(Pro)renin Revisited:
New Insights from Studies in Mast Cells

(Pro)renine:
Nieuwe inzichten uit studies met mestcellen

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ABSTRACT

Angiotensin synthesis at tissue sites is well-established, and interference with tissue angiotensin is now believed to underlie the beneficial effects of renin-angiotensin system blockers. At first it was thought that the renin required to synthesize angiotensin at tissue sites was also synthesized locally. Recent studies show, however, that this is not the case at important cardiovascular sites like the heart and vessel wall. Moreover, extrarenal sites that do express the renin gene release prorenin, the inactive precursor of renin, instead of renin. This chapter provides an update on the sources of (pro)renin in the body, lists the known stimulants and inhibitors of its production, and discusses the concept that prorenin rather than renin determines tissue angiotensin generation.

INTRODUCTION

Angiotensin (Ang) production at tissue sites is now well-established.[1] Such local production efficiently allows Ang II to stimulate its receptors,[2] with little Ang II spillover to blood. Yet, in contrast to what was originally believed, not all components required to generate Ang II locally are actually synthesized at tissue sites. In particular, renin is not expressed locally at important cardiovascular tissue sites like the heart and vessel wall.[3, 4] Since renin is indispensable for angiotensin generation, this implies that such tissues must sequestrate renin from blood. Alternatively, these tissues might sequester prorenin, particularly because the levels of this inactive renin precursor are much higher than those of renin.[5] To allow prorenin to contribute to angiotensin generation, it should be converted locally to renin.

Remarkably, it is prorenin, and not renin, that remains detectable in blood following a bilateral nephrectomy, although its levels are lower than in normal subjects. This suggests that the kidney is the main, if not the only, source of renin in the body, but that there are other tissues releasing prorenin into the circulation. Extrarenal prorenin might contribute to tissue angiotensin generation in the absence of renin/prorenin release from juxtaglomerular (JG) cells. This chapter provides an update on the sources of (pro)renin, lists the known stimulants and inhibitors of its production, and discusses the concept of prorenin activation at tissue sites.

KIDNEY

Juxtaglomerular cells

The JG epithelioid cells, located in the walls of renal afferent arterioles at the entrance of the glomerular capillary network, are the main source of renin in the body. Renin is synthesized as preprorenin. Preprorenin is converted to prorenin on insertion into the endoplasmatic reticulum. The majority (75%) of prorenin is secreted constitutively, while the remainder is targeted to dense core secretory granules. In the secretory granules, an acidic pH is created by vacuolar H+-ATPases to optimize the activity of the proteases (cathepsin B, prohormone convertases) that cleave off the 43-amino acid prosegment to yield renin.[6] Glycosylation affects the amount of prorenin trafficking to dense core secretory vesicles.[7] Acute stimulation of renin release leads to an increase in the release of mature renin secretory granules that contain only renin, while chronic stimulation leads to an increased release of both renin and prorenin. This is due to activation of the renin synthetic pathway and/or the recruitment of new juxtaglomerular cells. The granular volume and the number of granules per cell usually remain the same. Yet, chronic stimulation causes more prorenin to be converted to renin, leading to an increased renin/prorenin ratio in plasma.[5]

Well-known stimulants of renin secretion are the sympathetic nervous system (via β1-adrenoceptor stimulation), low renal perfusion pressure and sodium depletion. Stimulation of Ang II type 1 (AT_1) receptors receptors inhibits renin release, and thus, during renin-angiotensin system (RAS) blockade, renin levels will rise. β_1-adrenoceptors activate adenylyl cyclase, inducing exocytosis of the renin-containing granules via increased cAMP levels. Other adenylyl cyclase activators, like prostaglandin E_2, prostaglandin I_2, adrenomedullin, dopamine and calcitonin gene-related peptide also stimulate renin release. Nitric oxide (NO), through activation of soluble guanylyl cyclase, induces the generation of cGMP, which, by blocking phosphodiesterase, indirectly increases the levels of cAMP. Thus, NO also stimulates renin secretion. Conversely, activation of particular guanylate cyclases by natriuretic peptides inhibits renin secretion, possibly via a process involving cGMP-dependent protein kinase II. Paradoxically, calcium inhibits exocytosis, and therefore, calcium-mobilizing hormones like Ang II, endothelin and vasopressin, as well as α_1-adrenoceptor agonists, will block renin secretion. Recent studies have provided the molecular basis of this phenomenon: Ca^{2+} directly reduces the cAMP levels through interference with the calcium-sensitive isoforms of adenylyl cyclase (AC5 and AC6) that are expressed in the JG cells.[8]

Collecting duct

The principal cells of the collecting duct synthesize renin, and remarkably, Ang II stimulates renin expression at this site,[9] as opposed to its inhibitory effects in JG cells. Using angiotensinogen from proximal tubule cells as a substrate, collecting duct-derived renin may contribute to the generation of Ang II that acts in the proximal and distal tubulus. This Ang II augments H^+ secretion and Na^+ absorption, possibly by interfering with vacuolar H^+-ATPase activity .[10] Kang et al.[11] recently suggested that prorenin is stored in the collecting duct, and that such prorenin is the source of the elevated prorenin levels found in diabetic subjects.

ADRENAL

The adrenal gland contains the highest renin concentration next to the kidney, and the adrenal renin-angiotensin system (RAS) is a well-known regulator of aldosterone production. Renin protein and renin mRNA occur predominantly (>90%) in the steroid-producing cells of the zona glomerulosa.[12] A low-sodium diet increases, whereas a high-sodium diet decreases the adrenal renin content. Adrenocorticotrophic hormone (ACTH), cAMP and high potassium all stimulate adrenal renin, and the strongest stimulus of adrenal renin is nephrectomy, possibly because ACTH and potassium increase post-nephrectomy. Cultured zona glomerulosa cells predominantly contain renin, but release prorenin. Ang II has a dual effect in glomerulosa cells: it weakly increases (pro)renin synthesis and release, but simultaneously it blocks the ACTH-induced stimulatory effects on (pro)renin production.[13]

Analysis of adrenal RNA revealed the existence of 2 renin transcripts in the adrenal: the known full-length transcript, and a transcript lacking exon 1.[14] Translation of this second mRNA results in a truncated prorenin, lacking the endoplasmatic reticulum signal and part of the prosegment. Such prorenin will remain in the cell, and might display catalytic activity. Possibly, it accumulates in the mitochondria,[14] i.e., the site of aldosterone synthesis. Its function, if any, is not known.

EYE

As discussed above, chronic stimulation of the RAS generally increases renal prorenin to renin conversion, thereby decreasing the relative amount of prorenin in the circulation. There are some exceptions to this rule. A very striking example is diabetes mellitus complicated by retinopathy and nephropathy.[15] Prorenin levels increase with the severity of retinopathy, and it has therefore been speculated that this prorenin originates, at least in part, in the eye.[16, 17] Indeed, the prorenin levels in ocular fluids are up to 100 times higher than expected on the basis of the plasma protein content of these fluids, and prorenin was higher in vitreous fluid from eyes with proliferative diabetic retinopathy, than in eyes of non-diabetic subjects with spontaneous retinal detachment.[16] Renin in ocular fluids was low or undetectable. Furthermore, human retina and pigment epithelium-choroid contained renin mRNA,[18] whereas renin synthesis was subsequently confined to the macroglial Müller cells in the rat eye.[19] The endfeet of these cells make numerous contacts with retinal blood vessels, thereby suggesting that ocular prorenin is involved in neovascularization. Whether prorenin is released from ocular tissue sites into the blood remains to be proven. Based on the existing prorenin levels in the eye,[16, 17] it seems impossible however, given the low ocular blood flow, that the eye is a major contributant to the rise in prorenin in diabetics with retinopathy.

MAST CELLS

Silver et al.[20] discovered that in the rat heart, renin co-stained with mast cells. Also, when culturing the human mastocytoma cell line HMC-1, stimulation with a mast cell degranulator released Ang I-generating activity. Mackins et al. subsequently showed that the coronary effluent of the isolated perfused guinea pig heart contained Ang I-generating activity after exposure of the heart to the mast cell degranulator compound 48/80.[21] Moreover, mast cell-derived Ang I-generating activity appeared to contribute to the norepinephrine release and arrhythmias occurring after ischemia and reperfusion in guinea pig and mouse hearts. These data contrast with earlier studies failing to detect renin in the heart after a bilateral nephrectomy,[3, 22] and thus definite proof should now be obtained to ascertain that the mast cell-derived Ang I-generating activity in the coronary effluent truly represents renin.

REPRODUCTIVE SYSTEM

The reproductive system is a major source of prorenin. In men, prorenin is produced in the testis. In women, the ovaries, the uterus and, during pregnancy, the placenta are sources of prorenin. During pregnancy, a distinction should be made between prorenin-secreting tissues of maternal and of embryonic origin. Of maternal origin are the decidua, which corresponds with the endometrium in the non-pregnant state, and the ovaries. Of embryonic origin are the placenta, the amnion and the chorion.

Ovaries

The ovary secretes prorenin into the circulation and produces high plasma prorenin levels during early pregnancy and in women with hyperstimulated cycles.[23] The rise in prorenin is directly related to the number of ovarian follicles, and theca cell-stimulation by luteinizing hormone (LH) or human chorionic gonadotropin (hCG) is responsible for this prorenin release.[24] The release depends on de novo prorenin synthesis via the adenylyl cyclase-cAMP pathway, and both protein kinase C (PKC) activation and tumor necrosis factor α (TNFα) inhibit it.[25] Studies during pregnancy in a woman with primary ovarian failure[26] provided definite proof for the ovarian origin of prorenin. Follicular fluid prorenin levels are >10 times higher than those in plasma, and correlate inversely with follicular atresia.[27] This suggests that prorenin affects follicular development and oocyte maturation.

Uterus/Decidua

Decidual cells contain and release prorenin, but not renin.[28, 29] The total renin content of decidual homogenates is 3-fold larger than that in endometrial homogenates, suggesting that pregnancy enhances renin production by the cells lining the uterus. Decidual prorenin release occurs constitutively, as decidual cells do not contain dense core vesicles. The adenylyl cyclase-cAMP pathway, relaxin and endothelin stimulate prorenin production.[28, 30, 31] PKC potentiated the effect of cAMP,[28] while interleukin-1β and TNF-α inhibited the release of prorenin.[32] Decidual (pro)renin production has been linked to preeclampsia, e.g. because decidual tissue-derived angiotensin acts in placental tissue.[33]

Placenta

Prorenin secreted from the placenta is released into the maternal but not the fetal circulation.[34] It may thus contribute to the elevated plasma prorenin levels observed later in pregnancy, after the initial theca cell-dependent prorenin rise. Total renin in the maternal effluent from the perfused placenta consisted for <10% of renin, whereas in placental tissue the renin percentage was ≈50%. This finding is consistent with the idea of prorenin activation at tissue sites, either in vitro or in vivo. β_1- and, to a lesser extent, β_2-adrenergic stimulation, hCG as well as cAMP stimulated placental prorenin synthesis and release, while Ang II blocked it.[35, 36] Placental prorenin release might be regulated in a paracrine fashion as hCG

is also secreted from the placenta.

Amnion and chorion

The amnion lines the amniotic cavity containing the amniotic fluid, while the chorion is adherent to the maternal side of the amnion. The total renin concentration in amniotic fluid is on average ≈16 times the plasma concentration found in the second and third trimester of human pregnancy, and the major (>95%) form of renin in amniotic fluid is prorenin.[37] Interestingly, the prorenin levels measured in gestational sacs during the first trimester, when the chorionic cavity is much larger than the amniotic cavity, were among the highest ever detected: close to 1 µg/mL.[37] This suggests a possible role of prorenin in embryonic and fetal development. Most likely, the chorion leave is the primary source of this prorenin, since cultured chorionic, but not amnionic cells, synthesize and released prorenin.[38] This release is constitutive, and stimulated by cAMP, PKC and calcium.[39]

Testis

The testis secretes prorenin, but not renin,[40] and Leydig cells are the most likely source of this prorenin.[41] An important stimulant of testicular prorenin release is hCG.[42]

SUBMANDIBULAR GLAND

Humans and rats have only one renin gene, whereas mice have two alternative genotypes at the renin locus: some strains have one copy (Ren1c, as found in the C57Bl/6 strain), whereas others have two renin genes (Ren1d and Ren2, as in DBA/2 and 129J). The mouse renin genes are expressed in distinct, although overlapping, tissue-specific and developmental patterns. For example, Ren1d and Ren2 are expressed at equivalent levels in the kidney, but Ren2 is expressed at high levels in the submandibular gland, and is under the control of testosterone. Ren2 knockout mice are healthy and viable, and display a normal blood pressure,[43] indicating that the remaining Ren1d in these mice is functionally equivalent to Ren1c. The Ren1 and Ren2 proteins share 97% amino acid similarity, but differ in their potential glycosylation sites. Ren2 has no consensus sites for asparagine-linked glycosylation, whereas Ren1c and Ren1d have three sites. Thus, Ren2 is nonglycosylated. Although this does not affect its biochemical properties, it reduces the stability of the protein. Deleting Ren1d results in sexually dimorphic hypotension,[44] accompanied by elevated prorenin levels and reduced renin levels in female mice. Thus, Ren2 only partially compensates the loss of Ren1d in females, whereas in males, via the androgen-responsive Ren2 gene, full compensation occurs. Ren1d knockout mice display a complete absence of juxtaglomerular cell granulation, in agreement with the fact that glycosylation determines prorenin trafficking to dense core secretory vesicles.[7] Most likely, Ren2 is constitutively secreted in the inactive form, although the low levels of renin in Ren1d knockout mice

suggest that some prorenin-renin conversion does occur in these animals. Such conversion is also believed to take place in the submandibular gland. Yet, submandibular renin does not make its way into the circulation in large quantities. Interestingly, Ren2 expression in the rat ((mRen2)27 rat) results in severe hypertension, most likely due to the fact that mouse Ren2 renin reacts as well with rat angiotensinogen as rat renin.[45]

EXTRARENAL PRORENIN AND TISSUE ANGIOTENSIN GENERATION

Virtually all extrarenal renin-expressing tissues synthesize and release prorenin. Renin release rarely ocurs. Combined with the fact that 75% of JG cell preprorenin, after its conversion to prorenin in the endoplasmatic reticulum, is released in the inactive form, it is clear why we have such high prorenin levels.[6] The function of prorenin is however unknown. It seems reasonable to assume that, if prorenin has a function, this depends on its ability to generate angiotensins.

Evidence for prorenin-renin conversion outside the kidney is not available.[46] Non-proteolytic activation of prorenin (i.e., activation without actual removal of the prosegment) might provide an alternative. This is a reversible process. It can best be imagined as an unfolding of the prosegment from the enzymatic cleft. This unfolding consists of at least two steps. In the first step the prosegment moves out of the enzymatic cleft, and in the second step the renin part of the molecule assumes its enzymatically active conformation. Non-proteolytic activation can be induced by exposure to low pH and cold, called acid activation and cryoactivation, respectively.[15] Acid activation leads to complete activity of prorenin, cryoactivation to partial (~15%) activity. Non-proteolytically activated prorenin is enzymatically fully active, and can be recognized by monoclonal antibodies that are specific for the active site. Kinetic studies of the non-proteolytic activation process have indicated that an equilibrium exists between the closed (inactive) and open (active) forms of prorenin. The inactivation step is highly temperature-dependent and occurs very rapidly at neutral pH and 37°C. Consequently, under physiological conditions <2% of prorenin is in the open, active form. This percentage is sufficient to explain the low plasma 'renin' and angiotensin levels in anephrics, but will not result in significant angiotensin production at tissue sites.

The discovery of the (pro)renin receptor[47] has changed this view. This receptor, a 350-amino acid protein with a single transmembrane domain, was first identified on cultured human mesangial cells. The receptor binds prorenin with higher affinity than renin.[48] Binding induces a conformational change in the prorenin molecule allowing it to display catalytic activity without being cleaved to renin (i.e., prorenin is non-proteolytically activated). Interestingly, an 8.9 kDa fragment of the (pro)renin receptor called M8-9 is known to co-precipitate with a vacuolar H^+-ATPase (V-ATPase).[49] V-ATPases play important roles in acidification of intracellular compartments and cellular pH homeostasis (e.g., in the secretory granules of the JG cells[6] and in the tubulus[10]), thereby providing a potential link between the (pro)renin receptor and acid activation. Since there is only one gene for the

Production site	Release into the blood	Stimulators	Inhibitors	Ref.
Kidney	+			
JG cells		β_1-AR agonist, PGI_2, PGE_2, NO, adrenomedulin, dopamine, CGRP, cAMP	α-AR agonist, Ang II, endothelin, vasopressin, ANP, Na^+, cGMP, Ca^+	6,8
Collecting duct		Ang II		9
Adrenal	+			
Zona glomerulosa		ACTH, K^+, cAMP, Ang II	Ang II, Na^+	12,13
Eye	?			
Muller cells		?	?	16,19
Mast cells	?	compound 48/80 (degranulator)	cromolyn, lodoxamine (stabilizer)	20,21
Ovary	+			
Thecal cells		hCG, LH, cAMP	TNF-α, PKC	24,25
Uterus	-			
Myometrium/ decidual cells		relaxin, endothelin, cAMP, PKC	TNF-α, IL-1β	30-32, 36
Placenta	+	hCG, β_1/β_2 -AR agonist,cAMP	Ang II	35, 36
Chorion		cAMP, PKC, Ca^{2+}		38, 39

Table 1*:* Renin/prorenin production sites and the known stimulators and inhibitors of this production. Abbreviations: ACTH, adrenocorticotrophic hormone; ANP, atrial natriuretic peptide; AR, adrenoceptor; cAMP, cyclic 3',5'-adenosine monophosphate; cGMP, cyclic 3',5'-guanosine monophosphate; CGRP, calcitonin gene-related peptide; hCG, human chorionic gonadotropin; IL-1β, interleukin-1β; LH, luteinizing hormone; NO, nitric oxide; PG, prostaglandin; PKC, protein kinase C; TNFα, tumor necrosis factor α.

(pro)renin receptor and the M8-9 protein, it is likely that both proteins derive from the same transcript. The M8-9 fragment corresponds to the cytoplasmic domain, the transmembrane domain and part of the extracellular domain of the receptor.

After the discovery of the receptor, (pro)renin receptor antagonists were designed based on the idea that the prosegment contains a handle region which binds to the receptor.[50] These (peptidic) antagonists (also known as 'handle region peptides', HRP) mimic the handle region, and thus will bind to the receptor instead of prorenin, thus blocking receptor-mediated prorenin activation. In support of this concept, HRP infusion normalized the elevated renal angiotensin content in diabetic rats[50] without affecting blood pressure. Concomitantly, the development of diabetic nephropathy was prevented, suggesting that these phenomena depend on prorenin-induced tissue angiotensin generation. For reasons that are currently unknown, HRP infusion did not affect angiotensin levels in circulating blood, nor in tissues of healthy control rats. This was unexpected, since circulating angiotensins are largely derived from tissue sites, and prorenin is easily detectable in rats. Furthermore, since renin also binds to the receptor, it remains to be determined whether HRP interferes with renin binding, and what percentage of the receptors is occupied by renin, particularly in the kidney.

Surprisingly, renin and prorenin were also found to induce signaling via the (pro)renin receptor, resulting in p42/p44 and p38 mitogen-activated protein kinase activation.[47, 51] These effects occurred in an angiotensin-independent manner and required high (pro)renin concentrations. The importance of this phenomenon in humans in vivo is unknown.

CONCLUSION

The kidney is the main, if not the only, source of renin in the body. Yet, several organs release prorenin into the circulation, and prorenin remains detectable in humans at relatively high levels following a bilateral nephrectomy. For many years, prorenin was considered to be just the inactive precursor of renin, having no function of its own. However, the high prorenin levels in ocular fluids, follicular fluid and amniotic fluid, as well as the elevated plasma prorenin levels in subjects with diabetes complicated by microvascular complications suggested otherwise. The discovery of the (pro)renin receptor now offers a solution. The 'inactive' renin precursor gains Ang I-generating activity by binding to this receptor, without undergoing proteolytic cleavage. Thus, it might be prorenin, rather than renin, that contributes to tissue angiotensin generation. This concept is attractive, because it puts the much higher prorenin than renin levels in plasma into perspective. Nevertheless, there still are some uncertainties. For instance, tissue angiotensin levels in animals are low to undetectable following a bilateral nephrectomy.[3] This may relate to the fact that in animals, more than in humans, prorenin is kidney-derived. An alternative explanation is

that the different glycosylation patterns of renal and extrarenal prorenin determine their tissue uptake and/or receptor binding.[46] Furthermore, prorenin exerts direct effects via its receptor, in an angiotensin-independent manner. The relevance of this phenomenon remains to be verified in humans. Based on these observations, a new class of drugs might eventually emerge, i.e., the (pro)renin receptor blockers, which prevent both angiotensin generation at tissue sites and (pro)renin-induced, angiotensin-independent effects.

AIM OF THE THESIS

Extrarenal renin synthesis is highly controversial. Most studies claiming extrarenal renin expression report the release of prorenin, the inactive precursor of renin, rather than renin release. This may explain, at least in part, why humans have much more prorenin than renin. Nevertheless, recent studies suggest that mast cells in cardiac and pulmonary tissue are a new source of renin. Simultaneously, a (pro)renin receptor has been discovered which, upon binding, allows prorenin to display enzymatic activity. This receptor also binds renin.

Blockers of the renin-angiotensin system (RAS) predominantly increase renin, because they attenuate the negative feedback effect of angiotensin II on renin release. The rise in renin during renin inhibition with aliskiren has been suggested to be larger than during other types of RAS blockade. In addition, the beneficial effects of aliskiren last longer than expected on the basis of its half life.

Given the above controversies, the following aims were defined:

1) to quantify the amount of extrarenal renin/prorenin in the human body, and to evaluate the kinetics of renin/prorenin binding to their receptor as well as the possibility that this results in angiotensin generation at tissue sites (Chapters 2 & 3);

2) to investigate whether mast cells release renin/prorenin, and whether mast cell-derived renin/prorenin contributes to cardiac and pulmonary angiotensin generation, both under normal - and pathophysiological conditions (Chapters 4 & 5);

3) to study two characteristics of renin inhibition, i.e., an excessive renin rise and a long-lasting effect, and to evaluate a newly designed prorenin kit (Chapters 6 & 7).

Chapter 2
Renin and prorenin disappearance in humans post-nephrectomy: Evidence for binding?

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ABSTRACT

To study the distribution of kidney-derived renin-angiotensin system (RAS) components in humans, we monitored the decline in plasma prorenin, renin, angiotensin (Ang) I and Ang II post-nephrectomy. Prorenin and renin decreased biphasically, prorenin displaying a slower elimination. The distribution half life was similar for both. Angiotensins followed the disappearance of renin. Within two days post-nephrectomy, stable plasma concentrations at 5-10% (renin and angiotensins) and 25-30% (prorenin) of pre-nephrectomy levels were reached. The total amount of kidney-derived renin and prorenin in the body was ≈10 times as much as the amount in blood. Prorenin also originated at extrarenal sites. The renin levels in anephrics corresponded with the percentage of prorenin that in vitro has a so-called 'open conformation' (i.e., displays enzymatic activity), suggesting that renin in anephrics is in fact 'open' prorenin. Haemodialysis nor captopril significantly affected the level of any RAS component in anephrics. In conclusion, renal renin/prorenin enters tissue sites in humans, and renal renin is the main determinant of plasma angiotensins. Whether prorenin contributes to tissue angiotensin generation in humans remains to be determined.

INTRODUCTION

Angiotensin (Ang) production occurs at tissue sites rather than in circulating blood.[1, 52] Such tissue production efficiently results in the activation of Ang II type 1 (AT_1) receptors and/or Ang II type 2 (AT_2) receptors, and requires the local presence of renin, angiotensinogen and angiotensin converting enzyme (ACE). Yet, although ACE is indeed expressed locally in multiple organs, renin and angiotensinogen are not.[3, 22, 53, 54] Thus, to allow Ang I synthesis at tissue sites, renin and angiotensinogen need to be taken up from circulating blood. Indeed, both proteins diffuse into the interstitial fluid.[53, 55, 56] In addition, renin binds to receptors. Up to now, two of such receptors have been described: the mannose 6-phosphate (M6P) receptor (which is identical to the insulin-like growth factor II receptor) and the 'renin receptor'. M6P receptors bind any phosphomannosylated (M6P-containing) protein, and therefore cannot be considered as renin-specific receptors. These receptors most likely contribute to renin clearance.[46, 57, 58] Renin receptors bind renin on the cell surface.[47, 48]

Importantly, both receptors also bind prorenin, the inactive precursor of renin.[47, 48, 50] The renin receptor is therefore currently known as the (pro)renin receptor. M6P receptor-bound prorenin is internalized, and, as part of a clearance process, is converted to renin and subsequently degraded.[57, 59] (Pro)renin receptor-bound prorenin remains on the cell surface, and, due to a conformational change, becomes catalytically active. Recent studies suggest that prorenin, rather than renin, is the endogenous 'agonist' of this receptor.[48, 60] Consequently, tissue angiotensin generation may actually depend on prorenin.[15] Such a role for prorenin would explain not only why we have so much prorenin (its levels in blood are usually >10-fold higher than those of renin),[5] but also why some extrarenal organs selectively release prorenin into the circulation. Examples of prorenin-releasing organs are the ovary, the testis, the eye, and the adrenal.[14, 16, 23, 40]

Up to now, all evidence for prorenin contributing to tissue angiotensin production comes from animal studies.[50, 61-63] Such studies have made use of animals overexpressing prorenin or the (pro)renin receptor, and/or the application of a (pro)renin receptor antagonist, the so-called handle region peptide (HRP). Studies in humans on this topic are, for obvious reasons, much more difficult to perform, if not impossible.

In the present study, we set out to investigate the kinetics of renin and prorenin in humans, making use of subjects undergoing a complete nephrectomy, resulting in the removal of their last remaining kidney. A detailed analysis of the disappearance curves of both proteins provides insight into their tissue distribution. In addition we quantified the levels of renin-angiotensin system (RAS) components in subjects that had been anephric for several years, both before and after haemodialysis, and we studied the effect of captopril, ACE inhibitor, on these levels.

METHODS

The studies were approved by the Ethics Committee of the Erasmus MC, and all subjects gave their informed consent.

Subjects undergoing nephrectomy

Four subjects (3F, 1M; age 53-69 years) undergoing nephrectomy because of chronic pyelonephritis or renal adenocarcinoma participated in the study. One subject was taking 25 mg captopril twice daily. Blood samples for RAS component measurements were taken from an antecubital vein 30 minutes before, during (t=0) and 10, 20, 30, 60, 120, 240, 480, 720, 1440 and 2880 minutes after clamping of the renal vein and subsequent removal of the kidney.

Anephric subjects

Thirteen anephric subjects (9F, 4 M; age 24-79 years), who had been anephric for 1-17 years (mean 8 years), participated in the study. The reasons for nephrectomy were chronic pyelonephritis (n=4), chronic interstitial nephritis (n=1), chronic glomerulonephritis (n=1), renal adenocarcinoma (n=2), M. Goodpasture (n=1), polycystic kidneys (n=2), reflux nephropathy (n=1), and uncontrollable severe hypertension (n=1). All subjects were receiving maintenance haemodialysis, 2-3 times a week, with a Fresenius A2008C dialyzer and a disposable polyacrylonitryl membrane kidney. The subjects were seated for 30 minutes before the haemodialysis was started and remained seated during the procedure. Blood samples for RAS component measurements were taken from an antecubital vein immediately before and after haemodialysis.

On a second occasion, 5 anephric subjects (4 F, 1M; age 38-67 years) were given 50 mg captopril orally after they had been seated for 30 minutes. Haemodialysis was started 2 hours after captopril and lasted 4 hours. Blood samples for RAS component measurements were taken from an antecubital vein immediately before, and 2 hours and 6 hours after captopril was given. The patients remained seated throughout the entire period. Blood pressure was measured by standard sphygmomanometry at 0, 30, 45, 60, 90, 120 and 180 minutes after captopril had been taken. Heart rate was computed from a continuous electrocardiographic tracing.

For comparison, blood samples for renin, prorenin, Ang I and Ang II measurements were taken from 17 healthy controls (9F, 8M; age 21-60 years), after they had been seated for 30 minutes.

Blood for renin, prorenin, angiotensinogen, ACE and total protein measurements was centrifuged at 3000 g for 10 minutes at room temperature, and plasma was stored at -20°C.[64] Blood for angiotensin measurements was centrifuged at 3000 g for 10 minutes at 4°C, and plasma was stored at -70°C.[64]

Biochemical measurements

Renin and prorenin were measured by enzyme-kinetic assay (EKA).[65] To measure prorenin by EKA, it was first converted to renin by incubation with Sepharose-bound trypsin. Results of the enzyme-kinetic assay are expressed as mU/L using the WHO human kidney renin standard 68/356 as reference standard. The lower limit of detection was 0.5 mU/L. Renin + prorenin ('total renin') were also measured by immunoradiometric assay (IRMA), using the monoclonal antibodies R3-27-6 and R3-36-16.[66] These two antibodies recognize different epitopes of the renin molecule and react equally well with renin and prorenin. The results of this assay are also expressed as mU/L, and the lower limit of detection was 5 mU/L.

Angiotensinogen was determined as the maximum quantity of Ang I generated in the presence of excess human kidney renin.[67] The lower limit of detection was 1 nmol/L. Ang I and II were measured by radioimmunoassay after SepPak extraction and high-performance liquid chromatography separation. The lower limit of detection was 0.5 pmol/L for Ang I, and 0.25 pmol/L for Ang II, respectively.[1] ACE activity was measured with a commercial kit (ACEColor, Fujirebio, Tokyo, Japan). Total protein was measured with a routine laboratory method.

Statistical analysis

Data are presented as mean±SD or geometric mean and range. The plasma disappearance of RAS components after nephrectomy was studied by plotting the plasma levels semilogarithmically against time. The plasma levels reached 24-48 hours after nephrectomy were similar to those found in subjects who had been nephrectomized for several years, and these levels were therefore considered to be of non-renal origin. In order to correct for RAS components of non-renal origin, the plasma levels found 48 hours after nephrectomy were subtracted from the actual levels as measured during the first 12 hours. The plasma disappearance curves were then analyzed using a two-compartment exponential model. Differences between half lives were evaluated using Scheffé's test for multiple comparison. Associations were assessed by calculating Pearson's coefficient of correlation. Differences between plasma levels before and after haemodialysis or captopril were evaluated using Student's t-test for paired observations. Values were considered significant at $P<0.05$.

RESULTS

Subjects undergoing nephrectomy

RAS component levels at 0.5 hour prior to the clamping of the renal vein and the moment of clamping (t=0) were identical (Table 1). The plasma levels of prorenin, renin, Ang I and Ang II dropped rapidly after clamping of the renal vein and subsequent removal of the kidney (Figure 1 and Table 1). Prorenin levels reached a plateau at ≈25-30% of the levels at t=0, whereas renin, Ang I and Ang II decreased to levels <5% of the levels at t=0. Ang I and II were still above the detection limit after 48 hours (Figure 2 and Table 1). The disappearance curves were biphasic, and the half life of the fast component of the curve (1.0±0.2, 0.7±0.2, 0.6±0.2 and 0.7±0.3 hour for prorenin, renin, Ang I and Ang II, respectively) was identical for all 4 RAS components. The half life of the slow component (which corresponds with the effective plasma half life) was significantly ($P<0.05$) longer for prorenin (7.6±2.0 hours) than for the other 3 RAS components (2.8±0.6, 3.3±0.4 and 3.5±0.7 hours for renin, Ang I and Ang II, respectively). The area under the curve (AUC) for prorenin was 234 (geometric mean; range 61.6-1299) U/L.min, as opposed to 17.7 (10.4-45.0) U/L.min for kidney-derived renin.

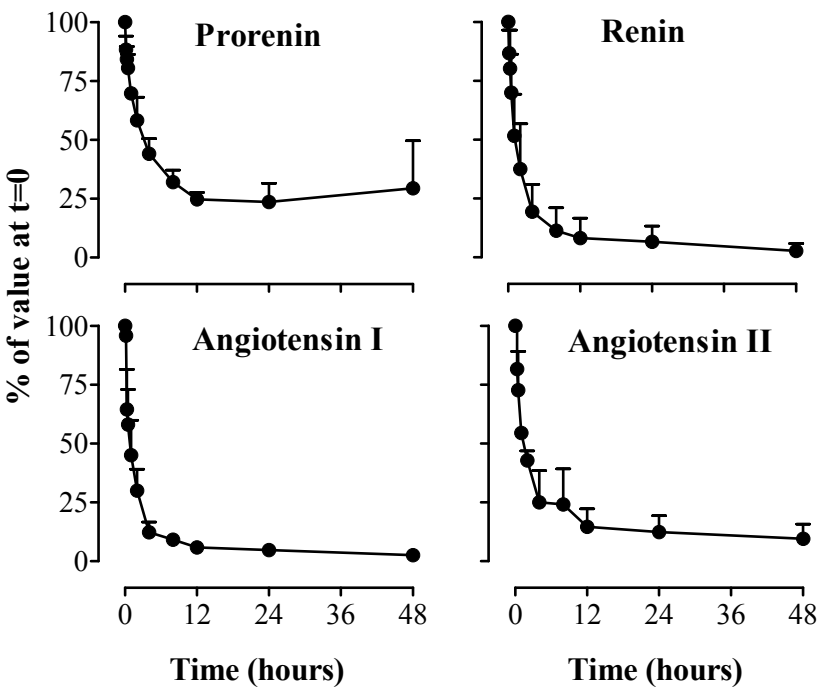

Figure 1. Plasma disappearance curves of prorenin, renin, angiotensin I and angiotensin II in patients after nephrectomy. Data (mean+SD of 4 subjects) have been expressed as a percentage of the values at t=0 (the time of renal vein clamping).

Table 1: Plasma prorenin, renin, angiotensin I and angiotensin II (geometric mean and range) before and after nephrectomy in 4 subjects. At t=0 the renal vein was clamped.

Time (hours)	Prorenin (mU/L)	Renin (mU/L)	Angiotensin I (pmol/L)	Angiotensin II (pmol/L)
-0.5	260 (92-1471)	55.7 (26.2-160)	69.1 (16.5-215)	12.8 (2.3-93.3)
0	278 (116-1656)	57.3 (26.4-145)	70.5 (17.6-187)	14.3 (3.8-77.2)
48	59.3 (15.5-107)	1.5 (0.6-3.1)	1.9 (0.5-4.1)	1.0 (0.6-1.4)

Table 2: RAS component plasma levels (geometric mean and range) in 13 anephric subjects (Nx) before and after heamodialysis and in 17 healthy controls.

RAS components	Nx before	Nx after	Controls
Prorenin (mU/L)	86.3 (25.5-537)	87.1 (20.7-676)	161 (75-548)
Renin (mU/L)	0.9 (0.3-4.4)	0.9 (0.3-4.0	13.2 (2.7-39.1)
Angiotensinogen (nmol/L)	2736 (1700-5277)	2879 (1737-6071)	not done
ACE (U/L)	11.8 (4.0-20.3)	13.5 (4.5-21.6)	not done
Angiotensin I (pmol/L)	4.5 (1.6-6.5)	5.2 (1.8-8.6)	21.8 (8.0-53)
Angiotensin II (pmol/L)	1.1 (0.4-2.8)	1.0 (0.3-4.3)	4.0 (1.2-8.5)

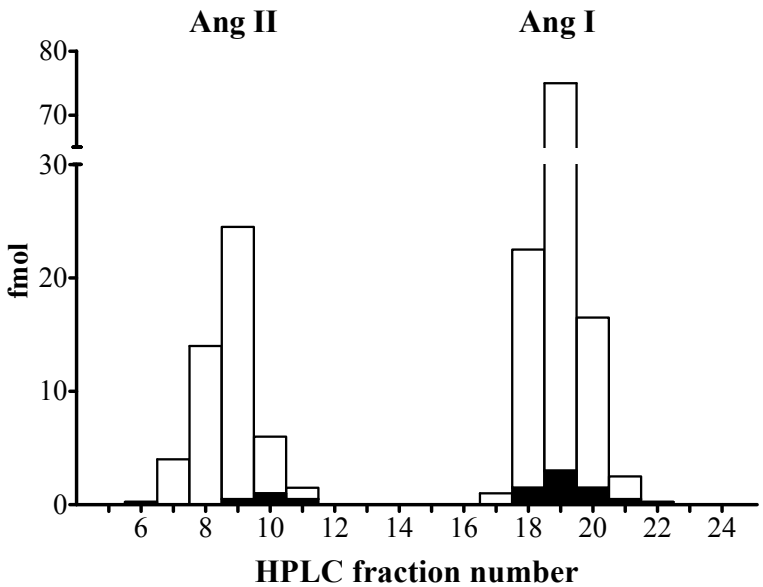

Figure 2. Angiotensin I and II levels found in HPLC fractions after SepPak extraction and HPLC separation of plasma obtained 30 minutes before (open bars) and 48 hours (closed bars) after nephrectomy.

Anephric subjects

Haemodialysis did not alter the levels of renin, prorenin, angiotensinogen, ACE, Ang I and Ang II (Table 2). EKA measurements were identical to IRMA measurements (Figure 3), confirming that the Ang I-generating activity measured in the EKA was entirely renin-dependent. The renin, Ang I and Ang II levels in anephric subjects were ≈5-10 times lower than those in healthy controls, whereas the prorenin levels in anephric subjects were ≈2 times lower than in healthy controls (Table 2). In anephric subjects, renin amounted to 2.0±0.8% of total renin, and correlated strongly with prorenin (Figure 4; r=0.97, P<0.001). In healthy controls, renin amounted to 8.4±4.0% of total renin (P<0.01 vs. anephric subjects), and correlated less well with prorenin (Figure 4; r=0.64, P<0.01). In 6 subjects (5F, 1M), total renin had also been measured 4 years prior to this study. Results were identical to those measured here (47±13 vs. 41±6 mU/L). The relationship between renin and Ang I and the relationship between Ang I and Ang II were identical in healthy and anephric subjects (Figure 5).

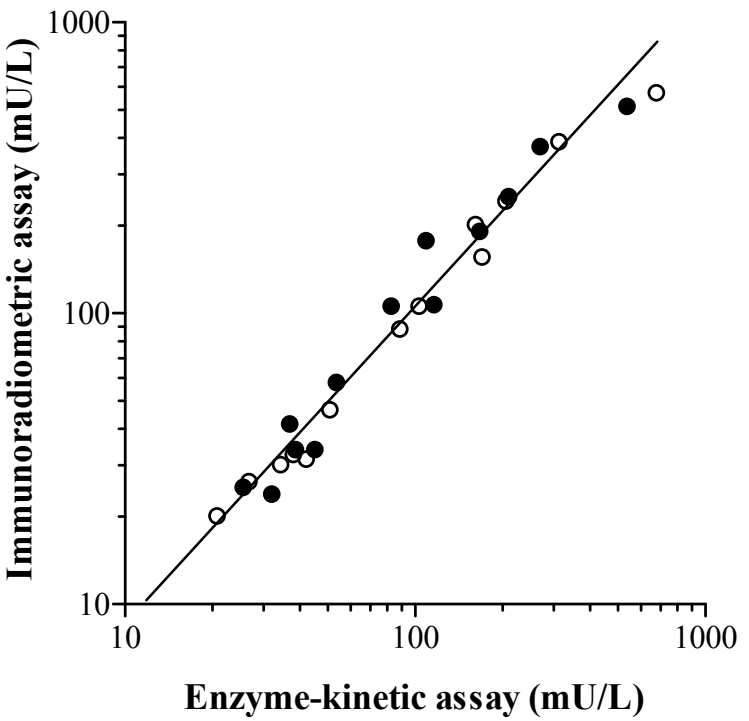

Figure 3. Comparison of the total renin concentration measured by enzyme-kinetic assay and by immunoradiometric assay in plasma samples obtained from 13 anephric subjects before (closed symbols) and after (open symbols) haemodialysis. The regression line is not significantly different from the line of identity (r=0.98; P<0.001).

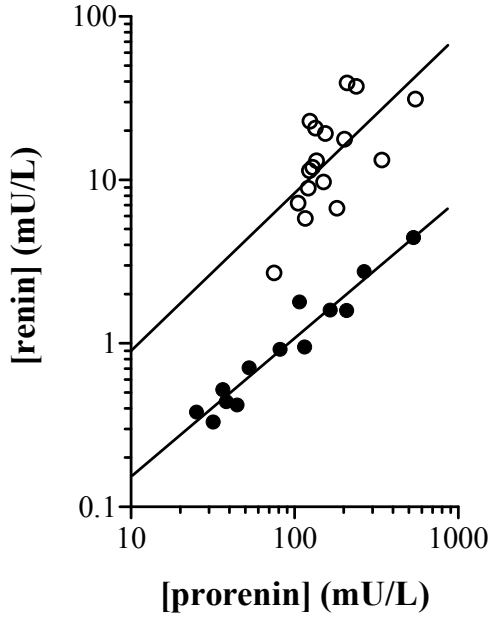

Figure 4. Relationship between renin and prorenin in healthy subjects (open symbols; log[renin]=0.96xlog[prorenin]-1.00, r=0.64, P<0.01) and anephric subjects (closed symbols; log[renin]=0.59xlog[prorenin]-0.94, r=0.97, P<0.001).

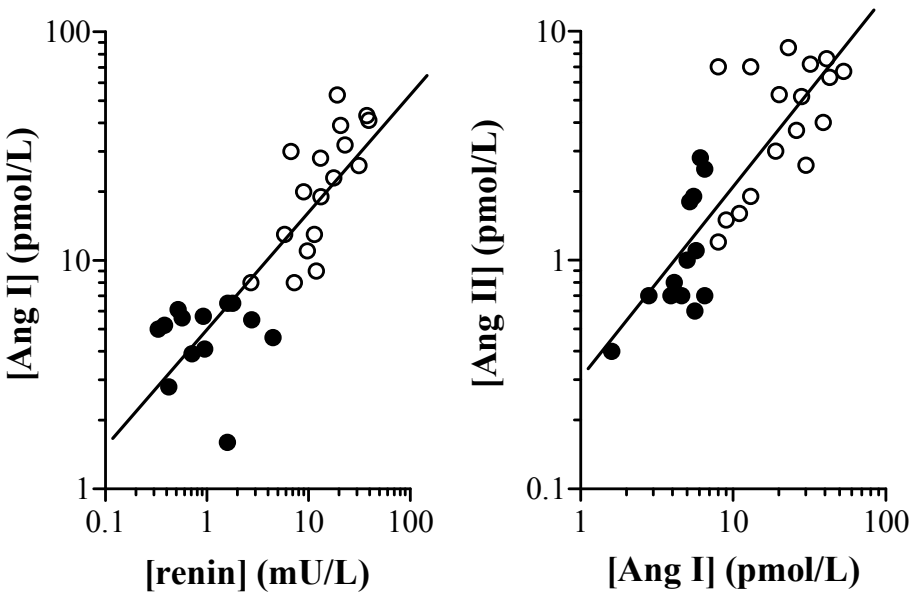

Figure 5. Relationship between renin and Ang I (left panel; log[Ang I]=0.62xlog[renin]+0.61, r=0.85, P<0.001) and between Ang I and Ang II (right panel; log[Ang II]=0.69xlog[Ang I]-0.35, r=0.75, P<0.001) in healthy subjects (open symbols) and anephric subjects (closed symbols).

Captopril reduced plasma Ang II in anephric subjects by 50-60% (P=NS) 2 hours after the dose. It did not alter the plasma levels of prorenin, renin, angiotensinogen, Ang I or total protein (Figure 6), nor did it affect blood pressure and heart rate (Figure 7).

DISCUSSION

This study demonstrates that in humans, kidney-derived renin and prorenin are not limited to the circulation. The distribution of both proteins over the body occurs relatively fast, with a half life of 0.5-1 hour, whereas their metabolism takes much longer, with a half life of several hours. The latter resembles data in cynomolgus monkeys and marmosets following infusion of prorenin.[68, 69] The elimination half life of prorenin in humans was twice as long as that of renin. This probably relates to the larger hepatic extraction of renin versus prorenin (hepatic vein/arterial concentration ratio 0.8±0.1 vs. 0.9±0.1, n=115, P<0.01; F.H.M. Derkx et al., unpublished observations). Differences in glycosylation most likely underlie these differences in hepatic extraction.[70] Since the liver blood flow corresponds with ≈25% of cardiac output (≈3 L plasma/min), it can be calculated that the hepatic clearances of renin and prorenin are 0.15 and 0.075 L/min, respectively. The AUC's of kidney-derived renin and prorenin in this study were 17.7 and 234 U/L.min. With this information it is possible to estimate the total amount of kidney-derived renin and prorenin in the body (=AUC x clearance): 3 and 18 U, respectively. This is roughly ten times the total amount of renin and prorenin in blood. Apparently, therefore, 90% of both proteins is localized outside the circulation, i.e., is present at tissue sites. This could be an underestimation, in case a significant amount of renin and prorenin is also cleared outside the liver. However, when infusing ^{125}I-labeled prorenin in rats, the radiolabeled protein reached hepatic levels that were 10 times higher than those in the kidney, while <1% of the dose accumulated in other organs, and thus, the liver is by far the most important prorenin/renin-clearing organ in the body.[68]

What might be the location of renin and prorenin outside the circulation? Both renin and angiotensinogen are known to enter the interstitium, reaching interstitial fluid levels in the rat heart that are comparable to those in blood.[53, 55, 71] Interstitial fluid amounts to ≈15% of tissue wet weight, and thus an equal distribution of renin and prorenin over the extracellular fluid space cannot fully explain total body renin and prorenin. Indeed, the tissue levels of renin (expressed per g wet weight) are often as high as its levels per mL blood,[1, 3, 65] which is impossible if tissue renin is limited to extracellular fluid and reaches concentrations in the interstitium that are identical to those in plasma. Therefore, an additional site must exist, which allows selective tissue accumulation of renin and prorenin. Nephrectomy studies in rats almost thirty years ago already suggested that arterial wall renin washout occurred more slowly than plasma renin washout, in full agreement with a 'renin binding site' in the vascular wall.[72] Two likely candidates for such binding sites are the M6P receptor,[46, 57, 58] and the recently discovered (pro)renin receptor.[47] Studies with antagonists of these receptors

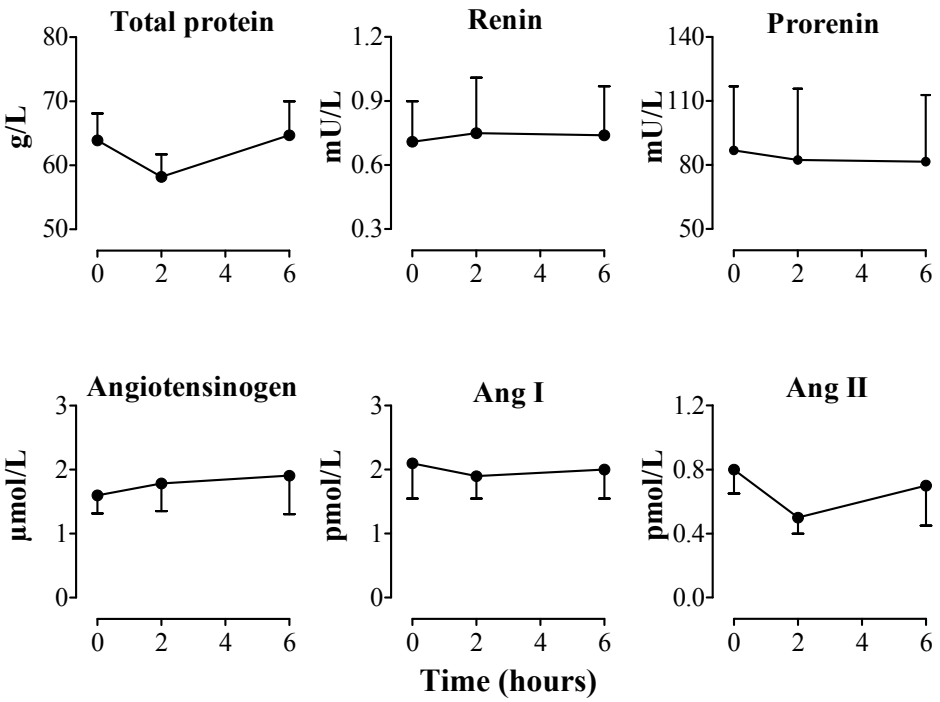

Figure 6. Plasma levels (mean+SD) of total protein, renin, prorenin, angiotensinogen, Ang I and Ang II in plasma of 5 anephric subjects given 50 mg captopril orally at t=0.

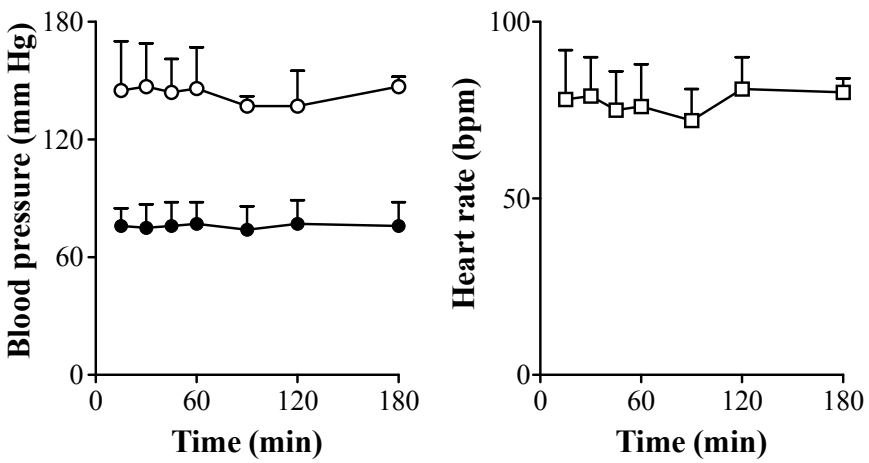

Figure 7. Systolic blood pressure (open circles), diastolic blood pressure (closed circles) and heart rate (squares) in 5 anephric subjects given 50 mg captopril orally at t=0. Data are mean+SD.

would be required to definitely settle this issue. An argument in favor of the (pro)renin receptor is that prorenin binding to this receptor allows prorenin to display enzymatic activity,[47, 48] thus explaining why the majority of 'tissue renin' is usually active. However, an alternative explanation for the latter is prorenin to renin conversion during the tissue homogenization and extraction procedure.

Importantly, Ang I and Ang II disappearance from plasma following nephrectomy closely resembled renin disappearance, suggesting that kidney-derived renin (and not prorenin) is the major determinant of circulating angiotensins. Approximately 24-48 hours after nephrectomy, the renin, Ang I and Ang II levels in plasma were identical to those in subjects who had been anephric for years, that is they were ≤10% of the levels before nephrectomy or in healthy controls. This is in full agreement with previous studies in anephric humans.[73] Prorenin did not decrease to such low levels. Its levels in anephric subjects were sometimes even in the normal range and remained stable over the years. Immunoreactive measurements confirmed that the prorenin levels measured by EKA truly resembled prorenin. Thus in humans, as has been suggested before,[74, 75] a major percentage of prorenin is of extrarenal origin. Although prorenin is enzymatically inactive, it is well-known that, depending on pH and temperature, prorenin is capable of undergoing a conformational change, involving the unfolding of the prosegment from the enzymatic cleft. Such unfolding is also known as 'non-proteolytic activation', and allows prorenin to become fully enzymatically active. Under in vitro conditions that mimick normal physiology as closely as possible (pH 7.4, 37°C), approximately 2% of prorenin is in this open conformation.[46, 48, 76] The present study shows that 2% of total renin in anephric subjects is active (as opposed to >8% in healthy controls). Moreover, 'renin' in anephric subjects correlated highly significantly with prorenin, whereas normally the renin-prorenin relationship is much less strong (Figure 4).[5] Taken together, these data suggest that 'renin' in anephric subjects is most likely open, non-proteolytically activated prorenin. Thus, the true renin levels in these subjects may actually be zero. This is in full agreement with human studies showing exclusive prorenin (and not renin) release from tissue sites,[35, 40] and animal studies showing no prorenin-renin conversion in the circulation following prorenin infusion or when overexpressing prorenin in the liver.[63, 69] A further possibility to attribute the Ang I-generating activity to prorenin is that (part of) plasma prorenin occurs in a truncated form, lacking a portion of the NH2 terminus of the prosegment.[77] Under such conditions, the prosegment will not fully cover the enzymatic cleft, and thus this truncated prorenin is enzymatically active.

The renin (=activated prorenin)-Ang I relationship in anephrics was identical to that in healthy controls, confirming that once prorenin has obtained its open conformation, its reaction with angiotensinogen is identical to that of renin. The Ang I-Ang II relationship was also normal, suggesting that the ACE levels in anephrics are not altered. Captopril tended to reduce Ang II in anephrics, but the differences were, due to the closeness of the Ang II values to the detection limit, not significant. No changes occurred in either renin or Ang I, thereby confirming the idea that the renin rise following captopril is entirely kidney-

dependent, and that the simultaneous Ang I rise is due to the rise in renin rather than its diminished conversion by ACE.[78] The same applies to the RAS component changes that normally occur after haemodialysis.[79] In contrast with a previous study,[80] we did not observe a change in blood pressure following captopril exposure in anephrics. This may relate to the difference in experimental setup: in that study captopril was applied in the fluid-depleted state, one hour after haemodialysis, whereas in the present study captopril was applied immediately prior to haemodialysis, i.e., in the fluid-repleted state. A captopril-induced blood pressure drop in anephrics might be due to a suppression of prorenin-dependent Ang II generation and/or bradykinin potentiation. To exclude the latter, similar studies should be performed in anephrics using a renin inhibitor or an AT_1 receptor antagonist.

Studies with the (pro)renin receptor blocker HRP in animals have suggested that, under pathological conditions, prorenin contributes to tissue angiotensin generation, but (virtually) not to angiotensin generation in the circulation.[50, 62] Our data support the latter, since the majority of plasma angiotensins (>90%) disappeared in parallel with plasma renin following a nephrectomy, and plasma angiotensin levels remained low in anephrics, despite the continued presence of prorenin. No conclusions can be drawn on the contribution of prorenin to tissue angiotensin generation from our current results. In rats and pigs, tissue angiotensin levels dropped to or below the detection limit following a nephrectomy.[3, 81, 82] As opposed to the situation in humans however, prorenin in these animals does not constitute 90% or more of total renin in plasma.[3, 62] Moreover, its levels dropped, at least in the pig, to almost the same degree as the renin levels after nephrectomy.[3] Thus, one explanation for the lack of tissue angiotensin following a nephrectomy in animals might be a lack of both renin and prorenin. This would imply that in animals, more than in humans, prorenin is kidney-derived. An alternative, although less likely explanation, is that only kidney-derived prorenin contributes to tissue angiotensin generation, for instance because the glycosylation pattern of non-renal prorenin does not allow its penetration (via receptor-binding?) at tissue sites. Indeed, as has been discussed, glycosylation determines (pro)renin clearance,[70] and M6P receptors do not bind non-glycosylated (pro)renin.[46, 57]

In summary, our study has revealed that kidney-derived renin and prorenin enter tissue sites in humans, and may thus contribute to local angiotensin production. A major percentage of prorenin is of extrarenal origin, and the strong correlation between ‘renin’ and prorenin in anephric subjects suggest that ‘renin’ in these subjects actually is non-proteolytically activated prorenin. Such activated, intact prorenin reacts normally with angiotensinogen, and thus, in combination with the doubled angiotensinogen levels and the unaltered ACE levels in anephric subjects, allows the generation of low levels of both Ang I and II in blood plasma following nephrectomy. To what degree prorenin also contributes to tissue angiotensin generation in humans remains to be determined.

Chapter 3

Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat VSMC's overexpressing the human (pro)renin receptor

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Based on:

Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJA, Burcklé CA, Müller DN, Bader M, Nguyen G, Danser AHJ., Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. *Journal of hypertension*. 2007 Dec; **25**(12): 2441-53.

ABSTRACT

Mannose 6-phosphate receptors (M6PR) bind both renin and prorenin, and such binding contributes to renin/prorenin clearance but not angiotensin generation. Here, we evaluated the kinetics of renin/prorenin binding to the recently discovered human (pro)renin receptor (h(P)RR), and the idea that such binding underlies tissue angiotensin generation. Vascular smooth muscle cells from control rats and transgenic rats with smooth muscle h(P)RR overexpression were incubated at 4ºC or 37ºC with human renin or prorenin. Incubation at 37ºC greatly increased binding, suggesting that (pro)renin-binding receptors cycle between the intracellular compartment and the cell surface. Blockade of the M6PR reduced binding by ≈50%. During M6PR blockade, h(P)RR cells bound twice as much prorenin as control cells, while renin binding was unaltered. Incubation of h(P)RR (but not control) cells with prorenin + angiotensinogen yielded more angiotensin than expected on the basis of the activity of soluble prorenin, whereas angiotensin generation during incubation of both cell types with renin + angiotensinogen was entirely due to soluble renin. The renin + angiotensinogen-induced vasoconstriction of isolated iliac arteries from control and transgenic rats was also due to soluble renin only. The recently proposed (P)RR antagonist HRP, which resembles part of the prosegment, blocked neither prorenin binding nor angiotensin generation. H(P)RR preferentially bind prorenin, and such binding results in angiotensin generation, most likely because binding results in prorenin activation.

INTRODUCTION

Prorenin is generally assumed to be nothing more than the inactive precursor of renin. Yet, its elevated levels in diabetes mellitus complicated by retinopathy and nephropathy suggest otherwise.[16, 83] Recently, the renal vasodilator response to captopril in diabetic subjects was reported to correlate better with plasma prorenin than with plasma renin.[84] Possibly therefore, prorenin contributes to tissue (e.g., renal) angiotensin generation. Studies in transgenic animals support this concept,[63, 85] but the underlying mechanism is still unknown. Evidence for prorenin to renin converting enzyme(s) outside the kidney is lacking. Thus, other mechanisms than proteolytic cleavage are required to understand why prorenin might contribute to tissue angiotensin production. Indeed, prorenin activation may also occur in a (reversible) non-proteolytic manner, i.e., not requiring the irreversible proteolytic cleavage step.[15] At low pH and temperature, prorenin is capable of undergoing a conformational change, involving the unfolding of the prosegment from the enzymatic cleft. Such non-proteolytically activated, 'open' prorenin is fully enzymatically active.[76, 86]

A few years ago Nguyen et al. identified a (pro)renin receptor ((P)RR) that binds both renin and prorenin.[47] Renin binding to the receptor enhanced the catalytic activity of renin 4-fold. Unexpectedly, prorenin, when bound to the receptor, displayed enzymatic activity without proteolytic removal of the prosegment. Apparently therefore, binding to the receptor induces comparable changes in the prorenin molecule as a decrease in pH or temperature. After the discovery of the receptor, a (P)RR antagonist was designed based on the idea that the prosegment contains a 'handle' region which binds to the receptor.[50] This (peptidic) antagonist (also known as 'handle region peptide', HRP) resembles the handle region, and thus will bind to the receptor instead of prorenin, thereby preventing receptor-mediated prorenin activation. In support of this concept, HRP infusion normalized the elevated renal angiotensin content in diabetic rats,[50] without affecting blood pressure.

A second receptor capable of binding renin and prorenin is the mannose-6 phosphate receptor (M6PR). M6PRs however, unlike (P)RRs, internalize renin and prorenin, and M6PR-binding of renin and prorenin has now been recognized as a clearance mechanism, not resulting in angiotensin generation.[59]

To further establish the role of the (P)RR, transgenic rats overexpressing the human (P)RR (h(P)RR) in vascular smooth muscle cells (VSMCs) have been created.[61] These rats display increased prorenin binding to vascular tissue and develop a cardiovascular phenotype with elevated systolic blood pressure and an augmentation in heart rate at an age of 7 months.

In the present study, aortic VSMCs were collected from these transgenic rats and their control littermates, to quantify the kinetics of renin and prorenin binding to the h(P)RR, and to investigate the idea that such binding underlies tissue angiotensin generation.

METHODS

Cell culture

All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the Guiding Principles of the American Physiological Society. Primary cultures of VSMCs were prepared from aortas of 6-week old transgenic rats with smooth muscle cell expression of the h(P)RR[61] and their control littermates.[87] In short, VSMCs were isolated from the aorta, plated and maintained at 37°C in a humidified 5% CO_2 incubator in supplemented SmBM-2 medium (Cambrex) containing 10% FBS. Cells were cultured to confluence in a 75 cm^2 flask (in supplemented SmBm-2 medium; passage 3-8), trypsinized and seeded into 12- or 24-well plates using the above medium. This yielded a confluent monolayer of ≈4 x 10^5 cells/cm^2 after 3 days. Before the start of the experiment the cells were cultured for 24 hours under serum-free conditions.

Human (pro)renin receptor expression

To verify whether h(P)RR overexpression had increased the (P)RR level in h(P)RR VSMCs, control and h(P)RR cells were lysed using Nonidet P-40 lysisbuffer and kept on ice for 15 minutes. Next, the cell lysates were centrifuged at 14000 g for 15 minutes at 4°C. Supernatants were collected and stored at -20°C until further analysis. Western blotting was performed with 20 µg of protein using antibody 1623 (diluted 1:1000), which recognizes the N-terminal domain of both the rat and human (P)RR. A peroxidase-conjugated antibody (goat anti-rabbit, 1:5000) was used to visualize the receptor protein.

Prorenin and renin binding kinetics

To study the time- and concentration-dependency of renin and prorenin binding, as well as the contribution of M6PRs and (P)RRs to this process, cells were incubated at 4°C or 37°C with 0-1200 U/L recombinant human prorenin or renin (a kind gift of Dr. S. Mathews, Hoffmann-LaRoche, Basel, Switzerland) for maximally 6 hours in the absence or presence of 10 mmol/L M6P (to block M6PR) and/or rat HRP (RILLKKMPSV-OH, to block rat (P)RR) or human HRP (IFLKRMPSI-OH, to block h(P)RR) (Biosyntan, Berlin, Germany; 1 nmol/L-1µmol/L). At the end of the incubation period the culture medium was removed. Each well was washed three times with 1 mL ice-cold PBS before the cells were lysed with ice-cold 0.2 % triton X-100 in PBS. Cell lysates were quickly frozen in liquid N2 and stored at -70°C. To investigate the intracellular presence of the (P)RR, incubations with prorenin were also performed after cell permeabilization with PBS containing 0.2 % saponin.

Angiotensin generation

To study whether (P)RR-binding of renin and prorenin results in facilitated angiotensin (Ang) I generation, cells were incubated at 37°C for maximally 4 hours with 150 nmol/L human angiotensinogen and 100 U/L human prorenin or 3-30 U/L human renin, in the absence or presence of 1 µmol/L HRP. Cells incubated without angiotensinogen, renin or prorenin served as control. Ang I generation under the above conditions was also monitored in the absence of cells, in order to correct for the enzymatic activity of soluble renin and prorenin. The first order rate constant for Ang I elimination was determined by incubating the cells with 100 nmol/L Ang I for maximally 4 hours. Medium samples (50 µL) were collected at 0, 1 and 4 hours, mixed with 2.5 µL inhibitor stock solution (containing 0.1 mmol/L aliskiren (a kind gift of Novartis Pharmaceuticals, Basel, Switzerland), 200 mmol/L EDTA and 0.2 mmol/L lisinopril), frozen in liquid N2, and stored at -70°C. Cells were collected and stored as described before.[57]

Functional studies

To study whether renin-induced, Ang II-mediated vasoconstriction involves Ang I generation by membrane-bound renin, iliac arteries were removed from 7-9-month old h(P)RR transgenic rats and their controls, and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. Artery segments (diameter ≈500 µm) were then mounted in Mulvany myographs (J.P. Trading) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2, and maintained at 37°C. Tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure as described by Mulvany and Halpern.[88] To determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid containing 10 µmol/L L-NAME for 30 min and exposed to Ang I (1 nmol/L-1 µmol/L), Ang II (1 nmol/L-1 µmol/L) or 100 U/L renin + 1 µmol/L angiotensinogen, with or without 0.1 µmol/L aliskiren. To monitor Ang I and/or II formation, 50 µL organ bath fluid was collected 10 minutes (i.e., at the time of maximum constriction) after the addition of Ang I or renin + angiotensinogen. The samples were rapidly mixed with 2.5 µL inhibitor solution and frozen in liquid N_2.

Due to the the low intrinsic activity of prorenin, combined with the fact that the vascular segments bind only a small fraction (<5%) of prorenin in organ bath fluid,[61] the Ang II generation in the organ bath setup at 100 U/L prorenin would be too low to induce vasoconstriction, even under conditions where all bound prorenin is active. Using substantially higher prorenin concentrations might solve this problem, but, given the relatively large volume of the organ bath, such concentrations are beyond our current stock. Therefore, we were unable to perform organ bath studies with prorenin.

Biochemical measurements

Renin and cell-activated prorenin were measured by enzyme kinetic assay (EKA).[46] Total (i.e., cell-activated plus nonactivated) prorenin was also measured by EKA, after prorenin had been converted to renin by incubation of the sample for 48 hours at 4°C with plasmin (0.5 caseinolytic U/mL). Protein content was determined according to Bradford. Ang I and II levels in medium were measured by radioimmunoassay (detection limit 40 and 20 fmol/mL, respectively). Ang I and II levels in cell homogenates were measured by radioimmunoassay after SepPak extraction and reversed-phase high-performance liquid chromatography separation (detection limit 4 and 2 fmol/10^6 cells, respectively).

Data analysis

Results are expressed as mean±SEM. Contractile responses are expressed as a percentage of the constriction to 100 mmol/L KCl. Angiotensin concentration-response curves (CRCs) were analyzed as described to obtain pEC_{50} ($^{10}logEC_{50}$) values.[89] Statistical analysis was performed using one-way or two-way ANOVA where appropriate. $P<0.05$ was considered significant.

RESULTS

Human (pro)renin receptor expression

Western blot analysis confirmed (P)RR protein expression in VSMCs at the expected size of 39 kD (Figure 1).[47] Expression was ≈3-fold higher in h(P)RR-overexpressing VSMCs than in control cells.

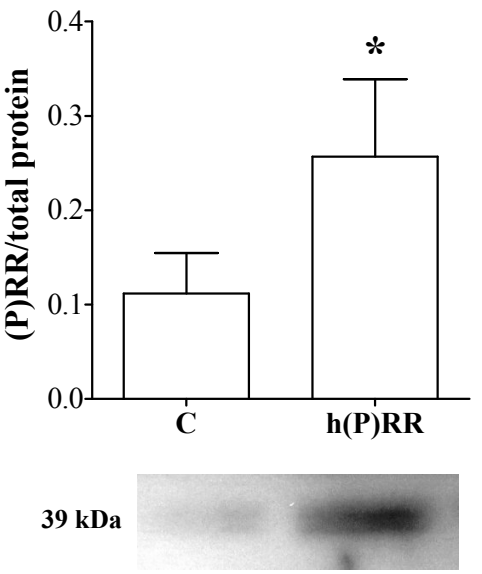

Figure 1. Quantification of (P)RR protein in control- and h(P)RR VSMCs. Western blotting with antibody 1623 revealed the expected (P)RR band at 39 kDa, with a higher expression in h(P)RR cells. Detected (P)RR protein (mean±SEM of 6 measurements) is expressed relative to total protein. *P<0.05 vs. control

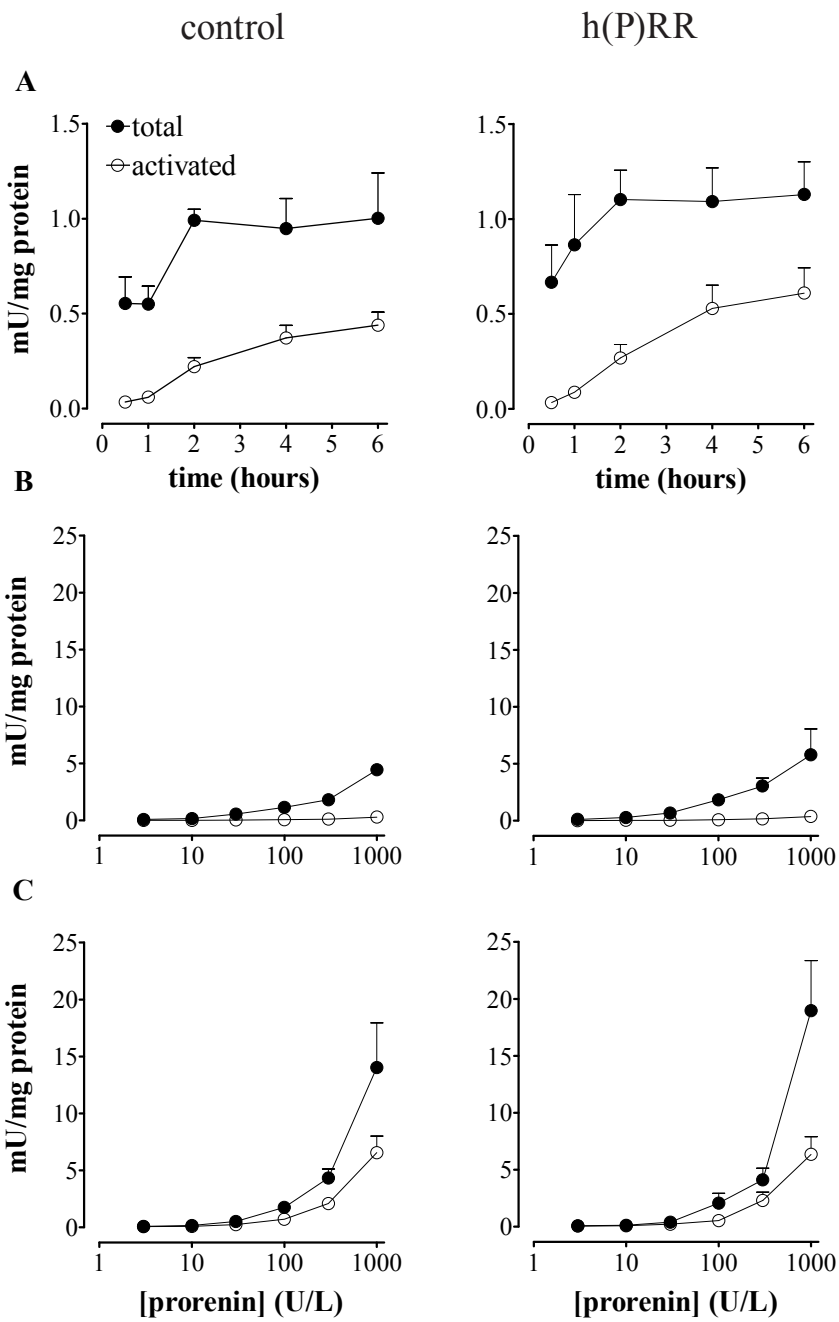

Figure 2. A, time-dependent increase in the levels of cell-associated total and activated prorenin during incubation of the cells at 37°C with 100 U/L prorenin. **B** and **C**, levels of cell-associated total and activated prorenin after incubation of the cells at 4°C (B) or 37°C (C) with 0-1000 U/L prorenin for 4 hours. Data (mean±SEM of 3-5 experiments) are expressed per mg protein. Left panels, control VSMCs, right panels, h(P)RR VSMCs.

Prorenin and renin binding kinetics

When VSMCs were incubated with 100 U/L prorenin at 37°C, the level of cell-associated total prorenin increased in both control and h(P)RR VSMCs in a time-dependent manner (Figure 2A; n=3 for both), plateau levels being reached after 2 hours. Cell-association of prorenin resulted in its activation. For both cell types, the levels of cell-associated activated prorenin at 6 hours did not differ from those at 4 hours. This suggests that the levels of cell-associated activated prorenin had reached a plateau after 4 hours. On the basis of these data, an incubation period of 4 hours was chosen for all further binding studies.

VSMCs bound prorenin in a concentration-dependent manner, both at 4°C (Figure 2B; n=5) and 37°C (Figure 2C; n=5). Activation of prorenin did not occur at 4°C. The levels of cell-associated total prorenin were ≈3-4-fold higher at 37°C than at 4°C. This suggests that prorenin binding at 37°C depends on recycling of membrane receptors and/or intracellular receptors.[46] In agreement with the latter concept, cell permeabilization increased prorenin binding at 4°C 3-4-fold (P<0.05), whereas no change occurred at 37°C (Figure 3; n=5).

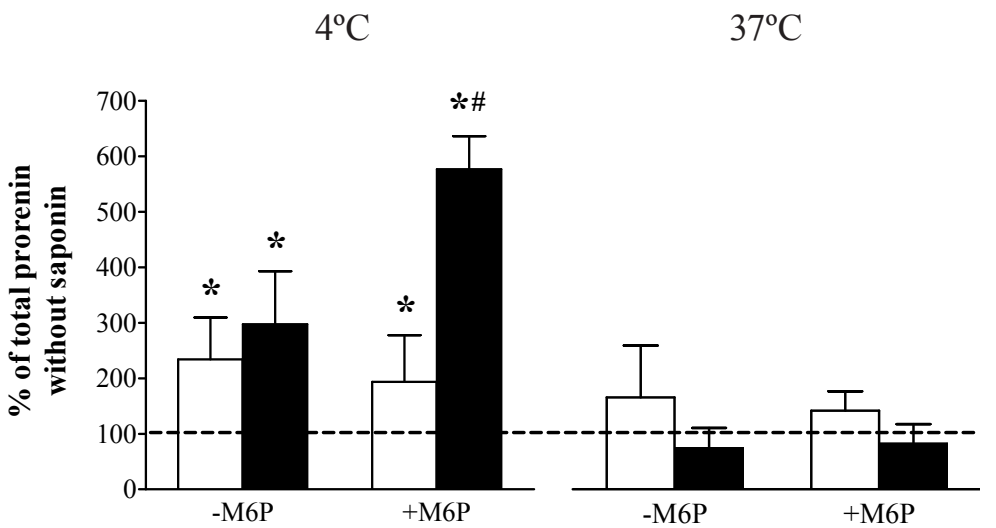

Figure 3. Levels of cell-associated total prorenin in cells permeabilized with 0.2% saponin after their incubation at 4°C (**left panel**) or 37°C (**right panel**) with 1000 U/L prorenin for 4 hours in the absence or presence of 10 mmol/L M6P. Data (mean±SEM of 5 experiments) are expressed as a percentage of the total prorenin level without prior permeabilization (dotted line). Open bars, control VSMCs, closed bars, h(P)RR VSMCs. *P<0.05 vs. 100%, #P<0.05 vs. control. Please note that, although M6P reduced prorenin binding at 37°C but not at 4°C (see also Figure 4), it did not block the effect of saponin. This indicates that the majority of the prorenin-binding receptors (M6PR and non-M6PR) in this study is located intracellularly. At 4°C, these intracellular receptors can only be reached by prorenin after cell permeabilization, whereas at 37°C they are reached equally well with and without permeabilization, most likely because at this temperature the receptors cycle between the intracellular compartment and the cell surface.

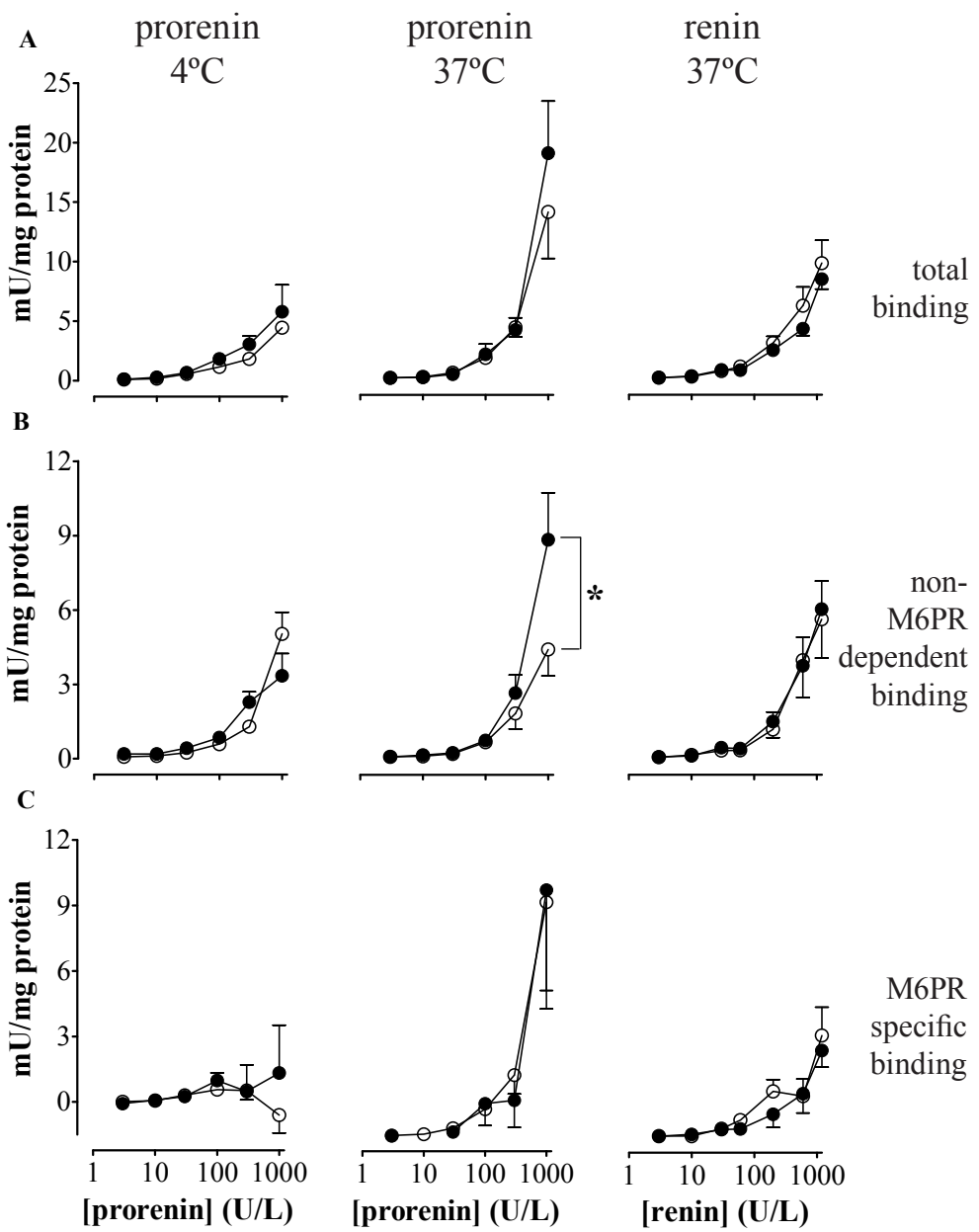

Figure 4. **A**, levels of cell-associated total prorenin or renin after incubation of VSMCs at 4°C or 37°C with 0-1200 U/L prorenin or renin for 4 hours. **B**, levels of cell-associated total prorenin or renin after incubation of VSMCs at 4°C or 37°C with 0-1200 U/L prorenin or renin for 4 hours in the presence of 10 mmol/L M6P. **C**, M6P receptor (M6PR)-specific binding (calculated from the difference between A and B) of total prorenin and renin after incubation of VSMCs at 4°C or 37°C with 0-1200 U/L prorenin or renin for 4 hours. Data (mean±SEM of 5 experiments) are expressed per mg protein. Open symbols, control VSMCs, closed symbols, h(P)RR VSMCs. *P<0.01, control vs. h(P)RR.

At 37°C, after incubation with the highest prorenin concentration in the medium (1000 U/L), the levels of cell-associated total prorenin were ≈25% higher in h(P)RR VSMCs than in control VSMCs (Figure 4A, middle panel; P=0.07). Parallel experiments performed in the presence of M6P (Figure 4B) revealed that this difference was not related to the M6PR, since a) M6PR-specific prorenin binding (calculated from the difference between the levels of cell-associated total prorenin with and without M6P; Figure 4C) was identical in both cell types, and b) the difference between the two cell types increased to ≈2-fold (P<0.01) in the presence of M6P (Figure 4B, middle panel). Thus, the consequences of h(P)RR overexpression could only be observed during M6PR blockade.

Importantly, no differences in prorenin binding were observed between the two cell types at 4°C (Figure 4A), and binding at this temperature was not significantly affected by M6P. Cell permeabilization at 4°C increased prorenin binding to h(P)RR VSMCs in the presence of M6P ≈6 times (as compared to ≈2 times in control VSMCs, P<0.05), thereby indicating that the majority of the h(P)RRs was located intracellularly (Figure 3).

Finally, renin binding at 37°C was identical in both cell types, and equaled prorenin binding in control VSMCs. Renin binding involved both M6PR- and M6PR-independent mechanisms (Figure 4C). Based on these data we conclude that, at least under our experimental conditions, renin did not bind to the h(P)RR, and that in the absence of the h(P)RR overexpression, renin and prorenin display a similar binding pattern. Apparently, only prorenin binds to the h(P)RR in cells expressing this receptor. Using the double reciprocal plot approach, assuming that the difference in prorenin binding between control- and h(P)RR VSMCs represents prorenin binding to the h(P)RR, the Kd for the binding of prorenin to its human receptor was estimated to be 469±131 U/L (or 5.9±1.6 nmol/L). The number of h(P)RRs/cell (Bmax) was ≈5000. When calculating the Kd (range 0.5-1.4 nmol/L, mean 1.1 nmol/L) and Bmax (range 4000-8000 sites/cell, mean ≈7500 sites/cell) for the M6P receptor-specific binding, the double reciprocal plot approach yielded identical values for renin and prorenin in control and h(P)RR cells.

Unexpectedly, on top of 10 mmol/L M6P, neither rat HRP (n=8) nor human HRP (n=7) affected prorenin binding, prorenin activation or renin binding in our experimental setup (Figure 5A, B). HRP (1 μmol/L) did not affect renin-induced Ang I generation in the EKA (data not shown).

Despite the increased total prorenin binding to h(P)RR VSMCs at 37°C, the level of cell-associated activated prorenin was identical in both cell types (Figure 2). Immunoradiometric measurements of renin confirmed this result (data not shown). Consequently, the percentage of cell-associated total prorenin that was activated tended to be lower in h(P)RR cells (36±11%) than in control cells (50±4%) (P=NS).

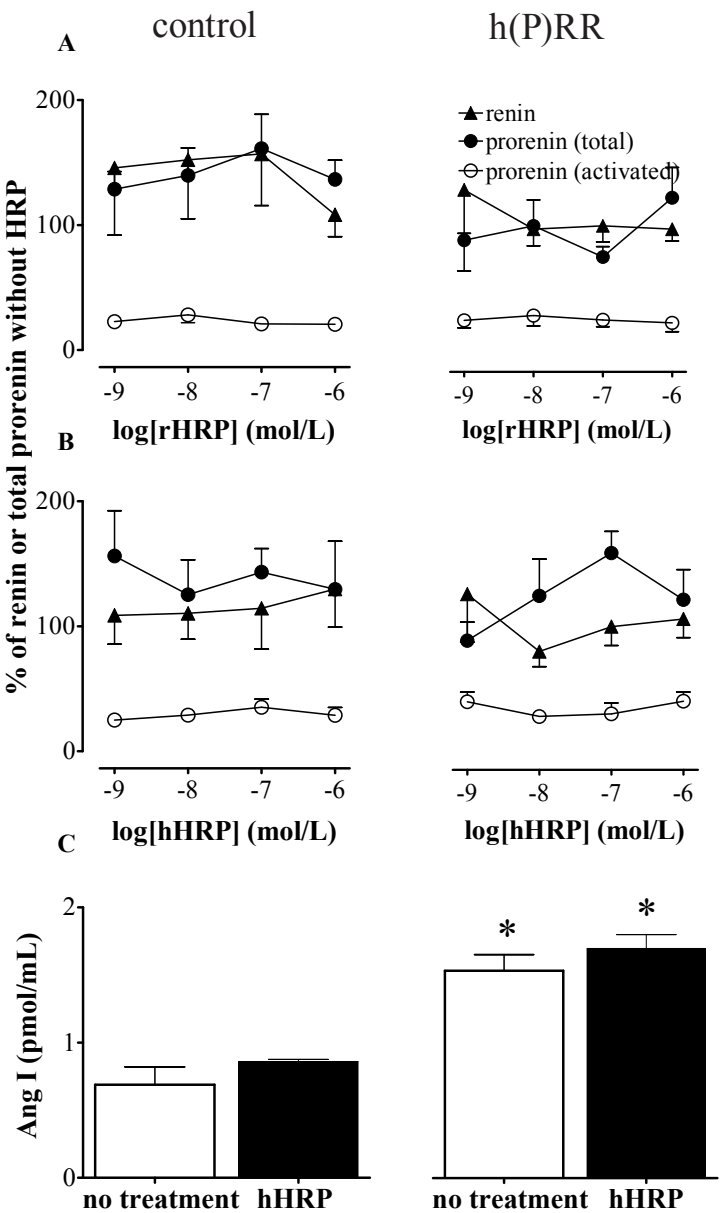

Figure 5. A and **B**, levels of cell-associated total prorenin, activated prorenin and renin after incubation of VSMCs at 37°C with 1000 U/L prorenin or renin for 4 hours in the presence of increasing concentrations of rat HRP (rHRP, **A**) or human HRP (hHRP, **B**). M6P (10 mmol/L) was present in all experiments. Prorenin and renin data (mean±SEM of 7-8 experiments) are expressed as a percentage of the levels of total prorenin and renin, respectively, in the absence of HRP. Left panels, control VSMCs, right panels, h(P)RR VSMCs. **C**, medium Ang I levels following a 4-hour incubation of VSMCs with 150 nmol/L angiotensinogen and 100 U/L prorenin at 37°C in the absence (no treatment) or presence of 1 µmol human HRP. Data are mean±SEM of 3 experiments. Left panels, control VSMCs, right panels, h(P)RR VSMCs. *P<0.05 vs. control cells.

Angiotensin generation

Figure 6 shows the Ang I and II levels in the medium following incubation of control and h(P)RR VSMCs at 37°C with angiotensinogen and prorenin (100 U/L) or renin (3, 10 or 30 U/L) for 0, 1 and 4 hours. Levels measured in the absence of angiotensinogen, renin or prorenin were at or below the detection limit. With prorenin, the medium Ang I levels of h(P)RR VSMCs after 1 hour and 4 hours of incubation were ≈3-fold higher than those of control VSMCs ($P<0.001$, n=4). For Ang II, a similar pattern was observed after 4 hours, although no significance was reached ($P=0.2$). Cellular Ang II levels after 4 hours of incubation with prorenin + angiotensinogen amounted to 3±1 and 10±2 fmol/well in control and h(P)RR VSMCs, respectively (P=NS). This amount corresponds with ≈5% of the total amount of Ang II in the medium. Cellular Ang I levels after 4 hours of incubation were 7±2 and 19±4 fmol/well ($P<0.01$). This amount corresponds with <1% of the total amount of Ang I in the medium.

With renin, the medium Ang I and II levels increased in both cell types in a similar manner (n=6). The levels reached after 1 hour of incubation clearly correlated with the renin concentration, but this relationship was no longer visible after 4 hours of incubation, due to the fact that after that time the angiotensinogen concentration in the medium had become too low to sustain the same degree of Ang I generation as after 1 hour of incubation.

To evaluate whether expression of the h(P)RR enhanced Ang I generation by prorenin and/or renin, experiments were also performed in the absence of cells. Without cells, 100 U/L prorenin, 3 U/L renin, 10 U/L renin and 30 U/L renin generated 2.2, 5.4, 17.3 and 34.5 pmol Ang I/mL per hour ('Ang I-generating capacity'; mean of 2 measurements each). From these data it can be calculated that 1.5±0.2% of prorenin displayed enzymatic activity. The first order rate constants for Ang I elimination (kel) were 0.8±0.2 and 0.9±0.2 min^{-1}, respectively (n=6), in control and h(P)RR cells (data not shown). Using the Ang I-generating capacity and the kel, the medium Ang I level during incubation with renin/prorenin and angiotensinogen was predicted as follows:

$$[\text{Ang I}]_{\text{predicted}} = [\text{Ang I-generating capacity}]/k_{el} \times [1-\exp(-k_{el} \times t]).$$

Figure 7 compares the predicted and measured medium Ang I levels after 1 hour of incubation. With renin, the predicted Ang I levels were identical to the measured levels. This was also the case for prorenin when incubated with control cells. For prorenin incubated with h(P)RR cells however, the measured Ang I levels were ≈2 times higher than the predicted Ang I levels. Apparently therefore, the presence of the h(P)RR doubled prorenin activity. Human HRP (1 μmol/L) did not affect the amount of Ang I generated by prorenin + angiotensinogen in control or h(P)RR VSMCs (Figure 5C; n=3).

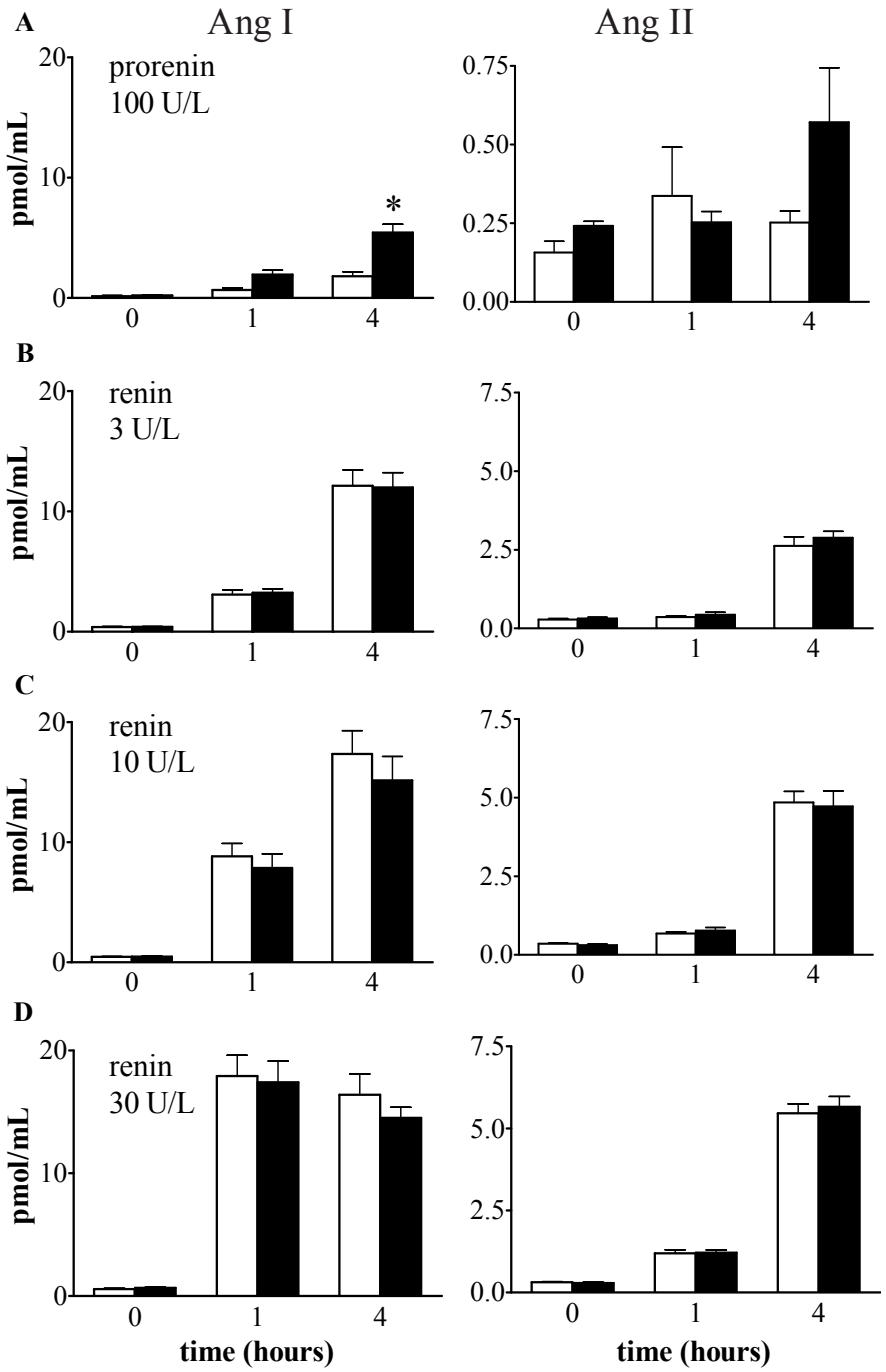

Figure 6. Medium Ang I and II levels following incubation of VSMCs with 150 nmol/L angiotensinogen and 100 U/L prorenin (**A**), 3 U/L renin (**B**), 10 U/L renin (**C**) or 30 U/L renin (D) at 37°C after 0, 1 and 4 hours. Data are mean±SEM of 4-6 experiments. Open bars, control VSMCs, closed bars, h(P)RR VSMCs. *P<0.001 vs. control cells.

Functional studies

Ang I and II constricted iliac artery segments obtained from 4 control rats in a concentration-dependent manner (pEC_{50} 7.6±0.8 and 7.8±0.5, respectively; E_{max} 104±40% and 108±29%, respectively; Figure 8A). Ang I and II CRCs in iliac artery segments of 5 h(P)RR transgenic rats were indistinguishable (pEC_{50} 7.5±0.6 and 7.7±0.4, respectively; E_{max} 61±29% and 96±25%, respectively; Figure 8B) from those in control rats. The contraction induced by

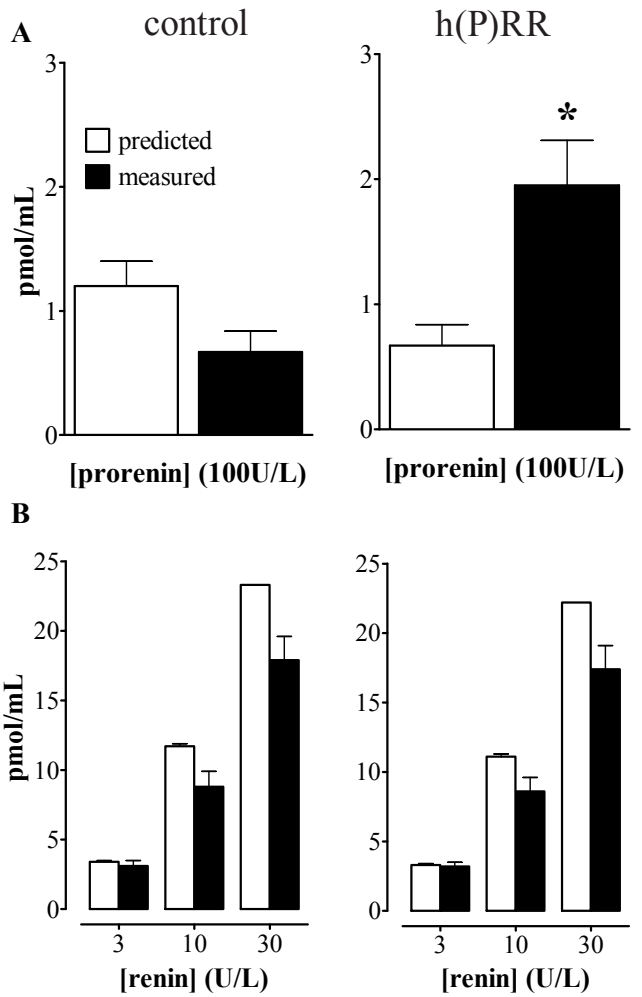

Figure 7. **A**, measured versus predicted medium Ang I levels following a 1-hour incubation of VSMCs with 150 nmol/L angiotensinogen and 100 U/L prorenin. **B**, measured versus predicted medium Ang I levels following a 1-hour incubation of VSMCs with 150 nmol/L angiotensinogen and 3, 10 or 30 U/L renin. Data are mean±SEM of 4-6 experiments. Left panels, control VSMCs, right panels, h(P)RR VSMCs. Please note that only in h(P)RR VSMCs incubated with prorenin, the measured levels were in all cases higher than the predicted levels. In all other situations, the measured levels were identical or lower than the predicted levels. *, measured level was higher than predicted level in all cases.

renin (100 U/L) + angiotensinogen (1 µmol/L) amounted to 41±20% and 28±25% in control and h(P)RR transgenic rats, respectively (Figure 8C; P=NS). Aliskiren fully prevented this contraction (P<0.01). Renin alone did not exert an effect. The Ang II levels in the organ bath fluid at the time of constriction following Ang I application amounted to <5% of the Ang I levels. Thus, for a given contraction, the organ bath fluid Ang II levels during Ang I exposure were 50-100 times lower than those during Ang II (<0.01). This relates to the fact that vasoconstriction is determined by tissue Ang II and not Ang II in the organ bath.

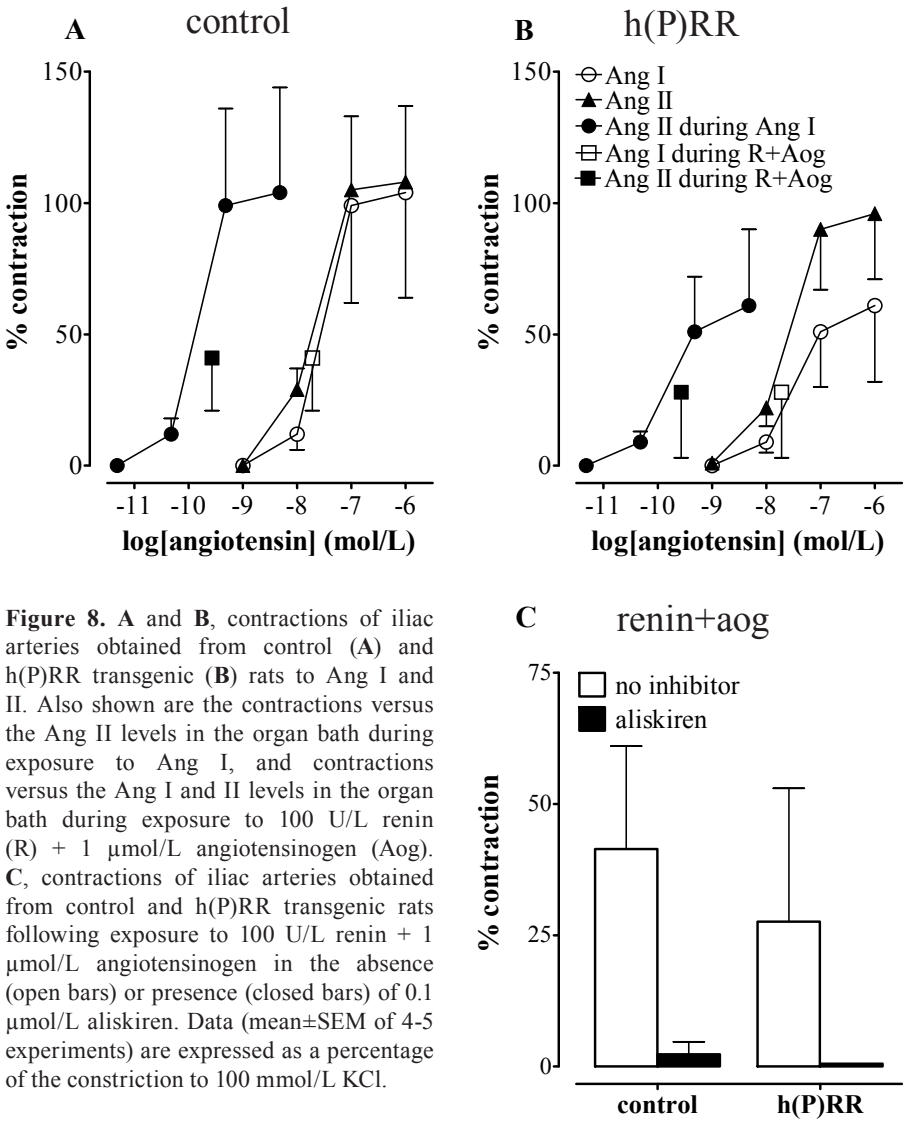

Figure 8. **A** and **B**, contractions of iliac arteries obtained from control (**A**) and h(P)RR transgenic (**B**) rats to Ang I and II. Also shown are the contractions versus the Ang II levels in the organ bath during exposure to Ang I, and contractions versus the Ang I and II levels in the organ bath during exposure to 100 U/L renin (R) + 1 µmol/L angiotensinogen (Aog). **C**, contractions of iliac arteries obtained from control and h(P)RR transgenic rats following exposure to 100 U/L renin + 1 µmol/L angiotensinogen in the absence (open bars) or presence (closed bars) of 0.1 µmol/L aliskiren. Data (mean±SEM of 4-5 experiments) are expressed as a percentage of the constriction to 100 mmol/L KCl.

A similar pattern might be expected for Ang I in organ bath fluid following the application of renin and angiotensinogen, provided that renin generates Ang I at tissue sites, e.g., after binding to the (P)RR. However, the organ bath fluid Ang I (and II) levels present during the contraction observed following renin and angiotensinogen application were identical to the organ bath fluid Ang I (and II) levels present in the organ bath at the same level of contraction following Ang I application. This finding argues against the idea of receptor-bound renin as a contributor to tissue Ang I generation, both in control- and transgenic rats. The organ bath fluid Ang I and II levels in the presence of aliskiren were below the detection limit (data not shown).

DISCUSSION

This study, making use of rat VSMCs overexpressing the h(P)RR, shows that the h(P)RR preferentially binds prorenin, and that prorenin binding to this receptor is associated with enhanced Ang I generation. Unexpectedly, the previously reported (P)RR antagonist, HRP,[50] did not block prorenin binding or prorenin-induced Ang I generation in h(P)RR VSMCs.

Rat VSMCs express M6PRs in comparable quantities (≈7500 sites/cell) as rat cardiomyocytes, rat fibroblasts, and human endothelial cells.[46, 57] These receptors bind and internalize both human renin and prorenin, as confirmed in the present study by the decrease in renin and prorenin binding in the presence of M6P. M6PRs largely occur intracellularly (in Golgi and endosomes),[46] and at 37°C, but not at 4°C, they cycle between the cell surface and the intracellular compartment. This explains why M6PR-mediated renin and prorenin uptake is particularly apparent at 37°C. Both renin and prorenin bind with high affinity (Kd≈1 nmol/L)[46, 57] to the M6PR, due to the fact they possess multiple M6P moieties, allowing them to interact with both M6P binding sites of the receptor.[90]

Excess M6P did not fully suppress renin and prorenin binding. This indicates that renin and prorenin also accumulate in VSMCs in a non-specific manner and/or via binding to a second, low-affinity receptor, like the rat (P)RR. The lack of effect of the rat (P)RR antagonist, rat HRP, argues against the latter. Overexpression of the h(P)RR did not affect M6PR-mediated binding of renin or prorenin, but greatly enhanced the M6PR-independent binding of prorenin at 37°C. The difference vs. non-h(P)RR- expressing cells was particularly apparent at high prorenin levels, and the Kd for this non-M6PR binding site was estimated to be ≈6 nmol/L. This value is in close agreement with the value calculated by Nabi et al. (8.0 nmol/L)[60] for the interaction between human prorenin and the recombinant h(P)RR, and with the Kd proposed by Nguyen[47] for the interaction between the h(P)RR and human renin (5.0-7.8 nmol/L). This observation, together with the increased (P)RR protein levels in h(P)RR VSMCs (Figure 1), strongly supports the idea that the enhanced prorenin accumulation is due to binding to the h(P)RR. Since such binding did not occur at 4°C, it appears that the majority of the (P)RRs is not located on the cell surface, but rather present intracellularly. Indeed, previous western hybridization studies in cardiomyocytes indicated

predominant (P)RR expression in the cytosol/microsome fraction.[51] The intracellular location of the receptor was not further evaluated in the present study.

Overexpression of the h(P)RR in VSMCs did not result in a significant increase in renin binding. Since several previous studies have already shown that human renin, at concentrations of 10-100 nmol/L,[47, 91] exerts effects via this receptor, the most likely explanation of our observation is that renin binds with lower affinity to the receptor than prorenin. Indeed, according to Nabi et al.,[60] the Kd of the h(P)RR for renin is 20 nmol/L, i.e., 3 times higher than our Kd for prorenin (and also 3-4 times higher than the Kd reported by Nguyen et al.).[47] Such a high Kd would be in agreement with the fact that even the highest renin concentration tested in our experimental setup (1200 U/L or ≈25 nmol/L) did not result in clearly distinguishable binding to the h(P)RR. We did not test higher renin concentrations, since a renin concentration of 25 nmol/L is already more than 4 orders of magnitude above the normal levels of renin in blood plasma (0.5 pmol/L).[5] For prorenin, the h(P)RR-induced increase in binding became apparent at concentrations of 300 U/L and higher. Although the prorenin levels in blood are approximately 10 times higher than those of renin, even a prorenin concentration of 300 U/L is still 3 orders of magnitude above the normal plasma prorenin levels. Thus, significant prorenin binding to the h(P)RR in vivo will most likely only occur at tissue sites where prorenin is produced locally, i.e., in the kidney, ovaries, testis, adrenal and eye.[19, 23, 92, 93] Studies in the kidney already support the idea of such prorenin-induced tissue angiotensin generation.[50] The high renin concentrations that would be required to induce (P)RR-dependent effects probably also only exist in the kidney.

Renin binding to the h(P)RR has been reported to induce a 4-fold increase of the catalytic efficiency of angiotensinogen-Ang I conversion, whereas prorenin became fully activated in a non-proteolytic manner.[47] We tested the increased catalytic activity of renin by quantifying Ang I and II generation during the incubation of VSMCs with angiotensinogen and renin, and during the constriction of rat iliac arteries when exposed to renin + angiotensinogen. Both with and without h(P)RR overexpression, Ang I generation by soluble renin was sufficient to fully explain the measured Ang I levels. Aliskiren blocked this generation, indicating that renin was the only Ang I-generating enzyme. Importantly, no evidence was obtained supporting the idea that the renin-induced, Ang II-dependent vasoconstriction involved Ang I generation by membrane-bound renin. At the same time, the well-established Ang I-II conversion at tissue sites did underlie the vasoconstriction induced by Ang I and renin + angiotensinogen.[2, 94]

Ang I and II generation also occurred during incubation of VSMCs with prorenin and angiotensinogen. In control VSMCs, this angiotensin generation could be fully explained on the basis of the activity of soluble prorenin. This activity is due to the fact that the prosegment is capable of unfolding from the enzymatic cleft, in a temperature- and pH-dependent manner.[15] Under physiological conditions (37°C, pH=7.4), <2% of prorenin displays catalytic activity. The 1.5% noted in this study is exactly within this range.

However, this activity was insufficient to explain the Ang I (and II) levels generated by VSMCs expressing the h(P)RR. Since these levels were <2 times higher than the levels in the medium of control cells or the levels expected on the basis of the 1.5% activity of soluble prorenin, it appears that the presence of the h(P)RR has resulted in a doubling of the enzyme's activity.

Previous studies have shown that prorenin binding to the M6PR results in internalization of the M6PR-prorenin complex, after which prorenin is rapidly activated in a proteolytic manner.[46, 56, 57] This conversion to renin did not result in either intra- or extracellular angiotensin generation, and therefore it was concluded that the activation step was part of a clearance process.[57] Indeed, cell-associated renin has been reported to be degraded intracellularly without being released back to the medium.[46, 56, 57] A significant percentage of cell-associated prorenin in the present study was activated (i.e., converted to renin), as expected on the basis of the M6PR-mediated internalization and activation of prorenin. The level of cell-associated activated prorenin was identical in control and h(P)RR VSMCs. This is not surprising in view of the identical number of M6PRs in both cell types. Thus, to explain the increased catalytic activity of prorenin in h(P)RR VSMCs, a mechanism other than M6PR-mediated proteolytic prorenin-renin conversion is required. Since prorenin binding to the (P)RR has been suggested to result in a conformational change, allowing prorenin to become enzymatically active in a non-proteolytic manner,[47] the most likely explanation of our data is that (P)RR binding of prorenin has indeed resulted in non-proteolytic activation. Unfortunately, cell lysis with triton X-100 (as applied in this study to collect cell lysates) destroys the delicate balance between inactive and non-proteolytically activated prorenin, thereby not allowing us to demonstrate this concept by EKA. The low cellular Ang I levels however, corresponding with <1% of the total amount of Ang I in the medium, fully support cell surface Ang I generation.

Given the higher affinity of prorenin (vs. renin) for the h(P)RR, it seems reasonable to assume that the prosegment facilitates binding, although this is clearly not the only binding determinant. Thus, we were surprised to see that even a 1 μmol/L concentration of the (P)RR antagonist HRP, which was designed on the basis of the prosegment of human prorenin,[50] affected neither prorenin binding nor Ang I generation. Apparently, HRP is either a relatively weak antagonist, requiring concentrations >1 μmol/L to exert blocking effects under our experimental conditions, or the in-vivo angiotensin-reducing effects that have been reported previously with this drug[50, 62] are exerted in an alternative, as yet unknown manner, not (only) involving the (P)RR.

In summary, the h(P)RR preferentially binds prorenin, suggesting that this inactive precursor of renin is its endogenous agonist. Prorenin binding resulted in Ang I generation, most likely because bound prorenin becomes activated non-proteolytically. Since binding occurred with low affinity, prorenin levels that are sufficiently high to allow (P)RR-dependent angiotensin generation are likely to occur only in tissues that synthesize prorenin locally, like the kidney and the adrenal. This could explain why HRP reduced

renal but not circulating Ang II in rats in vivo,[50] and why h(P)RR overexpression resulted in glomerulosclerosis and elevated aldosterone levels.[61, 95, 96] The lack of effect of HRP as a (P)RR antagonist in the present study warrants further investigations into the binding epitopes of prorenin. Finally, renin inhibitors, which are now being introduced in the clinical arena, will block the enzymatic activity of non-proteolytically activated prorenin to the same degree as that of renin. Therefore, these drugs are expected to suppress prorenin-induced tissue angiotensin production, particularly under conditions where prorenin levels are increased, e.g., in diabetic subjects with microvascular complications[83] and during RAS blockade.[5]

Chapter 4
Cardiac renin levels are not influenced by the amount of resident mast cells

Based on:

Krop M, van Veghel R, Garrelds IM, de Bruin RJ, van Gool JM, van den Meiracker AH, Thio M, van Daele PL, Danser, AH. Cardiac renin levels are not influenced by the amount of resident mast cells. *Hypertension* 2009 Aug; **54**(2): 315-21.

ABSTRACT

To investigate whether mast cells release renin in the heart, we studied renin and prorenin synthesis by such cells, using the human mast cell lines HMC-1 and LAD2, and fresh mast cells from mastocytosis patients. We also quantified the contribution of mast cells to cardiac renin levels in control and infarcted rat hearts. HMC-1 cells contained and released angiotensin I-generating activity, and the inhibition of this activity by the renin inhibitor aliskiren was comparable to that of recombinant human renin. Prorenin activation with trypsin increased angiotensin I-generating activity in the medium only, suggesting release but not storage of prorenin. The adenylyl cyclase activator forskolin, the cAMP analogue 8-db-cAMP and the degranulator compound 48/80 increased renin release, without affecting prorenin. Angiotensin II blocked the forskolin-induced renin release. Angiotensin I-generating activity was undetectable in LAD2 cells and fresh mast cells. Non-perfused rat hearts contained angiotensin I-generating activity, and aliskiren blocked ≈70% of this activity. A 30-min buffer perfusion washed away >70% of the aliskiren-inhibitable angiotensin I-generating activity. Prolonged buffer perfusion or compound 48/80 did not decrease cardiac angiotensin I-generating activity further, nor induced angiotensin I-generating activity-release in the perfusion buffer. Results in infarcted hearts were identical, despite the increased mast cell number in such hearts. In conclusion, HMC-1 cells release renin and prorenin, and the regulation of this release resembles that of renal renin. However, this is not a uniform property of all mast cells. Mast cells appear an unlikely source of renin in the heart, both under normal and pathophysiological conditions.

INTRODUCTION

The majority of cardiac angiotensin (Ang) II is produced at tissue sites by conversion of in-situ synthesized Ang I.[1] Yet, the renin required for this angiotensin generation is largely, if not completely, derived from the kidney, since after a bilateral nephrectomy, both cardiac renin and Ang II decrease to undetectable levels.[3, 22, 81] Circulating, kidney-derived renin diffuses into cardiac interstitial fluid,[56] and/or binds to 'renin receptors'. In support of this concept, the washout of renin from isolated perfused rat Langendorff hearts loaded with porcine renin followed a biphasic pattern: an initial, rapid ($t_{½}$<0.5 min) phase representing disappearance from the extracellular fluid compartment, and a secondary, slower ($t_{½}$≈3-4 minutes) phase, representing disappearance from tissue sites, possibly cell surface renin receptors.[53] Two of such receptors have recently been identified: the mannose 6-phosphate receptor (M6PR) and the (pro)renin receptor.[46-48, 58] M6PR's bind phospho-mannosylated proteins, including renin and its inactive precursor, prorenin. Binding to such receptors is followed by internalization, intracellular proteolytic cleavage of prorenin to renin, and subsequent proteolytic/hydrolytic removal or clearance.[46] M6PR's are therefore considered to act as clearance receptors for both renin and prorenin. (Pro)renin receptors bind renin and prorenin on the cell surface, without resulting in internalization.[47, 48]

Recently, the idea of cardiac renin being kidney-derived was challenged. Silver et al. provided evidence for the presence of renin in cardiac mast cells, using both the human mastocytoma (HMC-1) cell line and frozen tissue sections of buffer-perfused Sprague-Dawley rat hearts.[20] HMC-1 cells contained renin, as demonstrated by immunoblot and immunocytochemical analyses, and released Ang I-generating activity following their exposure to the mast cell degranulator compound 48/80 (48/80). The human renin inhibitor BILA2157 (IC_{50} 2.5 nmol/L),[97] at a concentration of 100 nmol/L, blocked part of this Ang I-generating activity. A monoclonal mouse anti-renin antibody recognized renin in mast cells in the rat heart. Moreover, in a follow-up study,[21] a bolus injection of 48/80 was reported to release Ang I-generating activity from Langendorff-perfused guinea pig hearts, and a comparable release occurred following exposure of the heart to 20 minutes of global ischemia and 30 minutes of reperfusion. BILA2157 not only blocked the Ang I-generating activity in the perfusate, but also prevented the norepinephrine overflow and arrhythmias occurring after ischemia. Thus, it was concluded that ischemia promotes cardiac angiotensin generation by mast cell-derived renin.

In the present study, we set out to confirm the renin synthesis and release by HMC-1 cells, applying both the selective human renin inhibitor aliskiren (IC_{50} 0.6 nmol/L)[98] and immunoradiometric assays making use of monoclonal antibodies that recognize renin's active site.[46] We also studied the regulation of mast cell renin release, using known stimulators and inhibitors of renal renin release, and we investigated to what degree these cells release prorenin. Results obtained in HMC-1 cells were compared with results in the mast cell line LAD2 and in primary mast cells isolated from mastocytosis patients.

Finally, we evaluated the washout of endogenous rat renin from the rat heart when perfused according to Langendorff, both with and without 48/80, in order to quantify the contribution of mast cells to cardiac renin levels. This was done both in control animals and animals subjected to myocardial infarction (MI), a procedure known to significantly increase the cardiac mast cell number.[21, 99]

METHODS

Cell culture studies

HMC-1 cells were a kind gift of dr. J.H. Butterfield (Mayo Clinic, Rochester, MN).[100] Cells were grown in 75 cm^2 culture flasks (37ºC, 5%CO_2) for 7 days using supplemented Iscove's modified Dulbecco's medium containing 10% heat-inactivated calf serum. Next, the cells were separated from the culture medium by centrifugation at 1500 g at room temperature. The culture medium was collected and stored at -20ºC, and the cells were resuspended in fresh medium at a concentration of ≈5 million cells per mL. Cells (0.5 mL) were then stimulated for up to 4 hours with compound 48/80 (20-100 µg/mL; final concentration in medium), the β-adrenoceptor agonist isoproterenol (1 nmol/L-1 µmol/L), the adenylyl cyclase activator forskolin (50 µmol/L), the membrane-permeable cAMP analogue 8-db-cAMP (1 mmol/L), Ang II (1 nmol/L-1 µmol/L), and/or the protein kinase C (PKC) inhibitor chelerythrine chloride (0.1-1 µmol/L) at 37ºC.[101] All drugs were obtained from Sigma and dissolved in water, except forskolin, which was dissolved in DMSO. Cells incubated with vehicle served as control. After the stimulation period, cells were separated from the stimulation medium by centrifugation at 1500 g, and the medium was collected and stored at -20ºC. The pellet was resuspended in lysis buffer (0.2% Triton-X in PBS), and also stored at -20ºC.

The human mast cell sarcoma cell line LAD2[102] was obtained from NIH (Bethesda, USA). Cells were cultured in StemPro 34 with nutrient supplement and 100 ng/mL stem cell factor for 7 days. Cells were then centrifuged and medium was collected and stored at -20°C. The pellet was resuspended in lysis buffer (≈1 million cells/mL), and also stored at -20°C.

Bone marrow aspirates were obtained from 2 women with systemic mastocytosis (age 67 and 41). Mast cells were isolated by FACS (FACS Aria, BD Biosciences) on the basis of CD117-PE-Cy7 and CD33-PE expression. Cells were resuspended in lysis buffer (≈0.8 and 0.2 million cells/mL, respectively) and stored at -20°C.

Animal studies

Ninety-one male Sprague-Dawley rats (Harlan), weighing 300-400 g, were housed in groups of 2 or 3 on a 12-hour light-dark cycle. Standard rat chow and water were available ad libitum. All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the Guiding Principles of the American Physiological Society. Sixty rats underwent coronary artery ligation as described before.[103] They were then allowed to recover for 2 weeks. Of the MI animals, 36 rats died within 24

hours after coronary ligation, and 1 animal died in the subsequent 2 weeks. Three MI rats were excluded from further analysis due to insufficient infarct size.

Isolated heart studies

Rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.). Blood (0.5 mL) was collected to measure plasma renin activity. Hearts were rapidly excised, cooled in ice-cold Krebs-Henseleit buffer (composition in mmol/L: NaCl 125, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 0.43, $MgCl_2$ 1.0, $CaCl_2$ 1.3 and D-glucose 9.1; pH 7.4) until contractions stopped, and then either frozen in liquid N_2, or prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O_2 / 5% CO_2) Krebs-Henseleit buffer at 37°C was perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mm Hg. A water-filled latex balloon was placed in the left ventricle via the left atrium to measure LVP. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 mm Hg during initial equilibration, and this volume was maintained throughout the experiment. Hearts were paced at 450 beats/min. Coronary flow was measured with an in-line flow probe (Transonic Systems, Ithaca, NY, USA). After 30 or 120 minutes of buffer perfusion, hearts were removed and frozen in liquid N_2. In a separate series of experiments, hearts were perfused with buffer for 30 minutes, and then exposed to a bolus injection of compound 48/80 (300 µg in 100 µL Krebs-Henseleit buffer). Coronary effluent was collected from 5 minutes before until 5 minutes after the exposure to compound 48/80, and stored at -20ºC. Five minutes after the exposure to compound 48/80, hearts were removed and also frozen in liquid N_2.

Biochemical measurements

Renin and prorenin were measured in medium and cell lysates by enzyme-kinetic assay (EKA) and/or immunoradiometric assay (IRMA; Cisbio), using recombinant human renin as a control. In order to allow its measurement by EKA or IRMA, prorenin was converted to renin by incubating the sample for 72 hours with trypsin-sepharose at 4°C, followed by 5 min centrifugation at 1300 rpm to remove trypsin-sepharose. This approach yielded the same result as prorenin activation via a 48-hour exposure at 4°C to plasmin (0.5 caseinolytic U/mL; data not shown). Renin in cardiac tissue and coronary effluent was measured as described before, using rat kidney renin and rat plasma renin as a control.[53] In short, hearts were homogenized in phosphate buffer (1:15, wt:vol), pH 7.4, and dialyzed for 48 hours at 4°C against 0.05 mol/L glycine buffer (pH 3.3), followed by a 24-hour dialysis against phosphate buffer. This procedure effectively removes all angiotensinase activity. Since the levels in coronary effluent were expected to be low, the effluent was concentrated 10-fold by centrifugal filtration (Millipore). Finally, to verify the contribution of renin to the Ang I-generating activity detected in the EKA, all EKA measurements were repeated in the presence of increasing concentrations of the renin inhibitor aliskiren (1 pmol-0.1 mmol/L; provided by Novartis Pharmaceuticals, Basel, Switzerland). Detection limits of the EKA

and IRMA were 0.05 ng Ang I/mL per hour, and 1 pg renin/mL, respectively, and 1 ng Ang I/mL per hour corresponds with 2.6 pg human renin/mL.

Data analysis

Results are expressed as mean±SEM. IC50 values were calculated as described before.[89] Statistical analysis was performed using one-way ANOVA. $P<0.05$ was considered significant.

RESULTS

Is HMC-1 cell-derived angiotensin I-generating activity due to renin?

The Ang I-generating activity of medium incubated without cells was below the detection limit of the assay. Cell lysates and medium of 48/80-stimulated cells did contain Ang I-generating activity. Comparison of these measurements with those by IRMA yielded the line of identity (Figure 1, left panel), suggesting that all Ang I-generating activity was due to renin. The inhibition curve obtained in the presence of increasing concentrations of aliskiren confirmed this view, since the IC_{50} values for Ang I-generating activity in medium and cell lysate (0.13±0.04 and 0.18±0.07 nmol/L; Figure 1, right panel) were comparable to that for recombinant human renin (IC_{50} 0.30 nmol/L). Full blockade of the Ang I-generating activity was observed at aliskiren concentrations of 0.1 µmol/L and higher, indicating that renin is the only Ang-I generating enzyme present.

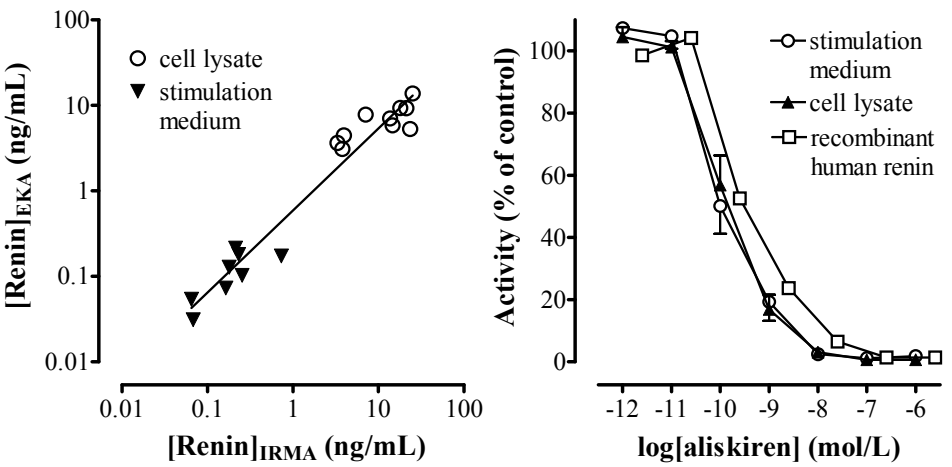

Figure 1. Left, comparison of the renin concentration in medium and lysates of HMC-1 cells measured by enzyme-kinetic assay (EKA) and immunoradiometric assay (IRMA). For the sake of clarity, EKA data in µg Ang I/mL per hour have been converted to ng renin/mL. The regression line ($\log[renin]_{EKA} = 0.96 \times \log[renin]_{IRMA} - 0.53$, $r=0.95$) was not significantly different from the line of identity. **Right**, concentration-dependent inhibition of the Ang I-generating activity by aliskiren in medium and lysates of 48/80-stimulated HMC-1 cells. Data (mean±SEM of 5 experiments) have been expressed as a percentage of the Ang I-generating activity in the absence of aliskiren ('control'). For comparison, results obtained with recombinant human renin are also shown.

Prorenin release by HMC-1 cells

Trypsin treatment increased the renin level in the culture medium, but not in the cell lysates or the stimulation medium of 48/80-stimulated cells (Figure 2). This indicates that only the culture medium contained prorenin. The stimulation medium of forskolin- (n=9) and 8-db-cAMP-stimulated (n=4) cells also did not contain prorenin (data not shown).

Regulation of renin release by HMC-1 cells

48/80 induced renin release into the medium in a concentration-dependent manner (Figure 2), without significantly lowering the renin levels in the cell lysates. The latter relates to the fact that the amount of renin released into the stimulation medium amounted to less than 10% of the levels in the cell lysates. Ang II and chelerythrine chloride, at concentrations up to 1 µmol/L, did not affect the 48/80-induced renin release into the medium, nor did these drugs affect renin release by themselves (Figure 3; n=4-5). Forskolin increased renin release into the medium to the same degree as 48/80 (Figure 3), and identical results were obtained with 8-db-cAMP (renin level in stimulation medium 209±36% of control; n=4). Ang II and chelerythrine chloride, both at a concentration of 1 µmol/L, fully prevented the forskolin-induced renin release (Figure 3). Isoproterenol, at concentrations up to 1 µmol/L, did not affect renin release (n=5, data not shown).

LAD2 and primary mast cells

Renin and prorenin levels were below detection limit in the culture medium and cell lysates of LAD2 cells, as well as in the cell lysates of primary mast cells obtained from 2 mastocytosis patients.

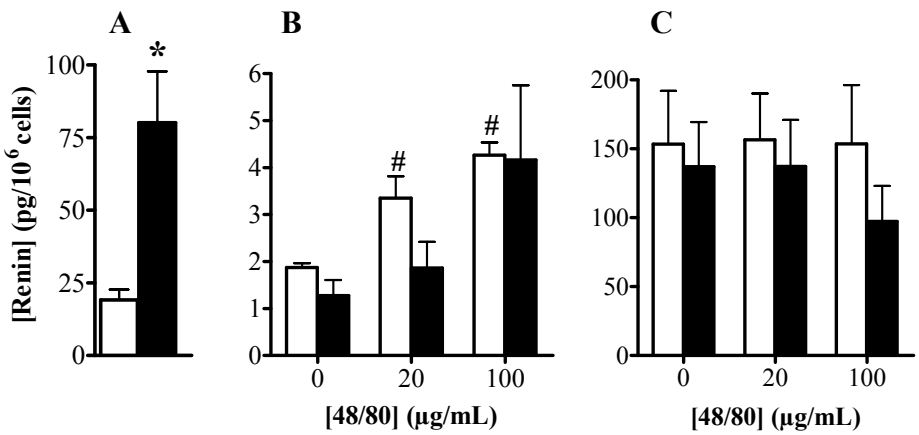

Figure 2. Renin in culture medium (**A**), stimulation medium (**B**) and lysates (**C**) of HMC-1 cells before (open bars) and after (black bars) trypsin activation of prorenin. Culture medium represents medium obtained from cells cultured for 7 days without stimulators. Stimulation medium represents medium of cells stimulated with 48/80 (0, 20 or 100 µg/mL) for 30 minutes. The lysates are derived from the 48/80-stimulated HMC-1 cells. Data are mean±SEM of 5 experiments. *P<0.05 vs. before trypsin activation, #P<0.05 vs. control.

Does renin in the rat heart originate in mast cells?

Non-perfused acidified rat heart homogenates contained Ang I-generating activity, and aliskiren blocked ≈70% of this activity at concentrations that fully blocked rat kidney and rat plasma renin (Figure 4). The IC_{50} values towards the cardiac, renal and plasma Ang I-generating activity were, respectively, 0.6±0.2, 0.2±0.1 and 0.2±0.1 μmol/L (P=NS), i.e., within the range of the previously published IC_{50} of aliskiren for rat renin (0.1 μmol/L).[98] Buffer perfusion greatly decreased the cardiac Ang I-generating activity, and under those conditions the effect of aliskiren, even at high concentrations, was marginal or absent (Figure 5, top left panel).

Renin-attributable Ang I-generating activity was calculated by subtracting the mean Ang I-generating activity in the presence of the 3 highest aliskiren concentrations from the Ang I-generating activity without aliskiren. Figure 5 (top right panel) shows that a 30-min buffer perfusion of the heart reduced the aliskiren-inhibitable (i.e., renin-attributable) Ang I-generating activity by >70%. Perfusion for longer periods (120 minutes) did not decrease this activity further, nor did exposure to 48/80 affect this activity. In the perfusion buffer, Ang I-generating activity was below the limit of detection both during 5 minutes before and during 5 minutes after exposure to 48/80. Stimulation with 48/80 reduced coronary flow from 8.7±0.6 to 4.4±0.4 mL/min (P<0.01). When performing these studies following MI (Figure 5, bottom panels), the results were identical, despite the increased mast cell content of the infarcted heart (Figure 6). Stimulation with 48/80 in MI hearts reduced coronary flow from 8.2±0.3 to 1.5±0.4 mL/min (P=0.02 vs. control hearts). The much larger effect of 48/80 in MI hearts also supports the concept of an increased mast cell content in these hearts.[99] However, Ang I-generating activity remained below the detection limit in the perfusion buffer of the MI hearts, both before and after 48/80 exposure.

DISCUSSION

This study confirms the observation by Silver et al.[20] that HMC-1 cells store and release Ang I-generating activity. Although mast cells have been reported earlier to synthesize the angiotensinogen-cleaving enzyme cathepsin D,[104] our study, making use of the selective human renin inhibitor aliskiren (IC_{50} for human cathepsin D: 5 μmol/L)[98] and monoclonal antibodies that recognize renin's active site, now demonstrates that renin is the sole contributor to the Ang I-generating activity in the lysates and medium of HMC-1 cells. The current findings also indicate that activation of the adenylyl cyclase-cAMP pathway stimulates renin release from HMC-1 cells, whereas interference with the Ca^{2+}-PKC pathway blocks this release. In addition, HCM-1 cells release prorenin in a constitutive manner, without storing the inactive precursor of renin intracellularly. Thus, the regulation of (pro)renin release in HMC-1 cells is identical to that in other renin-producing cells, including renal juxtaglomerular cells and adrenal glomerulosa cells.[8, 13, 105]

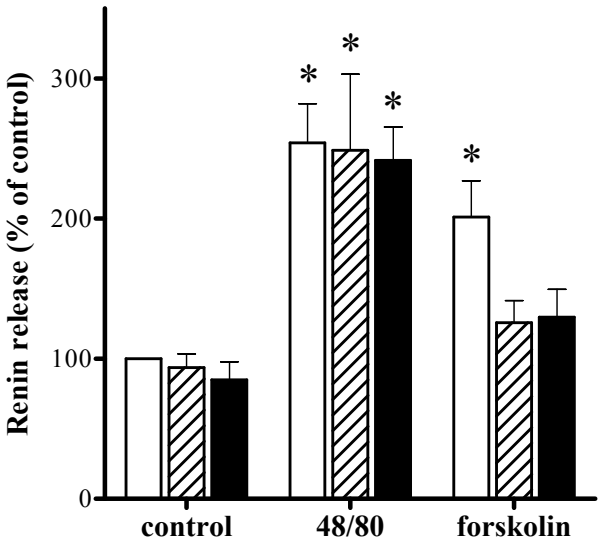

Figure 3. Renin release from HMC-1 cells induced by 100 µg/mL 48/80 or 50 µmol/L forskolin, in the absence (open bars) or presence of 1 µmol/L angiotensin II (hatched bars) or 1 µmol/L chelerythrine chloride (black bars). Data (mean±SEM of 4-10 experiments) are expressed as a percentage of the value obtained without the above agonists and antagonists. *P<0.05 vs. control.

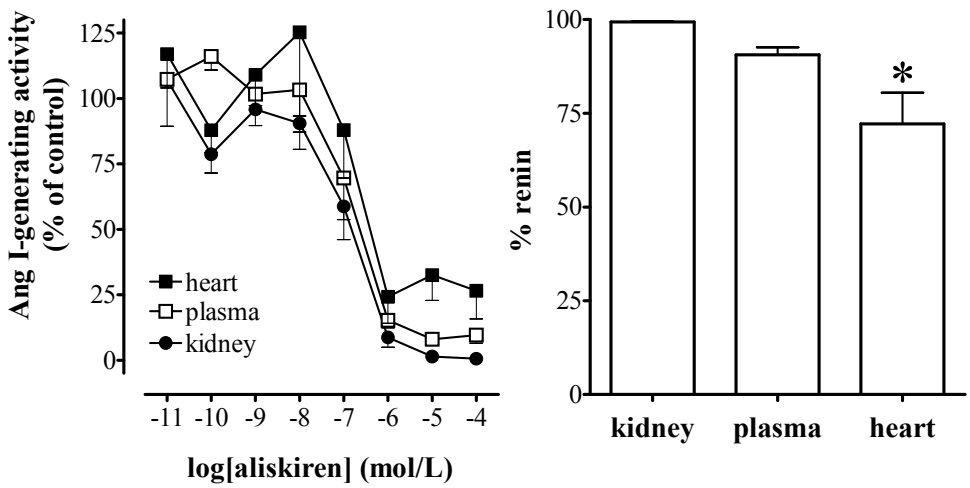

Figure 4. Concentration-dependent inhibition of the Ang I-generating activity by aliskiren in rat heart homogenates, plasma and kidney extracts (**left panel**) and the percentage of the Ang I-generating activity in these samples that is due to renin (**right panel**). Data (mean±SEM of 3-8 experiments) have been expressed as a percentage of the Ang I-generating activity in the absence of aliskiren ('control': 9±3, 26±15 and 47617±8918 ng Ang I/ml per hour, respectively). *P<0.05 vs. 100%.

Yet, activation of the adenylyl cyclase-cAMP pathway with the non-selective β-adrenoceptor agonist isoproterenol did not affect renin release from HMC-1 cells. This most likely relates to the mast cell-stabilizing action of β_2-adrenoceptors,[106] which are the predominant β-adrenoceptors on mast cells.[107] Such an effect would oppose the well-known β_1-adrenoceptor-mediated stimulation of renin release. Indeed, mast cell stabilizers (e.g., cromolyn and lodoxamide) suppress the release of Ang I-generating activity from mast cells.[21]

Furthermore, the mast cell degranulator compound 48/80 enhanced renin release from HMC-1 cells, and this effect could not be blocked by activation of the Ca^{2+}-PKC pathway. This is probably due to the non-specific action of basic secretagogues like 48/80, involving multiple receptors and/or direct intracellular actions.[108] Whether mast cell degranulators and stabilizers also interfere with renin release by non-HMC-1 cell renin-producing cells is currently unknown.

Unexpectedly, fresh mast cells obtained from mastocytosis patients and the mast cell line LAD2 did not contain or release Ang I-generating activity. Apparently therefore, HMC-1 cells are not representative for all mast cells, and thus the proposal that mast cells are a source of renin in the heart[20] needs to be viewed with care. This proposal also challenges the current concept that cardiac renin is strictly kidney-derived. This concept is based on the inability to detect renin mRNA at cardiac tissue sites,[109, 110] the disappearance of renin from the heart after bilateral nephrectomy,[3, 22] and the lack of renin synthesis by cardiac fibroblasts and myocytes.[54, 111] In fact, the majority of cardiac renin disappears within minutes when perfusing the heart with a renin-free buffer,[53] in full agreement with studies showing that cardiac renin is largely confined to the interstitial fluid compartment.[55, 71] Obviously, some retention may occur at sites that do not readily exchange with the extracellular fluid compartment. For instance, M6PR-internalized (pro)renin is cleared intracellularly (with a half life of several hours)[46, 57] without being released to the extracellular fluid.[56, 59] In addition, renin stored in mast cell granules would only be released after exposure to a mast cell degranulator.

To investigate these possibilities, in view of the discrepant findings in HMC-1 cells, LAD2 cells and fresh mast cells, we quantified cardiac renin after perfusing the rat heart with a renin-free buffer for several hours or following exposure of the heart to the mast cell degranulator 48/80. When expressed per g wet weight, non-buffer perfused rat hearts contained renin in quantities that were of the same order of magnitude as the levels per mL blood plasma. This is identical to the situation in the porcine and human heart.[3, 65] In our experiments, Ang I-generating activity was measured after acidification of the cardiac homogenates. This procedure effectively removes angiotensinases, but also activates prorenin.[3] We did not attempt to measure Ang I-generating activity without the acidification step, and thus the cardiac renin in the present study represents the sum of renin and prorenin. However, previous studies have already indicated that the increase in Ang I-generating activity of cardiac homogenates after acidification is marginal or

absent, thereby implying that the heart contains predominantly renin and virtually no prorenin.[3,65] About 25-30% of the Ang I-generating activity in the cardiac homogenates could not be inhibited by aliskiren. Thus, non-renin enzymes like cathepsin D also contributed to the Ang I-generating activity in the homogenized rat heart. This has been noted before,[111] but does not necessarily imply that such non-renin enzymes generate Ang I under in-vivo circumstances.

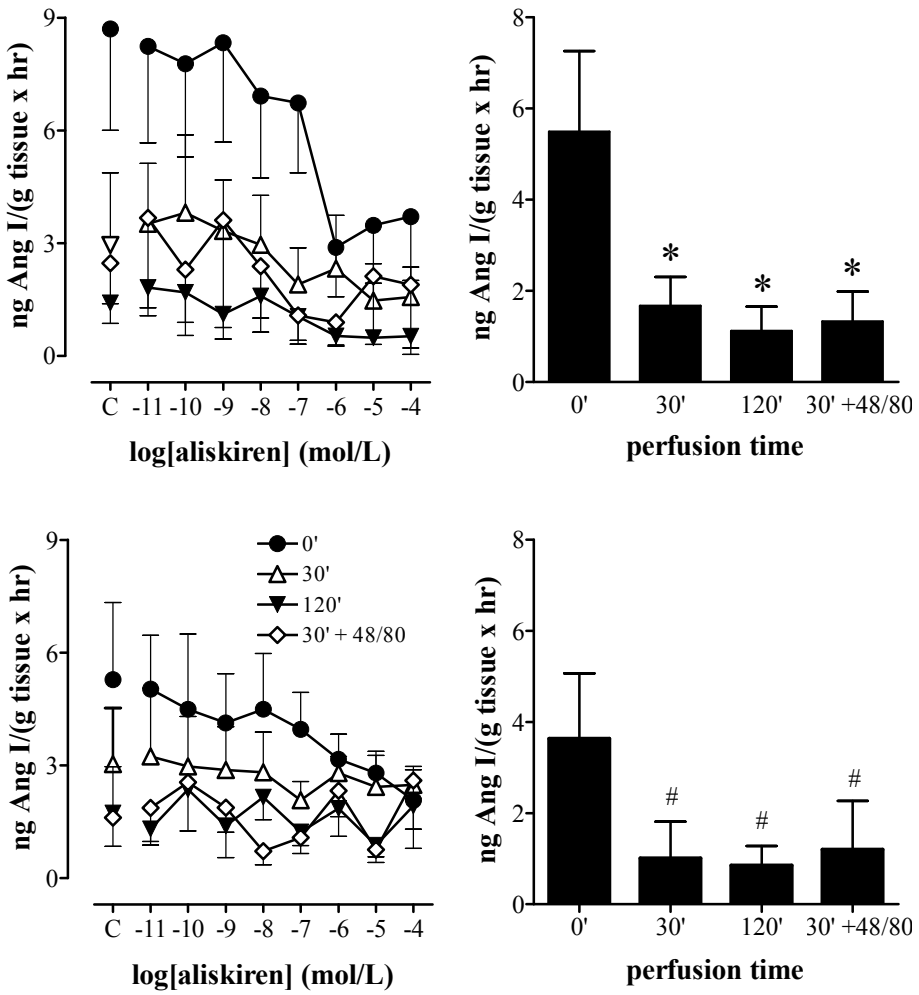

Figure 5. Concentration-dependent inhibition of the Ang I-generating activity by aliskiren in heart homogenates (**left panels**) and the cardiac renin levels calculated from these curves (**right panels**). **Top**, control rats; **bottom**, MI rats. Data represent non-buffer-perfused rat hearts (0'), and hearts that had been buffer-perfused for 30 min (30'), 120 min (120'), or 30 minutes followed by exposure to compound 48/80 (30'+ 48/80). Data are mean±SEM of 4-8 experiments. *P<0.05 vs. 0', #P<0.10 vs. 0'.

As expected, within 30 minutes after the start of buffer perfusion, more than 70% of cardiac renin (i.e., aliskiren-inhibitable Ang I-generating activity) disappeared. Longer perfusion did not decrease renin further, and exposure to 48/80, at quantities that were previously shown to release Ang I-generating activity in the coronary effluent of the isolated perfused guinea pig heart,[21] neither decreased the cardiac renin content, nor resulted in the appearance of detectable renin quantities in the coronary effluent. The cardiac renin content after 30 minutes of buffer perfusion amounted to ≈2.0 ng Ang I/g wet weight per hour. At an estimated rat heart weight of 1.5 gram, a coronary flow of 5 mL/min, and provided that the effluent in our assay was concentrated 10 times, this should have resulted in a coronary effluent renin level of 10 x (2.0 x 1.5)/(5 x 5) = 1.2 ng Ang I/mL per hour, had all cardiac renin been released within 5 minutes after the exposure of the heart to 48/80. Even if only 10% of this cardiac renin had been released, the renin level in the coronary perfusate would still have been above the detection limit of our assay. Since this was not the case, we conclude that the remaining cardiac renin was neither located in a compartment accessible to 48/80 (i.e., storage granules), nor in a compartment that allowed further washout. It might have been present in cardiac cells, e.g. after binding to and internalization by M6PR's.[46, 57] This finding, in combination with the rapid washout of angiotensinogen from cardiac tissue sites ($t_{½}$ <1 minute),[53, 112] strongly argues against the possibility of mast cell renin contributing to Ang I generation in the isolated rat heart. Such local angiotensin generation has been proposed in the isolated perfused guinea pig and mouse heart following ischemia and reperfusion (another condition resulting in mast cell degranulation),[21] although actual measurements of Ang I and II under these conditions were not performed.

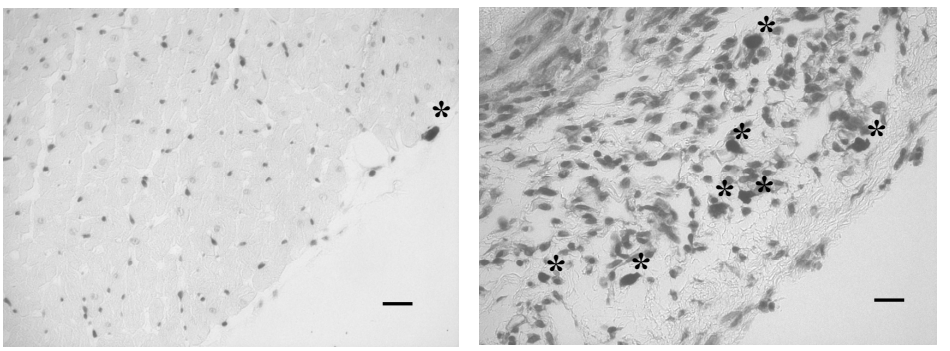

Figure 6. Mast cells (*), stained with toluidine blue,[20] in transversal slices of the cardiac left ventricle obtained from a control rat (**left panel**) and a rat that underwent a myocardial infarction 14 days prior to the removal of the heart (**right panel**). Hearts were perfused with Krebs-Henseleit buffer for 30 minutes, and fixed in 3.5-4% formaldehyde solution. Scale bar = 25 µm.

Importantly, 2 weeks following a MI, when mast cells have accumulated at cardiac tissue sites (Figure 6),[99] the findings on cardiac renin were identical to those in control hearts. If anything, renin tended to be lower in MI hearts, and again 48/80 did not induce renin release or lower cardiac renin. Nevertheless, the 48/80-induced coronary flow reduction was much stronger in infarcted hearts, suggesting that a factor other than renin was massively released from mast cells in infarcted hearts, e.g. serotonin.[113] However, the identity of this factor was not investigated in the present study.

Perspective

HMC-1 cells synthesize renin and prorenin, but this is not a uniform property of all human mast cells. Evidence to support the idea that mast cell-derived renin contributes to cardiac angiotensin production in humans is currently lacking, but seems unlikely in view of the current study and previous measurements in human heart tissue.[65] The present study also does not support such contribution in the infarcted rat heart, even though such hearts display an enhanced mast cell content.[99] This finding opposes observations on mast cell-derived renin synthesizing Ang I in buffer-perfused guinea pig and mouse hearts following ischemia and reperfusion.[21] A further complicating factor in the isolated perfused rat heart to allow such production would have been the low or undetectable levels of angiotensinogen at cardiac tissue sites following buffer perfusion (≈0.4 pmol/mL in interstitial fluid after 30 minutes of buffer perfusion, i.e. ≈3 orders of magnitude below the levels in blood plasma.[53] Using the equation V = Vmax x [S] / (Km + [S]), where Vmax = 2.0 ng Ang I/g per hour, Km = 2400 pmol/mL[53] and [S] = 0.4 pmol/mL, it can be calculated that the maximum degree of Ang I generation (under the unlikely condition that all cardiac renin had access to angiotensinogen) after 30 minutes is 0.3 pg Ang I/g per hour. This is far below the range required to result in physiologically relevant angiotensin concentrations. Clearly therefore, the role of mast cell-derived renin is unique for the guinea pig and mouse heart, and cannot be translated to the rat heart. In addition, the importance of mast cell-derived renin, if any, in humans needs to be reconsidered.

Chapter 5
Mast cell degranulation mediates bronchial contraction via serotonin and not via renin release

Based on:
Krop M, Özünal ZG, Chai W, de Vries R, Bouhuizen AM, Garrelds IM, Danser AHJ. Mast cell degranulation mediates bronchoconstriction via serotonin and not via renin release. Submitted 2009

ABSTRACT

To verify the recently proposed concept that mast cell-derived renin facilitates angiotensin (Ang) II-induced bronchoconstriction, bronchial rings from male Sprague-Dawley rats were mounted in Mulvany myographs, and exposed to the mast cell degranulator compound 48/80 (300 µg/mL), Ang I, Ang II, bradykinin or serotonin. Both in the absence or presence of the renin inhibitor aliskiren (10 µmol/ L), the ACE inhibitor captopril (10 µmol/L), the Ang II type 1 (AT_1) receptor blocker irbesartan (1 µmol/L), the mast cell stabilizer cromolyn (0.3 mmol/L), the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (0.1 µmol/L) or the α_1-adrenoceptor antagonist phentolamine (1 µmol/L). Bath fluid was collected to verify angiotensin generation. Bronchial tissue was homogenized to determine renin and angiotensinogen content. Compound 48/80 contracted bronchi to 24±4% of the KCl-induced contraction. Ketanserin fully abolished this effect, while cromolyn reduced the contraction to 16±5%. Aliskiren, captopril, irbesartan and phentolamine did not affect this response, and the Ang I and II levels in the bath fluid after 48/80 exposure were below the detection limit. Ang I and II equipotently contracted bronchi. Captopril shifted the Ang I curve ≈10-fold to the right, whereas irbesartan fully blocked the effect of Ang II. Bradykinin-induced constriction was shifted ≈100-fold to the left with captopril. Serotonin contracted bronchi, and ketanserin fully blocked this effect. Finally, bronchial tissue contained low Ang I-forming activity, but this could not be blocked by aliskiren, and the bronchial angiotensinogen content was below the detection limit. In conclusion, mast cell degranulation results in serotonin-induced bronchoconstriction, and is unlikely to involve renin-induced angiotensin generation.

INTRODUCTION

Mast cells have been proposed to synthesize and release renin.[20] This observation was made in the human mastocytoma cell line HMC-1. However, subsequent studies in the mast cell line LAD2 and in primary mast cells isolated from mastocytosis patients did not confirm such synthesis,[114] suggesting that renin synthesis is not a uniform property of mast cells.

Recently, the mast cell degranulator compound 48/80, when applied to isolated rat bronchial rings, was shown to elicit bronchoconstriction.[115] Since both a renin inhibitor, BILA2157, and the active metabolite of the angiotensin (Ang) II type 1 receptor antagonist losartan, EXP3174, prevented this constriction, it was concluded that angiotensin generated by mast cell-derived renin mediated this phenomenon. Indeed, exogenous Ang II was capable of constricting isolated rat bronchi. Remarkably however, the level of exogenous Ang II that was required to mimic the compound 48/80-induced constrictor response amounted to ≈0.1 μmol/L. This is roughly 5 orders of magnitude above the plasma levels of angiotensins. Given the well-established kinetics of the renin x angiotensinogen reaction,[116, 117] where micromolar angiotensinogen levels in blood plasma result in picomolar circulating Ang I and II levels,[64, 118] the generation of submicromolar concentrations of Ang II would require substantial concentrations of angiotensinogen and/or renin. Moreover, mast cells contain a wide range of other mediators, e.g., histamine and serotonin, which might also induce bronchoconstriction.[119] Finally, although BILA2157 is a human renin inhibitor,[97] it also blocks cathepsin D, and thus its antagonizing properties do not necessarily reflect rat renin inhibition. In addition, both losartan and its active metabolite EXP3174 have a wide range of non-AT_1 receptor-mediated effects[120-124] so that here too, blocking effects cannot with 100% certainty be attributed to AT_1 receptor antagonism.

Given these uncertainties, we set out to further investigate the compound 48/80-induced constriction of rat bronchi. Not only did we study the blocking effects of a renin inhibitor (aliskiren), an ACE inhibitor (captopril) and an AT_1 receptor blocker (irbesartan) on the constriction induced by compound 48/80 in rat bronchial rings, but we also verified angiotensin generation under these conditions, and determined the renin and angiotensinogen content of rat bronchi.

METHODS

Animals

Fortyfive male Sprague-Dawley rats (Harlan, The Netherlands), weighing 300-350 g, were housed in groups of 2 or 3 on a 12-hour light-dark cycle. Standard rat chow and water were available ad libitum. All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the Guiding Principles of the American Physiological Society.

Lungs were rapidly excised under pentobarbital anesthesia (60 mg/kg i.p.), and cooled in ice-cold Krebs solution (composition in mmol/L: NaCl 125, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 0.43, $MgCl_2$ 1.0, $CaCl_2$ 1.3 and D-glucose 9.1; pH 7.4). Bronchi were dissected free of parenchymal lung tissue, connective tissue, and fat, and either frozen immediately in liquid N_2, or stored overnight in Krebs solution at 4°C.

Myograph studies

Following overnight storage, the main bronchi were cut into segments of approximately 2 mm in length and mounted in Mulvany myographs (J.P. Trading, Aarhus, Denmark) with separated 6-mL organ baths containing oxygenated Krebs solution at 37°C. The Krebs solution was continuously aerated with 95% O_2 and 5% CO_2, and tissue responses were measured as changes in isometric force using a Harvard isometric transducer. Resting tension was set at 2 mN. Subsequently, tissue was exposed to 100 mmol/L KCl, to determine maximum contraction. The segments were then allowed to equilibrate in fresh buffer for 30 min.

Segments were incubated for 30 minutes with the renin inhibitor aliskiren (10 µmol/L), the AT_1 receptor blocker irbesartan (1 µmol/L), the ACE inhibitor captopril (10 µmol/L), the 5-$HT_{2A/2C}$ receptor antagonist ketanserin (0.1 µmol/L), the α_1-adrenoceptor antagonist phentolamine (1 µmol/L) or the mast cell stabilizer cromolyn (0.3 mmol/L). Thereafter, concentration-response curves (CRCs) were constructed to Ang I, Ang II, bradykinin or methacholine, or the segments were exposed to a single dose (300 µg/mL) of the mast cell degranulator compound 48/80. Aliskiren was a kind gift of Novartis Pharmaceuticals, Basel, Switzerland. All other chemicals were from Sigma.

To measure Ang I and II in organ bath fluid, 50 µL-samples were obtained from the organ bath at the time the vasoconstrictor response had reached a plateau. Samples were rapidly mixed with 10 µL angiotensinase inhibitor solution[89] and stored at -80°C.

Tissue preparation

Frozen bronchi from 5 rats were pooled and crushed using a mortar and 1:1 homogenized in phosphate buffer (pH 7.4). To convert prorenin to renin, and to remove angiotensinase activity,[53, 125] homogenates were dialyzed for 48 hours at 4°C against 0.05 mol/L glycine buffer (pH 3.3), followed by a 24-hour dialysis against phosphate buffer.

Biochemical measurements

Ang I-generating activity in bronchial homogenates was measured by incubating the acidified samples with excess sheep angiotensinogen in the absence or presence of 10 µmol/L aliskiren, a concentration that fully blocks rat renin.[114] Renin-dependent Ang I generation was defined as the amount of Ang I generation that could be blocked by this concentration of aliskiren. The detection limit was 0.20 ng Ang I/g per hour. Angiotensinogen was measured in non-acidified homogenates by determining the amount of Ang I generated following

exposure of the samples to excess recombinant human renin.[53, 125] The detection limit was 0.1 pmol/g. Ang I and II in organ bath fluid were measured with sensitive radioimmunoassay.[2] The detection limits were 40 and 20 fmol/mL, respectively.

Data analysis

Data are given as mean±SEM. Contractile responses are expressed as a percentage of the maximal contraction to 100 mmol/L KCl. CRCs were analyzed as described earlier[126] to obtain pEC_{50} ($-^{10}\log[EC_{50}]$) values. In the case of Ang I, Ang II and bradykinin, CRCs did not always reach a maximum (E_{max}) at the highest angiotensin concentration of 1.0 µmol/L. In such cases we determined the concentration required to obtain 2.5% of the K^+-induced contraction ($EC_{2.5\%K+}$), in order to calculate the $pEC_{2.5\%K+}$ value. Statistical analysis was performed by one-way ANOVA, followed by post hoc evaluation (according to Bonferroni). $P<0.05$ was considered significant.

RESULTS

Methacholine constricted bronchial rings in a concentration-dependent manner (pEC_{50} 6.5±0.2) to maximally 215±19% of the constriction to 100 mmol/L KCl (Figure 1; n=5). As compared to methacholine, Ang II only modestly constricted bronchial rings (n=9), and no clear E_{max} was reached (Figure 1A). This was not due to overnight storage of the bronchial rings, since the results in freshly obtained rings were identical (data not shown). The constrictor effects of Ang I (n=11) were comparable to those of Ang II, although its potency ($pEC_{2.5\%K+}$ 6.8±0.1 vs. 7.3±0.3) tended to be somewhat lower (Figure 1A). In agreement with this lower potency, the Ang II levels in the organ bath following the addition of 1 µmol/L Ang I were 0.2±0.1 µmol/L (n=5). Irbesartan fully blocked the effect of Ang II (n=3).

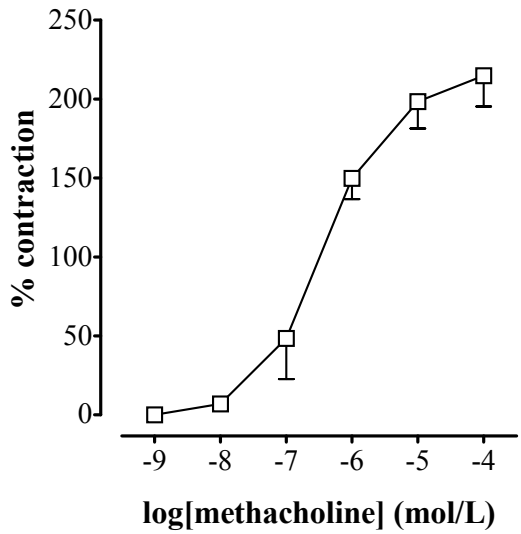

Figure 1. Effects of methacholine in rat bronchial rings. Values are mean±SEM of 5 experiments and have been expressed as a percentage of the response to 100 mmol/L KCl.

Captopril shifted the Ang I CRC ≈10 fold to the right ($pEC_{2.5\%K+}$ 5.9±0.4, n=5; P<0.05 vs. control). Bradykinin also constricted bronchial rings ($pEC_{2.5\%K+}$ 6.4±0.3, n=8; Figure 1B), and captopril potentiated bradykinin almost 100-fold ($pEC_{2.5\%K+}$ 8.1±0.3, n=5; P<0.01 vs. control). The maximum bradykinin-induced effects were comparable to those of Ang I and II.

Compound 48/80 constricted bronchial rings to 23.9±4.2% of the constriction to 100 mmol/L KCl (Figure 3; n=14). None of the applied renin-angiotensin system (RAS) blockers affected this constriction (Figure 3; n=6-7), nor did these blockers affect baseline tension (data not shown). In agreement with this obseration, the Ang I and II levels in the organ bath fluid following exposure to compound 48/80 were below the limit of detection, both with and without RAS blockers (n=4-8 for each condition).

Cromolyn (n=7) reduced the compound 48/80-induced bronchial constriction, whereas ketanserin (n=7) virtually abolished it (Figure 4, left panel). Phentolamine (n=5) was without effect. These data indicate that the compound 48/80-induced constriction depends on the release of serotonin. We therefore also verified the effect of serotonin. Indeed, serotonin constricted bronchial rings to maximally 54.8±17.5% of the constriction to 100 mmol/L KCl (Figure 4, right panel; n=6), and ketanserin (n=5) fully prevented this effect. By comparing the compound 48/80-induced bronchial constriction with the serotonin CRC, it could be estimated that mast cell degranulation had resulted in serotonin levels in the order of 0.5 µmol/L.

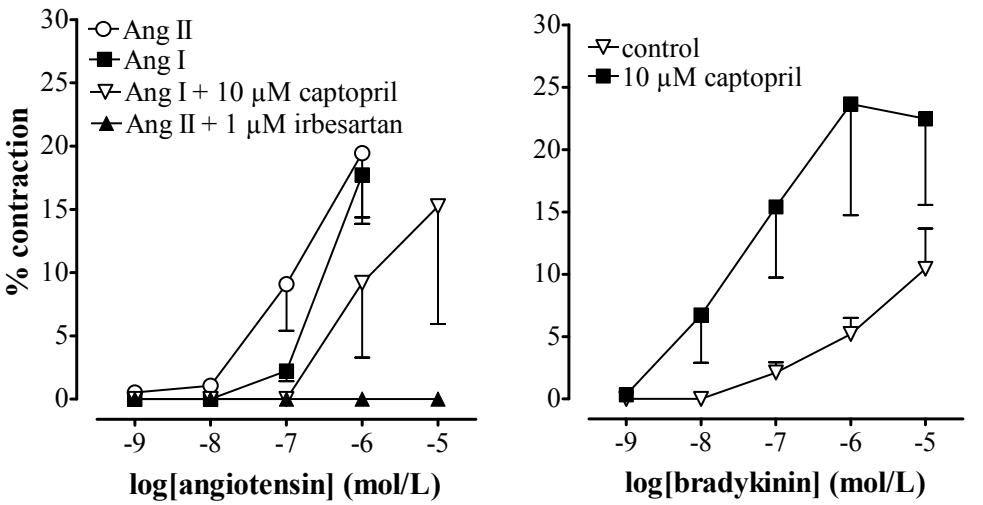

Figure 2. Effects of angiotensine (Ang) I and II (A) and bradykinin in rat bronchial rings, in the absence and presence of captopril and irbesartan. Values are mean±SEM of 3-11 experiments and have been expressed as a percentage of the response to 100 mmol/L KCl.

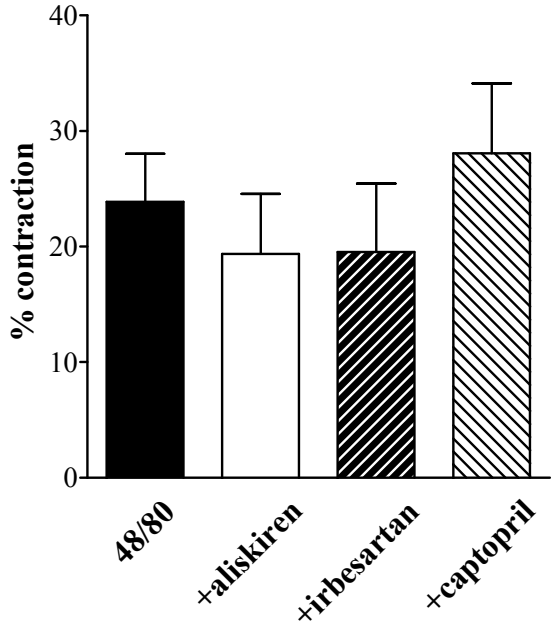

Figure 3. Effect of compound 48/80 (300 µg/mL) in rat bronchial rings in the absence or presence of aliskiren, captopril or irbesartan. Values are mean±SEM of 6-14 experiments and have been expressed as a percentage of the response to 100 mmol/L KCl.

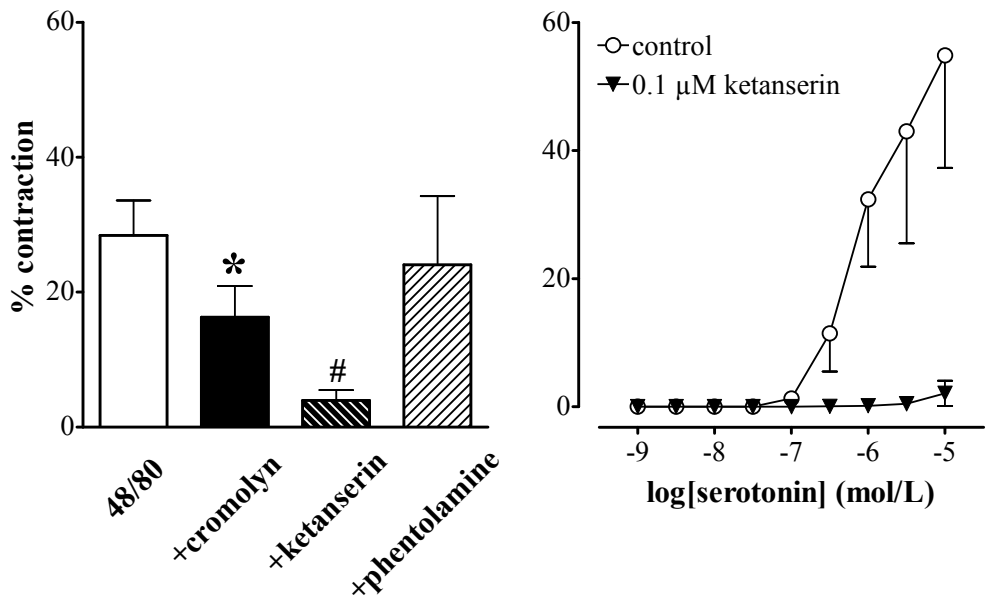

Figure 4. Effect of compound 48/80 (300 µg/mL, left panel) or serotonin (right panel) in rat bronchial rings in the absence or presence of cromolyn, ketanserin or phentolamine. Values are mean±SEM of 5-8 experiments and have been expressed as a percentage of the response to 100 mmol/L KCl. *P<0.05, #P<0.01 vs. control.

Finally, the Ang I-generating activity in acidified bronchial homogenates was 1.2±0.4 ng Ang I/g tissue per hour (n=3, representing pooled tissue of 5 rats each). This level was unaltered in the presence of 10 μmol/L aliskiren (1.2±0.3 ng Ang I/g tissue per hour), indicating that it was not due to renin. The angiotensinogen content of bronchi was below detection limit (n=3).

DISCUSSION

The current study shows that mast cell-derived renin is not involved in the compound 48/80-induced constriction of rat bronchial rings mounted in organ baths, in contrast to the proposal by Veerappan et al..[115] First, RAS blockade at 3 levels did not prevent this constriction. This was not due to inefficacy of the applied blockers, since, in the same preparation, the ACE inhibitor captopril significantly blocked the effects of Ang I and potentiated bradykinin, while the AT_1 receptor blocker irbesartan fully prevented the constrictor effects of Ang II. Second, Ang I and II were undetectable in the organ bath fluid after the addition of compound 48/80, although Ang II did appear in this fluid when Ang I was added. Third, neither renin nor angiotensinogen could be detected in bronchial homogenates in physiologically relevant amounts, in full agreement with the lack of renin mRNA in lung tissue.[127] The low level of Ang I-generating activity in these homogenates most likely represented non-renin enzymes (e.g., cathepsins) from intracellular sites that are capable of reacting with angiotensinogen, but that normally do not see angiotensinogen because they are located intracellularly.[3, 111]

Importantly, the 5-$HT_{2A/2C}$ receptor antagonist ketanserin fully prevented the compound 48/80-induced bronchoconstriction. Therefore, it seems more likely that mast cell-derived serotonin is responsible for the observed constriction, as has already been suggested by Ikawati et al..[113] To exclude the possibility that the α_1-adrenoceptor blocking properties of ketanserin were responsible for its effect, we also tested the α_1-adrenoceptor antagonist phentolamine. However, phentolamine did not affect the compound 48/80-induced constriction. Moreover, exogenous serotonin, like compound 48/80, effectively caused bronchoconstriction in a 5-$HT_{2A/2C}$ receptor-dependent manner. Thus indeed, stored serotonin, and not renin, induced bronchoconstriction in our experimental setup. The serotonin levels reached in our preparation following degranulation appeared to be in the order of 0.5 μmol/L.

What could explain the contradictory results in our study versus that of Veerappan et al.?[115] It is unlikely to be a methodological issue, since both the contractile response to methacholine and the compound 48/80-induced response were identical in the two studies. It might therefore relate to the use of BILA2157 and EXP3174 by Veerappan et al.,[115] as opposed to aliskiren, captopril and irbesartan in the present study. BILA2157 is a human renin inhibitor with an IC_{50} for human renin of 2.5 nmol/L.[97] At ≈100-fold higher concentrations, it also blocks cathepsin D. The IC_{50} of aliskiren for human renin is 0.6 nmol/L,[98] and 10.000-fold higher concentrations are required to also block cathepsin D. For aliskiren, detailed

information is available regarding its blocking effects towards rat, mouse and guinea pig renin.[98, 128] For BILA2157 no such information is available, and one possibility is that this drug, particularly in animals, blocks renin less effectively, and/or rather blocks the non-renin enzyme(s) (e.g., cathepsins) that are known to be released from mast cells.[129, 130] EXP3174, the active metabolite of losartan, is also well-known for mediating AT_1 receptor-independent effects.[120, 123] In particular, it increases the hKv1.5 current,[120] thereby potentially counteracting the serotonin-induced inhibition of these channels.[131] Such a mechanism could underlie the blockade of the compound 48/80-induced bronchoconstriction by EXP3174.

In conclusion, mast cell degranulation does not result in bronchoconstriction via renin release. Isolated bronchial tissue does not contain detectable levels of renin and angiotensinogen, and thus the local generation of angiotensin concentrations in the high nanomolar (or even low micromolar) range, i.e. the range at which angiotensins exert bronchoconstriction (Figure 1), is highly unlikely. This does not mean that locally produced angiotensin cannot be a novel therapeutic target in the management of airway disease, as suggested by Veerappan et al..[115] In fact, ACE inhibitors have recently been reported to decrease mortality in subjects hospitalized with a COPD exacerbation.[132] Given their strong potentiation of bradykinin-induced bronchoconstriction, as also evidenced in the present study, further work is needed to sort out the mechanism of this beneficial effect.

Chapter 6
Aliskiren accumulates in renin secretory granules and binds plasma prorenin

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Based on

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ABSTRACT

The vascular effects of aliskiren last longer than expected based on its half life, and this renin inhibitor has been reported to cause a greater renin rise than other types of renin-angiotensin system (RAS) blockade. To investigate whether aliskiren accumulation in secretory granules contributes to these phenomena, renin-synthesizing mast cells were incubated with aliskiren, washed and exposed to forskolin in medium without aliskiren. (Pro)renin concentrations were measured by renin- and prorenin-specific immunoradiometric assays, and renin activity was measured by enzyme-kinetic assay. Without aliskiren, the culture medium contained predominantly prorenin, the cells exclusively stored renin, and forskolin doubled renin release. Aliskiren dose-dependently bound to (pro)renin in the medium and cell lysates, and did not alter the effect of forskolin. The aliskiren concentrations required to bind prorenin were 1-2 orders of magnitude higher than those needed to bind renin. Blockade of cell lysate renin activity ranged from 27±15 to 79±5%, and these percentages were identical for the renin that was released by forskolin, indicating that they represented the same renin pool, i.e., the renin storage granules. Comparison of renin and prorenin measurements in blood samples obtained from human volunteers treated with aliskiren, both before and after prorenin activation, revealed that up to 30% of prorenin was detected in renin-specific assays. In conclusion, aliskiren accumulates in renin granules, thus allowing long-lasting RAS blockade beyond the half life of this drug. Aliskiren also binds to prorenin. This allows its detection as ‘renin’, and might explain, in part, the renin rise during renin inhibition.

INTRODUCTION

Renin inhibitors, like all renin-angiotensin system (RAS) blockers, increase the plasma concentration of renin because they attenuate the negative feedback effect of angiotensin (Ang) II on renin release.[133, 134] Whether they also increase kidney renin is still being debated.[133-135] The rise in blood plasma has been suggested to be larger than during other types of RAS blockade,[136] either because the degree of RAS blockade is superior during renin inhibition,[137] and/or because renin inhibitors increase the half life of renin.[138] It may also be an artifact, because renin inhibitors 'activate' renin's precursor, prorenin (Figure 1).[76] This 'activation' is due to the fact that the prorenin prosegment is capable of unfolding from the enzymatic cleft, in a pH- and temperature-dependent manner, thereby resulting in two prorenin conformations: a 'closed', inactive form, and an 'open' form that displays full enzymatic activity.[15] Under physiological conditions, <2% of prorenin is in the open conformation.[48] Renin inhibitors will bind to prorenin in the open conformation. Consequently, due to the presence of the renin inhibitor, the inactivation step (i.e., the return to the closed conformation) is now no longer possible, and thus, the equilibrium between the closed and open conformation will shift into the direction of the open conformation. Eventually, depending on the concentration of the inhibitor, a significant proportion of prorenin may be open ('non-proteolytic activation'), allowing its recognition by the active site-directed antibodies used in renin assays, despite the fact that the prosegment is still present.[76, 138, 139]

Unexpectedly, the vascular effects of aliskiren lasted much longer than expected on the basis of its circulating half life.[140,141] This could be due to accumulation of aliskiren-bound (pro)renin at tissue sites. In fact, long-lasting aliskiren accumulation has been demonstrated in the kidney,[128] although the exact site of accumulation could not be established. An attractive hypothesis is that aliskiren reaches the renin storage sites in juxtaglomerular (JG) cells, thus allowing the release of blocked renin even when aliskiren is no longer present in blood. Unfortunately, JG cells loose their capacity to store renin when cultured.[6] However, this does not apply to the recently described renin-synthesizing human mast cells (HMC-1).[20] These cells abundantly store renin, and release prorenin constitutively and renin in a regulated manner, thereby closely mimicking the in-vivo characteristics of JG cells.[114]

In the present study, we set out first to investigate aliskiren accumulation in renin secretory granules in HMC-1 cells, and second to determine to what degree the renin rise in humans post-aliskiren actually represents prorenin. The latter was possible by applying to plasma samples of aliskiren-treated volunteers not only the widely applied renin immunoreactive assays, but also a specific prorenin immunoreactive assay that is based on the recognition of the prosegment.[46, 138]

METHODS

Cell culture studies

HMC-1 cells, developed from a patient suffering from mast cell leukemia, were a kind gift of dr. J.H. Butterfield (Mayo Clinic, Rochester, MN).[20, 100] Cells were seeded in 25-cm^2 culture flasks at a concentration of 10^5 cells/mL and cultured for 7 days in 5 mL supplemented Iscove's modified Dulbecco's medium (IMDM) containing 10% calf serum, in the absence or presence of aliskiren (0.1 nmol/L-1 µmol/L, a kind gift of Novartis Pharmaceuticals, Basel, Switzerland) and/or 10 mmol/L mannose 6-phosphate (M6P). Next, cells were separated from the culture medium by centrifugation at 1500 g for 5 minutes at room temperature. The culture medium was collected and stored at -20ºC. The cells were washed in 10 mL ice-cold HBSS, centrifuged as described above, and, after removal of the supernatant, resuspended in fresh IMDM containing no aliskiren. Cells were then stimulated with the adenylyl cyclase activator forskolin (50 µmol/L; Sigma, dissolved in DMSO) or vehicle for 4 hours at 37ºC. After the stimulation period, cells were separated from the stimulation medium by centrifugation at 1500 g, and the medium was collected and stored at -20ºC. The pellet was resuspended in lysis buffer (0.2% Triton-X in PBS), and also stored at -20ºC.

Human studies

Plasma samples were obtained from 20 healthy subjects on a low-sodium diet (10 mmol daily sodium intake) each receiving 3 out of 4 escalating doses of aliskiren (75, 150, 300 or 600 mg).[142] Each subsequent aliskiren dose was given 2 days after the previous dose.

Biochemical measurements

Renin and prorenin were measured in plasma, medium and cell lysates by enzyme-kinetic assay (EKA) and/or immunoradiometric assay (IRMA; Cisbio), using recombinant human (pro)renin as a control. In order to allow its measurement by renin IRMA, prorenin was activated in a non-proteolytic manner by incubating the sample for 48 hours with 10 µmol/L aliskiren at 4°C. The renin inhibitor enters the enzymatic cleft in which the active site is located, thereby inducing a slow conformational change of the inactive (closed) form of the prorenin molecule into the active (open) form (Figure 1). This approach yields the same immunoreactive renin levels ('total renin') as proteolytic prorenin activation with trypsin[138] yet without removing the prosegment. In a select set of samples, prorenin was also measured on the basis of its prosegment, replacing the ^{125}I-labeled active site-directed monoclonal antibody of the Cisbio kit by a prosegment-directed ^{125}I-labeled monoclonal antibody (F258-37-B1) in the IRMA ('prorenin IRMA'). F258-37-B1 is directed against the C-terminal part (p20-p43) of the propeptide and does not react (<0.1%) with renin. F258-37-B1 also does not react (<0.1%) with intact, inactive prorenin.[46] However, it does react with prorenin after the above treatment of prorenin with aliskiren. Thus, the aliskiren-induced non-proteolytic conformational change, causing the propeptide to move to the surface of the

molecule, allows the recognition of prorenin by both the active site-directed antibody of the Cisbio kit, and the prosegment-directed antibody of the prorenin IRMA. Detection limits of the EKA, the renin IRMA and the prorenin IRMA were 0.1 ng Ang I/mL per hour, 1 pg renin/mL, and 5 pg prorenin/mL, respectively. Angiotensinogen in cell lysates and medium was measured as described before (detection limit 0.1 pmol/mL).[54] Protein was measured according to Bradford.

Data analysis

Results are expressed as mean±SEM. IC_{50} values were calculated as described before.[89] Ang I-generating activities obtained in the EKA were converted to renin concentrations based on the fact that 1 ng Ang I/mL per hour corresponds with 2.6 pg human renin/mL. Statistical analysis was performed using one-way ANOVA. $P<0.05$ was considered significant.

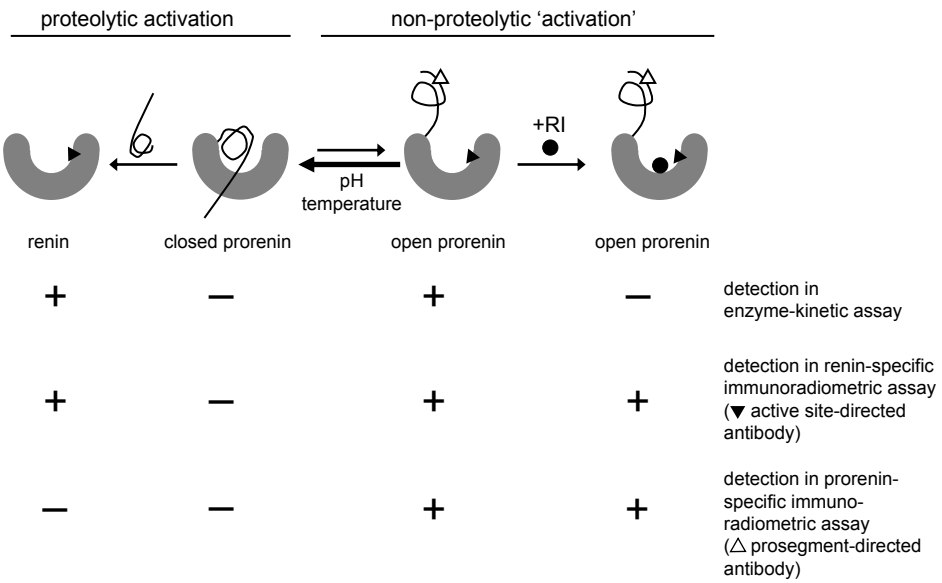

Figure 1. Scheme depicting proteolytic and non-proteolytic prorenin activation, the various prorenin configurations and the 3 types of assays that were applied in the present study. Classically, prorenin is determined by subtracting the renin measurement before prorenin activation from that after prorenin activation. The newly developed prosegment assay now allows the detection of prorenin also directly, on the basis of its prosegment. The prosegment can only be recognized when prorenin is in the 'open' conformation. Low temperatures and low pH shift the equilibrium between closed and open prorenin into the direction of the latter. A renin inhibitor (RI) is capable of binding to open prorenin, thereby preventing its return to the closed conformation. See text for further explanation.

RESULTS

Cell culture studies

Immunoreactive (pro)renin levels. In the absence of aliskiren, HMC-1 cells contained immunoreactive renin (Figure 2A), as measured with the Cisbio renin IRMA. The cellular total renin levels (i.e., the immunoreactive renin levels measured by Cisbio renin IRMA following prorenin activation) were identical to the immunoreactive renin levels without prorenin activation, suggesting that the cells did not store prorenin. Indeed, when using a prosegment-directed antibody, no signal was detected in the cell lysates (Figure 2B). The medium of non-aliskiren-exposed cells also contained immunoreactive renin. These levels increased ≈5-fold after prorenin activation (Figure 2C). This suggests that the cells predominantly released prorenin. The prosegment assay data confirmed this view (Figure 2D).

Neither the medium, nor the cells contained detectable amounts of angiotensinogen (n=4), thus demonstrating that HMC-1 cells cannot generate Ang II independently.

A 7-day incubation with aliskiren increased the cellular renin levels in a concentration-dependent manner (Figure 2A). The maximum increase in renin, reached at 1 μmol/L aliskiren, was ≈4-fold. Prorenin could not be detected in the cells at any of the tested aliskiren concentrations (Figure 2B). To investigate the possibility that the aliskiren-induced rise in cellular renin was due to the re-uptake of medium renin via M6P receptors (as part of a clearance process)[59] the experiments were repeated in the presence of excess M6P. The aliskiren-induced increase in cellular renin (2.8±0.2 fold, n=3) was unaltered in the presence of M6P (2.1±0.4 fold, n=3), and M6P did not alter the cellular renin levels in the absence or presence of aliskiren (122±22% and 88±14% of the levels without M6P, respectively, P=NS vs. 100%). Therefore, the aliskiren-induced increase in cellular renin is not due to reuptake of medium renin via M6P receptors.

In the medium, aliskiren also increased renin in a concentration-dependent manner (Figure 2C). This increase was much larger (maximally ≈20-fold) than that in the cells ($P<0.01$) and unaffected by M6P (data not shown). In parallel with this rise in secreted renin, the effect of prorenin activation on medium renin immunoreactivity diminished, until, at aliskiren concentrations of 0.1 μmol/L and higher, prorenin activation no longer yielded an increase in medium renin immunoreactivity. Yet, the prosegment assay still allowed the detection of intact, prosegment-containing prorenin (Figure 2D). This indicates that the 7-day incubation of the cells with aliskiren had resulted in non-proteolytic activation of prorenin, allowing its detection in both the Cisbio renin IRMA and the prosegment assay. Apparently, aliskiren concentrations of 0.1 μmol/L and higher were sufficient to fully 'open' all prorenin molecules in the medium during a 7-day incubation. Since prorenin activation in non-aliskiren-exposed cells resulted in a ≈5-fold rise of renin immunoreactivity, the 20-fold rise in 'renin' should be corrected for this detection of prorenin as renin. This implies that the true aliskiren-induced rise in medium renin is around 4-fold, i.e., identical to the

aliskiren-induced rise in cellular renin. Forskolin doubled renin release both in the absence and presence of aliskiren (P<0.01; Figure 3).

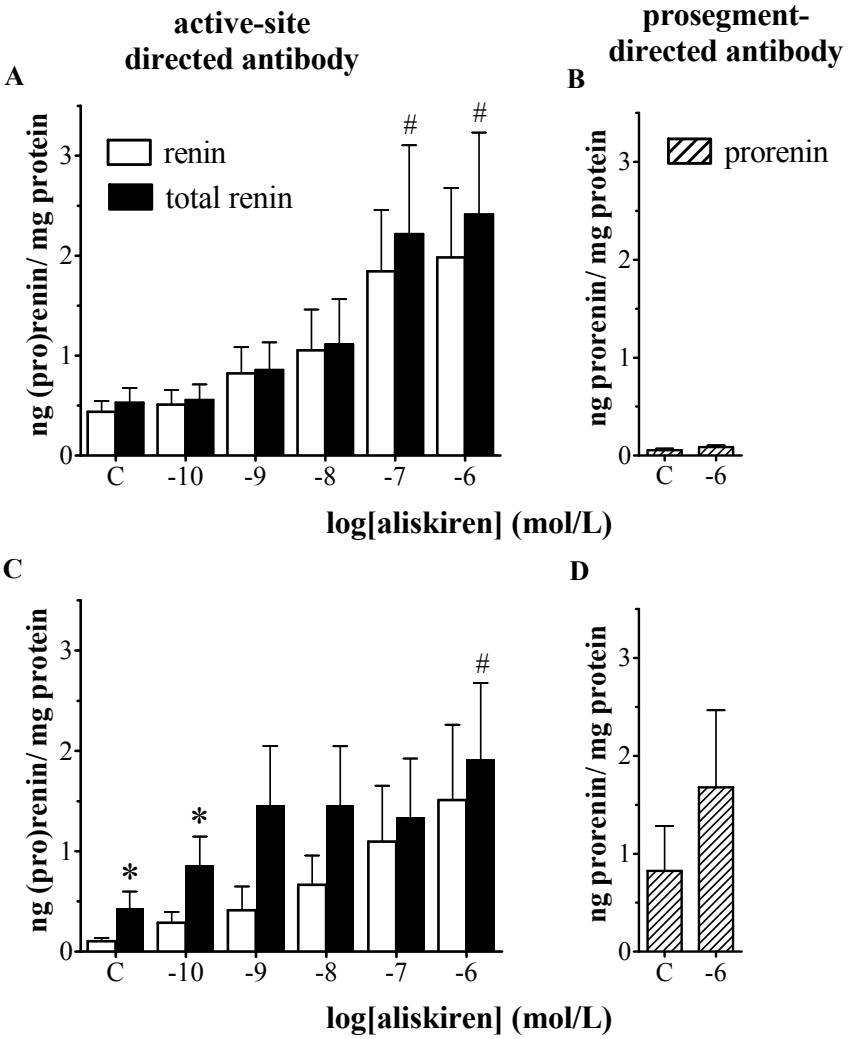

Figure 2. Levels of immunoreactive renin, total renin (=immunoreactive renin after prorenin activation) and prosegment-containing prorenin in the cell lysates (**panels A and B**) and culture medium (**panels C and D**) of HMC-1 cells following a 7-day incubation in the absence (C) or presence of aliskiren (0.1 nmol/L-1 µmol/L). Immunoreactive renin was measured with an antibody directed against the active site, while prosegment-containing prorenin was measured with an antibody against the prosegment. Data are mean±SEM of n=8. #P<0.05 vs. total renin control; *P<0.05 vs.corresponding immunoreactive renin.

Renin activity. Both cell lysate and culture medium contained Ang I-generating activity in the absence of aliskiren, and the activity in the culture medium was ≈10-fold lower than that in the cells (Figure 4, top panels). Aliskiren, when incubated for 7 days with the cells, did not alter the Ang I-generating activity in the cell lysates. It did however concentration-dependently reduce the Ang I-generating activity in the culture medium ($P<0.01$). To verify whether aliskiren had been degraded by the cells, Ang I-generating activity was also measured in culture medium of control cells (i.e., cells that that had not been incubated with aliskiren) after adding aliskiren to the incubation sample. As shown in Figure 4, the aliskiren-induced inhibition of Ang I-generating activity was identical under both conditions (IC_{50} 3.5±1.2 vs. 1.2±0.4 nmol/L; P=NS). Thus, a 7-day incubation had not degraded aliskiren.

Stimulating the cells with forskolin resulted in the appearance of Ang I-generating activity in the stimulation medium (Figure 4, lower panel). Applying forskolin after the 7-day incubation of the cells with aliskiren, revealed a concentration-dependent decrease in the released amount of Ang I-generating activity ($P<0.05$), despite the fact that the forskolin studies were performed without adding aliskiren again to the medium. Moreover, when incubating the stimulation medium with excess human renin, the Ang I-generating activity of this renin was 110±10% of that without stimulation medium (n=5). This indicates that the stimulation medium no longer contained aliskiren concentrations capable of blocking HMC-1 cell-released renin, i.e., that the wash procedure had effectively removed aliskiren.

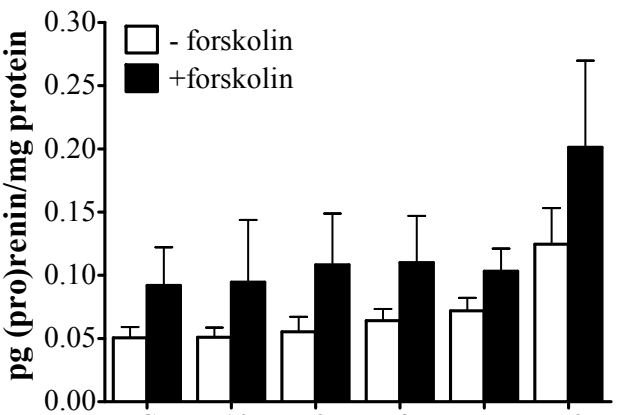

Figure 3. Immunoreactive renin levels in the stimulation medium of HMC-1 cells exposed to 50 µmol/L forskolin for 4 hours following a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L-1 µmol/L). Data are mean±SEM of n=5. Forskolin doubled the renin release at all aliskiren concentrations ($P<0.01$).

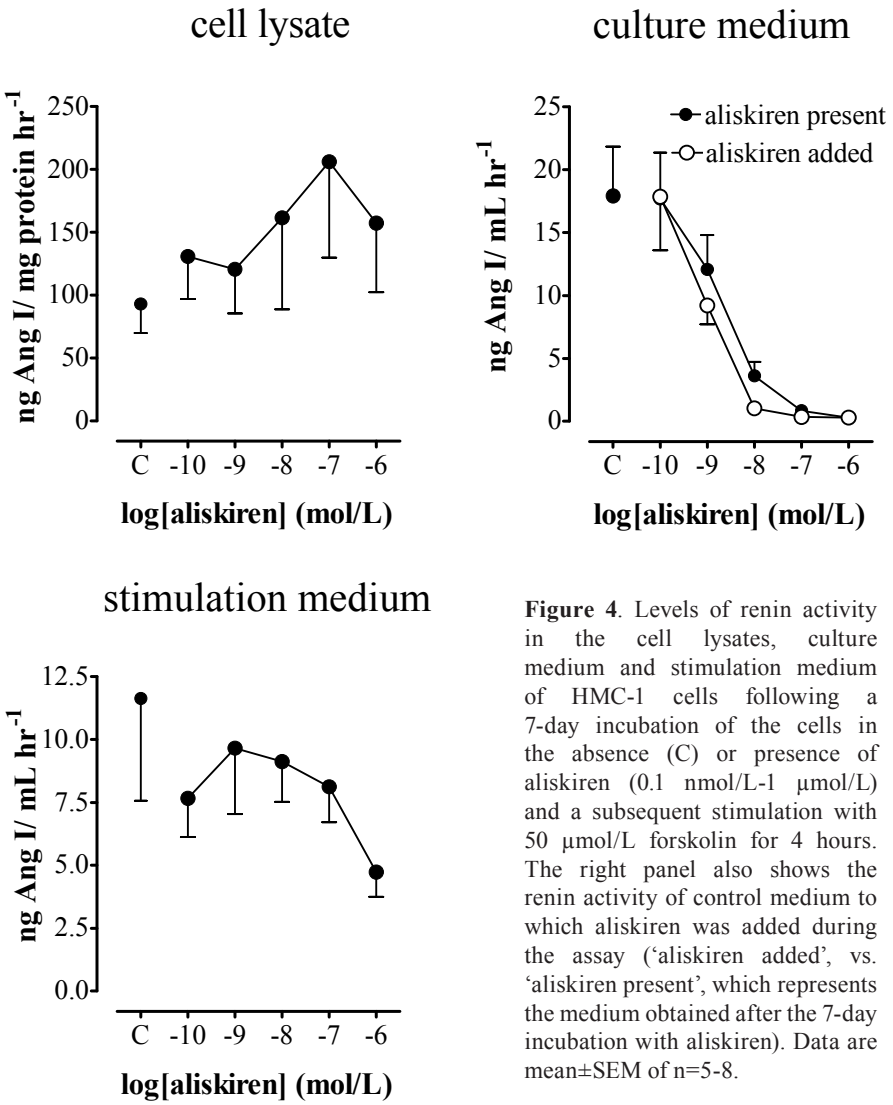

Figure 4. Levels of renin activity in the cell lysates, culture medium and stimulation medium of HMC-1 cells following a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L-1 µmol/L) and a subsequent stimulation with 50 µmol/L forskolin for 4 hours. The right panel also shows the renin activity of control medium to which aliskiren was added during the assay ('aliskiren added', vs. 'aliskiren present', which represents the medium obtained after the 7-day incubation with aliskiren). Data are mean±SEM of n=5-8.

The renin activity data should be viewed in the light of the increased immunoreactive renin levels during aliskiren exposure. Figure 5 therefore displays the amount of aliskiren-bound renin (i.e., inhibited renin) as a percentage of the total amount of renin in the cell lysate, culture medium and stimulation medium (i.e., 100% x (1 - [Ang I-generating activity x 2.6] / [immunoreactive renin])). It then becomes clear that aliskiren has also blocked intracellular renin. In fact, the curves for cell lysate renin and renin in the stimulation medium (Figure 5, left panels) were identical (P=NS). This demonstrates that they represent the same renin pool, i.e., the renin storage granules.

In the culture medium (Figure 5, right panel), a distinction was made between renin and prorenin. The prosegment assay data had indicated that the majority of culture medium total renin represented prorenin, both without and with aliskiren (Figure 2D). It was therefore assumed that, at all aliskiren concentrations, the percentage of total renin that represented renin was identical to that in the absence of aliskiren. The Ang I-generating activity data were compared with the renin levels that were thus calculated, in order to determine the amount of aliskiren-bound renin in the culture medium. The remainder of the total renin levels represented prorenin. To also calculate the amount of aliskiren-bound prorenin, these levels were compared with the amount of prorenin that was directly recognized in the renin IRMA because this recognition is based on the aliskiren-induced non-proteolytic activation of prorenin, i.e., on the binding of aliskiren to prorenin. As can be seen in Figure 5 (right panel), the aliskiren concentrations required to bind prorenin were 1-2 orders of magnitude higher than the concentrations needed to bind renin.

Human plasma samples

Figure 6 shows the immunoreactive (pro)renin levels in plasma of human subjects treated with aliskiren. These values are based on the Cisbio renin IRMA measurements before and after prorenin activation. According to these measurements, aliskiren treatment increased plasma renin in a dose-dependent manner, and no increases in prorenin occurred. If anything, with this assay prorenin even decreased at t=5 hours after the intake of the 2 highest aliskiren doses. In contrast, when measuring prorenin based on the presence of its prosegment ('true' prorenin), prorenin increases were observed, which were most prominent at 24 hours after the intake of aliskiren. The difference between the 2 prorenin measurements became significant at aliskiren doses of 150 mg and higher, and increased with each subsequent aliskiren dose. Importantly, the differences were larger at 24 hours than at 5 hours post-aliskiren, despite the fact that at 24 hours, the aliskiren concentration in blood was much lower than at 5 hours (Figure 6). When using the 'true' prorenin concentrations to recalculate the 'true' renin concentrations, by subtracting 'true' prorenin from the total renin level measured by Cisbio renin IRMA, it became apparent that the Cisbio renin IRMA overestimated renin by up to 30%.

DISCUSSION

This study reveals first that aliskiren accumulates in renin storage sites in renin-synthesizing cells, and second that a significant percentage of the in-vivo 'renin' rise after aliskiren is in fact non-proteolytically activated, intact prorenin. The latter has been suggested before,[139] and our data are the first to demonstrate the dose-dependency of this phenomenon, and to suggest that it also occurs in vivo. At low aliskiren doses, direct prorenin measurements (based on the detection of the prosegment) yielded results that were identical to the indirect prorenin measurements (calculated from the difference between the results of the renin

IRMA before and after prorenin activation). However, at aliskiren doses of 150 mg and higher, the indirect measurements yielded lower prorenin levels, and prorenin even appeared to decrease at 5 hours after the intake of aliskiren. The direct prorenin measurements now show that this is incorrect, and that, in fact, prorenin increases after the intake of aliskiren. This increase was of modest proportion in comparison with the increase in renin. Furthermore, it was most apparent at 24 hours after intake, as opposed to the rise in renin that was already maximal at 5 hours. These data are in full agreement with the fact that prorenin and renin release occur in a constitutive and regulated manner, respectively, and that RAS stimulation predominantly causes a rise in renin.[5]

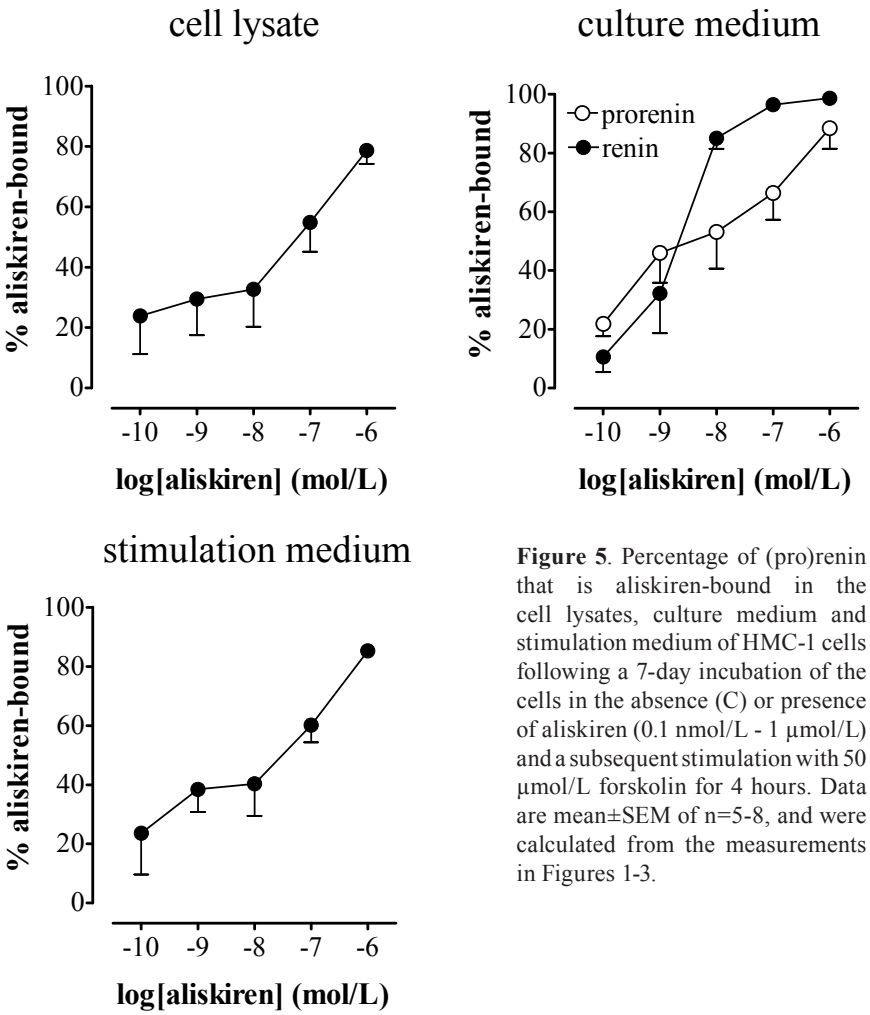

Figure 5. Percentage of (pro)renin that is aliskiren-bound in the cell lysates, culture medium and stimulation medium of HMC-1 cells following a 7-day incubation of the cells in the absence (C) or presence of aliskiren (0.1 nmol/L - 1 µmol/L) and a subsequent stimulation with 50 µmol/L forskolin for 4 hours. Data are mean±SEM of n=5-8, and were calculated from the measurements in Figures 1-3.

The mechanism underlying the underestimation of prorenin when using renin IRMAs involves aliskiren-binding to prorenin in the open conformation, thereby preventing its return to the closed conformation.[138] Only the former conformation will be recognized in renin IRMAs, and thus renin will be overestimated and prorenin underestimated when using these assays. Under normal physiological conditions, <2% of prorenin is in the open conformation, because at 37°C the inactivation step occurs rapidly.[76] Since at low temperature the equilibrium shifts considerably towards open prorenin, one may argue that the observations in this study are, at most, an ex-vivo phenomenon related to frozen storage of the samples. However, the aliskiren effect was most apparent at 24 and 48 hours after drug intake, and not at 5 hours after intake, when the plasma aliskiren levels are highest (and when the largest consequences of this phenomenon, if solely occurring during storage, should have occurred). This therefore suggests that the aliskiren binding to prorenin had already occurred in vivo.

In further support of this concept, aliskiren, when incubated with HMC-1 cells at 37°C for 7 days, concentration-dependently blocked both renin and prorenin in the medium, until at concentrations of 0.1 µmol/L and higher virtually all renin and prorenin had become aliskiren-bound. Such aliskiren concentrations do occur in vivo (Figure 6), and thus these results are clinically relevant. The concentrations required to fully block prorenin were 1-2 orders of magnitude higher than the concentrations needed to block renin. This relates to the small percentage of prorenin that is in the open conformation at 37°C. Since this percentage is higher at lower temperatures, it is much easier to 'activate' prorenin with aliskiren at 4°C than at 37°C (i.e., to fully shift the equilibrium between the closed and open conformation into the direction of the open, renin IRMA-recognisable conformation).[138] Aliskiren-binding to both renin and prorenin stabilizes the molecule, thereby increasing their half life by a factor of 2-3.[138] This explains why the medium and cellular levels of (pro)renin in the present study increased progressively when incubating the cells with increasing concentrations of aliskiren. In vivo, interference with the negative feedback loop would have been proposed to explain a rise in (pro)renin.[134] However, since we were unable to demonstrate angiotensinogen synthesis by these cells, this explanation cannot be applied here. In fact, incubation with either captopril (10 µmol/L) or the AT_1 receptor antagonist eprosartan (1 µmol/L) for 7 days did not increase renin (n=3, data not shown).

Finally, when exposing HMC-1 cells to forskolin after their 7-day incubation with aliskiren, the cells released aliskiren-bound renin. Thus, apparently, aliskiren is taken up by HMC-1 cells and reaches renin granules. Indeed, it has been suggested in both mast cells[143] and JG cells[144] that early secretory granules have the ability to take up extracellular substances, delivered through retrograde transport via early/recycling endosomes and surcompassing the Golgi network.[6] This is perhaps not surprising considering that the renin granules originate as lysosomes.[93] Experiments with excess M6P excluded the possibility that the cellular uptake of aliskiren represented internalization of aliskiren-bound renin from the medium via M6P receptors, which are known clearance receptors for both renin

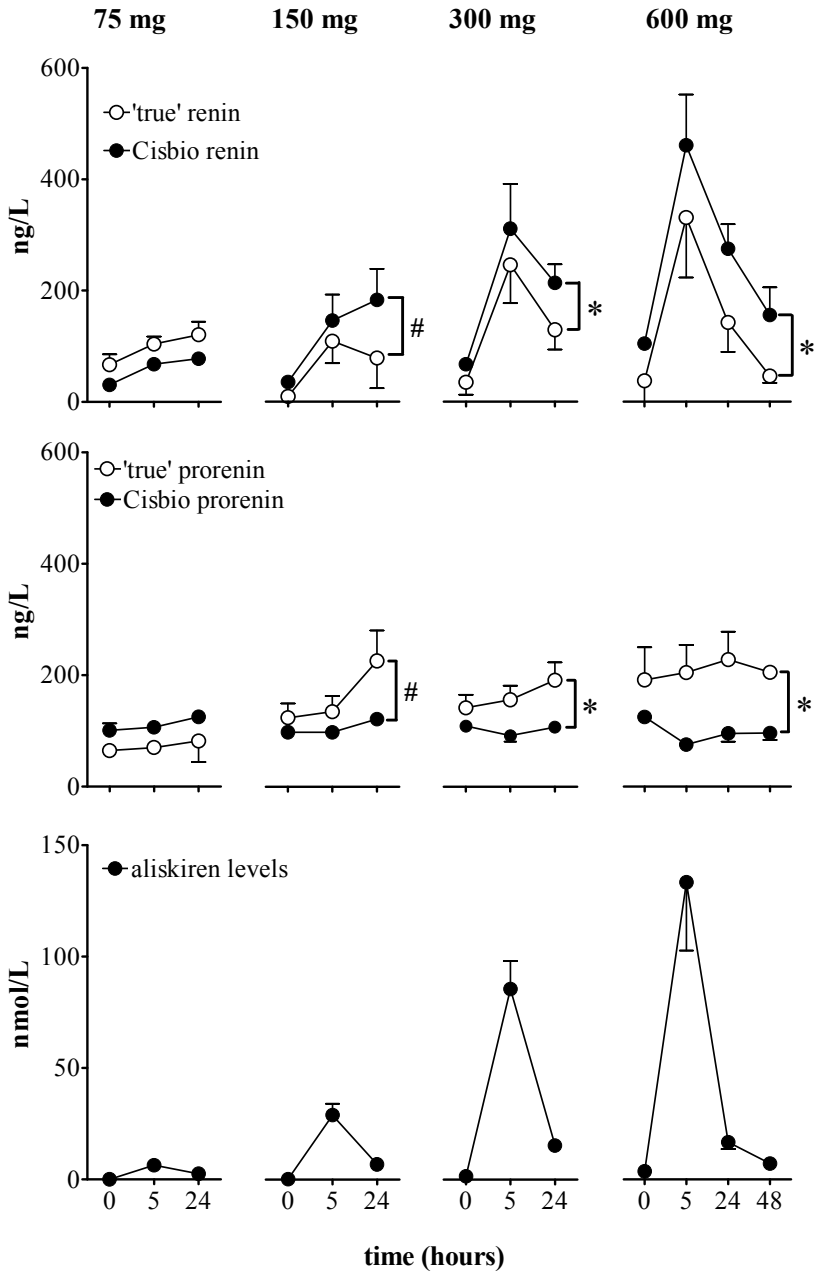

Figure 6. Renin (**top panel**), prorenin (**middle panel**) and aliskiren (**bottom panel**) levels in plasma in 20 subjects on a low sodium diet after aliskiren exposure (75-600 mg), measured with the Cisbio renin IRMA ('Cisbio' (pro)renin)) and/or a prosegment-directed assay ('true' (pro)renin)). For doses greater than 75 mg, time 0 usually occurred 48 hours post previous dose. Data are mean±SEM. #P<0.05 vs. Cisbio (pro)renin; *P<0.01 vs. Cisbio (pro)renin.

and prorenin.[57, 59] In-vivo studies in rats have already indicated that 3 weeks after stopping aliskiren treatment, when plasma levels are below the detection limit, the renal tissue levels of aliskiren are still well above its IC_{50}.[128] When combining these data with the present results, it seems reasonable to suggest that the renal 'reservoir' of aliskiren is in fact the JG cell. Thus, these observations might explain why the effects of aliskiren on blood pressure and renin activity last for several weeks after stopping treatment.[140, 141]

PERSPECTIVE

The cell culture and clinical data in the present study, when combined, convey two important messages. First, aliskiren binds intracellularly to stored renin, thus inhibiting the enzymatic activity of this enzyme prior to its secretion. The actual presence of aliskiren-bound renin in storage granules implies that the effect of the drug will continue even when drug levels in blood are low or undetectable (e.g., when a patient does not take his daily dose or when treatment has been stopped altogether). This phenomenon might therefore contribute to the long-lasting effects of aliskiren after stopping treatment.[140, 141] Obviously, since our results were obtained in mast cells, future in-vivo studies should now confirm aliskiren accumulation in JG cells, e.g., using fluorescently-tagged aliskiren.

Second, aliskiren also binds to prorenin. The cell culture data show that the concentrations required to accomplish this, at least at 37°C, are higher than those required for its binding to renin. Nevertheless, such concentrations do occur in blood during treatment, and thus this phenomenon is of clinical relevance. On the one hand, aliskiren-binding allows prorenin to be detected as renin in the commercially available renin IRMAs, and it thus explains, at least in part, the large rise in renin that has been noted during aliskiren treatment.[136] Simultaneously, given the tight binding of the molecule, it would allow aliskiren-bound prorenin to accumulate at tissue sites. Since prorenin, via binding to the (pro)renin receptor,[47, 48] has been suggested to be a major contributor to tissue angiotensin production, this would imply that aliskiren highly efficiently blocks tissue angiotensin generation. Such efficient interference with tissue angiotensin generation might for instance explain why the effects of aliskiren on renal plasma flow are much bigger than those of other RAS blockers.[142] In view of the long half life of aliskiren-bound prorenin,[138] it might also underlie the long-lasting effects of aliskiren after stopping treatment.

Chapter 7
Evaluation of a direct human prorenin assay

Based on:
Krop M, Derkx FHM, de Bruin RJA, van Gool JMG, Day D, Danser AHJ. Evaluation of a direct human prorenin assay. In preparation 2009.

ABSTRACT

Prorenin measurement requires prorenin-renin conversion by trypsin, thus allowing its detection in an active-site-specific immunoradiometric assay (IRMA). Pretreatment of prorenin with the renin inhibitor aliskiren induces a conformational change in the prorenin molecule, so that it can also be recognized by an antibody directed against the active site, although the prosegment is still present. Recently, a direct prorenin assay has been developed by Molecular Innovations. It applies an antibody (4B5-E3) against residues 30-43 of the prosegment. This assay measures prorenin directly, without requiring trypsin or aliskiren pretreatment. Here we applied this kit to 28 human plasma samples, and a comparison was made with the indirect prorenin measurements. We also verified whether the antibody interferes with prorenin-renin conversion by trypsin. Prorenin generated in CHO cells was used as a standard. Trypsin and aliskiren increased the renin levels detected with the renin IRMA from 24±7 to 144±31 and 185±37 pg/mL, resp. The prorenin levels calculated from these data correlated significantly ($P<0.001$) with the prorenin levels measured with the new prorenin kit. Trypsin reduced the amount of prorenin detected with the new kit by ≈70%. Thus, trypsin cleavage, at least in part, occurs at a site that still allows detection by 4B5-E3. Aliskiren reduced the prorenin levels measured with the new kit by ≈50%, indicating that the aliskiren-induced conformational change hampers prosegment recognition by 4B5-E3. Finally, 4B5-E3, at concentrations >10 nmol/L, reduced the increase in renin observed with the renin IRMA after trypsin conversion by ≈50%. However, since it also reduced renin detection at baseline by ≈50%, it appears that 4B5-E3 interferes with the binding of the active site-directed antibody used in the renin IRMA. In conclusion, the new kit allows rapid detection of prorenin, but should be used with care when measuring samples of aliskiren-treated patients.

INTRODUCTION

Direct prorenin assays currently do not exist. Yet, there is a need for such assays now that it has become clear that prorenin is more than just the inactive precursor of renin.[48, 50] Prorenin also originates at sites outside the kidney,[145, 146] and its levels are particularly elevated in diabetes[83] and pregnancy.[26, 147] Possibly, the elevated levels of prorenin in diabetes function as a marker of the microvascular complications of diabetes,[148] and thus the determination of plasma prorenin levels at an early stage may help to identify subjects who will develop nephropathy and/or retinopathy and require preventive treatment.

Currently, prorenin can only be measured indirectly, after its conversion to renin, by either an enzyme-kinetic assay, detecting angiotensin (Ang) I-generating activity, or an immunoradiometric assay, utilizing monoclonal antibodies that recognize renin's active site. Recently, a monoclonal antibody has been developed that recognizes an epitope near the putative cleavage site ($R_{43}L_{44}$) of prorenin,[149] i.e., the site where normally the 43-aminoacid prosegment is cut off. According to the manufacturer, this antibody does not detect renin. A kit has been developed that makes use of a capture antibody coated on a microtiter plate, the above mentioned cleavage site-directed primary antibody and a secondary antibody conjugated to horse radish peroxidase. The substrate tetramethylbenzidine (TMB) is used for color development at 450 nm, which is directly proportional to the concentration of prorenin in the sample.

The prorenin prosegment unfolds from the enzymatic cleft in a pH- and temperature-dependent manner, thereby resulting in two potential prorenin conformations: a 'closed', inactive form, and an 'open' form that displays full enzymatic activity.[15] Under physiological conditions, <2% of prorenin is in the open conformation.[46, 48, 76] The recently introduced renin inhibitors aliskiren binds to prorenin in the open conformation. As a consequence, due to the tight binding of the renin inhibitor, the inactivation step (i.e., the return to the closed conformation) is now no longer possible, and thus, the equilibrium between the closed and open conformation will shift in favor of the open conformation. Eventually, depending on the concentration of the inhibitor, a significant proportion of prorenin may be open ('non-proteolytic activation'), allowing its recognition by the active site-directed antibodies used in the above-mentioned direct renin assays, despite the fact that the prosegment is still present.[76, 138, 150] Therefore, the excessive immunoreactive renin rise that has been reported to occur following treatment with the renin inhibitor aliskiren[136] may be an assay artifact.[151]

Prorenin levels normally are ≈10-fold higher than those of renin.[5] Thus, even if only a small percentage of prorenin is in the open conformation, this might result in a significant contribution to Ang I generation in enzyme-kinetic assays and/or the recognition of prorenin as renin in renin immunoradiometric assays, particularly when samples have been frozen and thawed ('cryoactivation'), i.e., exposed to conditions allowing prorenin to obtain its 'open' conformation.[15, 86]

In the current investigation, we tested the new prorenin kit, using blood samples containing native prorenin, before and after exposure to the renin inhibitor aliskiren, and also after treatment with the prosegment-cleaving enzyme trypsin. Furthermore, we compared the Ang I-generating activity of blood plasma samples immediately after withdrawal with the activity in the same samples after they had been frozen and thawed, to evaluate the possibility that cryoactivation of prorenin is responsible for (part) of the Ang I-generating in blood samples that have been frozen.

MATERIAL AND METHODS

Plasma samples

Plasma samples were obtained from 28 hypertensive patients (12 women, 16 men, age 23-83 years, mean 57 years) and 6 healthy controls (2 women, 4 men; age 23-45 years, mean 31 years). Blood was collected in polystyrene tubes containing 6.25 mmol/L EDTA (final concentration). The samples were centrifuged at room temperature at 3000 g for 10 minutes, and plasma was either used immediately or stored at -20°C. None of the patients used aliskiren.

Biochemical measurements

Immunoreactive assays. Prorenin was measured indirectly with the Cisbio kit, and directly with the newly developed Molecular Innovations kit. In order to allow its recognition in the Cisbio kit, which makes use of an antibody recognizing renin's active site, prorenin was either converted to renin with immobilized trypsin (72 hours at 4°C), or the samples were exposed to 10 µmol/L aliskiren for 48 hours at 4°C. The latter procedure effectively converts all prorenin into the 'open' conformation, and yields identical renin results as trypsin treatment. Details of this approach have been described before.[138] In accordance with the instructions of the Molecular Innovations kit, 100 µL sample was incubated for 30 minutes with the capture antibody coated on a microtiter plate while shaking at 300 rpm. Next, the wells were washed with 300 µL wash buffer, and 100 µL primary antibody (4B5-E3) was added. Following another 30-min incubation at 300 rpm, the wells were washed again with 3 times 300 µL wash buffer, and incubated for 30 minutes with 100 µL secondary antibody at 300 rpm. Then another wash step followed and the amount of secondary antibody was determined by incubating the samples for 8 minutes with 100 µL TMB, and determining the absorbance at 450 nm after stopping the reaction with 50 µL 1 mol/L H_2SO_4. Recombinant human prorenin was used a standard, and the detection limit was 10 pg/mL (Figure 1).

Enzyme-kinetic assay. Ang I-generating activity was determined by incubating plasma with excess sheep angiotensinogen, either directly or after 18 hours at 37°C, in the presence of angiotensinase inhibitors. Imidazole buffer (final concentration 0.1 mol/L) was added to the incubation mixture to keep pH at 7.4.

Data analysis

Data are expressed as mean±SEM. Statistical significance was tested by one-way ANOVA, followed by Bonferroni's multiple comparison testing. $P<0.05$ was considered significant.

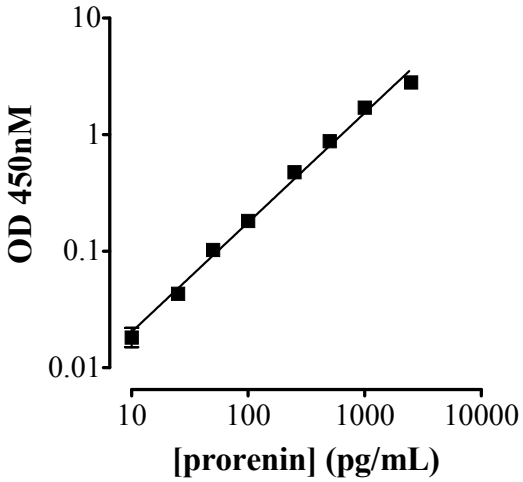

Figure 1. Typical standard curve obtained with recombinant human prorenin in the Molecular Innovations kit. Each data point represents the mean of a duplicate measurement, and has been corrected for the absorbance at 450 nm of a blanc not containing prorenin.

RESULTS

Plasma samples

Incubating human plasma samples containing excess recombinant human prorenin (≈10 ng/mL) with trypsin, yielded identical results in the Cisbio assay as incubating these samples with aliskiren (Figure 2, left panel; n=3). This confirms that both procedures allow full recognition of prorenin in this assay. Results obtained with the Molecular Innovations assay in untreated, excess recombinant human prorenin-containing plasma samples were identical to those in the Cisbio assay after trypsin or aliskiren treatment (Figure 2, right panel; n=3). Furthermore, as expected, trypsin treatment greatly decreased the amount of recombinant human prorenin detected with the Molecular Innovations assay in these samples (by 95±4%). Importantly, aliskiren exposure also reduced the amount of prorenin detected with the Molecular Innovations assay, by 58±4%. Thus, apparently, the conformational change induced by the renin inhibitor blocks the recognition of the $R_{43}L_{44}$ epitope by the monoclonal antibody applied in the new assay.

Results for endogenous prorenin (n=28) in the Molecular Innovations assay were comparable to that obtained with recombinant human prorenin (Figure 3): trypsin and aliskiren reduced the prorenin levels detected with this assay by 70±4 % and 50±6 %, respectively. The renin level detected with the Cisbio assay in the 28 samples was 24±7 pg/mL. After trypsin and aliskiren treatment this level increased to 144±31 and 185±37 pg/mL, respectively. The prorenin levels calculated from the difference amounted to 123±25 and 161±31 pg/mL, respectively, and correlated significantly with the prorenin levels measured in untreated samples with the Molecular Innovations assay (Figure 4).

Preincubating plasma samples with 10 nmol/L of the prosegment-directed monoclonal antibody for 24 hours at 4°C reduced the amount of renin recognized in the Cisbio assay following trypsin activation by 55±5 % (Figure 5; $P<0.05$, n=3). This suggests that the antibody blocks prosegment cleavage by trypsin. However, pretreatment of recombinant human renin (in buffer) with the monoclonal antibody (100 nmol/L) also reduced the amount of renin recognized in the Cisbio assay (by 80%). Thus, the 'reduction' in prorenin activation reflects interference of the antibody with the Cisbio assay per se rather than blockade of prorenin proteolysis by trypsin. Results obtained with the enzyme-kinetic assay revealed that the presence of the antibody interfered to a lesser extent with the enzymatic activity of renin (Figure 5), although renin activity was also reduced by ≈40% at 100 nmol/L of the monoclonal antibody.

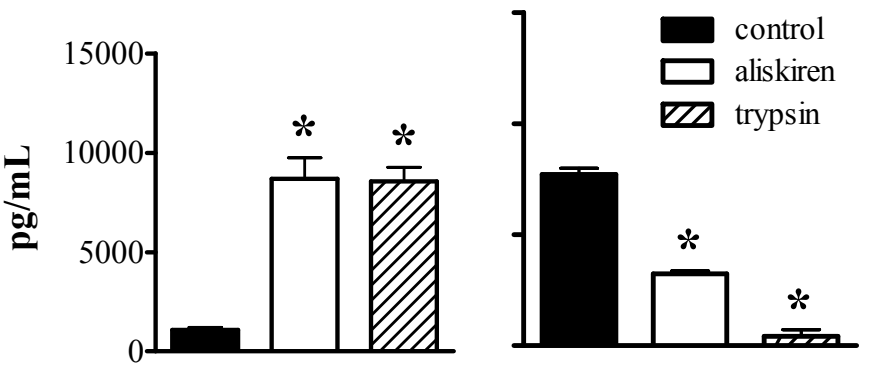

Figure 2. Detection of excess recombinant human prorenin added to human plasma with either the Cisbio renin kit (**left panel**) or the Molecular Innovations prorenin kit (**right panel**). Data (mean±SEM of n=3) were obtained without pretreatment (control), or after treatment of the sample with aliskiren or trypsin. *$P<0.01$ vs. control

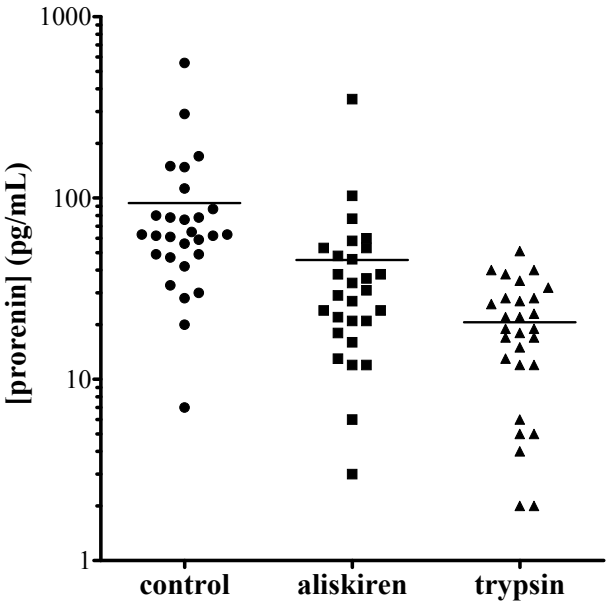

Figure 3. Prorenin levels in 28 human plasma samples determined with the Molecular Innovations kit. Data were obtained without pretreatment (control), or after treatment of the samples with aliskiren or trypsin. The bar represents the mean.

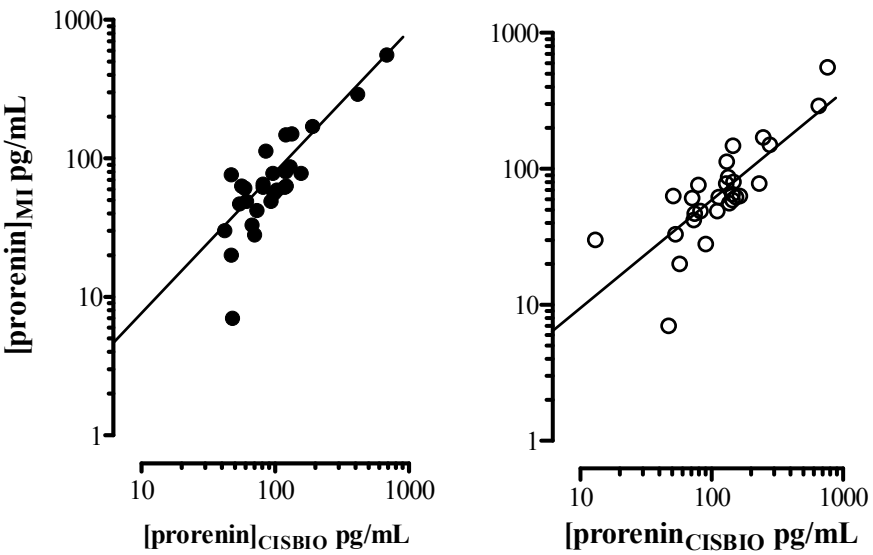

Figure 4. Correlation between the prorenin levels measured indirectly with the Cisbio renin kit using either aliskiren- (**left panel**; n=28, r=0.83, P<0.001) or trypsin-pretreated samples (**right panel**; n=28, r=0.80, P<0.001), with the prorenin levels measured with the Molecular Innovations (MI) kit in untreated samples.

Cryoactivation

When incubating freshly obtained plasma samples, from 6 healthy controls, with excess angiotensinogen at 37°C, Ang I generation was linear over time (Figure 6, top left panel). Results obtained in the same samples after they had been frozen and thawed were fully identical. All samples were also incubated under the same conditions after adding exogenous recombinant human renin. Ang I generation by exogenous renin (corrected for the activity of endogenous renin) was also linear over time and unaffected by freezing and thawing (Figure 6, bottom left panel). However, when performing the assay after the freshly obtained or frozen samples had been kept for 18 hours at 37°C (to inactivate open prorenin) the degree of Ang I generation was still linear, but lower, both for endogenous and exogenous renin, although the difference was significant only for samples that had been frozen and thawed (Figure 6, right panels). This appeared to be due to (pro)renin degradation, since the amount of total renin (i.e., the sum of renin and prorenin) detected in the plasma samples that had been frozen and subsequently exposed to an 18-hour incubation at 37°C amounted to 90±3% (no exogenous renin added) and 79±4% (with exogenous renin), respectively, of the level measured without incubation at 37°C (data not shown). The total renin half lives calculated from these % were 87±15 and 60±11 hours, respectively (P=NS). In contrast, total renin in the freshly obtained samples, also following the addition of exogenous renin, did not change over the 18 hour-period.

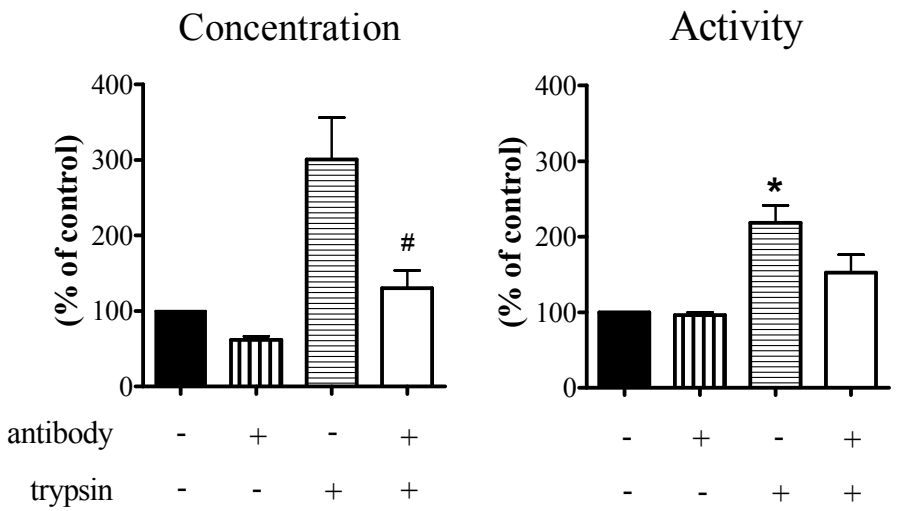

Figure 5. Immunoreactive renin levels detected with the Cisbio renin kit (**left panel**) and Ang I-generating activity determined with the enzyme-kinetic assay (**right panel**) in human plasma samples before and after trypsin treatment in the presence or absence of the antibody 4B5-E3 (10 nmol/L). Data (mean±SEM of n=3) are expressed as a percentage of the level in untreated samples. * P<0.05 vs. no pretreatment (=control); #P<0.05 vs. trypsin alone.

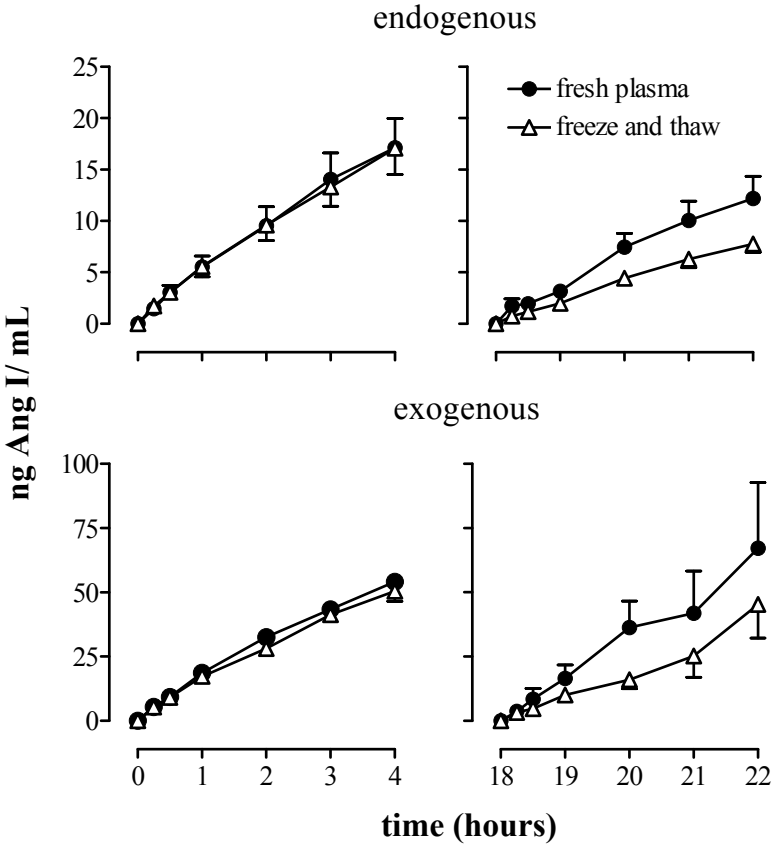

Figure 6. Ang I-generating activity by endogenous human renin (**top panels**) in plasma or by exogenous recombinant human renin added to these same plasma samples (**bottom panels**), determined either immediately (**left panels**) or after incubating the samples for 18 hours at 37°C (**right panels**). Data are mean±SEM of n=6, and represent freshly obtained plasma (closed circles) or the same plasma after it had been frozen and thawed once (open triangles).

DISCUSSION

This study shows that the newly developed prorenin kit allows a rapid and accurate detection of prorenin in human plasma samples. Results are comparable to those detected in an indirect manner with a renin-specific kit. Unfortunately, aliskiren interferes with the direct prorenin assay, lowering the amount of prorenin that can be detected under the conditions of the assay. Although this observation may help to obtain a better understanding of the kinetics of the non-proteolytic activation of prorenin,[152] it is clear that the contribution of aliskiren-induced prorenin 'activation' to the renin surge after aliskiren cannot be determined with

this new prorenin kit.

Trypsin-treatment reduced the amount of prorenin recognized in the Molecular Innovations kit by 70%. The fact that this percentage did not reach 100% suggests that trypsin-cleavage of the prosegment may not always occur at the same site, so that a small percentage of renin molecules still contain the $R_{43}L_{44}$ epitope recognized by the monoclonal antibody of the kit. Alternatively, the antibody may also detect correctly-cleaved renin, albeit with low affinity. This is evident from the studies showing that high concentrations ($\geq$ 10 nmol/L) of the antibody interfere with the direct renin assay and block renin's enzymatic activity. Clearly, since this required high antibody concentrations, and considering that renin levels are usually <10% of the levels of prorenin, this phenomenon is unlikely to be a problem when measuring prorenin with the new kit in blood plasma. In fact, the prorenin levels determined with the new kit tended to be lower than the prorenin levels determined indirectly with the Cisbio kit. The opposite should have been true when simultaneously detecting renin with the new kit.

A true comparison of the two types of prorenin measurement (direct and indirect) would ideally rely on the use of the same standard. Only when aliskiren-pretreated, intact prorenin could have been detected by the $R_{43}L_{44}$ antibody to the same degree as native prorenin this would have been possible. Unfortunately, however, this was not the case, and thus now in the Molecular Innovations assay we used untreated recombinant human prorenin as the standard, and in the Cisbio assay this same prorenin could only be detected after its treatment with either aliskiren or trypsin. In an earlier prorenin assay developed by us this problem did not exist, since in that assay the antibody that was used recognized the aminoacids 20-43 of the prosegment.[46, 138, 150] This antibody reacts with aliskiren-pretreated prorenin to the same degree as an active site-directed antibody.

Finally, we obtained no evidence for prorenin cryoactivation in samples of young volunteers that had been frozen and thawed once. Their Ang I-generating activity was identical to that in freshly obtained blood samples from the same subjects. Since cryoactivation is a reversible process, we had expected Ang I generation to decrease when keeping frozen and thawed plasma samples for a long period (18 hours) at 37°C. Although this did occur in such samples (and not in freshly obtained samples), it was due to (pro)renin destruction rather than prorenin inactivation. Apparently, freezing and thawing results in the activation of one or more (pro)renin-cleaving enzymes. The blood samples tested in our cryoactivation experiments were obtained from healthy subjects. Their renin and prorenin levels were in the normal range. Whether the absence of significant prorenin activation following freezing and thawing also holds true for plasma samples of subjects with high prorenin levels (.e.g., diabetics with microvascular complications) remains to be determined.

Chapter 8
Summary and General Discussion

SUMMARY

Chapter 1: Introduction and aim

Angiotensin (Ang) II production at tissue sites efficiently allows Ang II to stimulate its receptors, with little spillover to blood. In contrast to what was originally thought, not all components required to generate Ang II locally are synthesized at tissue sites. In particular, renin is not expressed locally at important cardiovascular tissue sites like the heart and the vessel wall. Because renin is indispensible for angiotensin generation, this implies that such sites must sequester kidney-derived renin from circulating blood. Alternatively, these tissue sites might sequester prorenin, also because the levels of this inactive precursor of renin are so much higher than those of renin.

The juxtaglomerular (JG) cells of the kidney are the main, if not only source of circulating renin. Other anatomical structures like the adrenal gland, the collecting duct, the eyes, and the reproductive system synthesize and release prorenin, but do not convert this into renin. In contrast, mast cells were recently suggested to release renin.

For unknown reasons, diabetes accompanied by microvascular complications (nephropathy and retinopathy) is associated with high prorenin levels. It seems reasonable to assume that the function of prorenin, if any, depends on its ability to generate angiotensins. However, evidence for prorenin-renin conversion outside the kidney is not available. Non-proteolytic activation of prorenin (i.e., a reversible, conformational change of the prorenin molecule allowing prorenin to display activity without undergoing cleavage of the prosegment) might provide an alternative. This is favored by low pH ('acid-activation', resulting in maximally ≈15% of all prorenin molecules being active) and cold ('cryoactivation', resulting in 100% activation). However, under physiological conditions (pH=7.4 and 37°C), the percentage of prorenin molecules displaying activity is <2%, and it is questionable whether in-vivo conditions allow a significant increase of this percentage.

The discovery of the (pro)renin receptor ((P)RR) has changed this view. This receptor binds prorenin with higher affinity than renin, and binding induces a conformational change in the prorenin molecule allowing it to display activity (i.e., similar to the changes induced by low pH and cold). In addition, at supraphysiological concentrations, prorenin induces signaling via this receptor, for instance inducing p42/44 and p38 MAP kinase activation. A second receptor capable of binding renin and prorenin is the mannose 6-phosphate receptor (M6PR). However, M6PRs, unlike (P)RRs, internalize renin and prorenin, and binding to these receptors has now been recognized as a clearance mechanism, not resulting in angiotensin generation.

In the present thesis, the following aims were addressed: 1) to quantify the amount of extrarenal renin/prorenin in the human body and to evaluate the kinetics of renin/prorenin binding to their receptor as well as the possibility that this results in angiotensin generation at tissue sites; 2) to investigate whether mast cells release renin/prorenin, and whether mast cell-derived (pro)renin contributes to cardiac and pulmonary angiotensin generation, both

under normal - and pathophysiological conditions; 3) to study two characteristics of renin inhibition, i.e., an excessive renin rise and a long-lasting effect, and to evaluate a newly designed prorenin kit.

Chapters 2 and 3: Prorenin distribution, binding and activity

In humans, prorenin and renin decrease biphasically after nephrectomy, with a similar half-life for distribution, but a longer elimination half-life for prorenin. Angiotensins followed the disappearance of renin. One-to-two days post-nephrectomy, stable plasma concentrations at 5-10% (renin and angiotensins) and 25-30% (prorenin) of the pre-nephrectomy levels were reached. These levels were comparable to those in patients who had been anephric for years. From the disappearance kinetics it could be calculated that normally ≈90% of kidney-derived renin and prorenin is present outside the blood compartment. The high prorenin levels in anephrics support extrarenal production of prorenin but not renin. The renin levels in anephrics correlated strongly with their prorenin levels and, in fact, corresponded with the percentage of prorenin that in vitro has a so-called 'open conformation' (i.e., displays enzymatic activity). This suggests that renin in anephrics is in fact 'open' prorenin. Haemodialysis nor captopril significantly affected the level of any renin-angiotensin system component in anephrics.

To study the kinetics of renin and prorenin binding to the (P)RR, vascular smooth muscle cells from rats overexpressing the human receptor (h(P)RR) or from their control littermates, were incubated with recombinant human renin or prorenin at 4°C and 37°C. Incubation at 37°C greatly increased binding, compared to incubation at 4°C, suggesting that (pro)renin-binding receptors cycle between intracellular compartments and the cell surface in a temperature-dependent manner. Blockade of the M6PR reduced binding by approximately 50%. During M6PR blockade, h(P)RR cells bound twice as much prorenin as control cells, while renin binding was unaltered. Incubation of h(P)RR (but not control) cells with prorenin and angiotensinogen yielded more angiotensin than expected on the basis of the activity of soluble prorenin, whereas angiotensin generation during incubation of both cell types with renin and angiotensinogen was entirely due to soluble (non-bound) renin. The renin-angiotensinogen-induced vasoconstriction of isolated iliac arteries from control and transgenic rats was also due to soluble renin only. The recently proposed (P)RR antagonist 'handle region peptide' (HRP), which resembles part of the prosegment, blocked neither prorenin binding nor angiotensin generation. So, the human (P)RR preferentially binds prorenin, and such binding results in angiotensin generation, most likely because binding allows prorenin activation.

Chapters 3 and 4: Mast cell-derived renin in heart and lungs

Mast cells are cells of the immune system that reside in all tissues. Recently, mast cells were suggested to release renin, both in the heart and in the lung. Indeed, when studying cells of the human mast cell line HMC-1, we observed that these cells stored renin and released

renin and prorenin. Both the degranulator compound 48/80 and activation of the adenylyl cyclase - cAMP pathway resulted in renin (but not prorenin) release, whereas Ang II blocked this release. These findings fully resemble renin release from renal JG cells. However, in LAD2 mast cells, or freshly isolated mast cells from patients with mastocytosis, no evidence for storage or release of renin or prorenin was found. Thus renin/prorenin synthesis may not be a uniform property of all mast cells.

Next, we investigated the contribution of mast cells to cardiac renin levels, both under control conditions and two weeks after a myocardial infarction (a condition that is known to significantly increase the amount of resident mast cells in the heart). Renin was measured in the coronary perfusate of isolated perfused rat hearts, as well as in the hearts following perfusion. Non-perfused hearts contained Ang I-generating activity, 70% of which could be blocked by the renin inhibitor aliskiren. A 30-minutes buffer perfusion washed away >70% of the aliskiren-inhibitable Ang I-generating activity. Prolonged buffer perfusion or compound 48/80 did not decrease cardiac Ang I-generating activity further, nor induced Ang I-generating activity-release in the perfusion buffer. Results in infarcted hearts were identical, despite the increased mast cell number in such hearts.

In rat main bronchi, mast cell degranulation with compound 48/80 contracted bronchi to 24±4% of the KCl-induced contraction. Blockade of the RAS at each level, i.e., inhibiting renin with aliskiren, ACE with captopril, or the AT_1 receptor with irbesartan, did not affect this response. The Ang I and II levels in the bath fluid after 48/80 exposure were below the detection limit, and freshly obtained bronchial tissue did not contain detectable Ang I-generating activity or angiotensinogen. Yet, Ang I and II equipotently contracted bronchi, and captopril shifted the Ang I curve ≈10-fold to the right, whereas irbesartan fully blocked the effect of Ang II. Thus, although Ang II is able to constrict bronchi, compound 48/80 does not result in sufficient Ang II generation to induce bronchoconstriction.

Mast cells contain a wide variety of mediators, including serotonin. Serotonin was capable of constricting bronchi, and the $5\text{-}HT_{2A/2C}$ receptor antagonist ketanserin fully blocked this effect. Importantly, ketanserin also abolished the compound 48/80-induced bronchoconstrcition, while mast cell stabilization with cromolyn reduced the constriction to 16±5% of that of KCl. Therefore, the compound 48/80-induced bronchoconstriction is due to the release of serotonin rather than renin, and mast cells are an unlikely source of renin, both in the heart and lungs.

Chapter 5 and 6: Renin inhibition and the measurement of renin and prorenin

The vascular effects of the human renin inhibitor aliskiren last much longer than expected based on its half life, and this renin inhibitor has been reported to cause a greater renin rise than other types of renin-angiotensin system blockers. To investigate whether aliskiren accumulation in secretory granules contributes to these phenomena, renin-synthesizing mast cells were incubated with increasing aliskiren concentrations for 7 days, washed and exposed to forskolin in medium without aliskiren.

Without aliskiren, the culture medium contained predominantly prorenin, the cells exclusively stored renin, and forskolin doubled renin release. Aliskiren dose-dependently bound to (pro)renin in the medium and cell lysates, but did not alter the effect of forskolin. The aliskiren concentrations required to bind prorenin were 1-2 orders of magnitude higher than those needed to bind renin. Blockade of cell lysate renin activity ranged from 27±15 to 79±5%, and these percentages were identical for the renin that was released by forskolin, indicating that they represented the same renin pool, i.e., the renin storage granules. Comparison of renin and prorenin measurements in blood samples obtained from human volunteers treated with aliskiren, both before and after prorenin activation, revealed that up to 30% of prorenin was detected in renin-specific assays. In conclusion, aliskiren accumulates in renin granules, thus allowing long-lasting renin-angiotensin system blockade beyond the half life of this drug. Aliskiren also binds to prorenin. This allows its detection as 'renin', and might explain, in part, the renin rise during renin inhibition.

Direct prorenin measurements would be very helpful to resolve the latter issue. We therefore examined a prorenin kit marketed by Molecular Innovations. This kit uses an antibody that recognizes the prosegment near the cleavage site. Results obtained with the new kit in human plasma samples were comparable to those obtained indirectly (following prorenin-renin conversion by trypsin, or after prorenin 'activation' by aliskiren) with a renin-specific assay. Furthermore, as expected, trypsin treatment greatly decreased the amount of prorenin recognized in the Molecular Innovations assay. Surprisingly, aliskiren exposure also reduced the amount of prorenin recognized in this assay. This suggests that the conformational change induced by the renin inhibitor blocks the recognition of the prosegment epitope, and thus that unfortunately, the new kit cannot be used to measure prorenin in aliskiren-treated patients.

Cryoactivation, as a consequence of the storage and thawing of plasma samples, might allow prorenin to contribute to the Ang I-generating capacity of human plasma samples. However, the Ang I-generating activity of plasma samples obtained from 6 healthy volunteers was identical, irrespective of whether the samples were measured immediately after blood withdrawal or after they had been frozen and thawed. Thus, under normal conditions, cryoactivation appears to be of no importance. Whether this also holds true for plasma samples containing high prorenin levels (e.g., from diabetics with nephropathy and/or retinopathy) remains to be proven.

GENERAL DISCUSSION

Prorenin distribution, binding and activity

There has been a lot of debate about the function of prorenin, ever since its discovery in amniotic fluid. The high prorenin concentrations in diabetics and pregnant women further fuelled the discussion. It is reasonable to speculate that the role of prorenin, if any, evolves around Ang I generation. Rodents displaying high (inducible) hepatic prorenin expression support this view,[153, 154] since they were hypertensive, had low renin levels (in agreement with the negative feedback of prorenin-dependent Ang II generation on renal renin release) and responded to RAS blockade. Moreover, this phenotype did not occur when expressing active site-mutated prorenin.[153] These animals exhibited no signs of glomerulosclerosis or cardiac fibrosis. In sharp contrast, an early rat model with liver-specific prorenin expression displayed severe renal lesions and hypertrophic cardiomyocytes in transgenic males only.[155] Renal renin content in these rats decreased, and plasma Ang I-generating activity increased, in line with the studies by Mercure et al. and Peters et al..[153, 154] Yet, these animals were normotensive. Based on this phenotype, it was proposed that prorenin itself is vasculotoxic. Whether RAS blockade could prevent this phenomenon has never been tested.

Although all three above studies imply that prorenin expression somehow resulted in Ang II generation, thereby subsequently suppressing renal renin release, it remains unclear whether this is due to the endogenous activity of prorenin, prorenin binding to the (P)RR and/or actual prorenin-renin conversion. When considering the first possibility, it should be kept in mind that even when <1% of prorenin displays activity, a several 100-fold rise in prorenin is already sufficient to increase Ang II without the need for prorenin-activating mechanisms. For instance, assuming that only 0.1% of prorenin displays activity under physiological conditions, a prorenin rise of 400-fold[155] will increase Ang I generation 4 times. Careful measurements of renin, prorenin, angiotensinogen and Ang I and II, preferably in blood and at the tissue level, are required to sort this out.

Reudelhuber's group has generated mice expressing human prorenin in the liver and human human angiotensinogen in the heart. These mice displayed increased cardiac (but not plasma) Ang I levels.[63] Since the renin-angiotensinogen reaction is highly species-specific (i.e., mouse renin does not react with human angiotensinogen, and vice versa), this approach elegantly demonstrates that apparently human prorenin is taken up in the heart and reacts locally with angiotensinogen. Importantly, the same phenomenon occurred when expressing a noncleavable variant of prorenin.[156] Thus, prorenin's activity at the tissue level did not depend on proteolytic removal of the prosegment. Tissue angiotensin generation by prorenin in humans is supported by the correlation between the plasma levels of prorenin and the renovascular response to captopril in diabetic subjects.[84]

The (P)RR provides an attractive explanation for tissue Ang I generation by intact prorenin, also because it is expressed ubiquitously in heart (coronary arteries[47] and cardiomyocytes[51]), brain, placenta, liver[47], kidney (renal artery[47], mesangial area of the

glomeruli and distal renal tubule[157]), retina[158] and monocytes.[159] Moreover, prorenin appears to be its preferred agonist.[48] However, the nanomolar affinity of the human (P)RR for prorenin (Kd ≈ 6 nmol/L) does not converge with the picomolar prorenin concentrations in plasma, and thus significant receptor binding may only occur when overexpressing prorenin (as in transgenic animals) or in organs expressing prorenin locally, like the kidney, eye, ovaries and placenta.[145] The prorenin concentrations in non-prorenin-synthesizing organs (heart, vessel wall) are unlikely to be much higher than those in blood plasma,[3, 22] and thus a (P)RR-mediated contribution of prorenin to Ang I generation at these sites seems unlikely. Nevertheless, a whole range of positive effects with the (P)RR blocker HRP has been described in both prorenin-expressing organs (kidney, eye)[50, 160-163] and organs that rely on (pro)renin uptake (heart).[62]

One possibility to explain these data is that the HRP effects are not due to interference with local Ang I generation, but rather represent blockade of the direct, angiotensin-independent effects of prorenin mediated via its receptor.[95, 164] Among others, prorenin stimulates signal transduction cascades resulting in the activation of ERK1/2[165] and p38 MAP kinase,[51] and the subsequent release of TGF-β1 and PAI-1.[166] Renin appears to do the same.[159] In view of the latter, it is difficult to understand why HRP (which selectively blocks prorenin, since it interferes with binding of the prosegment) would be effective at all, particularly in the kidney where renin levels far exceed those of prorenin. Possibly, HRP binding induces a conformational change in the receptor, no longer allowing renin (and prorenin) to bind.[167] This needs to be investigated in detail.

To complicate things further, the C-terminal part of the (P)RR is identical to an 8.9 kDa fragment being associated with vacuolar H^+-ATPase.[49] Consequently, the second name of the receptor is ATP6ap2 (ATPase, H^+ transporting, lysosomal accessory protein 2). Advani et al. recently reported that renin and prorenin increase vacuolar H+-ATPase activity, most likely via activation of the (P)RR.[168] Since vacuolar H^+-ATPase is a key mediator of urinary acidification, this implies that renin and prorenin influence the acidification process. This is of particular interest given the relationship between low pH and prorenin activation: possibly, the conformational change in the prorenin molecule induced by binding to the (P)RR involves vacuolar H+-ATPase activation? Moreover, in cultured collecting duct/distal tubule lineage Madin-Darby canine kidney cells, both renin and prorenin induced ERK1/2 phosphorylation, and the selective vacuolar H^+-ATPase inhibitor bafilomycin prevented this activation. Apparently, ERK1/2 phosphorylation in these cells depends on vacuolar H^+-ATPase activity. Given the abundant (P)RR expression in collecting ducts, distal convoluted tubules and distal tubules,[168] and in view of the recent observation that collecting duct principal cells might be the source of prorenin in diabetes,[11] future studies should now unravel the link between prorenin, its receptor, acidification and ERK1/2 activation in diabetes. In particular, a role for Ang II should be excluded. Among others, prorenin measurements in urine of diabetic patients may help to unravel the contribution of locally synthesized prorenin in the kidney.

A further potentially important area of prorenin investigation is pregnancy. Although it has been known for >2 decades that pregnant women have high prorenin levels, which originate in the ovaries and placenta, it is still unknown why prorenin is synthesized at all in the female reproductive tract, also because the regional angiotensinogen levels are low.[169] Decidual (pro)renin expression has been linked to preeclampsia.[33] Does this prorenin act in decidua and/or placenta? Do the prorenin levels in amniotic fluid reflect decidual prorenin expression? Is there a role for the (P)RR? Is prorenin detrimental only, or does it have a protective role, as suggested by Reudelhuber?[153]

The highest prorenin concentrations ever (1 μg/mL) were found in gestational sacs during the first trimester,[37] and thus prorenin and its receptor may have a role in embryonic and fetal development. Studies investigating the expression of prorenin, the (P)RR and other RAS components in decidua and placenta of normal pregnant women and women with preeclampsia will help to address these questions. Importantly, animals knockout for either renin,[44] angiotensinogen[170] or ACE[171] are viable, whereas (P)RR knockout animals are not.[172] This already suggests that the receptor has vital non-RAS-related properties, e.g. linked to vacuolar H^+-ATPase. Interestingly in this regard, patients with a mutation in their ATP6ap2 gene, resulting in an impairment of receptor-induced ERK1/2 phosphorylation (but not (pro)renin binding) suffered from X-linked mental retardation and epilepsy, without displaying cardiovascular or renal dysfunction.[173]

Finally, furin cleavage results in the generation of a soluble form of the receptor.[174] Although this soluble receptor is present in blood plasma, its concentrations are unknown. Thus, studies are required to sort out whether it is present in meaningful amounts, to what degree it binds renin/prorenin and what the consequences of such binding are. Possibly, the soluble receptor-(pro)renin complex acts as an agonist for an as yet unidentified, new receptor.[175] Alternatively, binding may alter the kinetics of the renin-angiotensinogen reaction.[47]

Mast cell-derived renin in heart and lungs

Mast cells play an important role in cardiovascular disorders, as evidenced by their significant increase at cardiac tissue sites after myocardial infarction,[99, 176-178] and their contribution to plaque progression and destabilization in apolipoprotein E-deficient mice.[179] Stabilization of mast cells[180] and the use of mast cell-deficient animals[181] diminished left ventricular remodeling under pathological conditions. Yet, whether this relates to mast cell-derived renin and/or chymase remains to be determined. After many years of research, the concept that chymase is an important Ang I-II converting enzyme in the heart holds no longer.[2, 125, 182, 183] Of course, this does not mean that chymase has no role in the heart.

Considering the wide range of mediators (proteases, histamine, serotonin, cytokines, chemokines and growth factors) released from mast cells,[119] it is challenging to firmly establish a cause-and-effect relationship of one specific mediator. Not surprisingly therefore, many other mast cell-derived components have been linked to cardiovascular

pathology as well: TNF-α, TGF-β1, interleukins, cytokines, and even preformed Ang II.[184-187] From this perspective, a mast cell stabilizer would be more beneficial than a selective blocker of one of the many pathways affected by mast cells-derived mediators.

Mast cells are also involved in the pathogenesis of asthma. In humans, unlike rats, mast cell-derived histamine is a potent bronchoconstrictor. Histamine is thought to be mainly involved in the primary reaction to allergic stimuli. Mast cells however are also indicated in the late phase inflammation and tissue remodeling.[188] Our observation that mast cells do not contribute to renin release in the rat bronchi, does not mean that locally produced angiotensin II cannot be a novel therapeutic target in the management of airway disease, as suggested by Veerappan et al..[115] In fact, ACE inhibitors have recently been reported to decrease mortality in subjects hospitalized with a COPD exacerbation.[132] Given their strong potentiation of bradykinin-induced bronchoconstriction, as also evidenced in our study, further work is needed to sort out the mechanism of this beneficial effect. At least from this perspective, renin inhibitors and/or AT_1 receptor blockers appear to be more attractive drugs to block the pulmonary RAS.

Our observation that mast cells abundantly store renin, and release prorenin constitutively and renin in a regulated manner, reveals that these cells closely mimick the in-vivo characteristics of JG cells.[114] This contrasts with most other extrarenal renin-expressing cells, which usually do not store renin and solely release prorenin.[145] Given that JG cells loose their capacity to store renin when cultured, HMC-1 cells might provide an unique alternative, allowing further studies on the enzymes involved in prorenin-renin conversion, and on the accumulation of a renin inhibitor in renin granules (for instance by using a fluorescently labeled variant of aliskiren). The latter study might be extended to in-vivo studies to also investigate aliskiren accumulation in JG cells.

Renin inhibition and the measurement of renin and prorenin

Our studies with aliskiren[151] demonstrate that the objections against the use of this drug, i.e., a limited effectiveness due to a reactive renin secretion,[136] are unfounded. Obviously, renin concentrations do rise during aliskiren treatment, as they do during every type of RAS blockade. However, the observation that these rises are 'excessive' is due biochemical phenomena: the detection of prorenin as renin,[151] and a prolonged half life of renin (and prorenin) following aliskiren binding.[138] When correcting for these phenomena the rise is unlikely to be as excessive as originally thought. Moreover, the aliskiren levels are high enough to suppress renin activity, even when it has increased excessively,[189] and no evidence was obtained for aliskiren-induced increases in blood pressure. At most, low-renin patients appeared to be somewhat resistant to aliskiren.[190]

The detection of prorenin as renin in renin immunoradiometric assays appeared to be the consequence of aliskiren-binding to prorenin in vivo, i.e., at 37°C. Prolonged incubation of prorenin with aliskiren at 37°C in vitro mimicked this observation. These findings support the concept of prorenin occurring in 2 conformations: a closed conformation displaying no

activity, and an open conformation that can generate Ang I. The latter will bind aliskiren, and due to the high affinity of the inhibitor, the inactivation step (i.e., the return to the closed conformation) is now no longer possible, so that the equilibrium between the closed and open conformation shifts into the direction of the open conformation. Clearly, our data support the occurrence of 'open' prorenin in humans in vivo. Sensitive prorenin assays, allowing a distinction between the open and closed conformation are required to obtain a more complete picture. The recently introduced Molecular Innovations assay, which allows the rapid detection of intact , closed prorenin, may be of help in this regard.

Since low temperature, like aliskiren, shifts the equilibrium between open and closed prorenin into the direction of the former, freezing and thawing of plasma samples theoretically will cause an increase in the levels of open prorenin, thus increasing the Ang I-generating capacity of plasma. However, the Ang I-generating activities in freshly obtained and frozen and thawed blood plasma samples (obtained from healthy volunteers) were identical. Thus, the impact of this phenomenon is apparently minimal, and it is safe to store plasma samples in the freezer before measuring their Ang I-generating activity, at least when it concerns plasma containing 'normal' prorenin levels. Whether this also holds for plasma samples of diabetic patients (containing high prorenin levels) remains to be determined.

To what degree renin inhibitors differ from other RAS blockers, and if so, whether this results in clinically meaningful, additional effects, is still uncertain. Often blockade of the (P)RR by aliskiren is put forward. However, aliskiren does not interfere with renin/prorenin binding to their receptor, nor does it block post-receptor signaling.[128, 159, 191] Yet, like many agonists, renin and prorenin downregulate their receptor, through interaction with the transcription factor promyelocytic zinc finger protein.[91, 191] Simultaneously, hypertension, Ang II (via AT_1 receptors) and/or oxidative stress[192] upregulate the receptor, and thus a complex picture arises in which the receptor may both increase and decrease in the face of changing blood pressure- and (pro)renin (and Ang II) levels during RAS blockade.[128, 192-194] If aliskiren, for a given decrease in blood pressure, causes a larger rise in renin/prorenin than ACE inhibitors and AT_1 receptor blockers, one might predict that the receptor downregulation is most prominent during renin inhibition. However, to sort this out, (P)RR expression should be studied following treatment with various RAS blockers at equi-hypotensive doses.

5-HT	5-hydroxy tryptamine or serotonin
8-db-cAMP	8-dibromo-cyclic 3'-5' adenosine monophosphate
AC	adenylyl cyclase
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
Ang	angiotensin
ANP	atrial natriuretic peptide;
ATP	3',5' adenosine triphosphate
$AT_{1/2}R$	angiotensin II type 1/2 receptor
cAMP	cyclic 3',5'-adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
CGRP	calcitonin gene-related peptide
COPD	chronic obstructive pulmonary disease
CRC	concentration response curve
E_{max}	maximum effect
EC_{50}	concentration where agonist reaches half of maximum effect
EKA	enzyme kinetic assay
ERK	extracellular signal regulated kinase
hCG	human chorionic gonadotropin
HMC-1	human mastocytoma cell line
HPLC	high pressure liquid chromatography
HRP	handle region peptide
IC_{50}	half-maximal inhibitory concentration
IL-1β	interleukin-1β
IMDM	Iscove's modified Dulbecco's medium
IRMA	immunoradiometric assay
JG	juxtaglomerular
K_M	Michealis-Menten constant
LH	luteinizing hormone;
M6P	mannose-6-phosphate
Nx	anephric subjects
NO	nitric oxide;
PAI-1	plasminogen activator inhibitor 1
PG	prostaglandin;
PKC	protein kinase C
(P)RR	(pro)renin receptor
RAS	renin-angiotensin system
RI	renin inhibitor
[S]	substrate concentration
SD	standard deviation
SEM	standard error of the mean
$t_{1/2}$	half-life
TNF-α	tumor necrosis factor α
$V_{(MAX)}$	(maximum) velocity
V-ATPase	vacuolar H+-ATPase
VSMC	vascular smooth muscle cell

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De auteur van dit proefschrift werd in 1978 geboren te Groningen. Na het behalen van zijn VWO diploma aan het NAC in Groningen, gevolgd door een kort uitstapje naar de Bouwkunde faculteit, besloot hij om Farmacie te gaan studeren aan de Rijksuniversiteit Groningen (RUG). Zijn eerste stage werd gedaan onder supervisie van Dr Reinoud Gosens, op de afdeling Moleculaire Farmacologie. Hier deed hij onderzoek naar de rol van MAP kinases in de contractie van luchtweg gladde spiercellen van koeien. Hierna volgde een tweede onderzoeksstage in het lab van Membraancelbiologie, onder leiding van Dr. Jan Willem Kok. Hier werd gekeken naar de internalisatie van AT_1 receptoren, met behulp van confocaal microscopie, in celsystemen waar AT_1 gekoppeld aan GFP tot expressie waren gebracht. Na een doctoraalscriptie geschreven te hebben over receptor dimerisatie, behaalde hij in 2005 zjn diploma farmaceutische wetenschappen.
Vanaf februari 2006 is hij werkzaam geweest als onderzoeker in opleiding op de afdeling Inwendige geneeskunde- sectie Farmacologie, vasculaire en metabole ziekten, van het Erasmus MC, onder leiding van Prof. dr. A.H. Jan Danser. Dit heeft uiteindelijk geresulteerd in dit proefschrift.

Full papers

Batenburg W.W., Krop M., Garrelds, I.M., de Vries R., de Bruin R.J.A., Burcklé C.A., Müller D.N., Bader M., Nguyen G., Danser A.H.J.. Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. *Journal of hypertension*. 2007 Dec; **25**(12): 2441-53.

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Krop M., Chai, W., Özünal Z.G., de Vries R., Buohuizen A.M., Garrelds I.M., Danser A.H.J.. Mast cell degrannulation mediates bronchial contraction via 5-HT and not via renin release. Submitted 2009.

Letters:

Krop M., van Esch J.H.M., Danser A.H.J.. Letter Regarding Article by Itoh et al, "Role of p90 Ribosomal S6 Kinase-Mediated Prorenin-Convertin Enzyme in Ischemic and Diabetic Myocardium". *Circulation*. 2006 Oct 24; **114**(17): E556.

Krop M., Danser A.H.J.. Letter Regarding Article by Campbell, "Response to Aliskiren Therapy will have minimal effect on intracellular renin of renin-producing cells." *Hypertension*. 2009 Feb; **53**(2): E18.

Abstracts:

Krop M., Garrelds I.M., Danser A.H.J.. Renin and prorenin release by human mast cells. *Journal of hypertension*. 2007 Jun; **25**(supl 2): S12.

Krop M., Garrelds I.M., Danser A.H.J.. Renin and prorenin release by human mast cells. *Hypertension*. 2007 Oct; **50**(4): E100.

Batenburg W.W., Krop M., Garrelds I.M., de Vries R., de Bruin R.J.A., Burklé C.A., Müller D.N., Bader M., Nguyen G., Danser A.H.J.. Prorenin is the endogenous agonist of the (pro)renin receptor: binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. *Hypertension.* 2007 Oct; **50**(4): E76.

Krop M., Garrelds I.M., Danser A.H.J.. Renin and prorenin release by human mast cells: does cardiac renin originate in mast cells? *Journal of the Renin Angiotensin-Aldosterone system*. 2008 March; **9**(supl 1): S7.

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Krop M, Chai W, Ozonal ZG, Bouhuizen AMB, Danser AHJ. Bronchial constriction following mast cell degranulation does not involve renin. *Journal of Hypertension*. 2009 Jun; **27**(supl 4): S344

Department:	Pharmacology, Vascular and Metabolic Diseases
Research school:	COEUR
PhD period:	Feb 2006-Nov 2009
Promotor/Supervisor:	Prof.dr. A.H.J. Danser

1. PhD training

	Year	Workload (ECTS)
General academic skills		
- Biomedical English writing and communication	2009	2.0
- Laboratory animal science	2005	3.0
Research skills	2008	4.0
- Statistics; classical methods for data nalysis		
In-depth courses		
- COUER courses	2006-2008	10.5
- Kidney Foundation winterschool	2007	1.2
Oral presentations	2007-2009	5.0
International conferences	2007-2009	9.6
Seminars and workshops		
- COUER seminnars	2006-2009	2.4

2. Teaching activities

	Year	Workload (ECTS
Supervising practicals	2006-2009	2.0

Hoofdstuk 1: Introductie en doel

Angiotensine (Ang) II is een hormoon dat een belangrijke rol speelt in de regulering van de bloeddruk- en de zouthuishouding. Productie van Ang II vindt vooral plaats in weefsels waar zich ook de receptoren voor Ang II bevinden. Echter, niet alle componenten die nodig zijn voor lokale Ang II productie worden ook lokaal geproduceerd. Dit geldt zeker voor renine, een enzym dat onmisbaar is voor de productie van angiotensines. Dus Ang II productie is afhankelijke van circulerend renine, en misschien ook van prorenine, de inactieve precursor van renine, die in hoge concentraties in het bloed voorkomt.

De juxtaglomerulaire cellen in de nier zijn de belangrijkste, en waarschijnlijk de enige bron van circulerend renine. Andere organen, zoals de bijnier, de ogen en het reproductieve systeem produceren prorenine net als de nier, maar zijn niet in staat om dit om te zetten naar actief renine, d.w.z. het verwijderen van het prosegment dat het actieve domein van afschermt. Er zijn echter recente aanwijzingen dat mestcellen, ontstekingscellen die vooral bekend zijn door productie van histamine bij acute allergische reactie, wel actief renine maken.

Bij diabetes wordt het ontstaan van microvasculaire complicaties, resulterend in nier- en oogschade, voorafgegaan door een stijging van de prorenine concentratie in plasma. Het is waarschijnlijk dat het effect van prorenine, als het al een effect heeft, afhankelijk is van de productie van angiotensines. Prorenine kan non-proteolytisch geactiveerd worden, d.w.z. het actieve domein kan blootgelegd worden door een conformatie verandering zonder dat het prosegment hiervoor verwijderd hoeft te worden. Dit gebeurt bij lage temperatuur en pH, echter, onder fysiologische omstandigheden is het percentage actief prorenine minder dan 2%.

In 2002 werd een receptor gevonden die zowel renine als prorenine kan binden, de zgn. (pro)renine receptor, (P)RR . Binding van prorenine aan de receptor zorgt voor een verandering naar een actieve conformatie, vergelijkbaar met die veroorzaakt door lage pH en temperatuur, waardoor angiotensine vorming mogelijk wordt. Bij extreem hoge concentraties (non-fysiologisch) activeert het ook intracellulaire kinases. Er is nog een tweede receptor, de mannose-6-fosfaat receptor (M6PR) die ook zowel renine als prorenine bindt. Echter, de M6PR zorgt alleen voor internalisatie van renine en prorenine, waarna intracellulair afbraak plaatsvindt. Deze receptoren zorgen dus voor klaring (eliminatie) van (pro)renine uit de bloedbaan, zonder dat dit resulteert in angiotensine vorming.

In deze thesis werden de volgende doelen gesteld: 1) het kwantificeren van de hoeveelheid renine/prorenine in mensen die niet afkomstig is uit de nier, en het bestuderen van de kinetiek van renine/prorenine binding aan zijn receptor en de daarmee potentieel samenhangende angiotensine vorming in weefsels; 2) te onderzoeken of mestcellen renine/prorenine bevatten, en of dit bijdraagt aan angiotensine vorming in het hart en de luchtpijp onder normale omstandigheden en tijdens ziekte; 3) de werkingsmechanismen van twee klinische observaties bij renine remming onderzoeken, d.w.z. de extreme stijging van renine concentraties in bloed en een langdurend aanhoudend klinisch effect in vergelijking met de halfwaardetijd, en ook

het evalueren van een nieuwe manier om direct prorenine te meten.

Hoofdstukken 2 en 3: Prorenine distributie, binding en activiteit

Na het verwijderen van beide nieren verdwijnen renine en prorenine uit het bloed volgens een bi-fasisch patroon, met een vergelijkbare distributie halfwaardetijd voor renine en prorenine, en een langere eliminatie halfwaardetijd voor prorenine. Angiotensines verdwijnen gelijk met de eliminatie van renine. Eén tot twee dagen na het verwijderen van de nieren worden stabiele plasma concentraties van 5-10% (renine and angiotensine) en 25-30% (prorenine) van het originele niveau bereikt. Deze niveaus zijn vergelijkbaar met die in patiënten die al jaren zonder nieren leven (anefrici). Uit deze data kan berekend worden dat ongeveer 90% van renine en prorenine afkomstig uit de nier, zich in weefsels bevindt. De hoge prorenine concentratie in plasma van anefrici geeft aan dat naast de nier, ook andere organen een belangrijke bijdrage leveren aan de hoeveelheid prorenine in het bloed. Er was een sterke correlatie tussen de renine en de prorenine concentraties in anefrici; het percentage renine correspondeert met het percentage prorenine dat in vitro een actieve conformatie aanneemt. Dit suggereert dat renine in anefrici in feite prorenine in de open conformatie is, en dat de nier de enige bron van renine is. Zowel hemodialyse, als captopril behandeling had geen effect op de renine-angiotensine componenten in anefrici.

Vasculaire gladde spiercellen van ratten met en zonder de humane prorenine receptor (h(P)RR) werden gebruikt om de receptorbinding van renine en prorenine te bestuderen. De cellen bonden veel meer humaan renine en prorenine bij 37°C dan bij 4°C. Dit suggereert dat de h(P)RR in een temperatuur-afhankelijke manier beweegt tussen intracellulaire compartimenten en plasma membraan, en dat de meerderheid van de receptoren zich in de cel bevindt. Blokkade van de M6PR reduceerde de binding tot ongeveer 50%. Tijdens M6PR blokkade bonden h(P)RR positieve cellen ongeveer twee keer zoveel prorenine als controle cellen, terwijl de renine binding niet verschilde tussen h(P)RR en controle cellen. Met cellen positief voor de h(P)RR zorgde incubatie met prorenine en angiotensinogeen voor meer angiotensine vorming dan verwacht op basis van de activiteit van vrij prorenine, in tegenstelling tot controle cellen. Hetzelfde experiment met renine leverde geen verschil op in activiteit tussen cellen met en zonder de receptor. De recent geïntroduceerde (P)RR antagonist ‘handle region peptide’ (HRP), een peptide dat gelijk is aan het prosegment van prorenine, was niet in staat om prorenine binding of angiotensine productie tegen te gaan. Dus, de humane (P)RR bindt het liefst prorenine, wat resulteert in angiotensine productie, waarschijnlijk omdat binding prorenine activeert.

Hoofdstukken 3 en 4: Renine uit mestcellen in het hart en de longen

Mestcellen zijn cellen van het immuunsysteem, die aanwezig zijn in alle weefsels. Een kenmerk van mestcellen is dat ze een heel scala aan signaalstoffen opslaan, en recent werd voorgesteld dat mestcellen ook renine afgeven in zowel het hart als de luchtpijp. De humane mestcellijn HMC-1 slaat inderdaad renine op, en geeft zowel renine als prorenine af. Zowel

de degranulatie-bevorderende stof compound 48/80 als directe activatie van adenylyl cyclase met forskoline resulteerden in afgifte van renine. Dit kon worden geremd door Ang II, wat in volledige overeenstemming is met de renine afgifte uit juxtaglomerulaire cellen. Echter, in LAD2 mestcellen, en in mestcellen geïsoleerd uit patiënten met mastocytose, werd geen renine of prorenine gevonden. Dus renine/prorenine productie is geen uniform kenmerk van alle mestcellen.

Ook waren we geïnteresseerd of mestcellen bijdragen aan de renine concentratie in het hart, zowel onder normale condities, als na een infarct (wat zorgt voor een toename van mestcellen in het hart). Geïsoleerde ratteharten werden geperfuseerd via de kransslagaders, en renine werd gemeten in zowel de perfusievloeistof als in de harten. Niet-geperfuseerde harten bevatten Ang I-genererende activiteit, waarvan 70% geblokkeerd kon worden met de renine remmer aliskiren. Deze 70% is dus renine, en de andere 30% word veroorzaakt door een enzym anders dan renine. Dertig minuten perfusie zorgde ervoor dat minder dan 30% van de originele concentratie renine nog aanwezig was. Dit percentage werd niet verlaagd door langer perfuseren, of door een bolus injectie met compound 48/80. Ook werd er geen Ang I-genererende activiteit gevonden in de perfusie buffer. Resultaten in de harten twee weken na een infarct waren identiek, hoewel het aantal mestcellen verhoogd was.

In rat bronchiën, zorgde mestcel degranulatie met compound 48/80 voor contractie. Blokkade van het renine-angiotensine systeem op verschillende niveaus, d.w.z., remming van renine met aliskiren, van ACE met captopril of van AT_1 receptoren met irbesartan, beïnvloedde de contractie niet. De Ang I en II concentraties in de badvloeistof waren onder detectielimiet, en vers long materiaal bevatte geen detecteerbare Ang I-genererende activiteit of angiotensinogeen. Ang I en II waren even potent in bronchoconstrictie, en captopril zorgde voor een shift van de Ang I curve, terwijl irbesartan volledig het effect van Ang II kon blokkeren. Dus, Ang II zorgt voor bronchoconstrictie, maar compound 48/80 zorgt niet voor voldoende Ang II om bronchoconstrictie te veroorzaken. Serotonine, een signaalstof die in mestcellen opgeslagen ligt, zorgde ook voor bronchoconstrictie, en de $5\text{-}HT_{2A/C}$ receptor antagonist ketanserine kon dit effect volledig blokkeren. Ketanserine kon ook de bronchoconstrictie veroorzaakt door compound 48/80 blokkeren, terwijl mestcelstabilisatie met cromoglicinezuur zorgde voor verminderde constrictie. Hieruit concluderen wij dat de bronchoconstrictie na compound 48/80 veroorzaakt wordt door serotonine, en niet door renine. Mestcellen zijn een onwaarschijnlijke bron van renine in zowel het hart als de longen.

Hoofdstukken 5 en 6: Renine remming en renine/prorenine metingen

Het bloeddrukverlagende effect van de renine remmer aliskiren houdt langer aan dan verwacht werd op basis van de halfwaardetijd. Dit wordt mogelijk veroorzaakt door intracellulaire accumulatie van aliskiren in de juxtaglomerulaire cellen waarin renine opslagen ligt. Dit werd onderzocht met behulp van de renine-producerende HMC-1 cellen, die 7 dagen aan verschillende concentraties aliskiren blootgesteld werden. Daarna werden de cellen gewassen, en blootgesteld aan forskoline, wat zorgt voor afgifte van opgeslagen renine.

Zonder aliskiren bevatte het kweekmedium vooral prorenine, lag er alleen renine opgeslagen in de cellen, en zorgde forskoline voor een verdubbeling van de renine afgifte. Aliskiren bond dosis-afhankelijk aan (pro)renine in het kweek medium en aan intracellulair renine, maar had geen effect op forskoline-geïnduceerde renine afgifte. De concentratie aliskiren die nodig was voor prorenine binding was tien- tot honderdvoudig die voor renine. Remming van de intracellulaire renine activiteit ging van 27±15% naar 79±5%, met oplopende concentraties aliskiren, en deze percentages waren identiek voor het renine dat na forskoline stimulatie afgegeven werd. Dit geeft aan dat ze uit dezelfde pool afkomstig waren, namelijk de intracellulaire opslagplaatsen voor renine.

Ook zorgt renine remming voor een grotere stijging van de renine concentratie in het bloed dan andere renine-angiotensine systeem blokkers. Dit kan komen doordat prorenine binding aan aliskiren zorgt voor een conformatieverandering waardoor prorenine door renine-specifieke antilichamen herkend wordt. Vergelijking van renine en prorenine metingen in bloed van vrijwilligers, behandeld met aliskiren, liet zien dat het percentage prorenine dat gedetecteerd werd in renine metingen, opliep tot wel 30% bij de hoogste concentratie aliskiren. Dus een gedeelte van de verhoogde renine concentratie na aliskiren behandeling, is eigenlijk prorenine wat foutief herkend wordt als renine.

Een manier om dit probleem goed op te lossen is om prorenine direct te meten. Daarom evalueerden we een nieuwe prorenine meting die gebruik maakt van een antilichaam dat het prosegment van prorenine herkent. Resultaten met deze meting waren vergelijkbaar met de indirecte methode (na prorenine-renine conversie met trypsine, of na prorenine 'activatie' met alsikiren) die gebruik maakt van renine-specifieke antilichamen. Zoals verwacht, zorgde trypsine behandeling dat prorenine bijna niet meer gedetecteerd werd in de directe prorenine assay, maar ook aliskiren behandeling zorgde voor een verminderde detectie. Dus de conformatieverandering die aliskiren teweeg brengt in prorenine, zorgt ervoor dat het prosegment minder goed herkend wordt door het antilichaam, waardoor de nieuwe meting niet geschikt is om prorenine concentraties te meten in patiënten die aliskiren gebruiken.

Prorenine activatie door lage temperatuur (cryo-activatie), na opslag en ontdooien van plasma monsters, kan ervoor zorgen dat prorenine bijdraagt aan de Ang I-genererende activiteit van plasma. Echter, de Ang I-genererende activiteit in plasma van 6 vrijwilligers was identiek na directe meting en na bevriezen en ontdooien van dezelfde monsters. Dus, onder normale condities lijkt cryo-activatie niet tot meetbare prorenine activatie te leiden. Of dit ook geldt voor plasma monsters met hele hoge prorenine spiegels (zoals aanwezig bij diabeten met microvasculaire complicaties) moet nog onderzocht worden.

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