartilage.[195] This may represent a novel research field in which to explore the role of RAS. It will be a major challenge to chart the versatile effect of Ang II, and possibly also other angiotensins, such as Ang-(1-7), on this route of MSC differentiation.

Table 1. Summary of beneficial or detrimental effects of angiotensin receptor signalling effects in bone marrow stem cells.

	HSC		**MSC**	
	beneficial	*detrimental*	*beneficial*	*detrimental*
AT_1 receptor	Improves HSC proliferation under hematopoietic stress[46] Pro-angiogenic EPC stimulation [74,75]	EPC apoptosis and senescence [77-79] Fibrocyte-related fibrosis [135,137,138]		Neointima formation or inflammation by VSMC-like progenitor cells [169-171] Promotes adipocyte formation [161] Inhibits cardiomyocyte formation [143] Inhibits neural repair by MSC after brain ischemia [146]
AT_2 receptor	Counteracts fibrocytes [135]		Promotes cardiomyocyte formation [142,143] Inhibits adipocyte formation [161] Improved neural repair by MSC after brain ischemia [146]	
Ang-(1-7)/Mas receptor	Improves HSC proliferation under hematopoietic stress [41,42,46,51] Increases early EPC [54]			

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Chapter 5

THE EFFECT OF A STABLE ANGIOTENSIN-(1-7) ANALOGUE ON PROGENITOR CELL RECRUITMENT AND CARDIOVASCULAR FUNCTION POST-MYOCARDIAL INFARCTION

Based on: *The effect of a stable angiotensin-(1-7) analogue on progenitor cell recruitment and cardiovascular function post-myocardial infarction.* Sevá Pessôa B, Moritz Becher P, van Veghel R, de Vries R, Tempel D, Sneep S, van Beusekom H, van der Velden VHJ, Westermann D, Jan Danser AH, Roks AJM. *Accepted: J Am Heart Assoc* 2015.

ABSTRACT

Background: Angiotensin-(1-7) improves cardiac function and remodelling after myocardial infarction (MI). This may involve recruitment of hematopoietic progenitor cells that support angiogenesis. However, angiotensin-(1-7) is rapidly metabolised in plasma and tissue. Here we investigated in mice the effect of a metabolically stable angiotensin-(1-7) analogue, cyclic angiotensin-(1-7), 1) on progenitor cell recruitment and 2) on the heart post-MI, when given in the angiogenesis phase of remodelling.

Methods and results: Angiogenic progenitor cell recruitment was measured by flow cytometry 24h and 72h after a daily bolus injection of cyclic angiotensin-(1-7) in healthy C57BL/6 mice. Furthermore, mice underwent MI or sham surgery, and subsequently received saline or 2 different doses of cyclic angiotensin-(1-7) for 3 or 9 weeks.

Cyclic angiotensin-(1-7) increased circulating hematopoietic progenitor cells at 24h but not 72h. Post-MI, cyclic angiotensin-(1-7) diminished cardiomyocyte hypertrophy, and reduced myogenic tone, without altering cardiovascular function or cardiac histology at 9 weeks. Importantly, cyclic angiotensin-(1-7)-treated mice presented reduced cardiac capillary density at 3 weeks after MI, but not after 9 weeks. Finally, cyclic angiotensin-(1-7) decreased tube formation by cultured HUVECs.

Conclusions: Our results suggest that cyclic angiotensin-(1-7), when given early after MI, recruits progenitor cells, but does not lead to improved angiogenesis, most likely because it simultaneously exerts anti-angiogenic effect in adult endothelial cells. Apparently, optimal treatment with cyclic angiotensin-(1-7) depends on the time point of onset of application after MI.

INTRODUCTION

The renin-angiotensin system (RAS) is a major physiological regulator of blood pressure and volume homeostasis and consequently it is involved in the pathogenesis of cardiovascular diseases.[1] The classical treatment of heart failure and hypertension is directed at inhibition of the angiotensin II (Ang II)-producing enzymes renin and angiotensin-converting enzyme (ACE), and of the Ang II type 1 (AT1) receptor. In an attempt to further optimize pharmacological RAS modulation, alternative intervention strategies have emerged and among them stimulation of angiotensin-(1-7) (Ang-(1-7)) function is one of the most attractive.[2] Ang-(1-7) is generated by ACE2. By stimulating its own receptor, the AT1-7/Mas receptor,[3] Ang-(1-7) exerts anti-fibrotic, anti-proliferative, anti-thrombotic, and anti-hypertensive effects, i.e., effects that generally oppose those of Ang II. .

The beneficial effects of Ang-(1-7) or ACE2 upregulation in cardiovascular tissue are well-documented. In rat models, Ang-(1-7) infusion or ACE2 overexpression preserves cardiac and endothelial function in heart ischemia and failure, prevents renal and cardiovascular anomalies in hypertension and diabetes, and exerts protective effects in cardiopulmonary disease.[3-8] A779, a Mas receptor antagonist, blocks Ang-(1-7)-stimulated collagen production in cardiac cells and attenuates the antihypertensive and antiproteinuric effects of captopril and hydralazine.[9-11] After myocardial infarction (MI), Ang-(1-7) prevented endothelial dysfunction[12] and alterations in the structure and hemodynamic function of the heart.[13] The anti-hypertrophic effect of Ang-(1-7) was also observed in rats displaying cardiac hypertrophy following isoproterenol exposure.[13] *In-vitro*, the hypertrophic effect of isoproterenol was blocked by AVE-0991, an Ang-(1-7) receptor agonist.[14] Moreover, Ang-(1-7) increases the formation of endothelial progenitor cells from bone-marrow (BM)-derived hematopoietic cells through AT1-7/Mas receptors *in vitro* as well as cardiac endothelial growth-factor-positive (VEGF+) cells *in vivo* in a mouse MI model,[15] and promotes tube formation in cultured pig BM-derived angiogenic

cells at low concentrations.[16] Studying the Ang-(1-7) – AT1-7/Mas receptor axis is therefore a crucial point for improving cardiac function and remodeling, in particular in relation to neovascularization.

Although beneficial effects of Ang-(1-7) have been observed in animal models of cardiovascular disease, its native form also stimulates AT1 receptors at high concentrations and is rapidly metabolized in tissue and plasma.[3] Consequently, Ang-(1-7) may not be optimal for use in patients. Hypothetically, cyclic Ang-(1-7) (cAng-(1-7)), an Ang-(1-7) analogue that is resistant to metabolism and acts as a specific agonist for AT1-7/Mas receptors, is a more promising compound for clinical cardiovascular therapy.[17] We recently showed beneficial effects of 8-week treatment of cAng-(1-7) on endothelial function and cardiac remodeling on MI rat model,[18] suggesting that cAng-(1-7) is a promising new agent in treating MI. In this study and our seminal study with native Ang-(1-7),[12] therapy was started 2 weeks after induction of MI. This might not be the optimal time point for stimulation of angiogenesis by recruitment of progenitor cells, which, in patients, takes place in the first week after MI.[19] Moreover, in the previous study with cAng-(1-7) the animals did not develop heart failure, possibly due to too small infarct size. Therefore, it is still not known if cAng-(1-7) can prevent heart failure, which remains a therapeutic challenge. On the basis of all previous data, we hypothesize that cAng-(1-7) will recruit angiogenic progenitor cells, and will improve cardiac neovascularization thereby suppressing early remodelling and preventing heart failure.

METHODS

Animal studies

Male wild-type C57Bl/6 mice were put on standard mouse chow and water, available *ad libitum*. Housing was at room temperature with a 12h light - 12h dark cycle. After at least one week of acclimatization in the caretaking facility, the mice either underwent a sham procedure or coronary ligation to induce left ventricular MI. Osmotic minipumps (model 1004, Alzet) were implanted

subcutaneously immediately after surgery for MI and the mice received saline, 5μg/kg/day (cAng-(1-7)-5) or 50μg/kg/day of cAng-(1-7) (cAng-(1-7)-50) continuously. For the 9 weeks group the animals were divided in sham, saline and 2 doses of cAng-(1-7) and for the 3 weeks groups we focused on the highest dose of cAng-(1-7) (50μg/kg/day of cAng-(1-7)) only. cAng-(1-7) was provided by Lanthiopharm BV, Groningen, the Netherlands and Tarix Pharmaceuticals, USA. After 3 or 9 weeks, hemodynamic function was measured with a 1F pressure-volume catheter (PVR-1035, Millar) under isoflurane anaesthesia.[20] To measure the effect of cAng-(1-7) on progenitor cell recruitment, two studies were performed in non-infarcted male wild-type C57Bl/6 mice, the first enabling us to get an impression of the time frame of effects and to make a comparison to native Ang-(1-7), and the second to confirm possible effects at the most relevant time point. In the first study, mice (n=4/group) received daily subcutaneous injections of saline, cAng-(1-7) (50μg/kg), or native Ang-(1-7) (500μg/kg). After 24h or 72h, the mice were euthanized and blood and bone-marrow were collected for fluorescence-activated cell sorting (FACS) analysis. Since in the first study cAng-(1-7) tended to increase c-kit-, Sca-1- and Flk-1-positive cells after 24 hours in blood, in the second study we focused on cAng-(1-7) (50μg/kg) effects vs. saline after 24 hours, and increased the n-number to 13/group. For both the first and second study, the percentage of progenitors in the total pool of mononuclear cells was first assessed. Data were normalized by expressing them as % of the mean % of the saline-treated animals.[21] All animal studies were performed according to Dutch guidelines and approved by the institutional animal care committee.

Cardiac histology

The hearts were collected and fixed in 4% paraformaldehyde solution. After fixation, the slices were dehydrated and paraffin-embedded. Gomori's staining was used to visualize individual cardiomyocytes. Only transversally cut cells in the surviving myocardium of the left ventricle showing a nucleus were used to determine the cardiomyocyte area. Sirius-red staining was used to determine

MI size and fibrosis. Fibrosis was scored outside the infarction and infarction border zone. Cardiac vascular density was assessed by Lectin staining (Isolectin B_4 (BSI-B_4), L5391, Sigma). Briefly, endogenous peroxidase was blocked with 0.3% hydrogen peroxide, and the lectin antibody was diluted in 1% bovine serum albumin in PBS were applied and incubated overnight at 4°C. Positive staining was visualized using diaminobenzidine, and nuclei were counterstained with haematoxylin. Negative controls were obtained by omitting the antibody from the incubation procedure.

Table 1: Animal data.

		Animals	BW(g)	HW/BW (mg/g)
3 weeks treatment	**Sham**	7	27.84±2.65	4.14±0.27
	MI+saline	5	27.44±2.73	4.98±0.73
	MI+cAng-(1-7)-50	8	26.50±1.62	5.69±0.60*
9 weeks treatment	**Sham**	12	30.38±3.50	4.45±0.67
	MI+saline	12	30.59±1.66	4.88±0.62
	MI+cAng-(1-7)-5	9	30.22±1.91	5.16±0.72
	MI+cAng-(1-7)-50	8	30.26±1.85	5.05±0.64

Data are expressed as mean ± SD. HW: heart weight, BW: body weight. MI + cAng-(1-7)-5: infarcted animals treated with 5µg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7). *p<0.05 *vs* Sham group (1-way ANOVA followed by Dunnett's post hoc test).

Vascular function

Thoracic aortic endothelial function was measured by Mulvany myograph as described previously.[18] Briefly, mice thoracic aortas (diameter ~800μm) were cut in ring segments of ~2 mm length. The aortic rings were mounted in Mulvany myograph organ baths and pre-incubated for 30 minutes after which tension was normalized by adjusting the diameter length to 90% of the length at which the equivalent of 100 mmHg blood pressure is reached. Subsequently, acetylcholine (Ach) concentration-response curves were constructed after preconstriction with U46619 (100 μmol/L).

Myogenic tone

Mesenteric arteries were used to determine the myogenic tone as described previously.[22] Briefly, each pressure level from 20 to 160 mmHg was maintained for 5 min, and the vessel diameter was then measured. Myogenic tone was expressed as $[(D_1 - D_2)/D_1]\cdot 100$, where D_1 is the difference in diameter between consecutives pressure points in Ca^{2+}-free buffer and D_2 is the difference in diameter in Ca^{2+}-containing buffer.

FACS analysis

Mononuclear cell suspensions (~$2x10^7$ cells/mL) of blood and BM were obtained by density centrifugation using Lympholyte-Mouse (CL5030, Cedarlane). Isolated cells were incubated with antibodies against Flk-1 (VEGF-R2, Ly-73, 560070, BD Pharmingen), c-kit (CD117, 553869, BD Pharmingen), Sca-1 (Ly6A/E, 553335, BD Pharmingen), following a previously used protocol that avoided overlap with Lin^- cells in the dot plots.[21] Flow cytometric analysis with a mononuclear gate was performed on a FACSCanto or BD LSR II (BD Biosciences). Data analysis occurred with the help of Flowjo and Infinicyte software.

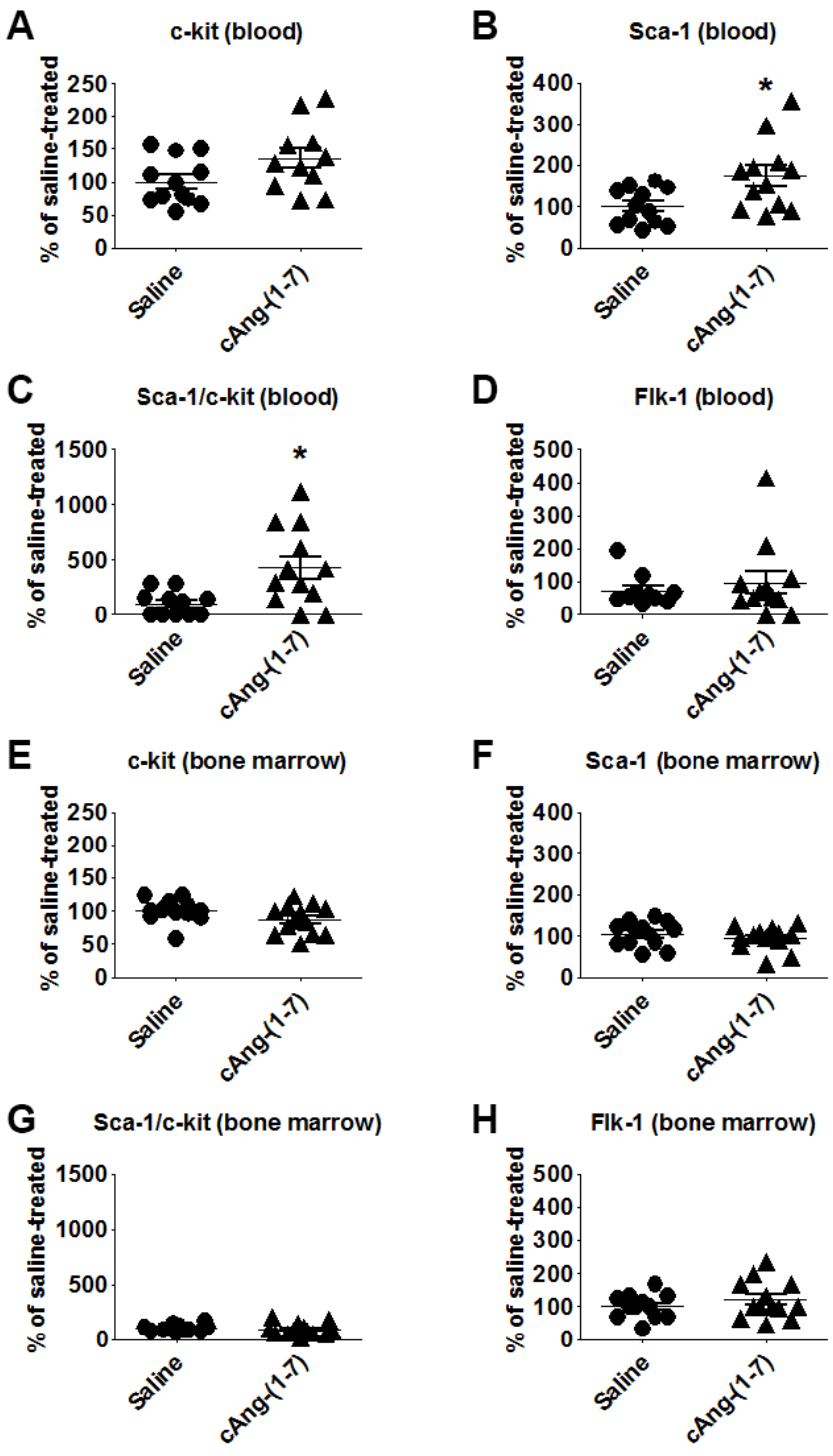

Figure 1. FACS analysis results of blood samples (A, B, C, and D) and bone marrow (E, F, G, and H) from non-infarcted animals treated with saline or cAng-(1-7) for 24h. A and E, percentage of hematopoietic progenitor cells (c-kit positive cells). B and F, percentage of positive cells for stem cells markers (Sca-1). C and G, percentage of double positive cells for Sca-1 and c-kit markers. D and H, percentage of positive cells for EPC marker (Flk-1 positive cells). cAng-(1-7): non-infarcted animals treated with 50µg/kg/day of cAng-(1-7). Data have been expressed as a percentage of the mean % in saline-treated animals (see Methods section). Percentages of measured cells out of total mononuclear cells in saline-treated mice were 0.24%, 2.00%, 0.20%, and 0.005% in blood samples versus 1.03%, 1.75%, 0.46%, and 0.08% in bone marrow samples for the c-kit, Sca-1, Flk-1, and Sca-1/c-kit double-positive cells respectively. *$p<0.05$ vs. saline (1-way ANOVA followed by Dunnett's post-hoc test).

Endothelial tube formation

Primary cultures of human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 medium (Lonza). Cells from passages 4 to 7 were used throughout the study. 15-well µ-slides (Ibidi GmbH) were coated with 10 µL of Matrigel (Cell Biolabs). To analyze the effect of cAng-(1-7) on endothelial tube formation *in vitro*, $3x10^3$ and $6x10^3$ cells per well were incubated with 10^{-10} to 10^{-7} M of cAng-(1-7) for 6 h. The total number of tubes was measured using CL Vision32 software (Clemex Analyse).

Statistical analysis

The results are expressed as mean±SD. One-way analysis of variance (ANOVA) followed by correction for multiple comparisons with post-hoc tests was used to compare the groups. Differences in concentration-response curves to ACh were tested by general linear model for repeated measures. $P<0.05$ was considered significant.

RESULTS

FACS analysis of progenitor cells

After a 24h treatment with cAng-(1-7), levels of cells positive for Sca-1 were significantly increased in blood samples of non-infarcted animals, and c-kit-positive cells also tended to increase (Fig. 1A, B). Double-positive cells were also significantly increased at this time point (Fig. 1C). In contrast, at 24h, cAng-(1-7)-treated animals presented no effects on levels of cells positive for the endothelial marker Flk-1 (Fig. 1D). No triple-labeled cells were observed, most likely due to the already very low amounts of Sca-1/ckit double-positive and Flk-1 single positive cells. Furthermore, after cAng-(1-7) treatment for 72h, the Flk-1-, c-kit- and Sca-1-positive cell levels were no longer different from those in saline-treated mice (Supplemental Material Fig. S1). In bone marrow, at 24h after injection, cAng-(1-7) treatment tended to decrease c-kit-single-positive

cells, but none of the cell types changed significantly. Native Ang-(1-7) did not change progenitor cell levels (n=4; data not shown).

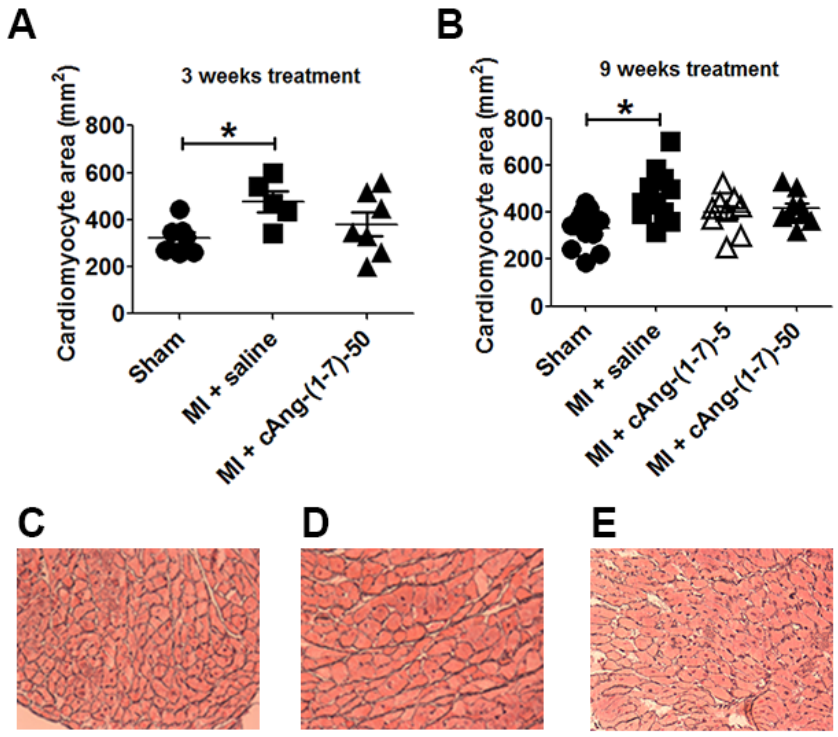

Figure 2. Histological analysis of ventricular cardiomyocyte area on 3 weeks (A) and 9 weeks (B) treated groups. Representative pictures of sham (C), saline (D) and cAng-(1-7)-treated animals (E). MI + cAng-(1-7)-5: infarcted animals treated with 5µg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7). *p<0.05 vs. sham (1-way ANOVA followed by Dunnett's post-hoc test).

Weight relations and histological characteristics

All animal groups showed similar body weights (BW) during the 3 and 9 weeks treatments. However, at 3 weeks, the heart-weight/body-weight ratio (HW/BW) tended to be higher in the MI + saline group than in the sham group (p=0.069; Table 1). Remarkably, a 3-week treatment with cAng-(1-7)-50 increased this ratio even further (Table 1), and results after 9 weeks were similar. However, significance was not reached due the small n-number per group. We therefore combined all MI animals into one group, and subdivided them according to treatment (saline vs. cAng(1-7)). Under those conditions, HW/BW was significantly increased after MI, and cAng(1-7) did not alter this. Repeating the analysis with tibia length instead of body weight yielded the same result (data not shown). However, left ventricular cardiomyocyte area was higher in the MI + saline group than in the sham group, and this difference disappeared in the

presence of cAng-(1-7) (Fig. 2A, B), although the difference between saline and cAng-(1-7)-treated MI animals could not be statistically confirmed. Coronary ligation led to large infarctions and increased intercellular fibrosis outside the infarct area, and both remained unaffected by cAng-(1-7) (Fig. 3). Vascular density near the infarcted area did not change after MI (Fig. 4), but tended to decrease in the surviving myocardium remote from the infarction area after saline treatment (Fig. 4C, not significant) 3 weeks after MI. Treatment with cAng-(1-7) led to a further decrease, becoming statistically significant as compared to sham-operated controls (Fig. 4C). 9 weeks after MI the differences in vascular density had disappeared (Fig. 4B, D).

Table 2: Hemodynamic data after 3 weeks treatment.

	3 weeks treatment		
		MI	
	Sham	**saline**	**cAng-(1-7)-50**
Animals	6	4	4
HR (min^{-1})	515.60 ± 42.76	534.30 ± 43.17	454.30 ± 84.07
SV (µL)	10.41 ± 4.54	4.65 ± 1.90*	3.26 ± 1.71*
CO (mL/min)	5201 ± 2197	2487 ± 978.9*	1883 ± 422.9*
EF (%)	49.63 ± 20.27	25.47 ± 12.48	19.35 ± 21.67

Data are expressed as mean ± SD. HR: heart rate. SV: stroke volume. CO: cardiac output. EF: ejection fraction. MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7). *$p<0.05$ *vs* Sham group (1-way ANOVA, Dunnett's post-hoc).

Hemodynamics

Both infarcted saline- and cAng-(1-7)-treated groups showed no differences with regard to blood pressure versus sham animals (Tables 2 and 3). Furthermore, heart rate, end-systolic and end-diastolic pressure, stroke

volume, cardiac output, ejection fraction, and cardiac contractility were not altered by MI nor by cAng-(1-7) treatment after 3 weeks (not shown) or 9 weeks (Table 3).

Vascular function and myogenic tone

The aortic endothelial function was not significantly altered by the infarction after 9 weeks and cAng-(1-7) treatment showed no additional effects (Fig. 5A). However, pressure-induced contraction of mesenteric arteries tended to be enhanced at 9 weeks after MI vs. non-infarcted animals, while cAng-(1-7) treatment prevented this effect (Fig. 5B).

Endothelial tube formation

HUVEC cultured for 6h formed less tubes in the presence of cAng-(1-7) treatment in a dose-dependent manner (Fig. 6: $p<0.001$ GLM-repeated measures for dose).

DISCUSSION

In the present study we explored if cAng-(1-7) would be able to improve recruitment of angiogenesis-supporting progenitor cells and thus improve neovascularization of the surviving myocardium after MI to prevent heart failure. The results show that although cAng-(1-7) can increase circulating hematopoietic progenitor cells after 24 hours, the effects lasted only shortly and had disappeared after 72 hours. Moreover, neither circulating Flk-1-positive cells, nor vascular density in the myocardium of infarcted mice increased after chronic cAng-(1-7) administration. Rather, vascular density was reduced by cAng-(1-7) 3 weeks after MI in the surviving hyperthrophied myocardium remotely from the scar tissue, and normalized again at 9 weeks after MI. cAng-(1-7) reduced myocyte hypertrophy and prevented the increase of peripheral myogenic tone without showing an effect on aortic endothelial function. Despite the large infarctions, hypertrophy and increased fibrosis, cardiac function was not worsened 9 weeks after MI so that an effect on heart

failure could not be effectively measured. Since myocyte dimensions were increased 9 weeks after MI but vascular density was similar to that of sham-operated animals, the surviving mice had apparently compensated for cardiac hypertrophy by increasing neovascularization, thus maintaining proper hemodynamic function. Implicitly, this means that cAng-(1-7) delayed restoration of vascular density, as observed at 3 weeks post-MI. This is most likely due to its anti-angiogenic effect on adult endothelial cells (reduced tube formation by HUVECs), its short-lasting (1 day) effect on Sca-1- and c-kit-positive progenitor cells, and the absence of an effect on Flk-1-positive cells. Since cAng-(1-7) demonstrates potentially beneficial effects such as reduction of hypertrophy and a decrease of myogenic tone, the compound is still eligible for exploration as a drug to prevent heart failure after MI. However, our results suggest that it is more optimal to administer cAng-(1-7) after the cardiac angiogenesis phase, as we did in our previous studies.[12, 18]

The increase of hematopoietic progenitor cells due to cAng-(1-7) is in accordance with previous studies showing that Ang-(1-7) analogues improve restoration of circulating blood cells in models of hematopoietic stress.[23-26] Our current results, which do not represent conditions of hematopoietic stress, indicate that cAng-(1-7) swiftly recruits progenitor cells causing a tendency of lowering of such cells in the bone marrow. Apparently, this recruitment does not result in de novo progenitor cell formation, so that the effect is shortlasting. We were unable to detect an increase in Flk-1-positive cells. It is therefore unlikely that cAng-(1-7) increases hematopoietic cells that support angiogenesis. Although our previous study predicted such an effect of native Ang-(1-7), showing an increase of cardiac cKit- and VEGF-positive cells,[15] native Ang-(1-7) also has anti-angiogenic properties in HUVEC, and on this basis it is now exploited as anti-cancer therapy.[27, 28] Conversely, Ang-(1-7) analogues have shown beneficial effects on wound repair, which is largely dependent on angiogenesis.[29] Apparently, HUVEC as a model for tube formation might be predictive for tumor and cardiac angiogenesis, but not for

all other vascular beds. An example is stroke, in which native Ang-(1-7) has shown benefit, although this was largely contributable to an anti-inflammatory effect.[30-32]

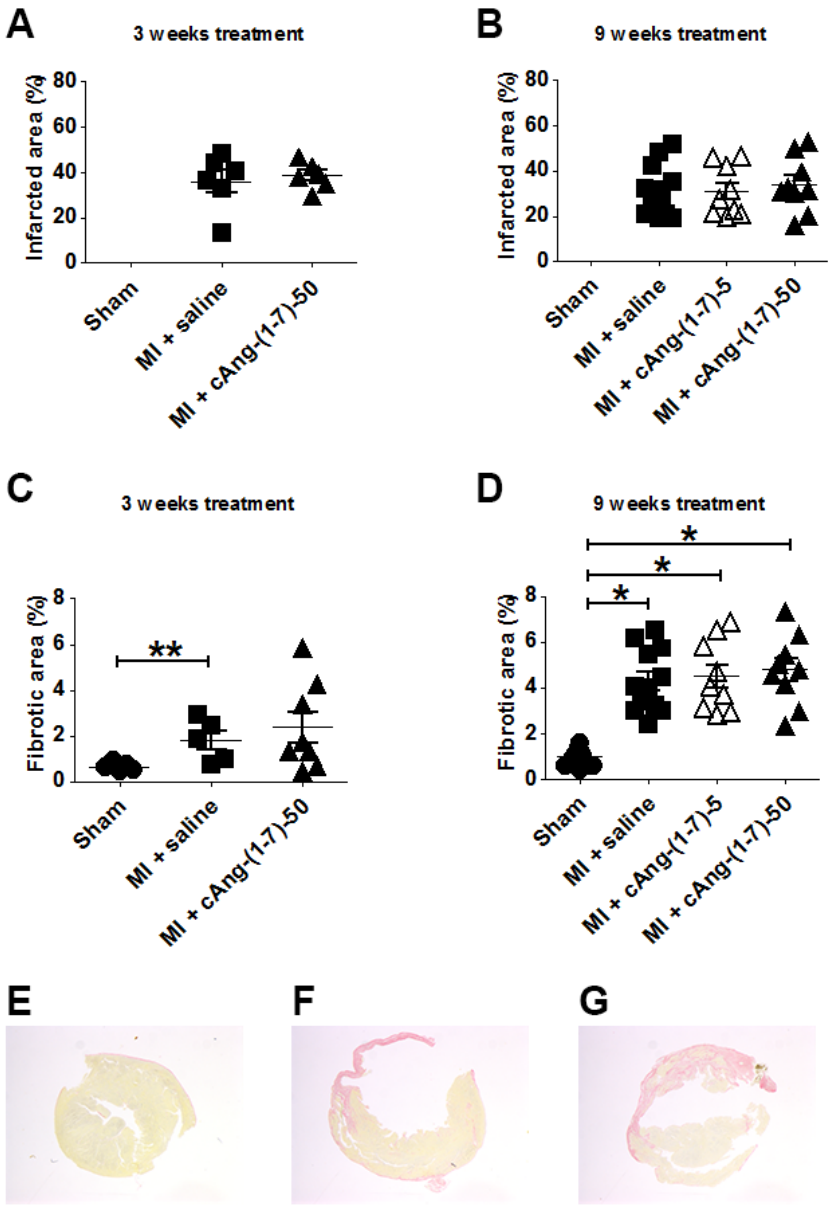

Figure 3. Histological analysis of myocardial infarction. Myocardial infarction area on 3 weeks (A) and 9 weeks (B) treated groups. Fibrosis on 3 weeks (C) and 9 weeks (D) treated groups. Representative pictures of sham (E), saline (F) and cAng-(1-7)-treted animals (G). MI + cAng-(1-7)-5: infarcted animals treated with 5μg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50μg/kg/day of cAng-(1-7). *p<0.05 vs. sham (1-way ANOVA followed by Dunnett's post-hoc test). **p<0.05 vs. sham (t-test followed by Mann-Whitney post-hoc test).

Table 3: Hemodynamic data after 9 weeks of treatment.

	9 weeks treatment			
		MI		
	Sham	**saline**	**cAng-(1-7)-5**	**cAng-(1-7)-50**
Animals	10	5	7	7
HR (min^{-1})	503.10±36.28	472.30±62.30	488.20±27.84	506.70±20.49
ESP (mmHg)	79.72±18.78	79.14±24.75	75.63±15.63	90.71±26.07
EDP (mmHg)	6.60±8.20	8.27±6.77	7.33±8.09	6.18±3.19
SV (µL)	8.83±2.84	7.06±3.24	10.14±3.95	6.27±2.37
CO (mL/min)	4584±1679	3441±1871	5012±2069	3190±1233
EF (%)	42.93±19.42	40.99±9.80	42.65±22.04	43.27±32.50
dP/dTmax (mmHg/s)	6514±1487	6692±2021	6050±747.1	6090±1495
dP/dTmin (mmHg/s)	-6391±2073	-7170±2046	-5405±1038	-6231±2941

Data are expressed as mean ± SD. HR: heart rate. ESP: end-systolic pressure. EDP: end-diastolic pressure. SV: stroke volume. CO: cardiac output. EF: ejection fraction. dP/dT max: peak rate of pressure rise. dP/dT min: peak rate of pressure decline. MI + cAng-(1-7)-5: infarcted animals treated with 5µg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7).

In the present study we observed a decrease in left ventricular myocyte size. This observation is in agreement with our recent study in a rat model in which treatment with cAng-(1-7) was started two weeks after MI.[18] The previous study showed a decrease of HW/BW after cAng-(1-7) treatment. This indicates that indeed the effect of the drug is more optimal when started later after MI. Still, fibrosis could not be prevented, which is in disagreement with effects of native Ang-(1-7) in the DOCA salt, angiotensin II infusion, and isoproterenol infusion models of cardiac hypertrophy.[33-36] However, these models are either blood pressure-dependent, and/or are not based on ischemia, or are models of lifetime overexpression of Ang-(1-7), which is not translatable

to clinical use in myocardial infarction. One study applying cyclodextrin-enveloped Ang-(1-7) shows reduced collagen I mRNA levels, but did not actually evaluate fibrosis nor mention the ventricular region that was sampled for PCR analysis, which precludes comparison.[37] A study with AVE0991, a claimed Ang-(1-7) analogue, shows that this drug can reduce fibrosis in infarcted hearts, which is partly reversible by A779; however the microphotographs in this study confusingly represent the infarcted zone.[38] Studies dedicated to effects of ischemia-induced fibrosis show that ACE2 overexpression can reduce fibrosis, without demonstrating however that this is due to enhanced Ang-(1-7) formation.[39, 40] Therefore, although ACE2/Ang-(1-7)/Mas stimulation has shown promise as an antifibrotic treatment in several conditions[41-43] the role of Ang-(1-7) in ischemia-induced fibrosis is still unclear. At this point we cannot determine if native Ang-(1-7) would be more effective against fibrosis than cAng-(1-7). Moreover, MI increases vascular permeability, leading to edema.[44] Since fibrosis and HW/BW were not different after cAng-(1-7) treatment, despite the reduced left ventricular myocyte size, it seems reasonable to conclude that the increased HW/BW was due to edema.

In our previous studies in rats we observed improvement of endothelial function.[12, 18] We were not able to observe this in the present study in mice, possibly because of the lack of clear endothelial dysfunction in the aortic segments used in the present study. However, we have observed a normalization of myogenic tone in mesenteric arteries. It is well known that after MI animals can develop an increased myogenic tone, and that blockade of AT1 receptors can abolish this increase instantaneously.[45] The effect of chronic Ang-(1-7) on myogenic tone was never studied before. We show that after MI also in the absence of heart failure myogenic tone has a tendency to increase, and that after MI the myogenic tone is effectively reduced by chronic cAng-(1-7) treatment. Both AT1 receptor signalling as well as myogenic tone are well-known to be modulated by NO released from the endothelium.[45]

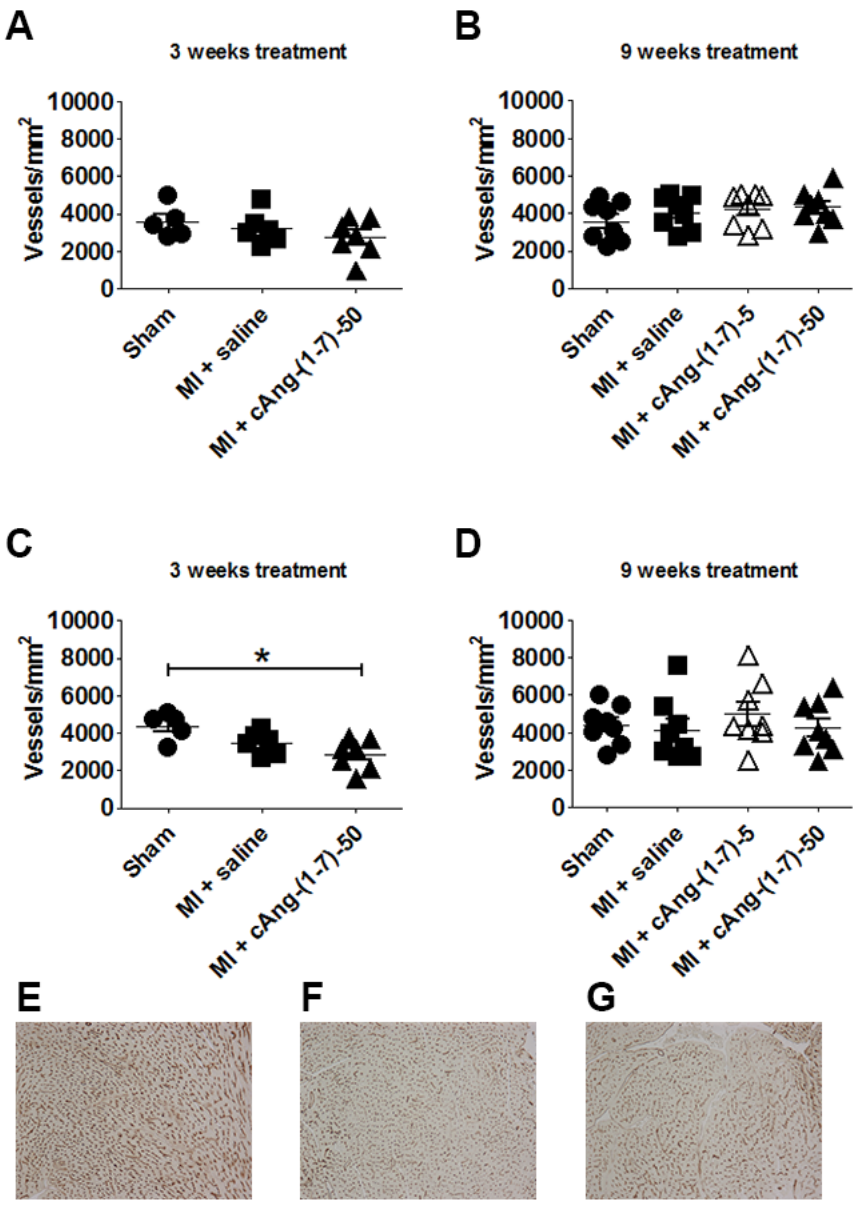

Figure 4. Cardiac vascular density determined by lectin staining. Vessel density at infarcted border area on 3 weeks (A) and 9 weeks treated groups (B). Vessel density at remote myocardium area on 3 weeks (C) and 9 weeks (D) treated groups. Representative pictures of sham (E), saline (F) and cAng-(1-7)-treated animals (G). MI + cAng-(1-7)-5: infarcted animals treated with 5µg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7). *p<0.05 vs. sham (1-way ANOVA followed by Dunnett's post-hoc test).

Furthermore, Ang-(1-7) antagonizes Ang II-induced vasoconstrictions in organ bath experiments independently from NO when given at concentrations > 1 µmol/L.[46, 47] This can involve inhibition of transactivation of epithelial growth factor receptors, which is importantly involved in myogenic tone.[48, 49] To our knowledge the effect of chronic Ang-(1-7) administration *in vivo* on *ex vivo*

vascular responses to Ang II, and its relation to plasma levels, has not been evaluated yet. Neither is there any direct evidence for the involvement of Mas receptors in myogenic tone. It is therefore not clear how Ang-(1-7)/Mas signalling decreases myogenic tone, but this might be a relevant question in relation to treatment of cardiovascular disease.

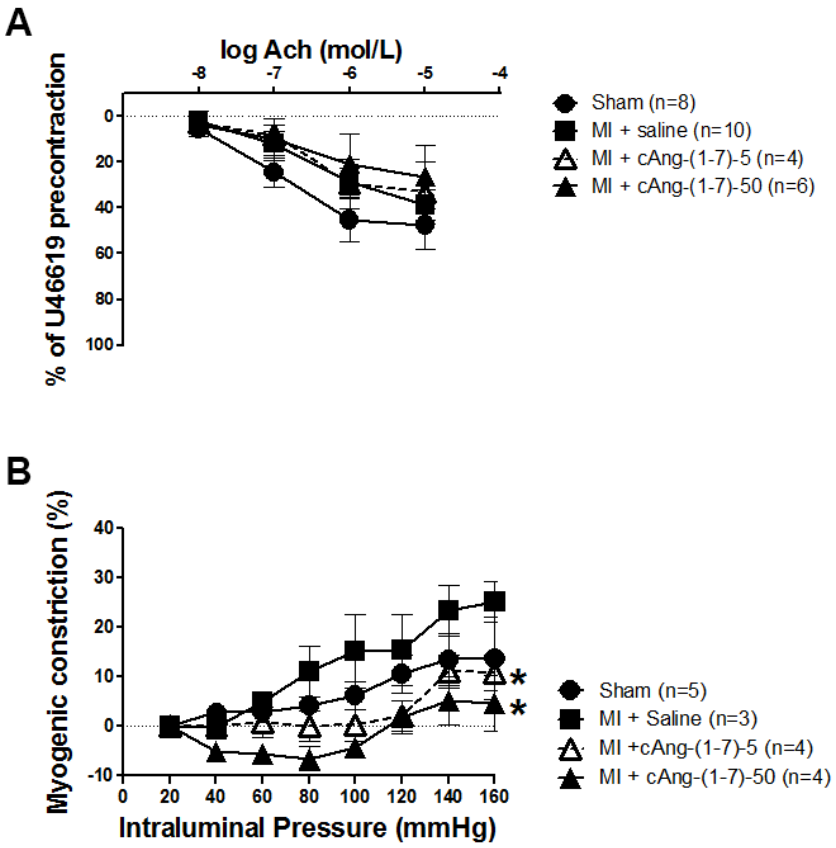

Figure 5. Aortic endothelial function and mesenteric myogenic tone on 9 weeks treated groups. A, concentration-response curve of U46619-preconstricted thoracic aorta to acetylcholine (Ach) on 9 weeks treatment groups. B, myogenic tone performed on mesenteric arteries from animals treated for 9 weeks. MI + cAng-(1-7)-5: infarcted animals treated with 5µg/kg/day of cAng-(1-7). MI + cAng-(1-7)-50: infarcted animals treated with 50µg/kg/day of cAng-(1-7). *p<0.05 vs. MI + saline (general linear model for repeated measures).

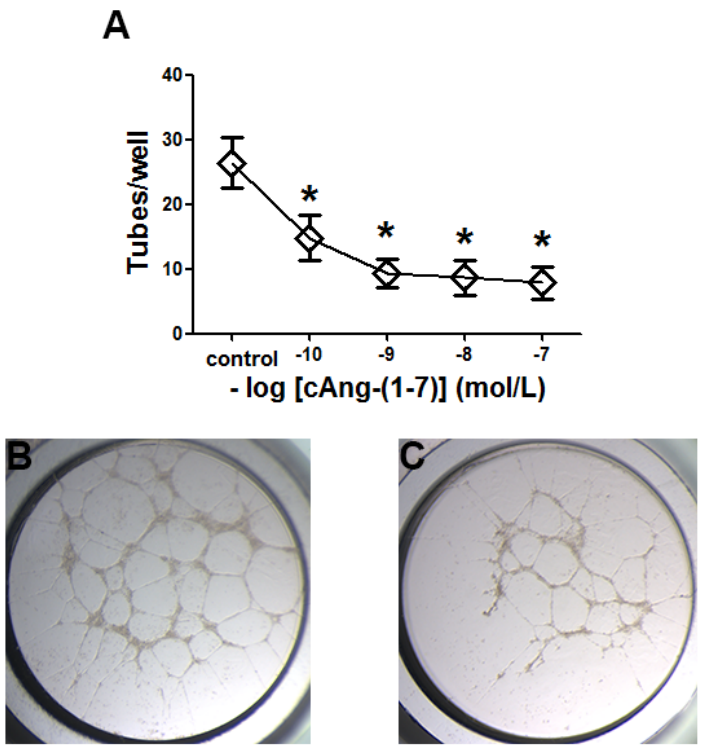

Figure 6. Tube formation assay performed on HUVEC cultured for 6h with cAng-(1-7) at densities of $3x10^3$ cells/well and $6x10^3$ cells/well. Representative pictures of control (B) and cAng-(1-7)-treted cells (C). n=8 (control); n=6 (cAng-(1-7) doses). *p<0.05 vs. control (1-way ANOVA followed by Dunnett's post-hoc test).

CONCLUSIONS

This study suggests that cAng-(1-7) given early after myocardial infarction might not lead to improved angiogenesis, mainly due to an anti-angiogenic effect on adult endothelial cells. It is recommended that after MI Ang-(1-7) analogues are administered after the neovascularization phase. However, the full potential of cAng-(1–7) as an experimental drug still needs to be explored in diverse cardiac diseases models, and be compared to effects of other Ang-(1-7)-Mas-stimulating drugs such as ACE2 activators, Nor-Leu3 Ang-(1-7), CGEN856S, cyclodextrin-encapsulated Ang-(1-7) and AVE0091.[2] Abolishment of myogenic tone, as a mechanism to reduce workload, is an important new topic in such studies.

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SUPPLEMENTAL MATERIAL

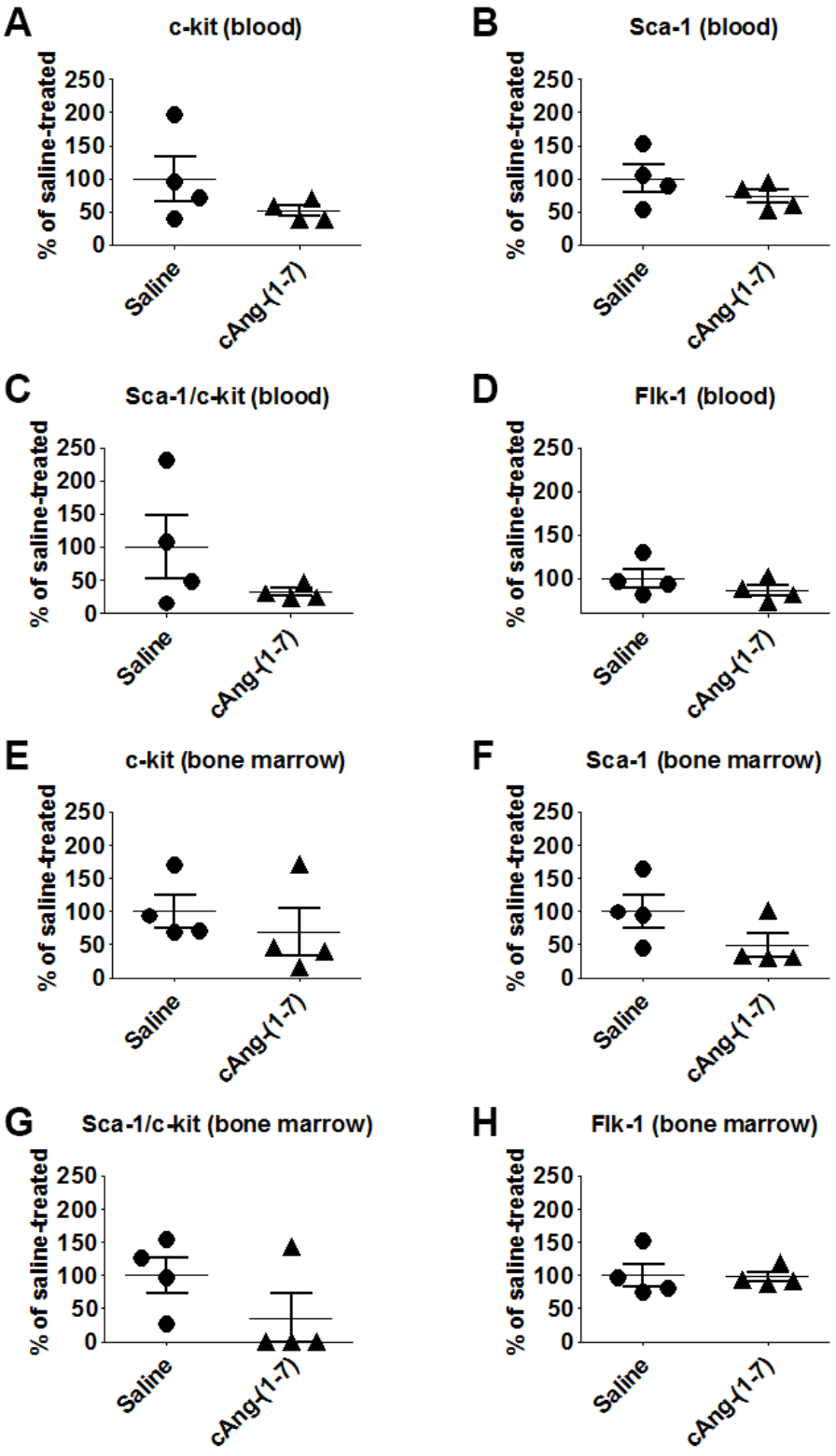

Figure S1. FACS analysis results of blood samples (A, B, C, and D) and bone marrow (E, F, G, and H) from non-infarcted animals treated with saline or cAng-(1-7) for 72h. A and E, percentage of hematopoietic progenitor cells (c-kit positive cells). B and F, percentage of positive cells for stem cells markers (Sca-1). C and G, percentage of double positive cells for Sca-1 and c-kit markers. D and H, percentage of positive cells for EPC marker (Flk-1 positive cells). cAng-(1-7): non-infarcted animals treated with 50µg/kg/day of cAng-(1-7). Data have been expressed as a percentage of the mean in saline-treated animals (see Methods section).

CHAPTER 6

DEVELOPMENT OF THE LOCAL RENIN-ANGIOTENSIN SYSTEM DURING DIFFERENTIATION OF MESENCHYMAL STEM CELLS

Based on: *Development of the local renin-angiotensin system during differentiation of mesenchymal stem cells.* Sevá Pessôa B, van der Eerden B, van der Leije C, Schreuders-Koedam M, Musterd-Bhagghoe U, Jan Danser AH, van Leeuwen H, Roks AJM. *Manuscript in preparation.*

Abstract

Introduction: The renin-angiotensin system (RAS) performs a pivotal role in cardiovascular homeostasis and disease. Chronic RAS activation, ie. the excessive increase of activity of the hormone angiotensin II (Ang II) through Ang II type 1 (AT_1) receptors, is the main mechanism for pathogenic effects. Ang II type 2 (AT_2) receptors and the hormone angiotensin-(1-7) (Ang-1-7), which acts through Mas receptors, counteract these AT_1 receptor-mediated effect. Recently, stem and progenitor cells have been implicated in cardiovascular disease and effects of RAS modulation, but not much is known how RAS is involved in diverse stages of progenitor cell differentiation. Our aim was to study the presence and activity of RAS components in stem cells in relation to their differentiation.

Methods: Bone marrow-derived human mesenchymal stem cells (hMSC) were cultured in propagation medium or differentiation medium (adipogenic and osteogenic). The time-resolved expression of RAS components (micro-array) and the response to Ang II and Ang-(1-7) was studied.

Results: hMSC on propagation medium showed expression of AT_1 receptors, angiotensinogen, angiotensin receptor-asscoiated protein (ATRAP), neutral endopeptidase (NEP) and prolyl endopeptidase (PEP) mRNA. Of these components, AT_1 receptors, angiotensinogen and NEP increased significantly after addition of differentiation medium, the increase beginning at various time points.AT_2 and Mas receptors, angiotensin-converting enzyme (ACE), ACE2, renin and chymase mRNA was not expressed. Undifferentiated hMSC on proliferation medium did not respond to Ang II. 3 days after addition of adipogenic medium hMSC responded to Ang II, as visualized by increased Erk and decreased Akt phosphorylation, while on osteogenic medium only Erk phosphorylation was increased. All Ang II effects were mediated by AT_1 receptors, not AT_2, whereas Ang-(1-7) had no effects. Ang II given at

concentrations of 1 and 100 nM dose-dependently increased differentiation of hMSC into adipocytes measured after 20 days.

Conclusion: Undifferentiated hMSC do not express functional AT_1 receptors. Differentiation initiates the capacity of Ang II-AT_1 signaling. The direction of differentiation (adipogenic vs osteogenic) determines which second messenger is involved. AT_2 and Mas receptor signaling was not induced by differentiation. A full expression spectrum of RAS compenents was never accomplished. Based on expression profile of the RAS components and receptor functionality differentiated hMSC resemble adult visceral fat adipocytes and osteoblasts.

INTRODUCTION

The renin-angiotensin system (RAS) is known as a major system involved in the pathogenesis of cardiovascular diseases, mostly via the hormone angiotensin II (Ang II) through Ang II type 1 (AT_1) receptors.[1] On the other hand, Ang II type 2 (AT_2) receptors and the hormone angiotensin-(1-7) (Ang-1-7), which acts through Mas receptors, counteract these AT_1-mediated effect.[2] The effects of angiotensins take place not only directly through modulation of cardiovascular tissue and function, but also in association with modulation of extracellular matrix, adipose tissue, inflammatory cells, and glucose metabolism.[3] This implies that many cell types other than cardiovascular might be involved.

Since the presence of RAS components in bone marrow (BM) cells was confirmed, studies have shown effects of RAS modulation on proliferation of stem and progenitor cells.[4] Among others, these studies determined the inhibitory effect of Ang II on adipogenesis in pre-adipocytes, which might involve even effects on mesenchymal stem cells (MSC) that are either undifferentiated or in an early phase of differentiation.[5-8] Adipose tissue is believed to play an important role in cardiovascular disease, as it would contribute to RAS activation and to metabolic disease.[9] Well-known are also the effects of Ang II on endothelial progenitor cells, in which the hormone can either stimulate angiogenesis or cause senescence, thus prohibiting proper angiogenic function, depending on the duration of stimulation.[10] In addition, we have previously shown that Ang-(1-7)/Mas signaling promotes formation of endothelial progenitor cells from blood and bone marrow-derived mononuclear cells *in vitro*, while according to Than et al. such signaling may also regulate pre-adipocytes via activation of the PI3K/Akt pathway and inhibition of MAPK/ERK pathways.[11,12]

The consequent results of such studies open new possibilities to optimize therapies using RAS modulation as a way to favorably change the phenotype of stem and progenitor cells. However, for the better exploration of this potential it

is important to determine how the function of the AT_1 and AT_2 receptors, as well as the hormone Ang-1-7 and its receptor Mas, relate to the stage of development of stem cells during their differentiation phases from becoming progenitor cells up until the point at which differentiation has been completed. As to yet this is an unexplored terrain. In the present study we therefore explored expression of RAS components starting from undifferentiated MSC up until a progressed phase of differentiation. In addition, we have studied effects of Ang II and Ang-(1-7), and the involvement of the relevant angiotensin receptors. To determine whether development of a functional RAS is dependent on the course of differentiation towards diverse cell types, we have compared two differentiation conditions. The first condition is differentiation towards adipocytes, from which it is known that the RAS should at some point in the differentiation process become involved. The second condition is differentiation towards osteogenic cells. This might be important for cardiovascular disease in relation to calcification of arteries involving aging of vascular smooth muscle cells, leading to an osteogenic phenotype.[13] PI3K/Akt and MAPK/ERK signaling were used as a read-out for angiotensin signaling, since it is known that these pathways are involved in angiotensin-mediated growth and differentiation effects in pre-adipocytes.[12]

METHODS

Propagation of MSC

Human MSCs (hMSC) derived from BM (Lonza Benelux BV, Breda, Netherlands, cat.#PT2501) were characterized and selected by flow cytometry-based sorting using specific MSC markers (CD105, CD166, CD29, and CD44), and exclusion on hematopoeitic lineage markers (CD14, CD34 and CD45), showing multiple differentiation abilities under an atmosphere of 5% CO_2, incubated at 37°C. Medium was changed every 3-4 days. The growth medium used for proliferation consisted of mesenchymal stem cell growth medium (hMSC medium), i.e. α-MEM with 10% heat inactivated fetal calf serum (HI-

FCS), 2% penicillin-streptomycin (PLS, 5000 U/ml penicillin and 5000 U/ml of streptomycin) and 1.8 mM calcium chloride. This medium keeps the MSC undifferentiated.

Differentiation of MSC towards adipocytes

To produce differentiation medium we combined α-MEM (without phenol red and calcium chloride; Invitrogen), with 10% charcoal-treated fetal calf serum (CT FCS, Invitrogen), 2% penicillin-streptomycin (PLS, 5000 U/ml penicillin and 5000 U/ml of streptomycin) and 0.18% 1 M calcium chloride. Subsequently, pH was adjusted to 7.5 with 10 M sodium hydroxide. hMSCs were differentiated into lipid vesicle-containing adipocytes within 1 to 2 weeks by incubation with 1 mM dexamethasone, 30 mM indomethacin, and 250 mM 3-isobutyl-1-methylxanthine. Refreshments took place 3 times a week. The adipogenic differentiation was assessed by histology of lipid-containing vesicles, Oil Red-O staining (ORO). At different intervals (Day 5, 10, 14, 17 and 20) after treatment with adipocytic growth factors, the wells were checked for the presence of adipocytes. Sufficient amounts of adipocytes to perform reliable analyses (10% or more of total number of cells) were present from day 17 to 20 after addition of adipogenic medium, assuming stationary quantities. At each of the two time points we performed quantitative analyses from three independent experiments with duplo observations. To this end, cells were washed twice with PBS and fixed with 10% formalin in PBS. After washing once with 60% iso-propanol, the wells were incubated for 30 minutes with filtered ORO working solution which contains 3 parts of saturated ORO solution with 2 parts of Milli-Q water, filtered through a 0.2 μm filter. For cell count DAPI nuclear staining was used. Cell counting was performed on pictures taken from each well, using 3 pictures per well. Pictures were obtained with a fluorescence microscope (Axiovert 200M, 100x, Axiovision 4.6 software). Since the numbers of adipocytes showed considerable variability from culture cluster were expressed as % of adipocytes in cultures that were not treated with angiotensin (baseline control).

Differentiation of MSC towards osteogenic cells

To evoke osteogenesis hMSC medium was supplemented with 0.1 µM dexamethasone and 10 mM M β-glycerophosphate. Refreshments took place 3 times a week.

Erk and Akt phosphorylation experiments

To test angiotensin signalling, cells were exposed to hMSC, adipogenic or osteogenic differentiation medium for 3 days. To compare propagation medium with differentiation medium (p)ERK and (p)Akt measurement the cells were stimulated for 5 or 15 minutes with 100 nML of the angiotensins as indicated in the results. To test the involvement of angiotensin receptor subtypes, cells were preincubated with the AT_1 receptor antagonist irbesartan (100 nM) or the AT_2 antagonist PD123319 (100 nM) for 10 minutes before Ang II (1 and 100 nM) was added. hMSC homogenate was used to quantify ERK (Cell Signaling, cat.#9102S, rabbit, 1:2000 diluted), phosphorylated ERK (pERK) (Cell Signaling, cat.#9102S, rabbit, 1:2000 diluted), Akt (Cell Signaling, cat.#9272, rabbit, 1:1000 diluted), phosphorylated Akt (pAkt) (Cell Signaling, cat.#4060, rabbit, 1:2000 diluted) proteins by standard Western blot techniques under denaturating conditions. To verify the uniformity of the protein load and transfer efficiency across the test samples, the membranes were re-probed for β-actin (Millipore cat.#MAB1501, clone C4, 1:50000 diluted). The secondary anti-body goat anti-rabbit (BioRad) was used at 1:2000 dilution. Signal were detected by an enhanced chemiluminescent substrate for detection of HRP (SuperSignal CL-HRP substrate system, Thermo Scientific, cat.#32106). Exposed films were scanned and then analyzed quantitatively with ImageJ software (rsb.info.nih.gov/ij/). Band densities of the protein of interest were corrected for β-actin expression. The average of these corrected values for samples taken before stimulation was set at 100%, and subsequently values of all samples were expressed relatively to this level. For the time-resolved western blot determining angiotensin-induced phosphorylation 4 individual

experiments for adipogenic, osteogenic as well as propagation medium were performed with single observations of each treatment condition per 6-well culture plate (baseline, Ang II, and Ang-(1-7); 5 and 15 minutes for each treatment). Therefore, no variance could be obtained for the baseline controls that were set at 100% for this experiment. For the experiments applying Ang II in combination with antagonists, all measurement were performed in duplo per plate, allowing calculation of the variance of the baseline controls.

Micro array protocol

Microarray analysis was performed by means of Illumina Human HT-12 v3 BeadChip (Illumina, Inc, Eindhoven, The Netherlands) human whole-genome expression array, as described before.[14] In short, RNA of 3 biologic replicates for baseline conditions under propagation medium (0 hour) and each time point after addition of differentiation medium was isolated condition. The Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) was used for RNA amplification, after which single-stranded cDNA was generated with the use of T7 oligo(dT) primer followed by double-strand synthesis. Subsequently, biotin-labeled cRNA was produced with the use of T7 RNA polymerase, and 750 ng was hybridized for each array according to the standard protocol, using streptavadine-Cy3 label for signal quantification (GE Healthcare, Piscataway, NJ, USA). Slides were scanned on an iScan and analyzed using GenomeStudio (both from Illumina, Inc.). Background was subtracted from the raw data using GenomeStudioV2010.1 (Gene Expression Module 1.6.0, Illumina), and data were processed using the Bioconductor R2.10.0 Lumi package (www.bioconductor.org). The data were as transformed by variance stabilization and quantile normalization. Probes that were detected at least five times in the experiments (Illumina detection p value < .01) were considered to be expressed and were further analyzed. The RAS components available on his array were angiotensinogen, angiotensin-converting enzyme (ACE), ACE2, AT_1 receptor, AT_2 receptor, Mas receptor, prolylendopeptidase (PEP), neutral endopeptidase (NEP), and angiotensin receptor-associated

protein (ATRAP). The mean level of expression of detectable genes was set on 1 for undifferentiated hMSC (i.e. the 0 hour point), and all mRNA levels are expressed relatively to this initial level.

qPCR protocol

Total RNA isolation was performed in samples taken from hMSC cultures, taken from a different donor, on proliferation medium (day 0), and day 9 and 19 after addition of differentiation medium, using the NucleoSpin RNA II kit (Machery- Nagel). RNA was reverse transcribed by use of the Quantitect Rev. Transcription Kit (Qiagen). Four nanograms of cDNA was amplified by real-time polymerase chain reaction (qPCR). Each reaction was performed in duplo with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: human MAS1 (Forward 5'-TGTGGAGAGGTGCCTGTCAGTCC-3'; Reverse 5'-GCCCACAGAAGGGCACAGACC-3'), human AGTR-1 (AT_1 receptor) (Forward 5'-GTCCCGCCTTCGACGCACAA-3'; Reverse 5'-AACTGGCCAAGCCTGCCAGC-3'), human AGTR-2 (AT_2 receptor) (Forward 5'-TCCAACAGAAGCTCCGCAGTGTG-3'; Reverse 5'-AGAGAACTGCTTTTCCGGCAAGACA-3'). β-actin correction was performed as an endogenous control (Ct values ≥31 were excluded). The relative amount of genomic DNA in DNA samples was determined as $RQ = 2^{(-\Delta\Delta Ct)}$.

Statistical analysis

The change in mRNA levels as detected by the micro-array was tested with the general linear model for repeated measures (GLM-RM) on one factor, being the individual gene product, using the mRNA levels at each time point as dependents. To test if at each time point after addition of differentiation medium the mRNA levels were different from those before treatment (the 0 hour time point), one-way ANOVA with Dunnett post-hoc test was used. For the time-resolved western blot determining angiotensin-induced phosphorylation one-sample t-test against the value 100 was used. For the experiments

applying Ang II in combination with antagonists one-way ANOVA with Dunnett post-hoc test against control was applied.

RESULTS

Time-resolved expression pattern of RAS components

hMSC showed only detectable levels of AT_1 receptor, angiotensinogen, ATRAP, NEP and PEP which changed all five after addition of adipogenic medium ($p<0.05$, GLM-RM). NEP and AT_1 receptor mRNA expression significantly increased starting resp. from 1 and 2 days after addition of adipogenic medium (Figure 1). Angiotensinogen mRNA levels were in general very low, sometimes undetectable, before and the first days after addition of adipogenic medium, increasing significantly, and being always detectable, from day 7 (Figure 1). Levels of the angiotensin receptor - modulating protein ATRAP were always detectable and although changing significantly overall in time ($p<0.05$ GLM-RM and one-way ANOVA without Dunnett post-hoc test), levels remained relatively constant, differing only significantly, but always very modestly, from undifferentiated cells 12 hours, 1 day and 25 days after addition of adipogenic medium (Figure 1). In addition, changes in ATRAP and PEP expression were inconsistent, showing increases as well as decreases depending on the time point. Levels of ACE, ACE2, renin, chymase, cathepsin G, AT_2 receptor and Mas receptor mRNA remained beyond the detection limit. To assess which of the receptors might be important for effects of angiotensins on adipogenic hMSC, the presence of the angiotensin receptors was checked in duplo samples from an independent experiment with qPCR on propagation medium (day 0), and day 9 and day 19 after addition of adipogenic medium. This study confirmed the array data, showing detectable level of AT_1 receptors (Ct ranging from 26.3 to 27.5), and levels that increased by 3.8 and 4.4 times on day 9 and day 19 respectively, whereas mRNA of AT_2 receptor was very low (Ct value ~ 34) and Mas receptor mRNA was undetectable.

When looking at changes in RAS components after addition of osteogenic medium, similar results were obtained as for adipogenic medium (Figure 2). ATRAP expression increased (GLM-RM $p<0.05$), but again this change was modest. Also, qPCR for the various receptors performed in duplo samples obtained from an independent experiments obtained in hMSC of a different donor confirmed an increase of AT_1 receptors of 1.8 and 10.8 fold of baseline at days 9 and 19 respectively, whereas AT_2 and Mas receptor mRNA was very low or undetectable.

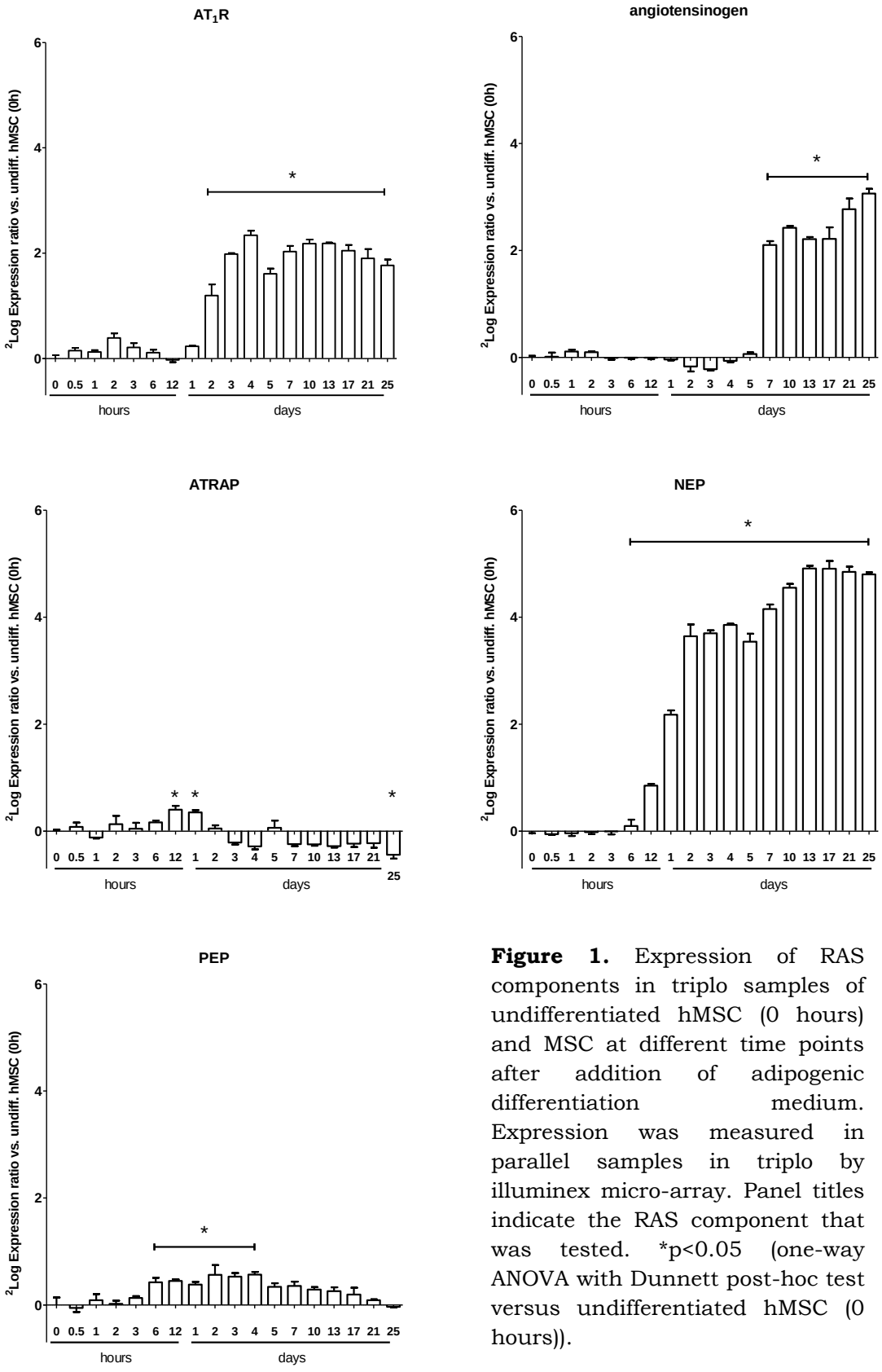

Figure 1. Expression of RAS components in triplo samples of undifferentiated hMSC (0 hours) and MSC at different time points after addition of adipogenic differentiation medium. Expression was measured in parallel samples in triplo by illuminex micro-array. Panel titles indicate the RAS component that was tested. *p<0.05 (one-way ANOVA with Dunnett post-hoc test versus undifferentiated hMSC (0 hours)).

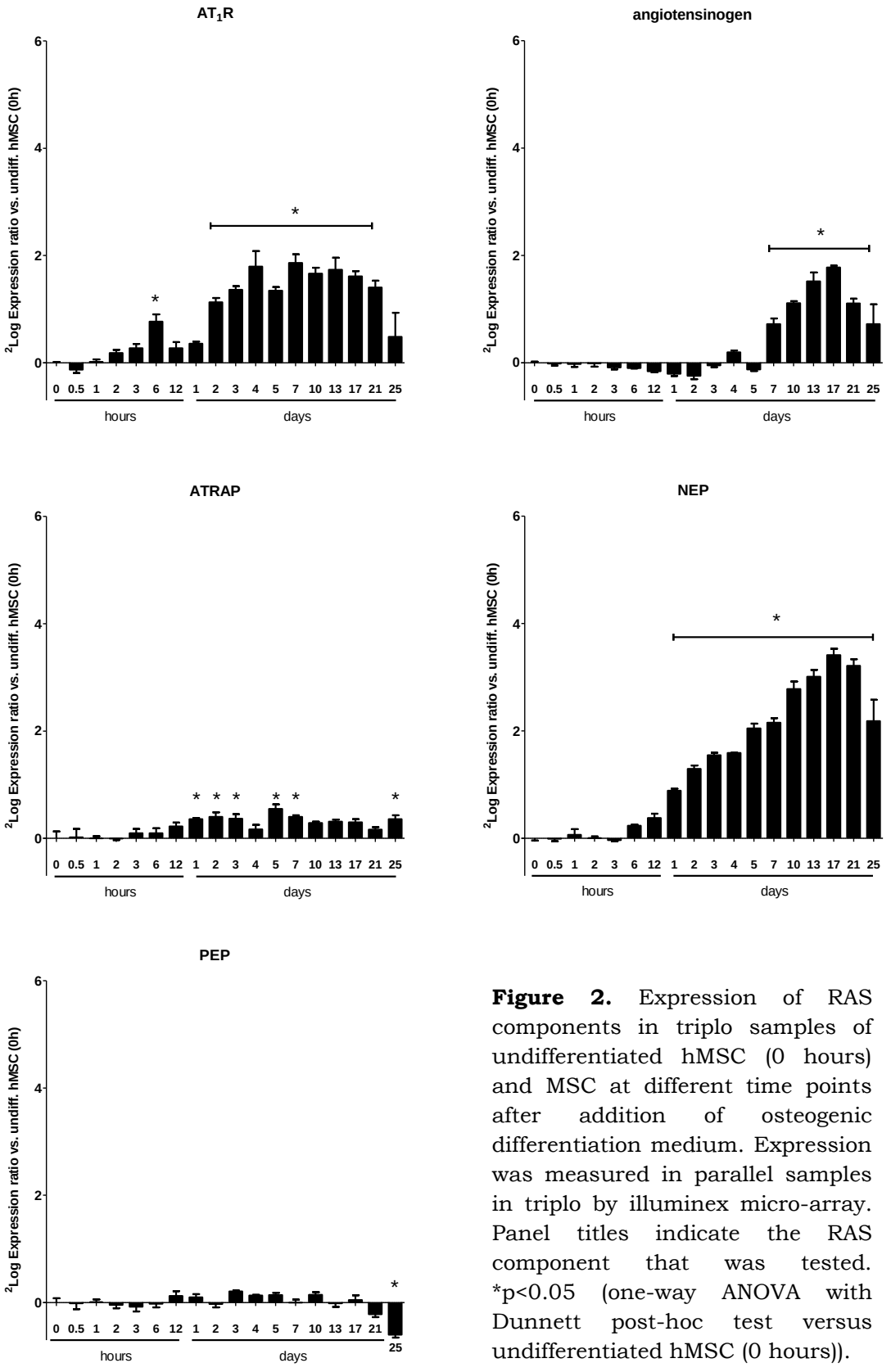

Figure 2. Expression of RAS components in triplo samples of undifferentiated hMSC (0 hours) and MSC at different time points after addition of osteogenic differentiation medium. Expression was measured in parallel samples in triplo by illuminex micro-array. Panel titles indicate the RAS component that was tested. *p<0.05 (one-way ANOVA with Dunnett post-hoc test versus undifferentiated hMSC (0 hours)).

ERK and Akt phosphorylation during Ang II and Ang-(1-7) propagation and differentiation phases

Ang II-exposed cells appeared to present lower pERK/ERK ratios when grown on propagation medium (Figure 3A). Nevertheless, taken separately, ERK and pERK were unchanged when hMSC were not differentiated (Figure 3A). No changes in Akt or pAkt were observed (Figure 3C). Also, Ang-(1-7) did not changes (p)ERK or (p)Akt (Figure 3B,D). On adipogenic or osteogenic medium, however, Ang II unambiguously increased pERK and pERK/ERK (Figure 4A, 5A), an effect that appeared optimal between 5 and 15 minutes after addition of Ang II. Moreover, Akt phosphorylation tended to decrease (Figure 4C: $p=0.06$ pAkt/Akt 15 minutes vs. value 100%, one sample t-test, Figure 5C) 15 minutes after addition of Ang II. Although Ang-(1-7) appeared to result in lower pERK levels in hMSC under osteogenic circumstances, this was not observed when correcting for total ERK levels (Figure 4B). Also, under all other conditions Ang-(1-7) did not have any effect (Figure 4D, Figure 5B, 5D). Based on this outcome and for further functional validation that of the micro-array, experiments to explore involved receptors as a of the micro-array results were performed on adipogenic medium with Ang II only, using 15 minutes as the time point of read-out.

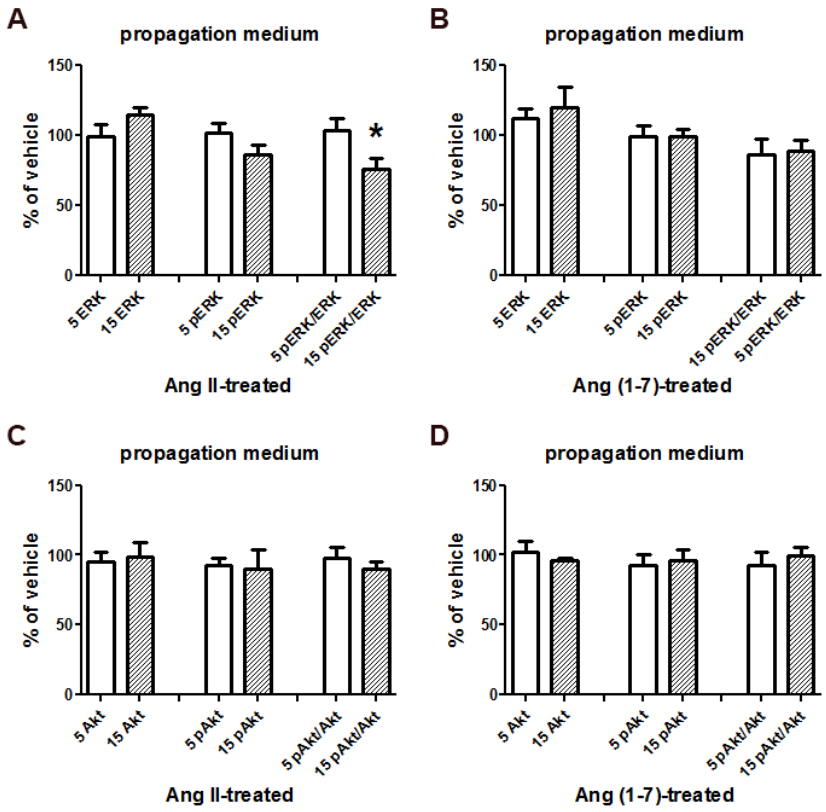

Figure 3. Analysis of ERK and Akt pathways during the propagation conditions. The cells were treated with Ang II (A and C) or Ang-(1-7) (B and D) for 5 or 15 minutes. N=4 observations per time point *: p<0.05, one-sample t-test versus % of control.

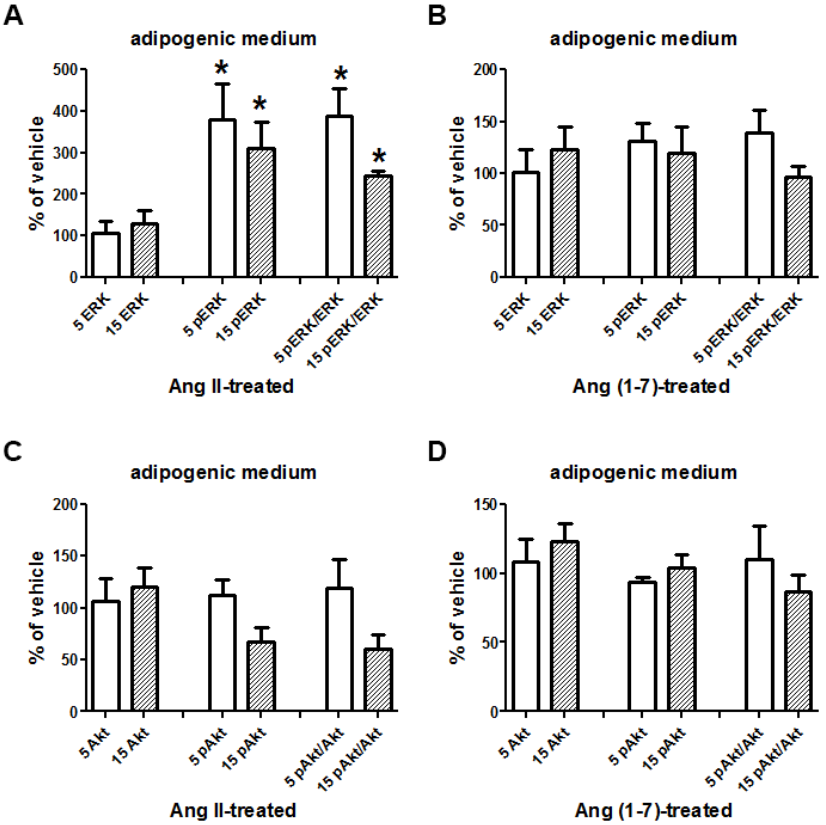

Figure 4. Analysis of ERK and Akt expression during the adipogenic differentiation condition. Adipogenic medium cultured cells were treated with Ang II (A and C) or Ang-(1-7) (B and D). The exposition to Ang II or Ang-(1-7) were done for 5 or 15 minutes. N=4 observations per time point *: p<0.05, one-sample t-test versus the value of 100%.

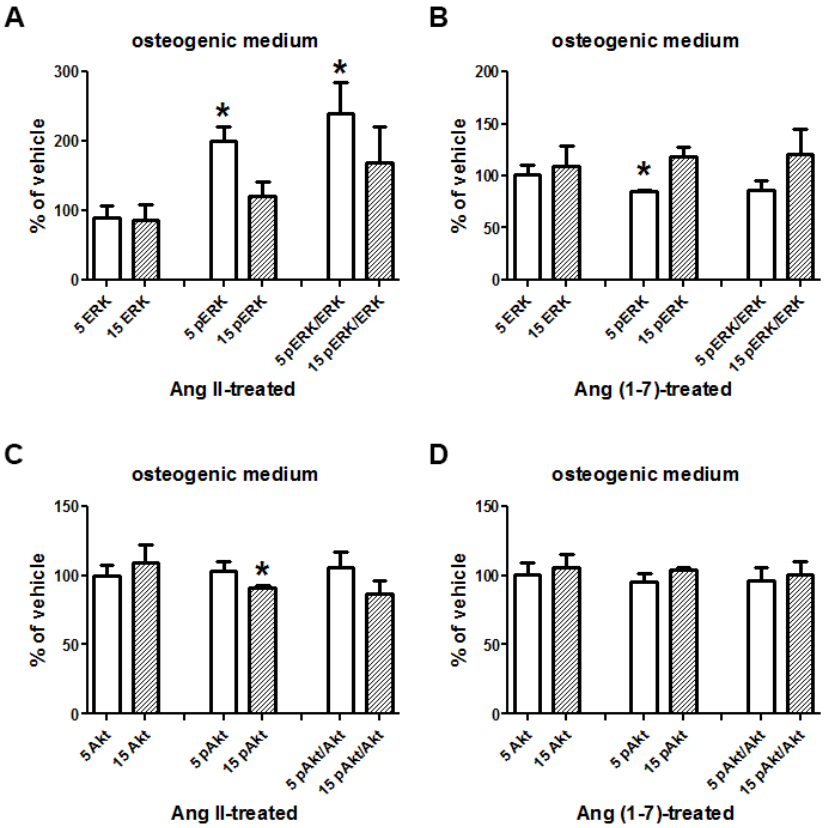

Figure 5. Analysis of ERK and Akt expression during the osteogenic differentiation condition. Osteogenic medium cultured cells were treated with Ang II (A and C) or Ang-(1-7) (B and D). The exposition to Ang II or Ang-(1-7) were done for 5 or 15 minutes. N=4 observations per time point *: $p<0.05$, one-value t-test versus % of control.

Receptor subtypes involved in the Ang II effects

Western blot analysis revealed that Ang II, given for 15 minutes, dose-dependently increased ERK phosphorylation versus baseline, i.e. cells not treated with Ang II, significance being detectable at the concentration of 100 nM Ang II (Figure 6A). Antagonists PD123319 and irbesartan changed by their own pERK/ERK levels ($p<0.05$ one-way ANOVA, Dunnett post-hoc test). PD123319 did not block the Ang II response, whereas irbesartan did. Moreover, Ang II treatment decreased Akt phosphorylation (Figure 6B). The antagonists did not have an effect on their own (P=NS, one-way ANOVA), but irbesartan blocked the response to 1 nM Ang II, while PD123319 did not affect the Ang II responses.

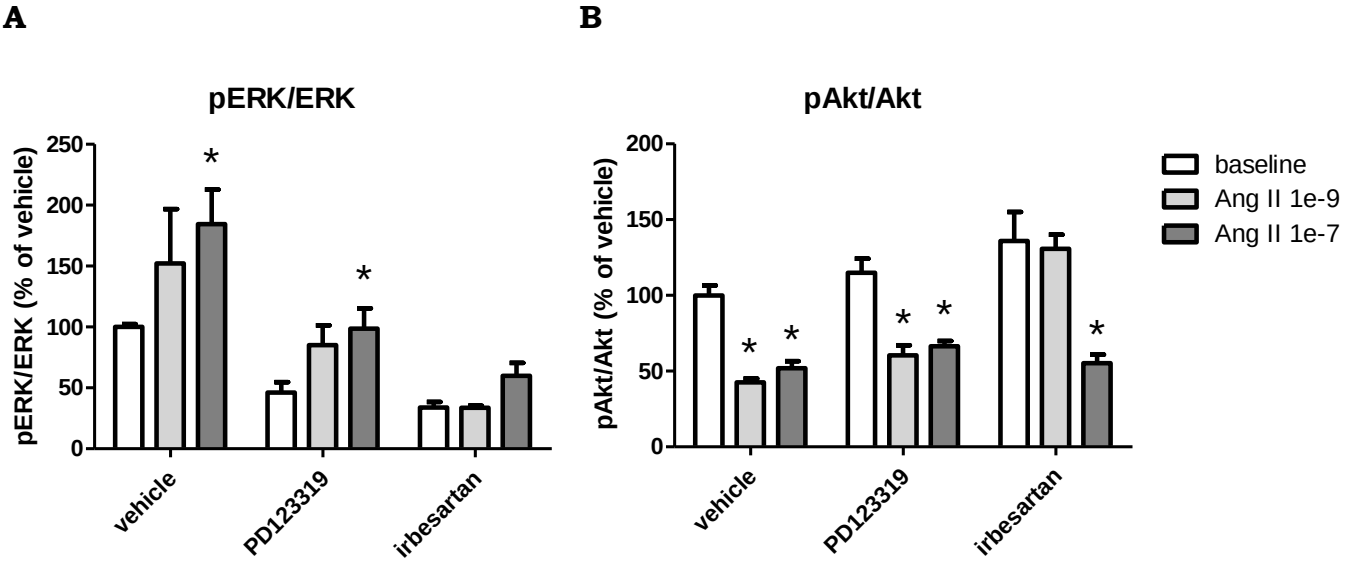

Figure 6. Western blot analysis of ERK/pERK (A) and Akt/pAkt (B). Cells were exposed to 1 nM or 100 nM Ang II, with AT_1 and AT_2 receptors blockers (n=4 per experiment, except irbesartan + 1 nM Ang II: n=3), or without (n=4 for 1 nM, and n=8 for 100 nM Ang II). *: $p<0.05$, one-way ANOVA with Dunnett post-hoc vs. baseline. testvalue t-test versus baseline. *: $p<0.05$, one-way ANOVA versus baseline.

Effects of Ang II and Ang-(1-7) on MSC differentiation into adipocytes

Adipocyte numbers assessment at day 17 and 20 after addition of adipogenic medium to hMSC (Figure 7A) showed that only Ang II given at 1 or 10 nM each 2 days significantly increased adipocyte development (Figure 7B), whilst Ang-(1-7) had no effect (Figure 7C).

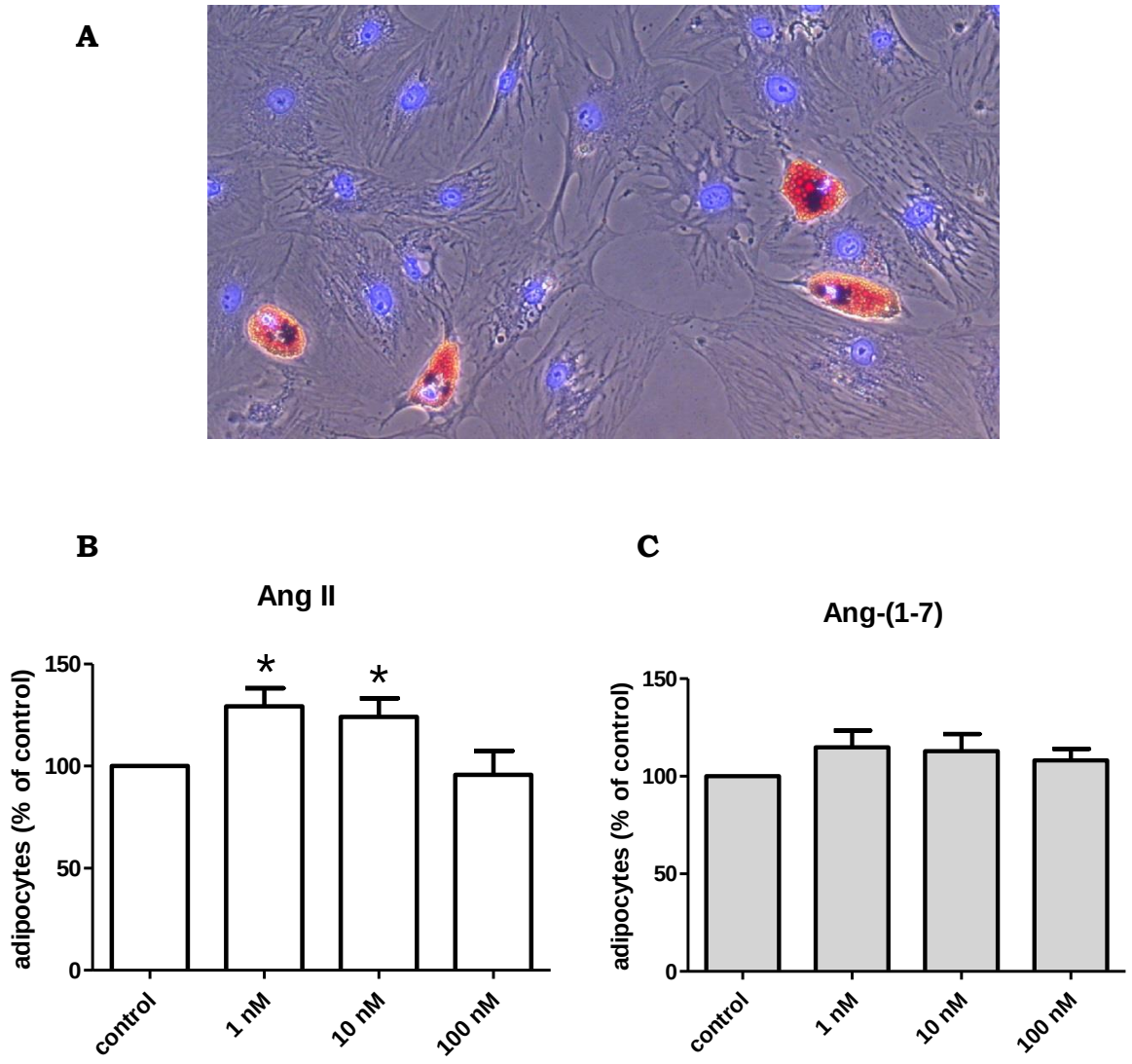

Figure 7. Oil Red-O staining on hMSC grown in adipogenic differentiation medium for 20 days (A). Cells were exposed to 1 nM, 10 nM or 100 nM of Ang II (B), Ang-(1-7) (C), or vehicle (control) for 17 to 20 days. The number of positive cells were compared to control, which was set at 100%. Positive ORO cells were counted and divided by the amount of DAPI-stained nuclei. N=6 observations per concentration obtained in 3 independent experiments *: $p<0.05$, one-value t-test against the value 100.

DISCUSSION

Prompted by the possibility to optimize therapies using RAS modulation by targeting of stem and progenitor cells, we set out to study if angiotensin effects depends on the differentiation process. Our present findings demonstrate that stem cell marker-selected, early undifferentiated BM-derived hMSC, do not express RAS components. Although AT_1 receptors and ATRAP were demonstrated at the mRNA level, AT_1 receptor signaling could not be observed, implying that protein levels might be very low. AT_1 receptors, however, rapidly increased upon addition of differentiation medium, permitting signaling. This effect seems to be ubiquitous: it is observed on adipogenic as well as osteogenic medium, and AT_1 signaling has been observed by others in endothelial progenitor cells, as discussed previously.[10,15] Another important conclusion is that under the conditions that were used here hMSC do not develop a complete RAS since Mas and AT_2 receptors, ACE, ACE2, renin, chymase and cathepsin G do not appear to be expressed. Also, functional AT_2 and Mas receptors do not appear to be present during development into adipogenic or osteogenic cells, which is confirmed by our functional experiments. The finding is in contrast with an earlier report showing that rat culture-selected, adherent rat BM-derived cells, which were high in CD90 but low in haematopoietic markers CD4 and CD11b, expressed more RAS members.[16] However, the use of CD90, or thymus cell antigen-1, as an MSC marker is questionable, since it is used also as a hematopoietic lineage marker, and in addition shows species-related differences.[17,18] The results implicate that cells forming the niche of MSC express a wide array of RAS components, and selection markers importantly influence the interpretation of results.

When focusing on adipogenic differentiation, in general, the AT_1 receptor appears to inhibit differentiation of adult adipose tissue, leading to increased adipose tissue, which can be reduced by blockade of AT_1 signaling.[19] AT_2 receptors appear to have the opposite effect of AT_1 receptors, but only act in the presence of high lipid levels, as found in ApoE knockout mice.[19] For adipocytes

it is known that the RAS components that are expressed display regional differences.[20] In comparison to isolated human adipocytes, the RAS in our *in vitro* differentiated BM-derived hMSC appear to largely mimick visceral (perirenal, omental and mammary) adipocytes, which show both angiotensinogen and AT_1 receptor expression, but not AT_2 receptors.[21,22] Adult human adipocytes also express ACE, unlike hMSC-derived cells in our study, but are low on chymase and do not show renin expression, which resembles the phenotype described in the current study. Our observations in adipogenic differentiation of hMSC are largely opposite to those reported in an earlier publication by Matsushita et al., who observed a lowering of AT_1 receptors and angiotensinogen, and an increase of AT_2 receptors during differentiation, and also demonstrated ACE and renin expression.[23] These findings were previously reported to be also in contrast with other studies.[20] These contrasts might have different explanations. Firstly, this previous study did not report the number cycles needed to observe PCR signals, making it hard to determine if expression levels were relevant. Secondly, the origin, purity and donor preconditions of the hMSC were not reported, but this might be relevant as well: AT_2 receptors are expressed in whole adipose tissue or in 3T3-L1 cells, a mouse fibroblast with adipogenic ability when grown under the specific adipogenic medium, and, in contrast to what is observed *in vivo* in ApoE knockout mice, lacking this receptor in an otherwise wildtype genotype increases adipogenesis.[12,20,24-26] Thirdly, the AT_2 antagonist PD123319 partly blocks Ang II in cultured human adipocytes or hMSC-derived adipocytes when both compounds were used in very high concentrations (1 or 10 μM).[23,27] Unfortunately, more relevant, lower concentrations, which waarant specifity, were not used. However, AT_2 receptor expression appears to be lost during the development from a preadipocyte into an adipocyte.[28] Therefore, the role of AT_2 receptors in relation to the cell type in which it is present, the location of the adipose tissue compartment, and the metabolic conditions in the organism needs to be further explored.

Similarly, according to a previous study Mas receptor appear to be present on subcutaneous (pre)adipocytes,[12] but we could not detect mRNA for

Mas receptor in hMSC-derived adipocytes. Unfortunately, in this previous study Ang-(1-7) and A779 were used in very high concentrations of 1 and 10 μM respectively, raising concerns as to the specificity and physiological relevance of such findings. In addition, subcutaneous fat and visceral fat are different in their role in (patho)physiology, where visceral fat relates to the classical concept of obesity-related cardiovascular disease risk, and it is known that also the RAS is differentially expressed in these two compartments.[29] When studying visceral fat it has been shown that Mas receptor knockout in mice leads to adaptation of this tissue and Ang-(1-7) was reported to stimulate glucose uptake in adipocytes derived thereof, which was reversed by the Ang-(1-7) antagonist A779.[30,31] Nevertheless, to our knowledge the Mas receptor, although present,[32] was not extensively quantified in visceral adipose tissue, nor has it been demonstrated on isolated visceral adipocytes. We did not observe Ang-(1-7) signaling. Also, in the present study we have also looked at expression of NEP and PEP. Both enzyme metabolize Ang II into Ang-(1-7).[33] PEP did not seem to develop a specific expression pattern after addition of differentiation medium. However, NEP clearly showed an increase. Since Ang-(1-7) signaling was not present, it seems logical to conclude that if NEP acted here as a modulator of Ang II signaling. However, The relative time course of expression of RAS-related does not seem to follow a pattern that suggests any specific relationship with local Ang II generation; angiotensinogen is increasing much later than NEP, and renin, ACE, chymase and cathepsin G are anyhow not expressed in differentiating hMSC. Also, ATRAP is not showing strong changes or any specific pattern in relation to AT1 receptors or other RAS members. Therefore, there does not seem to be any concerted regulation of the various genes.

In summary, hMSC-derived seem to develop an angiotensin receptor profile that predominantly resembles that of visceral fat-derived adipocytes, and not that of subcutaneous adipocytes or the mouse fibroblast-derived 3T3-L1. It seems most likely that effects of AT_2 and Mas receptor in visceral fat do not occur within the adipocytes but rather in communication with other cell

types within the niche, or even with other organs as suggested before.[20] The extrapolation of receptor interaction studies performed in 3T3-L1 or skin-derived adipocytes is therefore not a straightforward task. Also, the use of very high angiotensin or receptor antagonist concentrations should in general be avoided.

The role of the RAS in osteogenic cells is far less studied than in the case of lipid metabolism. Studies in humans to the effect of classical RAS inhibitors on bone fractures appear to point at protection of bone but are not uniformly confirmative.[34] Yet, in animal models Ang II appears to increase the formation of osteoclasts from BM-derived mononuclear cells, and to induce an osteoclasts-activating phenotype of human osteoblasts, all through AT_1 receptors.[35] Differentiated osteoblast from newborn mice contain both AT_1 and AT_2 receptors, whereas osteoclasts contain only low numbers of AT_1 receptors.[36] In osteoblasts from humans Ang II was reported to induce pERK and pAkt, which takes place through AT_1 receptors only.[35] Also in osteoblast-rich cell populations isolated from newborn rat calvaria Ang II responses only take place via AT_1 receptors.[37] Paradoxically, in mice with an activated RAS due to transgenic overexpression of human renin and angiotensinogen the induction of osteoclast activation by osteoblasts is AT_2 receptor-dependent, and inhibited by AT_1 receptors.[36] Thus far, an explanation for this conversion is lacking. Our differentiated hMSC, which should obtain an osteoblast-like phenotype, only express AT_1 receptors, which resembles features of human and rat, but not mouse osteoblasts. Differentiated hMSC lack the effect of Ang II on pAkt observed in human osteoblasts, although until now osteoblasts were only tested with a high concentration of 1 μM Ang II.[35]

Osteogenic differentiation is also a feature of vascular smooth muscle cell (VSMC) induced by high phosphate levels and promoted by cellular aging.[13,38] In a model mimicking vascular calcification in chronic kidney disease the AT_1 receptor antagonist olmesartan has shown anti-calcification effects.[39] Although that might relate to attenuation of increased plasma phosphate levels, it has been shown that in cultured human VSMC 100 μM olmesartan decreases

calcium deposition in the cells in the absence of Ang II. Others have shown that Ang II given at relevant concentrations increases the calcifying effect of receptor activator of nuclear factor κB ligand (RANKL) in VSMC *in vitro* and in ovariectomized, ApoE knockout mice.[40] Also, a possible involvement of magnesium channels and of calcification inhibitor matrix Gla protein has been proposed.[3,41]

The presence of Mas receptors or of angiotensin-(1-7) have never been tested directly on osteogenic cells, although the peptide reduces calcification markers in a rat model of vascular calcification induced by vitamin D_3 plus nicotine. Whether this was a local vascular effect of Ang-(1-7) and if it involved Mas receptors was not clear from this study.[42] Furthermore, osteoclastogenesis induced by prostate tumor cells in an metastasis model in SCID mice is reduced by Ang-(1-7), but this is presumably an indirect effect exerted *via* the tumor cells.[43] As to date there has not been a comprehensive comparative study of expression of RAS components in differentiated osteogenic vs. differentiating hMSC, and the role of MSC in vascular calcification is not known, Nevertheless, hMSC might be an appropriate model to further study the role of RAS under osteogenic or calcifying conditions.

With respect to studies exploring other differentiation paths of MSC, we have recently summarized the involvement of RAS in effects of MSC on vascular and cardiac progenitor cells, and on healing properties in the stroke model.[4] Several studies have shown that AT_1, AT_2, and Mas receptors influence the behavior of progenitor cells, but for all these studies the same question applies: are local receptors in the cells involved, or is their niche of origin and homing determining the impact of the RAS? The answer to this question can importantly contribute to the design of interventional strategies that combine, either obligatory or voluntarily, the stem cell with RAS modulating therapies.

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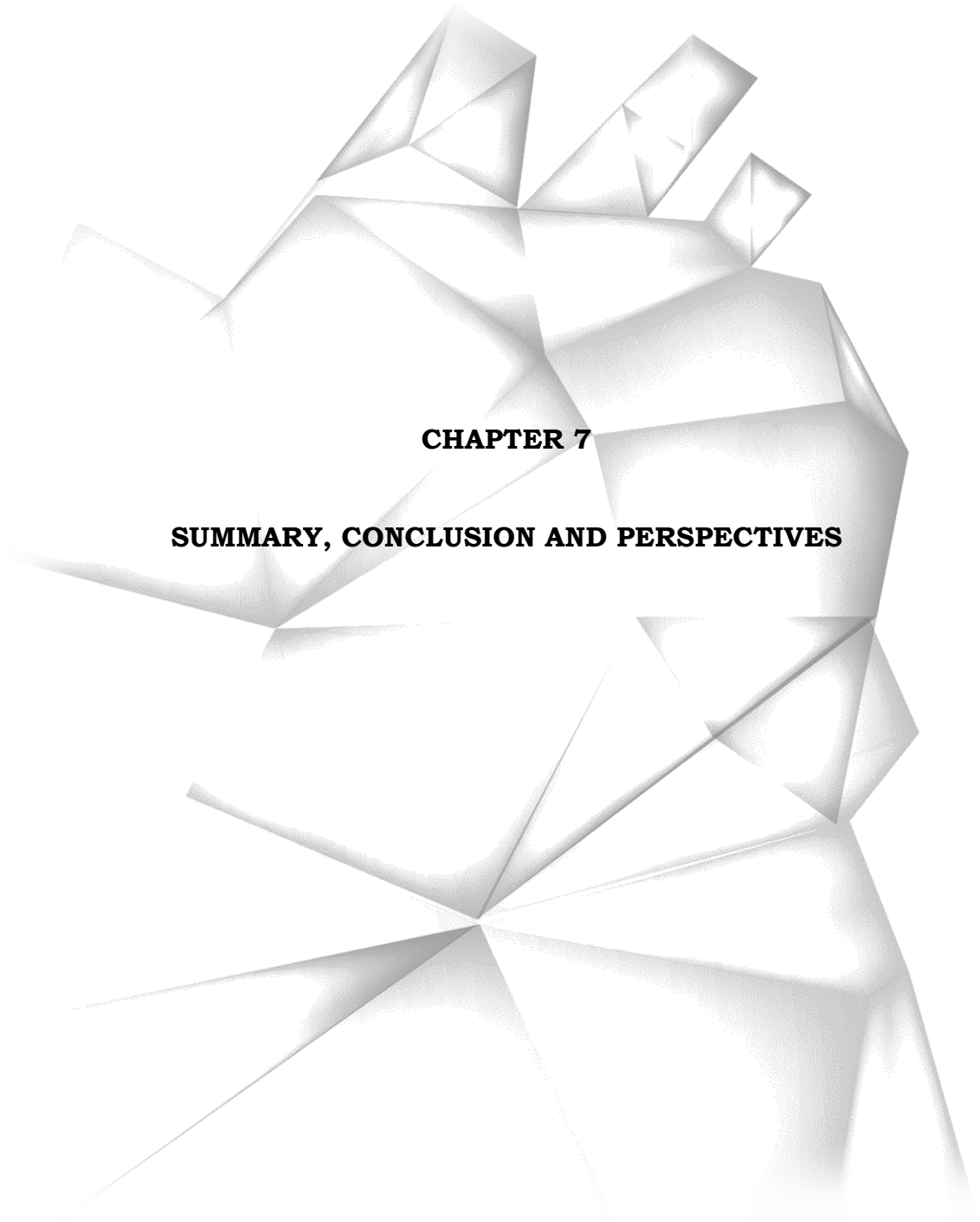

CHAPTER 7

SUMMARY, CONCLUSION AND PERSPECTIVES

SUMMARY AND CONCLUSION

It is widely accepted that the renin-angiotensin system (RAS) plays a major role in the progression of cardiovascular and kidney disease, for instance by influencing blood pressure and fluid homeostasis. Classically, this role concerns angiotensin (Ang) II-Ang II type 1 (AT_1) receptor-aldosterone interaction. Yet, new drugs are now emerging that act beyond this classical axis. This is important because currently existing drugs (ACE inhibitors, AT_1 receptor antagonists and mineralocorticoid receptor antagonists), when given separately, often result in a so-called Ang II or aldosterone escape, while in combination their side-effect profile is unattractive. This thesis evaluated the various novel concepts, i.e., the interference with the 'alternative RAS'.

Chapter 1 summarizes our current knowledge regarding the alternative RAS, focusing among others on renin, Ang II type 2 (AT_2) receptors, angiotensin-(1-7) (Ang-(1-7)) and the Mas receptor. Subsequently, **Chapter 2** describes the results obtained with the new renin inhibitor VTP-27999 in salt-depleted healthy volunteers. Although on the one hand, given its identical potency but much larger bioavailability, VTP-27999 may block renin at lower oral doses than aliskiren, too much renin inhibition with VTP-27999 turned out to be detrimental. Surprisingly, this was accompanied by a rise in extrarenal RAS activity, due to the fact that VTP-27999, like aliskiren, accumulates in the kidney. This results in excessive renal RAS suppression, inducing massive renin rises that can no longer be suppressed after 24 hours by the remaining VTP-27999 levels in blood. Clearly therefore, renin inhibition has an upper limit, and more is not necessarily better.

In **Chapter 3**, we analyzed the contribution of the AT_2 receptor to the Ang II-induced vasoconstrictor effects in the four core genotype mouse model. This allowed us to establish that the dilator effects of AT_2 receptors, occurring exclusively in women, depend on both female sex hormones and the XX chromosome sex complement. The latter was also required for the non-NO-mediated dilator effect of acetylcholine. Since AT_2 receptors induce vasodilation via bradykinin type 2 receptors, which also link to NO and non-NO dilator

pathways, these two findings may be related. To what degree these phenomena contribute to the cardiovascular protection in premenopausal women remains to be determined. Moreover, they raise the possibility of sex-specific therapies, despite the fact that, at this moment, there is no evidence that RAS blockers act differently in males and females.

Chapter 4 discusses the potential role of the RAS in the regulation of hematopoiesis and tissue regeneration by progenitor cells. It concludes that Ang-(1-7), via Mas-receptor activation, affects the proliferation and differentiation of stem cells. **Chapter 5** has investigated this possibility in the post-myocardial infarction model in mice, making use of a stable Ang-(1-7) analogue, cyclic Ang-(1-7). This compound diminished cardiomyocyte hypertrophy and myogenic tone, without affecting cardiovascular function. It increased circulating progenitor cells, albeit without endothelial markers. Unexpectedly, restoration of cardiac capillary density lagged behind in cyclic Ang-(1-7)-treated animals. Moreover, the drug reduced tube formation by endothelial cells *in vitro*. In conclusion, cyclic angiotensin-(1-7), when given early after myocardial infarction, recruits progenitor cells, but this does not lead to improved angiogenesis, most likely because it simultaneously exerts an anti-angiogenic effect in adult endothelial cells.

Finally, **Chapter 6** shows that Ang II effects mediated via AT_1 receptors were absent in mesenchymal stem cells, but appeared as soon as these cells became adipogenic or osteogenic progenitor cells. Ang-(1-7)-induced effects could not be observed under any condition, and the same was true for AT_2 receptor-mediated effects. The underlying reason appeared to be that adipogenic and osteogenic progenitor cells exclusively express AT_1 receptors at sufficient levels to warrant receptor signaling.

PERSPECTIVES

Many years ago, Packer asked the question why the kidneys release renin in patients with heart failure, the answer being that they do everything possible to preserve renal function and glomerular filtration, apparently at the expense of the hemodynamic burden on the heart.[1] This situation mimics our findings with VTP-27999, where the kidneys also respond to renal RAS suppression by releasing very large quantities of renin, resulting in elevations (above pre-drug levels) of plasma renin activity, Ang II and aldosterone. This implies that renin inhibitors should be dosed optimally and not necessarily maximally. Moreover, future studies should now investigate to what degree this phenomenon also underlies the detrimental effects of dual RAS blockade with RAS blockers other than renin inhibitors.

Simultaneously, we need to know whether females respond differently to RAS blockade as compared to males. If AT_2 receptor-mediated vasodilation truly occurs in premenopausal women only, their preferred RAS blocker might be an AT_1 receptor antagonist, because such a drug would allow exclusive AT_2 receptor stimulation. Our data do not strongly support a role for AT_2 receptor agonists as antihypertensive agents, except perhaps in premenopausal women. In fact, AT_2 receptors may even be contractile in men (Chapter 3), and might also induce pain.[2]

Our results suggest that Ang-(1-7) might be more effective after the angiogenic remodeling phase when it concerns post-myocardial infarction treatment.[3] The anti-angiogenic effect of such compounds could also be interesting for the treatment of cancer, as shown by other researchers,[4,5] but the occurrence of cardiovascular events requiring an angiogenic response might limit the clinical use of Ang-(1-7) in such patients. Moreover, after >25 years of preclinical research on the effects of Ang-(1-7)/Mas axis stimulation, we now need clinical data comparing such stimulators with classical RAS blockers, either alone or on top of these drugs. This would also require the definite choice of an optimal Mas receptor stimulant, taking into consideration the short half-life of Ang-(1-7) itself.

Rodgers et al.[6] have suggested that Ang-(1-7) offers great potential in stimulating platelet recovery following myelosuppression by improving hematopoietic progenitor cell pools. In addition, we made similar observations for endothelial progenitor cells.[7] Obviously, the underlying signaling pathways should be scrutinized further. Moreover, as our current data indicate, this should always be performed in the context of comparison with effects on adult cells. Remaining issues to be studied are effects on progenitor cells evoked through angiotensin effects on the surrounding niche, such as bone marrow stroma cells or cardiovascular cells. Additionally, we need to know the impact of cardiovascular and metabolic diseases on the responses of stem and progenitor cells to angiotensins.

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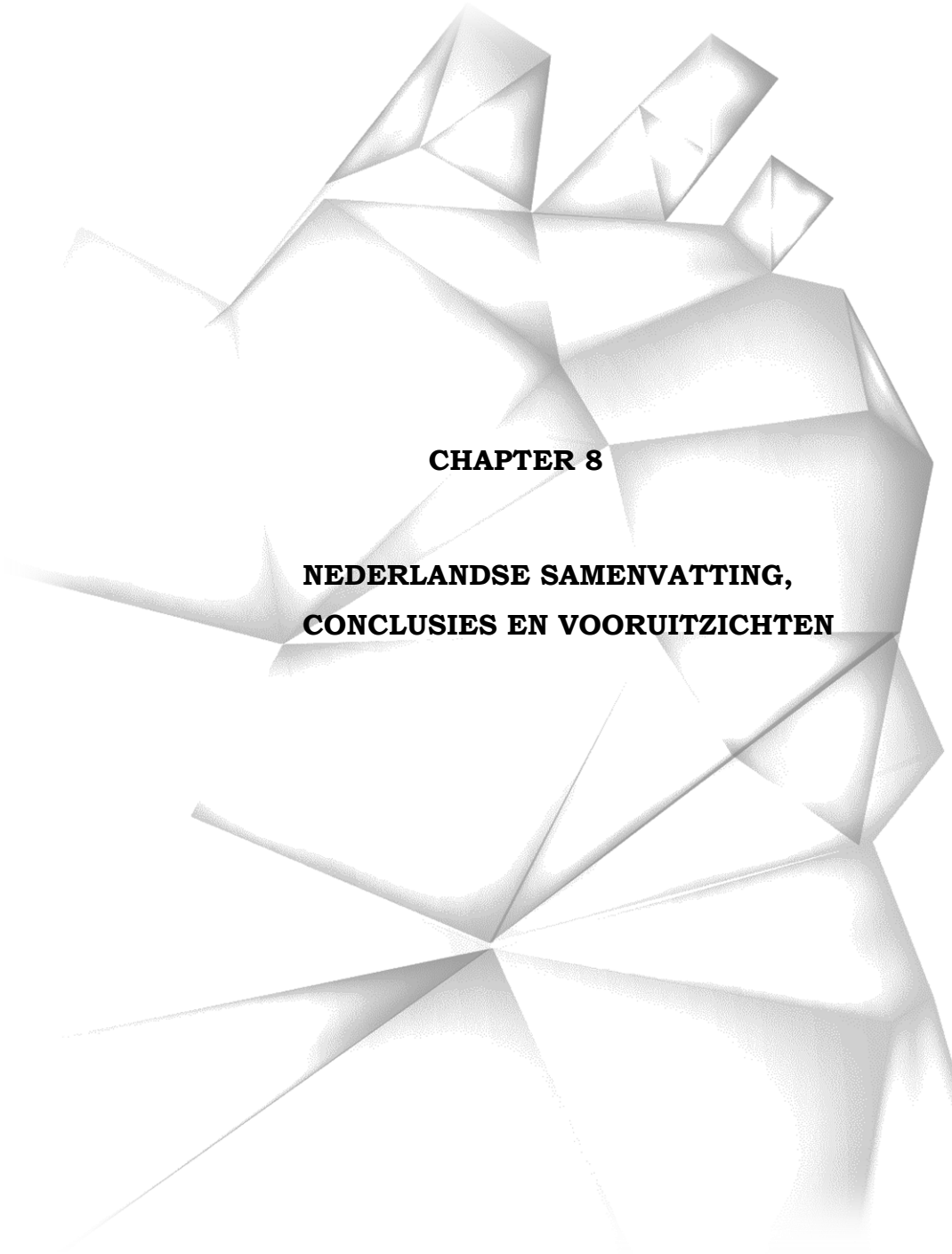

CHAPTER 8

NEDERLANDSE SAMENVATTING, CONCLUSIES EN VOORUITZICHTEN

SAMENVATTING EN CONCLUSIES

Het is een algemeen geaccepteerd gegeven dat het renine-angiotensine systeem (RAS) een grote rol speelt bij het ontstaan van cardiovasculaire en renale ziekten, bijvoorbeeld door de beïnvloeding van de bloeddruk middels regulatie van bloedvatmotoriek en de waterbalans. Van oudsher concentreert deze rol zich op de angiotensine (Ang) II-Ang II type 1 (AT_1) receptor-aldosteron interactie. Er zijn nu echter nieuwe medicijnen die ook aangrijpen buiten deze traditionele as. Dit is belangrijk, want de bestaande geneesmiddelen (ACE remmers, AT_1 receptor antagonisten en mineralocorticoïd receptor antagonisten), indien apart gegeven, resulteren niet in een blijvende onderdrukking van (de effecten van) Ang II of aldosteron door het optreden van compenserende mechanismen. Bovendien is er sprake van een zeer ongunstig bijwerkingenprofiel wanneer een combinatie van deze 'klassieke' medicijnen gebruikt wordt. Dit proefschrift evalueert de diverse nieuwe concepten, en met name het ingrijpen op dit 'alternatieve RAS'.

Hoofdstuk 1 bevat een beknopt overzicht van de huidige kennis over het alternatieve RAS, waarbij de nadruk ligt op renine, Ang II type 2 (AT_2) receptoren, angiotensine-(1-7) (Ang-(1-7)) en de Mas receptor. Vervolgens beschrijft **hoofdstuk 2** de resultaten die behaald zijn met de nieuwe renine remmer VTP-27999 in gezonde vrijwilligers tijdens een laag zout dieet. Hoewel enerzijds, vanwege een gelijke potentie bij een veel hogere biologische beschikbaarheid, VTP-27999 renine bij een lagere orale dosis blokkeert dan aliskiren, blijkt teveel renine remming door VTP-27999 nadelig te zijn. Verrassend genoeg ging dit gepaard met een toename van RAS activiteit buiten de nier. Dit heeft te maken met het feit dat VTP-27999, zoals aliskiren, zich ophoopt in de nier. Een dergelijke ophoping resulteert in excessieve renale RAS onderdrukking, zodat, dankzij het wegvallen van de lokale negatieve feedback van Ang II, er een enorme afgifte van renine op gang komt. Na 24 uur zijn de overgebleven hoeveelheden VTP-27999 in het bloed niet langer voldoende om dit renine onderdrukt te houden. Hierdoor is het duidelijk dat renine remming

een bovengrens heeft, en dat een hogere dosis renine remmer niet noodzakelijkerwijs beter is.

In **hoofdstuk 3** is het aandeel van de AT_2 receptor in de door Ang II veroorzaakte bloedvat-vernauwende effecten onderzocht in het zogenaamde 'four-core genotype' muismodel. Dit is een muismodel waarbij de invloed van geslachtschromosomen onafhankelijk van het geslacht zelf kan worden bestudeerd. Dit hielp ons om vast te stellen dat de verwijdende effecten van AT_2 receptoren, die alleen bij vrouwen voorkomen, afhankelijk zijn van zowel vrouwelijke geslachtshormonen als van de aanwezigheid van beide X-chromosomen. Dit laatste was ook een vereiste voor het non-NO-gemedieerde bloedvatverwijdende effect van acetylcholine. Aangezien AT_2 receptoren bloedvatverwijding veroorzaken middels bradykinine type 2 receptoren, welke ook gelieerd zijn aan NO en niet-NO verwijdende mechanismen, zouden deze twee bevindingen gerelateerd kunnen zijn. In hoeverre deze fenomenen bijdragen aan de cardiovasculaire bescherming die geldt voor premenopausale vrouwen dient nog nader onderzocht te worden. Wel bieden ze de mogelijkheid voor geslacht-specifieke therapieën, ondanks het feit dat er momenteel geen bewijs bestaat dat RAS remmers zich anders gedragen in mannen en vrouwen.

Hoofdstuk 4 bespreekt de potentiële rol van het RAS in de regulatie van bloedcelvorming en weefselvernieuwing door stamcellen. De conclusie luidt dat Ang-(1-7), via Mas-receptor activatie, de proliferatie en differentiatie van stamcellen beïnvloedt. **Hoofdstuk 5** onderzocht de implicaties van deze mogelijkheid in het post-myocardiale infarctmodel in muizen, waarbij gebruik gemaakt is van een stabiel Ang-(1-7) analoog, cyclisch Ang-(1-7). Deze stof verminderde de hypertrofie van cardiomyocyten en de myogene tonus van arteriolen na het infarct, zonder echter de pompfunctie van het hart te beïnvloeden. Cyclisch Ang-(1-7) vergrootte het aantal bloedcelvormende circulerende stamcellen, maar niet die met endotheliale eigenschappen. Een onverwacht effect was dat het herstel van de cardiale capillaire dichtheid achterbleef in de dieren die met cyclisch Ang-(1-7) behandeld waren. Bovendien verminderde het medicijn de zogenaamde tube vorming, i.e., de primitieve

voorlopers van bloedvaten, door endotheelcellen *in vitro*. Hieruit kunnen we concluderen dat cyclisch angiotensine-(1-7), wanneer het na een hartinfarct toegediend wordt, stamcellen activeert, maar dat dit niet leidt tot een verbeterde angiogenese, waarschijnlijk omdat het tegelijkertijd een anti-angiogeen effect heeft op volwassen endotheelcellen.

Tenslotte wordt in **hoofdstuk 6** aangetoond dat de Ang II effecten die gemedieerd worden via AT_1 receptoren afwezig waren in mesenchymale stamcellen, maar wel optraden zodra deze cellen adipogene of osteogene stamcellen werden. Ang-(1-7)-geïnduceerde effecten werden onder geen enkele omstandigheid gevonden, en hetzelfde gold voor AT_2 receptor-gemedieerde effecten. De onderliggende reden hiervoor lijkt te zijn dat adipogene en osteogene stamcellen alleen AT_1 receptoren in voldoende mate tot expressie brengen om receptor signallering in gang te zetten.

VOORUITZICHTEN

Vele jaren geleden stelde dr. Milton Packer de vraag waarom zoveel renine door de nier geproduceerd wordt in patiënten met hartfalen. Het antwoord luidde dat al het mogelijke gedaan wordt om de nierfunctie en glomerulaire filtratie te behouden, schijnbaar ten koste van de hemodynamische belasting van het hart.[1] Deze situatie komt overeen met onze bevindingen met VTP-27999, waar de nieren ook reageren op renale RAS onderdrukking door de afgifte van enorme hoeveelheden renine, hetgeen resulteert in verhoging (boven pre-medicatieniveau) van de plasma renine activiteit, Ang II en aldosteron. Dit wijst erop dat renine remmers vooral optimaal en niet per se maximaal gedoseerd zouden moeten worden. Bovendien zouden toekomstige studies nu moeten onderzoeken of ditzelfde fenomeen wellicht ten grondslag ligt aan de nadelige effecten van duale RAS blokkade met andere RAS blokkers dan renine remmers.

Tegelijkertijd moet nu onderzocht worden of vrouwen daadwerkelijk anders reageren op RAS blokkade dan mannen. Als AT_2 receptor-gemedieerde vasodilatatie inderdaad alleen voorkomt bij premenopausale vrouwen, zou bij hen de voorkeur uitgaan naar het gebruik van een AT_1 receptor antagonist als RAS blokker, omdat een dergelijk medicijn de mogelijkheid biedt van exclusieve AT_2 receptor stimulatie. Onze data ondersteunen niet de brede toepassing van AT_2 receptor agonisten als antihypertensieve middelen, behalve wellicht bij premenopausale vrouwen. AT_2 receptor stimulatie kan zelfs constrictie van bloedvaten veroorzaken bij mannen (Hoofdstuk 3), en ook pijnprikkels verhogen.[2]

Onze resultaten suggereren dat Ang-(1-7) analoga effectiever zullen zijn na de angiogene remodelleringsfase van de hartspier die normaal volgt op het hartinfarct.[3] De anti-angiogene eigenschappen van dergelijke stoffen kunnen ook interessant zijn voor de behandeling van kanker, hetgeen reeds door diverse onderzoekers aangetoond is.[4,5] Echter, indien cardiovasculaire gebeurtenissen bij patiënten gelijktijdig een angiogene respons vereisen, zou dit het klinisch gebruik van Ang-(1-7) analoga beperken. Na meer dan 25 jaar preklinisch onderzoek naar de effecten van Ang-(1-7)/Mas receptor stimulantia

is er bovendien sterkte behoefte aan klinische data die zulke stimulantia vergelijken met de traditionele RAS blokkers, zowel bij enkelvoudig gebruik als in combinatie. In dat geval dient er ook een definitieve keuze gemaakt te worden voor de optimale Mas receptor agonist, waarbij de korte halfwaardetijd van Ang-(1-7) zelf in beschouwing genomen moet worden.

Rodgers et al.[6] hebben geopperd dat Ang-(1-7) veel potentie heeft wat betreft het induceren van bloedplaatjesherstel na myelosuppressie, met name door het verhogen van de hematopoiëtische stamcelvoorraden. Eerder vonden we zelf dat de stof ook de groei van endotheel stamcellen bevordert.[7] Uiteraard dienen de onderliggende signaleringscascades eerst verder ontrafeld te worden. Zoals onze huidige resultaten al aangeven, dient dit altijd uitgevoerd te worden in de context van een vergelijking met de effecten op volwassen cellen. Wat tevens nog nader onderzocht dient te worden is hoe stamcellen de effecten van angiotensines op de omliggende niche (o.m. beenmerg stroma cellen of cardiovasculaire cellen) beïnvloeden. Daarnaast dienen we te weten wat de impact van cardiovasculaire en metabole ziekten is op de reacties van stamcellen op angiotensines.

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PHD PORTFOLIO

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Thesis	The alternative renin-angiotensin system: exploration of its therapeutic potential
Erasmus MC Department	Internal Medicine, Division of Pharmacology and Vascular Medicine
Research School	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotor	Prof. dr. A. H. J. Danser
Co- Promotor	Dr. A. J. M. Roks
PhD period	March/2011 - February/2015

COURSES	18.5 credits	Year
Principles of laboratory animal course	3.0	2011
COEUR Cardiovascular Medicine	1.5	2011
COEUR Pathophysiology of ischemic heart disease	1.5	2012
Biomedical English Writing and Communication	4.0	2012
NHS Cardiac Function and Adaption	2.0	2012
COEUR Cardiovascular Pharmacology	1.5	2013
COEUR Heart failure research	1.5	2014
COEUR Molecular biology in atherosclerosis and cardiovascular research	1.5	2014
NHS Vascular Biology	2.0	2014

RESEARCH SEMINARS	4.8 credits	Year
COEUR - The First Erasmus MC Meeting of the Interdisciplinary Working Group on Healthy Aging	0.4	2011
COEUR - Diagnosis and risk of ischemic heart disease	0.4	2011

COEUR - Imaging Carotid Arteries: structure and function	0.4	2011
COEUR - Heart valve Implantation	0.4	2012
COEUR - Symposium Focus on Aging	0.5	2012
Dutch Pharmacology day anno 2012	0.5	2012
COEUR - Biomarkers in Cardiovascular Disease	0.4	2012
COEUR - The cardiovascular system in women, a relation with female sex hormones	0.4	2012
COEUR - Pulmonary-Right ventricle Interaction	0.4	2013
COEUR - Salt	0.4	2014
COEUR - Symposium Current Cardiac and Vascular Aging Research at EMC	0.4	2014
COUER – Arterial Thrombosis in acute ischemic stroke	0.2	2014

LECTURES	**0.7 credits**	**Year**
COEUR - Neurovascular Pharmacology: Prof. Carlos Villalon (*The role of dopamine D2, but not D3 or D4, receptor subtypes, in quinpirole-induced inhibition of the cardioaccelerator sympathetic outflow in pithed rats*) and Prof. Jan de Hoon (*Calcitonin gene-related peptide; from basic to clinical pharmacology*)	0.2	2012
COEUR – Pharmacology: Prof. Robson AS Santos (*Time-Resolved Quantitative phosphoprotenomics*)	0.1	2012
COEUR - Dr. Matej Durik (*The Game of Genes, and its consequences*)	0.1	2014
COEUR - Dr. Suang Koid (*Mechanism of cardioprotection by aliskiren*)	0.1	2014
COEUR - Dr. Candice M Thomas (*The intracellular renin-angiotensin system in the heart*)	0.1	2014
COEUR - Dr. Sajadeh Eftekhari (*Distribution of CGRP and CGRP receptor in the trigeminovascular sytem and CNS*)	0.1	2014

CONGRESSES/SYMPOSIUMS	**9.6 credits**	**Year**
Erasmus MC PhD Day - Rotterdam (The Netherlands)	0.3	2011
Wetenschapsdagen Interne Geneeskunde - Antwerpen (Belgium)	0.6	2012
Erasmus MC PhD Day – Rotterdam (The Netherlands)**	0.3	2012
Nederlandse Vereniging voor Farmacologie - Rhythms in Pharmacology – Zeist (The Netherlands)	0.3	2013
23rd European Meeting on Hypertension & Cardiovascular protection - Milan (Italy)*	1.2	2013
3rd ISH New Investigators Symposium - New Orleans (USA)*	0.3	2013
High Blood Pressure Research – Scientific Sessions 2013 - New Orleans (USA)*	1.2	2013
Dutch Medicines Days 2013 - Ede (The Netherlands)**	0.9	2013
Wetenschapsdagen Interne Geneeskunde - Antwerpen (Belgium)*	0.6	2014
Gordon Research Seminar – Angiotensin - Barga (Italy)*	0.6	2014
Gordon Research Conference – Angiotensin - Barga (Italy)*	1.8	2014
PhysPhar 2014 – 2nd Benelux Congress on Physiology and Pharmacology - Maastricht (The Netherlands)**	0.6	2014
COEUR PhD Day 2014 - Rotterdam (The Netherlands)**	0.3	2014
Wetenschapsdagen Interne Geneeskunde - Antwerpen (Belgium)*	0.6	2015

** poster presentation / ** oral presentation*

TEACHING (SUPERVISING PRACTICUM)	**5.2 credits**	**Year**
Pharmacological control of the autonomous nervous system (Farmacologische beïnvloeding van het autonome zenuwstelsel)	4.8	2011-2014
Pharmacology of isolated blood vessels (COEUR Cursus 3 Farmacologie van geïsoleerde bloedvaten)	0.4	2012-2013

TOTAL - EUROPEAN CREDIT TRANSFER AND ACCUMULATION SYSTEM (ECTS)	**38.8 credits**	**2011-2015**

LIST OF PUBLICATIONS

Antioxidant SOD mimetic prevents NADPH oxidase-induced oxidative stress and renal damage in the early stage of experimental diabetes and hypertension. Peixoto EBMI, **Pessôa BS**, Biswas SK, Lopes de Faria JB. *Am J Nephrol*. 2009; 29:309-318 (DOI:10.1159/000163767).

The renin-angiotensin system, bone marrow and progenitor cells. Durik M, **Sevá Pessôa B**, Roks AJM. *Clin Sci (Lond)*. 2012; 123:205-223 (DOI:10.1042/CS20110660).

Spironolactone improves nephropathy by enhancing glucose-6-phosphate dehydrogenase activity and reducing oxidative stress in diabetic hypertensive rat. **Pessôa BS***, Peixoto EB*, Papadimitriou A, Lopes de Faria JM, Lopes de Faria JB. *J Renin Angiotensin Aldosterone Syst*. 2012; 13:56-66 (DOI:10.1177/1470320311422581).

Key developments in renin-angiotensin-aldosterone system inhibition. **Sevá Pessôa B**, van der Lubbe N, Verdonk K, Roks AJ, Hoorn EJ, Danser AH. *Nat Rev Nephrol*. 2013; 9:26-36 (DOI:10.1038/nrneph.2012.249).

Multiple ascending dose study with the new renin inhibitor VTP-27999: nephrocentric consequences of too much renin inhibition. Balcarek J*, **Sevá Pessôa B***, Bryson C, Azizi M, Ménard J, Garrelds IM, McGeehan G, Reeves RA, Griffith SG, Danser AH, Gregg R. *Hypertension*. 2014; 63:942-50. (DOI:10.1161/HYPERTENSIONAHA.113.02893).

Effect of a stable angiotensin-(1-7) analogue on progenitor cell recruitment and cardiovascular function post-myocardial infarction. **Pessôa, Bruno Sevá**, Moritz Becher P, van Veghel R, de Vries R, Tempel D, Sneep S, van Beusekom H, van der Velden VHJ, Westermann D, Danser AHJ, Roks, AJM. *J Am Heart Assoc*. 2015; accepted.

***** these authors contributed equally

CURRICULUM VITAE

Bruno Sevá Pessôa was born on November 13th 1981 in Campinas, Brazil. In 2008 he finished his study at Biology Institute, State University of Campinas, Brazil. After defending his Master thesis titled “Mineralocorticoid receptor blockade ameliorates nephropathy by increasing glucose-6-phosphate dehydrogenase activity and reducing oxidative stress in diabetic hypertensive rats” and graduating in 2010, at Faculty of Medical Sciences, State University of Campinas, he worked as Science and Biology teacher. In 2011 he came to The Netherlands and started his work as a PhD candidate at the Division of Vascular Medicine and Pharmacology, of the Department Internal Medicine, at the Erasmus Medical Center, Rotterdam. Under the supervision of Dr. A. H. Jan Danser and Dr. A. J. M. Roks he did his research on therapeutic exploration of the renin-angiotensin system.

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