Prorenin and the Heart

The Mannose 6-Phosphate Connection

Prorenine en het Hart

De Mannose-6-fosfaat Connectie

Proefschrift

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Table of Contents

Where did it start?

(1)

General Introduction

The renin-angiotensin system and aim of the thesis

General Introduction

Cardiac hypertrophy, i.e., enlargement of individual myocytes that results in an increase in cardiac mass, is part of the proces of remodelling of a heart under long-term stress and is an effective phenotypic adaptation to compensate for increased (systolic and diastolic) left ventricular wall stress. Increased wall stress has several independent causes, e.g. physical training, hypertension or the presence of scar tissue after a myocardial infarction. In pathological situations cardiomyocyte hypertrophy is a mechanism to compensate for the loss of individual cardiomyocytes due to necrosis (i.e., unregulated cell death) and/or apoptosis (i.e., regulated cell suicide). This process is often accompanied by fibrosis (i.e., collagen deposition) resulting in increased stiffness. Cardiac remodeling, although advantageous at start, may ultimately evolve into overcompensation and end-stage heart failure.

The renin-angiotensin system (RAS, see figure 1) is one of the important regulators of blood pressure and cardiac hypertrophy. Originally, the RAS was thought of as an endocrine system (i.e., the active endproduct, angiotensin II, acts at a distant location), required to regulate blood pressure and retention of Na^+ and H_2O. According to this concept, kidney-derived renin cleaves liver-derived angiotensinogen in the circulation, thereby leading to the release of angiotensin I.

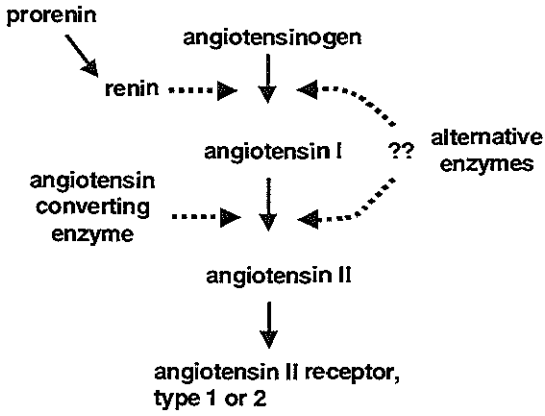

Figure 1: Scheme of the renin-angiotensin system.

This peptide of 10 amino acids is then converted to the octapeptide angiotensin II by angiotensin-converting enzyme (ACE), an enzyme that is abundantly present on the endothelial cells lining the vasculature. Angiotensin II subsequently binds to angiotensin II receptors resulting in contraction of vascular smooth muscle cells in the vessel wall, aldosterone synthesis and release by the adrenal, enhancement of thirst and vasopressin secretion in the brain, and Na^+ and H_2O reabsorption in the kidney.[1] In these processes mainly type 1 angiotensin receptors (AT_1 receptors) are involved.[1] Likewise, angiotensin II binding to AT_1 receptors on cardiac cells can lead to hypertrophy of these cells.[1]

It is generally thought that the levels of renin are the main rate-limiting factor for angiotensin II generation. Renin levels fluctuate depending on its regulated release from juxtaglomerular cells in the kidney. The main determinants of renin release are the sympathetic nervous system, angiotensin II, blood pressure and the tubular Na^+ concentration. Like many secreted proteases, renin is synthesized as a larger inactive precursor, preprorenin. Preprorenin contains a signal peptide directing the protein to a cellular excretion route. During maturation, ending in the *trans*-Golgi network, the signal peptide is removed and carbohydrates are added to prorenin. Following its synthesis, prorenin is sorted to secretory granula , where it is stored, after proteolytic cleavage to renin. Part of prorenin escapes proteolytic cleavage to renin and is constitutively released into the circulation. Normally, the blood plasma concentration of prorenin in humans is 10-fold the concentration of renin.[2]
In native prorenin, the prosegment covers the active site of renin (Figure 2). The prosegment can be temporarily displaced by "unfolding", a process known as non-proteolytic activation. Under physiological conditions (pH 7.4 and 37°C) about 2% of prorenin is in the active, open conformation.[3,4] At lower temperatures, or in acid milieu (as present in endosomal and lysosomal vesicles) this percentage is higher.[4,5]

ACE is a transmembrane protein with its two active domains located extracellularly.[6] Following cleavage of its membrane anchor, it is also present as a soluble enzyme in blood plasma. ACE activity, which may vary by a factor of 5 between individuals, is regulated in part by ACE gene polymorphisms.[7]

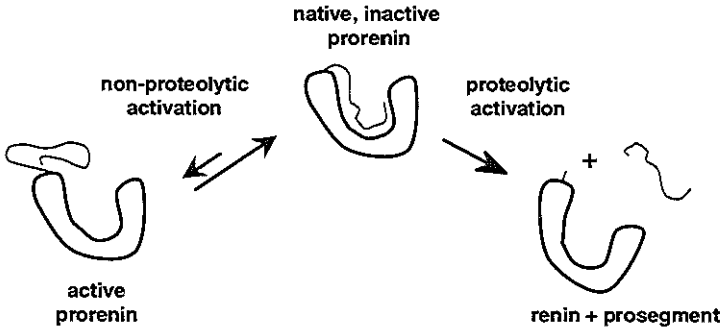

Figure 2: "Closed" (top) and "open" (left) conformation of prorenin. Temporal non-proteolytic activation of prorenin leads to exposure of the active site and display of intrinsic activity. Proteolytic removal of the prosegment generates renin (right).

However, in view of its ubiquitous presence, it is generally assumed that these differences in ACE concentrations have little influence on the angiotensin II levels in blood. In this regard, it is of interest that the ACE upregulation that occurs under certain pathological conditions[8-10] has never convincingly been associated with enhanced angiotensin II generation.[11]

The concentration of angiotensinogen in blood is relatively stable and approximates the Michaelis-Menten constant (K_m) for renin, so that plasma angiotensin production is sensitive to small changes in angiotensinogen concentration. Both renin and angiotensinogen are capable of crossing the endothelial barrier and their interstitial fluid concentrations most likely resemble the levels in the circulation.[12-15]

Angiotensinogen appears to be the only substrate of renin, although there are other enzymes (e.g., cathepsin D) capable of cleaving off angiotensin I. In contrast, ACE is a relatively non-specific protease and, among others, also cleaves the potent vasodilators bradykinin and substance P. Angiotensins in extracellular fluids are degraded within seconds by several proteases ("angiotensinases").

The recognition of the importance of the RAS with regard to blood pressure regulation and its contribution to cardiovascular diseases, like the development of

cardiac hypertrophy and/or heart failure, led to the search and synthesis of angiotensin receptor blockers and inhibitors of ACE and renin. ACE inhibitors (prototype: captopril) were the first to be used on a large clinical scale. Pfeffer et al. provided experimental data on the improvement of ventricular function and survival of infarcted rats following chronic treatment with ACE inhibitors.[16] The animal data were confirmed in large clinical trials (e.g., SOLVD and SAVE).[17,18] Importantly, the beneficial effects that were observed were greater than could be expected on the basis of the decrease in blood pressure that was caused by the ACE inhibitor. Recently, this was confirmed in the HOPE trial.[19,20]
Consequently, the idea has arisen that, in addition to the circulating endocrine RAS, local tissue-specific angiotensin II-generating systems may exist, for instance in the heart and blood vessel wall.[21,22] Locally generated angiotensin II is now believed to influence cell number, cell size, and extracellular matrix composition, and to act as an inflammatory agent, thereby enhancing the destabilization of atherosclerotic plaques. [21-23] The tissue sites where angiotensin is generated (i.e., extra- and/or intracellular) and exerts its multiple actions are still under investigation.

Local generation of angiotensin II could not only depend on the local synthesis of renin, angiotensinogen and ACE, but also on the uptake of one or more of these proteins from the circulation. With regard to renin, uptake of its inactive precursor, prorenin, is also feasible, particularly as prorenin is present in blood plasma at much higher concentrations than renin. Capture of prorenin by tissues should however be followed by local activation (proteolytic or non-proteolytic), on the cell surface and/or intracellularly. Evidence for this concept is currently lacking, except for *in vitro* data supporting the presence of (pro)renin binding proteins and/or receptors, for instance mannose 6-phosphate/IGFII receptors, on isolated cells.[24-28]

AT_1 receptor blockers (prototype: losartan) appear to be as successful as ACE inhibitors in clinical trials (e.g. ELITE II).[29] Importantly, the AT_1 receptor blockers have less side effects than ACE inhibitors, thus resulting in better compliance. This is mainly related to the fact that AT receptor blockers, unlike ACE inhibitors, do not interfere with bradykinin metabolism. Renin inhibitors are currently not available for clinical use, mainly due to their low oral bioavailability and high costs of synthesis.[30]

Studies investigating the underlying mechanisms of local angiotensin II generation may eventually lead to new treatment possibilities. For instance, if prorenin is proteolytically activated in a cardiac-specific manner, then interruption of this mechanism might enhance or replace ACE inhibition and/or AT_1 receptor blockade. Also, interference with the presumed (pro)renin binding might be worthwhile to investigate.

Aim of thesis

The knowledge concerning the formation of angiotensins at cardiac tissue sites in relation to the presence and origin of cardiac renin, angiotensinogen and ACE is evaluated in chapter 2. To gain insight in the functional importance of locally generated angiotensin II, the response of human forearm blood flow to infusion of either angiotensin I or angiotensin II was investigated (Chapter 3). To extend our results in the perfused isolated rat heart,[31] experiments were performed to detect *de novo* synthesis of RAS components by neonatal rat cardiomyocytes and -fibroblasts under basal conditions and after stretch (Chapter 4). In addition, we characterized the binding and activation of human recombinant prorenin via mannose 6-phosphate/IGFII receptors on the surface of human endothelial cells, and neonatal rat cardiomyocytes and -fibroblasts (Chapters 5 and 6). To validate our results obtained with human recombinant prorenin, neonatal rat cardiomyocytes were also incubated with human (pro)renin- containing body fluids (Chapter 7). The latter studies also addressed the importance of soluble mannose 6-phosphate/IGFII receptors.

Finally, since 1) under certain conditions mannose 6-phosphate/IGFII receptor activation initiates transcellular signaling pathways,[32] and 2) renin binding to glomerular mesangial cells leads to plasminogen activator inhibitor type-1 release and an increase in ^{3}H-thymidine incorporation,[25] we investigated whether prorenin binding and/or uptake by rat cardiomyocytes, in the presence or absence of angiotensinogen, resulted in a cellular response (Chapter 8). In these latter studies we also investigated intra- and extracellular angiotensin II generation and compared the effects of prorenin with those obtained with angiotensin II in parallel experiments.

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Where did it start?

(2)

The renin-angiotensin system of the heart

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Is there a local renin-angiotensin system in the heart?

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Is there a local renin–angiotensin system in the heart?

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Abstract

The existence of a local renin–angiotensin system in the heart is still a controversial issue. This review discusses the evidence, obtained from studies in cardiac cells, in isolated perfused hearts and in intact animals and humans, both under normal and pathological conditions, for local production of prorenin, renin, angiotensinogen, angiotensin-converting enzyme, angiotensin I and angiotensin II at cardiac tissue sites. In addition, the role of alternative angiotensin-generating enzymes (cathepsin, chymase) and the possibility of (pro)renin uptake from the circulation is evaluated.

Keywords: Angiotensin; ACE inhibitors; Interstitial space; Myocytes; Renin angiotensin system

1. Introduction

The renin–angiotensin system (RAS) has traditionally been viewed as a circulating system. Kidney-derived renin cleaves liver-derived angiotensinogen to form angiotensin (Ang) I in circulating blood. Subsequently, Ang I is converted into Ang II, the main effector peptide of the RAS, by angiotensin-converting enzyme (ACE) located at the luminal side of the endothelium. Ang II exerts its effects via stimulation of Ang II receptors, of which at least two types have been described, AT_1 and AT_2. Based upon discrepancies, observed more than 15 years ago, between RAS blocker-induced changes in the circulating levels of RAS components and the blood pressure-lowering effects of these drugs, it was proposed that so-called local renin–angiotensin systems exist in addition to the circulating RAS. Subsequent studies showing the presence of RAS components at tissue sites appeared to confirm this theory. However, one has to keep in mind that the mere presence of RAS components in tissue cannot be taken as direct evidence for their local production. With regard to the heart, more convincing evidence for local Ang II production comes from the recently discovered beneficial effects of the ACE inhibitors in heart failure, which are independent, at least partly, of their effect on blood pressure [1–4]. Local Ang II production may depend on (1) in-situ synthesis of all RAS components required for Ang II production (i.e., renin, angiotensinogen and ACE), (2) uptake of these components from the circulation, or (3) a combination in-situ synthesis and uptake of RAS components (e.g., uptake of circulating renin and angiotensinogen in combination with local synthesis of ACE). The latter two definitions would no longer require in-situ synthesis of renin, so that one should better speak of a system generating Ang II locally rather than a local RAS. This review addresses the question whether a local RAS exists in the heart.

2. Studies in cardiac cells

The uncertainties concerning local synthesis arising from tissue measurements (i.e., local synthesis vs. uptake from circulating blood) can be avoided largely when measurements are made in cells cultured in the absence of serum. The use of serum-free medium is necessary to

exclude the uptake of RAS components present in serum. However, serum-free conditions are always preceded by cell-culturing conditions in the presence of serum, so that even under 'serum-free' conditions RAS components may be detected in cells that have been sequestered from the serum to which the cells were exposed earlier. Table I summarizes the findings in cardiac cells that are discussed below.

2.1. Renin

Several attempts have been made to measure renin, both at the mRNA and protein level, in myocytes and fibroblasts obtained from hearts of neonatal and adult rats. Some [5–7], but not all [8] authors were able to detect renin mRNA, using sensitive PCRs. Renin immunoreactivity was present in the perinuclear region in neonatal rat cardiomyocytes and fibroblasts [5], or throughout the cytoplasm in adult rat cardiomyocytes and non-myocytes [6]. In these latter two studies, no distinction between renin and its inactive precursor, prorenin, was made, nor was (pro)renin release into the culture medium investigated. This is remarkable, since most so-called renin-expressing extrarenal cells produce prorenin rather than renin [9–12]. These cells do not store prorenin, and secrete it in a constitutive manner. Studies with cardiac cells where renin and prorenin (the latter after in-vitro activation to renin) were measured enzyme-kinetically, were unable to support either the release of (pro)renin into the medium [13] or the intracellular presence of (pro)renin [13,14]. The lack of (pro)renin release in combination with the low to undetectable renin mRNA levels in cardiac cells does not support the concept of (pro)renin synthesis by these cells. The discrepancy between the absence of renin-dependent Ang I-generating activity in cardiac cells on the one hand, and the presence of immunoreactive renin in these cells on the other hand can be explained as follows: 1) the polyclonal antibodies used to demonstrate intracellular renin immunohistochemically [5,6] may have crossreacted with renin-like enzymes such as cathepsin D [15], or 2) the cells may indeed have contained renin, not due to local synthesis but due to uptake from the serum to which the cells were exposed prior to the serum-free period. In support of the latter explanation we have recently demonstrated that neonatal rat cardiac cells are capable of internalizing both renin and prorenin [16]. Internalized prorenin is rapidly activated to renin. The amount of serum to which the cells were exposed, the serum source, the duration of exposure to serum, and the intracellular half life of internalized (pro)renin may have differed between studies, and this might explain why under certain conditions renin still is present within cells, whereas in others it is not.

2.2. Angiotensinogen

Studies on angiotensinogen synthesis by cardiac cells are scarce. Angiotensinogen mRNA has been demonstrated in both neonatal and adult rat cardiac cells by several investigators [5–8,17–20]. However, the actual presence of the angiotensinogen protein has been investigated in two studies only. Dostal et al. described positive immunoreactive staining in the perinuclear region for angiotensinogen in both neonatal rat cardiomyocytes and fibroblasts, without investigating its release into the medium [5]. Van Kesteren et al., who measured angiotensinogen by radioimmunoassay after its conversion to Ang I by renin, was unable to detect angiotensinogen in neonatal rat cardiac cells or in the conditioned medium of these cells [13]. All other cells described to synthesize angiotensinogen secrete this substrate constitutively, without storing it intracellularly [21–23]. In vivo, angiotensinogen is also limited to the extracellular fluid compartment, and not located in cells [14,24,25]. Thus, despite the reports demonstrating angiotensinogen mRNA in cardiac cells with the help of sensitive PCRs, evidence for the release of angiotensinogen from these cells is lacking.

Table 1
Presence of renin, angiotensinogen and ACE (mRNA and/or protein) in serum-deprived cardiac myocytes and fibroblasts vs. their release into or uptake from the medium[a]

	mRNA	Protein		Release into medium	Uptake from medium[b]
		Immunohistochemical measurement	Enzyme-kinetic measurement		
Myocytes					
(Pro)renin	±[c]	Yes	No	No	Yes
Angiotensinogen	±	Yes	No	No	Not done
ACE	Yes	Yes	Yes	Not done	Not done
Fibroblasts					
(Pro)renin	±	Yes	No	No	Yes
Angiotensinogen	±	Yes	No	No	Not done
ACE	Yes	Yes	Yes	Not done	Not done

[a] Data are taken from Refs. [5–8,13,14,16–20,26–29].
[b] Studied after the addition of exogenous protein to the medium under serum-free conditions.
[c] ± denotes a lack of consensus in the existing literature.

2.3. ACE

ACE, a cell membrane-bound enzyme, has been demonstrated in cardiac cells by enzyme-kinetic and immunohistochemical methods [13,26–29]. Both neonatal rat cardiomyocytes and fibroblasts generate Ang II when incubated with Ang I under serum-free conditions, and ACE inhibitors fully inhibit this Ang II generation [13,29]. The presence of ACE protein or activity in cardiac cells correlates well with the demonstration of ACE mRNA in these cells [5–8].

2.4. Angiotensin I and II

According to several studies, serum-deprived cardiac cells release angiotensins into the culture medium. The Ang I and II levels in the medium, however, showed huge variations, from <10 fmol/ml to >1000 fmol/ml [13,17,26,28,30]. Part of these discrepancies may be due to the fact that angiotensins were sometimes measured by direct radioimmunoassays (i.e., without prior purification and/or separation from material crossreacting with the Ang I and II antibodies applied in these assays). This approach will lead to an overestimation of the "true" angiotensin levels, or even to the detection of angiotensins in medium that does not contain angiotensins [31]. It should also be kept in mind that, in view of the cardiac angiotensin levels measured in vivo (Ang I, ≈5 fmol/g wet weight; Ang II, ≈20 fmol/g wet weight) [24,32–34], even levels of 5–10 fmol/ml are very high, since in most studies medium was collected from only 1–10 million cells, with an estimated wet weight of less than 10 mg [35]. Sadoshima et al. [17] found the Ang II concentration in the medium of serum-deprived cardiomyocytes to increase nearly 100-fold upon stretch (Table 2). This Ang II, which is assumed to be responsible for the hypertrophic [8,17–19,28,30] or apoptotic [20] response of cardiomyocytes after stretch, appeared to originate from intracellular storage sites, since its release was not affected by captopril and not accompanied by Ang I release [17]. Immunoelectron microscopy confirmed the existence of secretory granule-like structures containing Ang II in ventricular cardiomyocytes [17]. Dostal et al. [26] did not observe these granule-like structures and localized intracellular Ang II in the perinuclear region of neonatal rat cardiomyocytes and fibroblasts. Stretch is assumed to cause an upregulation of RAS components in cardiomyocytes, and this would explain why the Ang II levels in the medium are also elevated 20–24 h after the initiation of stretch [20]. However, the reports on elevated renin – and ACE mRNA levels were not supported by protein measurements [17,28], suggesting that increases in expression may not be translated to the protein level. In addition, not all authors were able to observe a rise in Ang II following stretch [13,30]. Taken together therefore, the initial report by Sadoshima et al. on Ang II release after stretch has not been unequivocally confirmed by others. It is possible that differences in experimental conditions have played a role (Table 2). Furthermore, the Ang II in intracellular storage sites may have been derived, via AT_1

Table 2
Effect of stretch on renin–angiotensin system components in cardiomyocytes[a]

Study	Presence of serum	Cyclic/static stretch	Renin	Angiotensinogen	ACE	Ang II release
Sadoshima et al., 1993 [17]	No	Static	No change in renin-like activity	mRNA ↑	No change in ACE-like activity	100-fold increase to ≈450 pmol/l after 10 min
Malhotra et al., 1994 [7]	No	Static	mRNA ↑	mRNA ↑	mRNA ↑	–[b]
Shyu et al., 1995 [18]	No	Cyclic	–	mRNA ↑	–	–
Yamazaki et al., 1995 [30]	No	Static	–	–	–	Undetectable
Miyata et al., 1996 [29]	No	Static	–	–	No change in ACE-like activity	2-fold increase to ≈30 pmol/l after 24 h
Tamura et al., 1998 [19]	No	Static	–	mRNA ↑	–	–
Liang et al., 1998 [8]	Yes	Cyclic	mRNA undetectable	mRNA ↑	mRNA ↑	–
Leri et al., 1998 [20]	No	Static	–	mRNA ↑	–	4-fold increase to ≈10 pmol/l after 1 hr and after 24 h
van Kesteren et al., 1999 [13]	No	Cyclic	–	–	–	undetectable

[a] All studies employed neonatal rat cardiomyocytes, except the study by Leri et al., in which adult rat cardiomyocytes were used.
[b] –: not studied.

receptor-mediated endocytosis [36], from the serum-containing medium used to culture the cells prior to stretch.

3. Studies in isolated perfused hearts

The isolated perfused heart has been used widely to study the effects of RAS blockers on coronary flow and cardiac function. In these studies, it is generally assumed that all RAS components are present in cardiac tissue and that Ang II is generated continuously. However, evidence to proof this notion is currently lacking. Studies investigating the presence of RAS components in the isolated perfused heart are scarce, despite the fact that an isolated perfused preparation is ideal to study local synthesis. The buffers used to perfuse the heart are free of renin and angiotensinogen, thereby eliminating the problems arising from in-vivo measurements, when the heart is perfused with blood containing these components.

3.1. Renin

Renin or prorenin release by the heart has never been demonstrated. Angiotensin release from isolated perfused hearts occurred only after the addition of renin to the perfusion fluid [37–40], thereby demonstrating that (1) renin is not present in the isolated buffer-perfused heart, and (2) renin is the only enzyme involved in cardiac Ang I generation. The kinetics of renin uptake into the heart have been studied extensively by de Lannoy et al., using a modified version of the rat Langendorff heart, allowing separate collection of both coronary effluent and interstitial fluid [38]. Renin could not be demonstrated in either the coronary effluent or the interstitial fluid of buffer-perfused hearts. When renin was added to the perfusion buffer, it slowly entered the interstitial space, reaching steady-state levels in interstitial fluid comparable to those in coronary effluent after approximately 30 min. Calculations on the basis of the steady-state renin levels in coronary effluent, interstitial fluid and cardiac tissue, revealed that the majority of cardiac renin was present in extracellular fluid. After stopping the renin infusion, the washout of renin from the heart followed a biphasic pattern, suggesting that renin may also be present in an additional compartment. In support of this finding, Müller et al. observed Ang II release from isolated hearts of rats overexpressing the human angiotensinogen gene even after discontinuation of renin infusion, at a time when renin had already disappeared from the coronary perfusate [40]. It is therefore possible that some renin is located outside the extracellular fluid compartment, for instance bound to the membrane of vascular or cardiac cells. This notion is in agreement with the recent observation that both endothelial cells [41] and cardiac cells [16] are capable of binding renin and prorenin. Prorenin kinetics in the isolated perfused heart were comparable to those of renin; release of activated prorenin into either the coronary effluent or interstitial fluid could not be demonstrated [42].

3.2. Angiotensinogen

Angiotensinogen release from isolated buffer-perfused rat Langendorff hearts has been investigated in two studies. Lindpaintner et al. [37] observed a rapid decline of the angiotensinogen levels in coronary effluent to levels that were <1% of the levels in blood plasma. De Lannoy et al. [38] were unable to demonstrate angiotensinogen in coronary effluent, but found low angiotensinogen levels (corresponding to <0.1% of the plasma levels of angiotensinogen) in interstitial fluid, which decreased even further (to levels below the detection limit) during prolonged buffer perfusion of the heart. The most likely explanation for these findings is that some blood-derived angiotensinogen is still present in the isolated heart preparation (for instance in the interstitial space), which is slowly washed away during perfusion with buffer. In support of this assumption, angiotensinogen, when added to the perfusion buffer of the isolated rat heart, entered the interstitial space [38]. Steady-state levels comparable to those in coronary effluent were reached 30–40 min after the start of the angiotensinogen perfusion. The steady-state tissue levels of angiotensinogen were also compatible with its presence in extracellular fluid. Following discontinuation of the angiotensinogen perfusion, angiotensinogen disappeared monophasically from cardiac extracellular fluid. This outcome contrasts with the findings on renin, which disappeared in a biphasic manner, and suggests that angiotensinogen is limited to one compartment (the extracellular fluid) only. Indeed, binding of angiotensinogen to cardiac or vascular membranes could not be demonstrated [24,43].

3.3. ACE

Many investigators have shown that Ang I is converted to Ang II in the isolated perfused rat heart [39,40,44–46]. ACE inhibitors prevented the Ang I–II conversion completely. Thus, there is no doubt that ACE is present and functionally active in the isolated perfused heart. The conversion of arterially delivered Ang I was usually low (<10%) and appeared to depend on both endothelial and extra-endothelial ACE [46].

3.4. Angiotensin I and II

Buffer-perfused rat hearts do not release Ang I or Ang II, unless renin is added to the perfusion buffer [37–39]. Renin-induced angiotensin release diminishes rapidly, suggesting that it depends on a limited amount of trapped plasma-derived angiotensinogen [37,38]. Cardiac angiotensin release during renin infusion can only be maintained over longer time periods by adding angiotensinogen simultaneously to the perfusion buffer [38,39], or by using

hearts of rats overexpressing the angiotensinogen gene [40]. Interestingly, angiotensin release via coronary effluent reached a steady state after 30–40 min of combined renin and angiotensinogen perfusion, whereas renin and angiotensinogen in coronary effluent had reached a steady-state level within 5 min after the start of their infusion into the perfusion buffer. This finding, in combination with the fact that angiotensin release continues after discontinuation of the renin perfusion, at a time when renin is no longer present in coronary effluent [40], strongly suggests that cardiac angiotensin release depends largely on tissue-bound renin. The most likely binding site for renin involved in the release of Ang I into the intravascular compartment is the vascular wall. Angiotensin production also occurred in cardiac interstitial fluid, and under steady-state conditions, the interstitial fluid Ang I and II levels were 2–3 times higher than those in coronary effluent [38,39]. In view of the low interstitial fluid flow (50–100 times lower than the coronary flow) and the extensive metabolism of angiotensin in the vascular wall, it is unlikely that interstitial angiotensin production contributed importantly to the angiotensin levels in the coronary effluent. Finally, Ang II was present in cardiac tissue during combined renin and angiotensinogen perfusion, at levels higher than those expected on the basis of the Ang II levels in extracellular fluid [39]. This was not the case for Ang I: per gram tissue the amount of Ang I was as high as expected on the basis of the assumption that Ang I is present only in extracellular fluid. Thus, cardiac Ang I is largely confined to the coronary vascular bed and the interstitial fluid compartment, whereas cardiac Ang II is also located outside these compartments. Tissue Ang II may therefore be present in cells, either because it is synthesized intracellularly, or because, following its synthesis outside the cell, it is rapidly internalized via AT_1 receptors [36].

4. Studies in intact animals and humans

Results from in-vivo studies on the tissue levels of RAS components are difficult to interpret because these levels might be partly or wholly contributed to the presence of blood in tissues. Even when the levels are considerably higher per gram of tissue than per millilitre of plasma, one should keep in mind that an active uptake process (e.g., receptor binding) rather than local synthesis may underlie these high levels.

4.1. Renin

Renin mRNA concentrations in normal hearts are close to or below the detection limit [47–50], suggesting that under normal circumstances cardiac renin synthesis may not occur. We compared the renin levels in the heart with its level in blood plasma in normal and nephrectomized pigs [24]. Ang I-generating activity of cardiac tissue was identified as renin by its inhibition with a specific active site-directed renin inhibitor. The levels of renin in cardiac tissue (expressed per gram wet weight) were similar to those in blood plasma (expressed per ml plasma) and could therefore not be attributed to trapped blood plasma. However, both in cardiac tissue and in plasma renin fell to undetectable levels after nephrectomy. These data, which were confirmed in the rat heart by Katz et al. [25], suggest that most, if not all, renin present in the normal heart originates from the kidney. Apparently, the heart is capable of sequestrating renin from the circulation. Renin may either diffuse into the interstitial space [38] or bind to the recently described renin receptor(s) and/or renin binding proteins [16,41,43,51–53]. In support of the latter, we [24] and others [43] found renin to be enriched in a purified membrane fraction prepared from either left ventricular tissue or mesenteric arteries. Such enrichment in cardiac and vascular tissue is in agreement with the existence of a renin receptor. It is currently not known what cardiac cells are responsible for the binding of renin. Based on studies in isolated cells [16,41], endothelial cells as well as cardiomyocytes and fibroblasts might be involved in the uptake process. Interestingly, these cells were not only capable of binding renin, but bound prorenin as well. Moreover, following binding, renin and prorenin were internalized, and prorenin was activated to renin. These findings may explain why in the normal heart virtually no prorenin can be detected [24,53]: prorenin taken up by the heart from the circulation may have been activated locally to renin. The receptor involved in the binding and internalization process of (pro)renin and prorenin activation appeared to be the mannose 6-phosphate receptor [16,41]. Renin acquires phosphomannosyl residues during its biosynthesis that enable it to bind to this receptor [54]. Katz et al. [25] found that, following bilateral nephrectomy, high-mannose renin glycoforms disappeared from the heart at a much slower rate than from plasma, thereby indirectly confirming that (phosphorylated) oligosaccharide attachments to renin determine its binding to cardiac tissue.

4.2. Angiotensinogen

Angiotensinogen mRNA can be detected in the heart [49,55–57], at levels that are <0.1% of the angiotensinogen mRNA levels in the liver [57]. The angiotensinogen concentrations in porcine cardiac tissue are 10–25% of the levels in plasma, a figure compatible with the diffusion of angiotensinogen from plasma into the interstitium [24]. In hearts of humans and rats, the angiotensinogen levels, measured by enzyme-kinetic assay, were lower than expected on the basis of diffusional equilibrium between plasma and tissue [25,53], either because these hearts were washed with buffer after their removal from the body [25], or because significant consumption of angiotensinogen had occurred at cardiac tissue sites [53]. In support of the latter possibility, a negative correlation was found between angiotensinogen and renin in human

hearts [53]. Despite the low levels of angiotensinogen that could be demonstrated in the human heart by enzyme-kinetic assay [53], Sawa et al. [58] found intense immunoreactivity for angiotensinogen in the atrial muscles, the muscles of the conduction system and those of the subendocardial layers of human autopsy hearts. Remarkably however, despite the intense immunoreactivity in myocardial cells, Sawa and colleagues were unable to show positive immunostaining of angiotensinogen in the liver. Taken together therefore, although the data for angiotensinogen are less clear than those for renin, it appears that the majority of cardiac angiotensinogen is derived from the circulation. Most likely angiotensinogen diffuses freely from plasma into the interstitial space, where cleavage by interstitial fluid renin and/or membrane-bound renin may occur.

4.3. ACE

ACE mRNA is readily detectable in cardiac tissue [45,59,60]. ACE has been demonstrated in the heart by autoradiography [61], using a radiolabelled ACE inhibitor, as well as by measurement of its activity in cardiac homogenates [45,62]. Most likely, cardiac ACE is normally limited to the coronary vascular endothelial cells and the endocardium [63].

4.4. Angiotensin I and II

Many groups have reported on the presence of Ang I and II in cardiac tissue [24,32–34]. In most cases the tissue levels (expressed per g wet weight) were similar to or higher than the concomitant plasma levels (expressed per ml plasma). This, however, cannot be taken as definite evidence for angiotensin production at cardiac tissue sites, since both Ang I and Ang II may have been actively sequestered from the circulating blood. According to two studies [24,32], cardiac angiotensin levels decreased to levels close to or below the detection limit following a bilateral nephrectomy, whereas a third study recently reported no change in cardiac angiotensin levels after nephrectomy [33]. The most likely explanation for these differences is that in the latter study cardiac angiotensin levels were measured in rats 24 h after nephrectomy, while the effects of nephrectomy on cardiac Ang II, at least in the rat, become apparent only at 48 h after nephrectomy [32]. Moreover, a bilateral nephrectomy is known to be accompanied by the release of large amounts of renin from the kidney, and this may have resulted in correspondingly high myocardial renin levels immediately during and after surgery [14,25].

What are the possible sources of Ang I and Ang II in cardiac tissue? Ang I in cardiac tissue might be derived from Ang I in the coronary artery, from Ang I generated in the coronary circulation by the reaction of circulating renin with circulating angiotensinogen ('plasma renin activity', PRA), or from Ang I synthesized in situ in cardiac tissue. Cardiac Ang II might be derived from Ang II in the coronary artery and, via conversion, from the above three sources of Ang I.

In order to quantify the contribution of each of these sources, we have measured the steady-state tissue and plasma levels of endogenous and radiolabelled Ang I and Ang II, as well as PRA, during infusions of ^{125}I-labelled Ang I and Ang II [34]. Great care was taken to measure intact ^{125}I-labelled and endogenous Ang I and Ang II rather than tissue radioactivity or immunoreactive angiotensin levels. The body does not distinguish between radiolabelled and endogenous angiotensins [64], and thus the steady-state levels of ^{125}I-labelled Ang I and Ang II present in cardiac tissue during the infusion of these radiolabelled peptides are a measure for the uptake of angiotensins from the circulation. The contribution of angiotensins generated in the coronary circulation by PRA can be calculated by assuming that PRA-derived angiotensins are taken up by the heart in the same way as radiolabelled angiotensins.

The results indicated that, under steady-state conditions, the cardiac ^{125}I-Ang I concentrations are less than 5% of its levels in plasma, whereas the concentrations of cardiac ^{125}I-Ang II are approximately 90% of plasma ^{125}I-Ang II. At the same time, the cardiac tissue concentration of endogenous Ang I was similar to the plasma concentration of endogenous Ang I, while the cardiac tissue concentration of endogenous Ang II was 4–5 times higher than the plasma concentration of endogenous Ang II (Fig. 1). Taking into consideration the small amounts of angiotensins generated by PRA in the coronary circulation, it can be calculated that over 90% of the Ang I in tissue is synthesized in the tissue itself and not derived from the circulation. Moreover, more than 75% of the Ang II in tissue is also synthesized in the tissue and its source is in-situ synthesized Ang I rather than Ang I from the circulation. Interestingly, locally synthesized Ang I, but not locally synthesized Ang II, was found to be released into the coronary circulation [65,66]. This suggests either that Ang I produced at tissue sites enters the blood at a level distal to the site where Ang I-to-II conversion occurs, or that Ang II produced in the tissue cannot leave the tissue, for instance because, following its formation, it rapidly binds to angiotensin receptors.

Summarizing, the majority of cardiac Ang I and Ang II is synthesized at tissue sites by kidney-derived renin. Locally synthesized Ang II is kept in the tissue, whereas locally synthesized Ang I is capable of reaching the coronary circulation.

5. Cardiac renin-, angiotensinogen- and ACE synthesis under pathological conditions

Although synthesis of renin and angiotensinogen at cardiac tissue sites does not appear to occur under normal circumstances, it is possible that the renin and angioten-

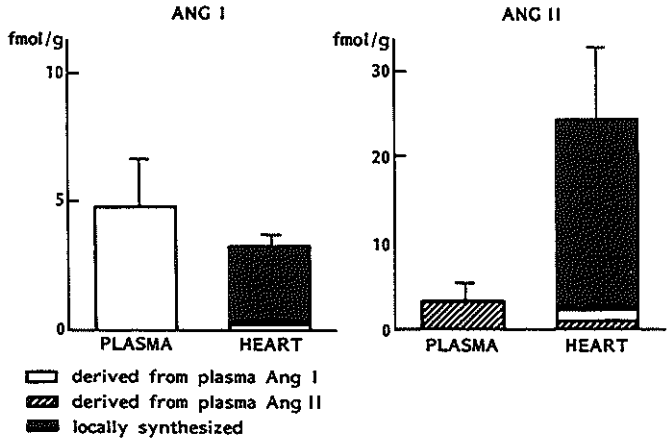

Fig. 1. Plasma and cardiac tissue levels of angiotensin (Ang) I and II in the pig. The majority of cardiac Ang I and II is synthesized locally at cardiac tissue sites. The contribution of plasma renin activity in the coronary vascular bed to the cardiac tissue levels of Ang I and II is too small to be shown. Data are taken from Ref. [34].

sinogen gene are switched on in response to pathological conditions. In addition, changes in ACE gene expression may occur. Most studies investigating the cardiac RAS under pathological conditions have determined changes at the mRNA level only. In view of the low to undetectable levels of renin and angiotensinogen mRNA in control hearts, as well as the uncertainties with regard to transcriptional regulation, it is difficult to establish the value of increased mRNA levels in the diseased heart. Boer et al. [50] and Pieruzzi et al. [67] described increases in renin mRNA in the rat heart in response to volume overload. These increases could only be demonstrated after 37 and 40 PCR cycles, and were not confirmed by Iwai et al. [57]. No change in cardiac angiotensinogen mRNA was found in the volume overload model [50,57]. Passier et al. found increased renin mRNA levels in the infarct zone following coronary artery ligation, and no change in angiotensinogen mRNA levels in either the infarcted and noninfarcted zone [49]. In contrast, Lindpaintner et al. reported a transient activation of angiotensinogen mRNA in the noninfarcted left ventricle of rats after a coronary artery ligation [56]. Heller et al. studied myocardial renin–angiotensinogen dynamics during pressure-induced cardiac hypertrophy, and found cardiac renin to vary directly with plasma renin [14]. Similarly, the increases in plasma renin occurring in subjects with end-stage heart failure were found to be accompanied by parallel increases in cardiac renin [53]. Thus, on the basis of renin protein measurements in cardiac tissue no evidence was obtained for significant cardiac renin production under pathological conditions. As far as angiotensinogen is concerned, decreased rather than increased levels were found in failing hearts, suggesting local consumption by cardiac renin [53]. Thus, demonstration of significant angiotensinogen production in the heart under pathological conditions is difficult, since increased consumption may mask local production. Finally, with regard to ACE, changes in its mRNA levels have been observed in diseased hearts that are supported by protein measurements. Both ACE protein and ACE mRNA increase following myocardial infarction, as well as during pressure – and volume overload-induced left ventricular hypertrophy [45,57,59,60,67]. Under these conditions, the localization of ACE may no longer be limited to the endothelium. In humans, following myocardial infarction, ACE can be detected in the remaining viable cardiomyocytes near the infarct scar of the aneurysmal left ventricle, as well as in fibroblasts, vascular smooth muscle cells, and macrophages in the scar area itself [68]. In rats, following coronary occlusion, ACE was demonstrated in fibroblasts in the healthy hypertrophying part of the heart [69].

6. Site of tissue angiotensin generation

Tissue angiotensin generation may occur in interstitial fluid, on the cell membrane, or within cells (Fig. 2).

6.1. Interstitial fluid

According to our studies in the isolated rat heart, circulating renin and angiotensinogen are able to reach the interstitial space [38]. This offers the possibility of angiotensin generation within this fluid. Indeed, during combined renin/angiotensinogen perfusion of the Langen-

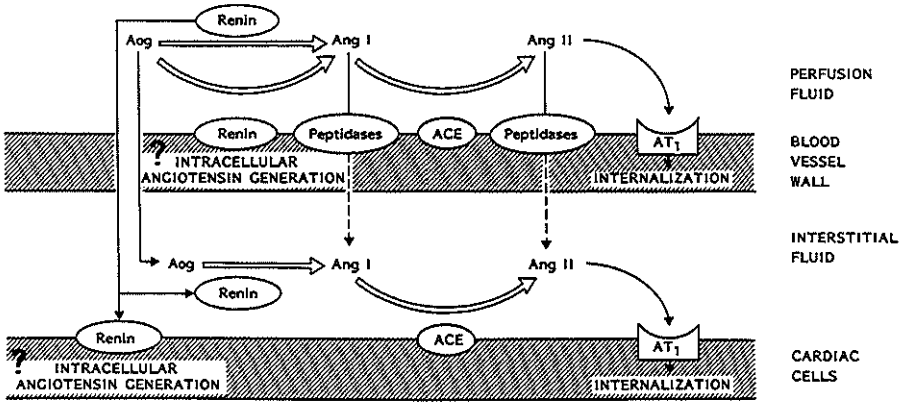

Fig. 2. Proposed scheme of angiotensin (Ang) I and II production in the heart [39]. Intravascular and interstitial compartments as well as blood vessel wall and cardiac cells (endothelial cells, myocytes, fibroblasts, macrophages) are depicted. Circulating renin and angiotensinogen (Aog) both enter the interstitial fluid compartment. Renin may also bind to the vascular wall and cardiac cells. ACE is present on endothelial cells and cardiac cells. Ang I and II are metabolized by peptidases while passing through the vascular wall. Ang I and II in the interstitial fluid are mainly generated outside the vascular fluid compartment. Tissue Ang I and II generation may occur not only in the interstitial fluid or on the cell surface but also within cells, for instance after renin uptake by the cells. Binding of Ang II to the AT_1 receptor is followed by internalization of the AT_1 receptor–Ang II complex.

dorff heart, we found the levels of Ang I and Ang II in interstitial fluid to be two–three times higher than the levels measured simultaneously in the intravascular compartment [38,39]. Data from in-vivo studies in the dog also demonstrated that the cardiac interstitial angiotensin levels are higher than the plasma levels of these peptides [70]. Since diffusion of intact Ang I and II from the intravascular compartment to the interstitial compartment is marginal (most likely because of rapid metabolism of angiotensins in the vascular wall [38,39]), the high interstitial levels can be taken as evidence for interstitial angiotensin generation.

6.2. Cell membrane

In support of a role for membrane-bound renin in local angiotensin generation, we [38,65] and others [66] observed that, both in vivo and in vitro, the amount of Ang I released by the heart via coronary effluent was too high to be explained by the renin–angiotensinogen reaction occurring in intravascular fluid during coronary passage. Moreover, in the isolated Langendorff heart preparation perfused with renin and angiotensinogen, Ang I release via coronary effluent reached a steady-state level long after renin and angiotensinogen had reached a steady state in this fluid [38], and angiotensin release continued after discontinuation of the renin perfusion [40]. These data suggest that tissue-bound renin rather than extracellular fluid renin is responsible for the high Ang I levels in coronary effluent. Endothelial cells, vascular smooth muscle cells, cardiomyocytes and cardiac fibroblasts may all be involved in the binding process.

6.3. Intracellular compartment

Direct evidence for intracellular angiotensin generation is not available. Renin dialysis into cultured cardiomyocytes leads to a decrease in the conductance of the adjacent myocytes [71]. The reduction of conductance was amplified when renin was infused together with angiotensinogen and attenuated when a renin inhibitor was co-administered, suggesting that these effects are mediated by renin-dependent angiotensin II formation within the cell. Our data on renin and prorenin internalization [16,41] might explain how renin normally enters the cell. When, concurrently with (pro)renin, angiotensinogen is taken up from the interstitial fluid via bulk fluid endocytosis, a scenario for intracellular angiotensin generation is provided (Fig. 3). Additional proof for intracellular angiotensin generation comes from studies where the extracellular and tissue levels of Ang II were measured during Ang II perfusion and during renin/angiotensinogen perfusion of the isolated rat Langendorff heart in the absence or presence of the AT_1 receptor antagonist losartan [39]. During these infusions the heart is exposed to arterially delivered and locally generated Ang II, respectively. Losartan did not affect the extracellular Ang II levels during both infusions. It did however reduce the tissue Ang II levels during Ang II perfusion to almost undetectable levels, whereas tissue Ang II during renin/angiotensinogen perfusion was not affected. It appears therefore that arterially delivered Ang II binds to AT_1 receptors at cardiac tissue sites and that losartan interferes with this process, thereby reducing the tissue Ang II levels. Locally synthesized Ang II present at tissue sites is not affected by

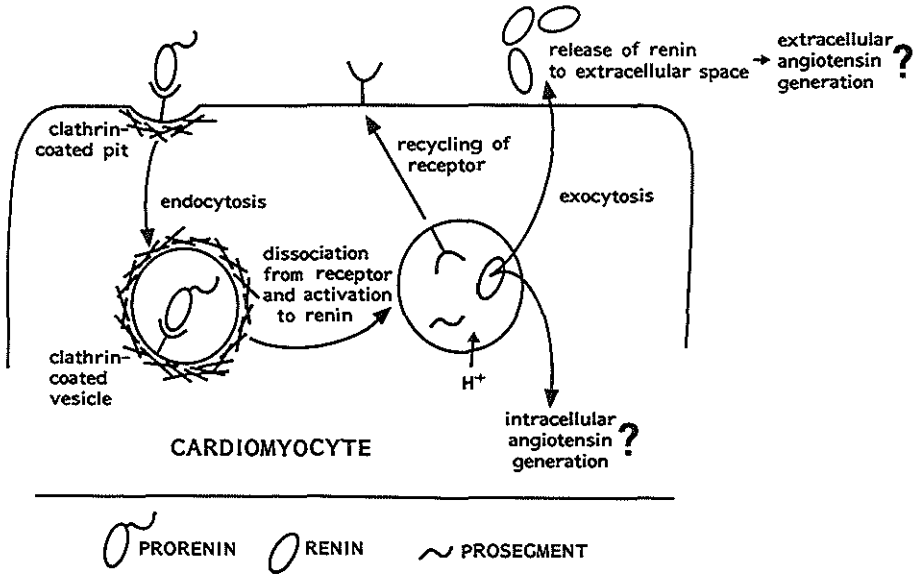

Fig. 3. Binding and activation of prorenin by cardiomyocytes. After binding to the cell surface, the receptor–prorenin complex is internalized in a clathrin-coated pit, that pinches off to become a coated vesicle. The clathrin coat then depolymerizes, thereby leading to the formation of an endosome. Prorenin is activated to renin and dissociates from the receptor due to the acid environment in the endosome. The receptor returns to the cell surface, and the activated prorenin might participate in either intracellular or extracellular angiotensin generation.

losartan, and thus may have been generated at a site that cannot be reached by losartan, i.e. the intracellular compartment.

7. Mannose 6-phosphate receptors and cardiac (pro)renin binding

In view of the absence of significant renin synthesis at cardiac tissue sites, one may speculate that the heart possesses specific mechanisms to sequester (pro)renin from the circulation. Several groups have reported on the existence of (pro)renin binding proteins and/or receptors [16,41,43,51–53,72–74]. An intracellular renin-binding protein (RnBP) was discovered in the early eighties in humans, rats and pigs [72–74]. Binding to this RnBP reduces the Ang I-generating activity of renin by >80%. Recently, this RnBP was found to be equal to the enzyme *N*-acyl-D-glucosamine 2-epimerase, indicating that it might be involved in the intracellular processing of renin rather than renin uptake [75]. Subsequently, using chemical cross-linking, two vascular RnBPs were identified by Campbell and colleagues in membranes isolated from rat mesenteric arteries or cultured rat aortic smooth muscle cells [43]. Interestingly, binding to these RnBPs was inhibited by a specific, active site-directed renin inhibitor, suggesting that the active site of the renin molecule might be involved in the binding process. Nguyen et al. and Sealey et al., with the use of radiolabelled (pro)renin, demonstrated high-affinity renin binding sites/receptors ($K_d \approx 1$ nM) in human mesangial cells and in membranes prepared from rat tissues [51,52]. In the rat, these binding sites bound prorenin and renin equally well, which suggests that neither the prosegment nor the active site is involved in the binding process [51]. This contrasts with Campbell's findings.

If binding does not involve the prosegment or the active site, a further possibility would be binding to the carbohydrate portions which both proteins contain. Renin and prorenin display isoelectric heterogeneity; up to five or six forms with different isoelectric points have been described in rats and humans [76–79]. This heterogeneity most likely results from differential glycosylation (glycoforms). The carbohydrate portion appears to be involved in the clearance of renin by the liver, since deglycosylation greatly (>90%) reduced the hepatic uptake of renin [80]. We recently observed that the mannose 6-phosphate signal, present on both renin and prorenin, determines (pro)renin binding and internalization by cardiac and endothelial cells [16,41]. Most likely therefore, it is the mannose 6-phosphate receptor (MPR) which is involved in this process.

MPRs function in the process of intracellular lysosomal enzyme sorting [81,82]. At present, two different MPRs have been identified: a large MPR (mol. wt. 300 kDa), which binds ligand independent of divalent cations (cation-independent or CI-MPR), and a small MPR (mol. wt. 46 kDa), which requires divalent cations for optimal binding (cation-dependent or CD-MPR) [83,84]. In 1987, it was discovered that the CI-MPR and the insulin-like growth factor II receptor are the same protein [85]. Thus, the CI-MPR is now also known as the M6P/IGFII receptor. This receptor binds IGFII, which is non-glycosylated, and phosphomannosylated proteins at distinct sites (Fig. 4) [86,87]. The M6P/IGFII receptor is involved in the activation processes of several precursor proteins, such as procathepsin D and the latent form of transforming growth factor beta [88,89]. In view of the fact that we observed not only MPR-dependent binding and internalization of renin and prorenin, but also activation of prorenin to renin, it seems logical to assume that the MPR involved in these processes is the M6P/IGFII receptor.

The M6P/IGFII receptor consists of a large extracellular domain, containing 15 repeat regions, and a small cytoplasmic domain (Fig. 4). The extracytoplasmic domain of the CD-MPR is similar to each of the repeating units of the extracellular domain of the M6P/IGFII receptor, suggesting that the two receptors may be derived from a common ancestor [90]. The M6P/IGFII receptor exists as a monomer, whereas the CD-MPR exists as a monomer, dimer or tetramer [84,91]. MPRs cycle constitutively among the Golgi, endosomes, and the plasma membrane. The majority (90%) of the M6P/IGFII receptors is located in a late endosomal/prelysosomal compartment, with the rest being distributed over the plasma membrane, early endosomes, and the Golgi [92]. Extracellular lysosomal enzymes which bind to the cell surface M6P/IGFII receptor are internalized via clathrin-coated pits. They dissociate from the receptor in acidified endosomal compartments and are subsequently delivered to lysosomes. The receptor is then reutilized; it can undergo many rounds of ligand delivery [92–95]. Binding and internalization of IGFII to the M6P/IGFII receptor results in the lysosomal degradation of this ligand [96]. In addition, IGFII mediates growth-stimulatory responses via this receptor [97]. At present it is not clear what the function of the M6P/IGFII receptor with regard to (pro)renin is: clearance, coupling to second messengers [52], or facilitation of local angiotensin production (Fig. 3)? The idea of prorenin contributing to local angiotensin production is attractive, in view of the fact that the prorenin concentrations in the circulation are tenfold higher than those of renin.

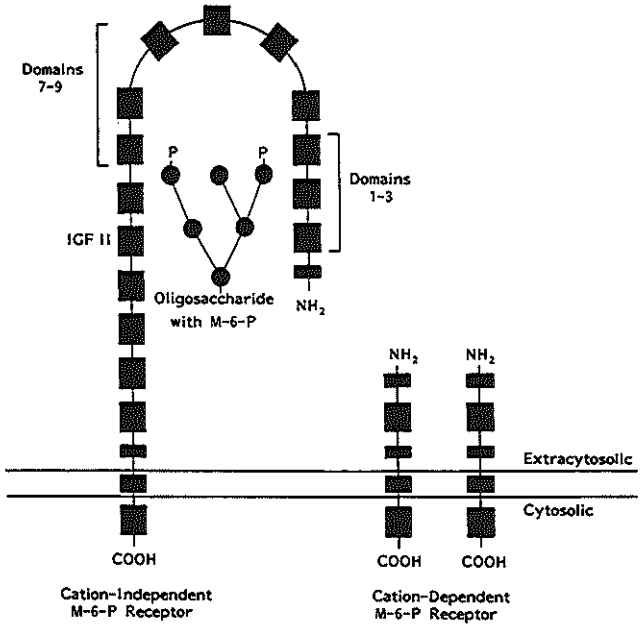

Fig. 4. Schematic representation of the mannose 6-phosphate receptors. The two ligands of the cation-independent mannose 6-phosphate are depicted. Arginine residues at positions 435 and 1334 in domains 3 and 9, respectively, are essential for high-affinity binding of mannose 6-phosphate. Sequences in domain 11 are involved in binding insulin-like growth factor (IGF) II. Modified from Refs. [81,82].

8. Role of alternative angiotensin generating pathways

It has been suggested, on the basis of in-vitro experiments, that Ang II synthesis may occur independently of renin and ACE. The candidates that are generally assumed to replace renin and ACE are cathepsin D and chymase, respectively. Cathepsin D is a lysosomal enzyme that cleaves angiotensinogen, unlike renin, at low pH [98,99]. Consequently, measurement of Ang I-generating activity at acidic pH will yield results that are representative for lysosomal cathepsin D rather than renin. Studies in which Ang I-generating activity is quantitated as a measure for renin activity should therefore always be performed in the absence and presence of specific renin inhibitors to correct for non-renin-dependent Ang I generation. Evidence that cathepsin D is of importance in vivo is currently lacking. Under circumstances where cardiac angiotensinogen levels are high and cardiac renin levels are low or undetectable (e.g., after nephrectomy), cardiac Ang I and II levels are close to or below the detection limit [24,32]. Moreover, in human heart homogenates renin levels correlated negatively with angiotensinogen levels, thereby suggesting angiotensinogen consumption by renin at cardiac tissue sites [53]. Studies in the isolated perfused heart also do not support a role for cathepsin D, since angiotensin generation in this preparation only occurred after the addition of renin to the perfusion buffer [37–40].

Chymase is a serine protease present in the cardiac interstitium, and cardiac mast cells and endothelial cells are sites of chymase biosynthesis and storage [100]. Remarkably, chymase is the main enzyme in human heart homogenates responsible for Ang I–II conversion [101]. In contrast, Ang I–II conversion in the coronary vascular bed of intact humans and pigs depends on ACE only [65,102,103]. This raises the question whether chymase is of importance in vivo. Kokkonen et al. [104] have suggested that interstitial fluid contains an endogenous inhibitor of chymase, α_1-antitrypsin, which would normally suppress any chymase-dependent Ang I–II conversion. However, the inhibitory effect of α_1-antitrypsin may be limited to tissue homogenates only, since it could not be demonstrated in an intact preparation [105]. Cardiac chymase mRNA levels are unaltered in subjects with heart failure [60]. More detailed knowledge on the in-vivo role of chymase will be obtained once specific chymase inhibitors are available.

9. Regulation of cardiac angiotensin generation

All RAS components are present in cardiac tissue, and both Ang I and II are generated in the heart. However, the renin responsible for this local angiotensin production originates from the circulation and is therefore kidney-derived. Thus, a local RAS in the sense that all RAS components are synthesized in situ does not appear to exist in the normal heart. This does not mean that cardiac angiotensin synthesis occurs in parallel with angiotensin generation in the circulation. There still are many ways by which the heart may regulate its Ang I and II concentrations independent of the circulating levels of these RAS components. Membrane binding could be a mechanism by which renal renin is sequestered in the heart. The density of the binding sites involved in the uptake process may vary, and this could modify the cardiac production of Ang I and II. Interestingly, the inactive precursor of renin, prorenin, also binds to these binding sites and becomes activated following internalization [16,41]. It is not yet known what enzyme(s) is/are responsible for the prorenin-to-renin conversion step. Their concentration may decrease or increase under different circumstances. In addition, one has to keep in mind that normally the circulating levels of prorenin are approximately 10-fold higher than those of renin.

Cardiac ACE levels will also influence local Ang II production. These are determined, at least in part, by the so-called insertion/deletion polymorphism [62]. Furthermore, enzymatic degradation of Ang II and AT_1 receptor-mediated endocytosis could influence the Ang II concentrations at the cellular and subcellular level. Finally, under certain pathological conditions renin and/or angiotensinogen may be produced in the heart itself and this would create the possibility for the heart to regulate its own Ang II production, independent of kidney and liver.

More detailed knowledge on the actual sites of Ang II production in the heart and on its regulation under pathological conditions will further illuminate the role of the RAS in cardiac function, growth, and remodelling, and will help us to understand the beneficial cardiac effects of ACE inhibitors, AT_1 receptor antagonists and renin inhibitors.

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The importance of *in situ* angiotensin generation

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Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm.

Saris J.J., van Dijk M.A., Kroon I., Schalekamp M.A.D.H. and Danser A.H.J.

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Functional Importance of Angiotensin-Converting Enzyme–Dependent In Situ Angiotensin II Generation in the Human Forearm

Jasper J. Saris, Marjan A. van Dijk, Ingrid Kroon, Maarten A.D.H. Schalekamp, A.H. Jan Danser

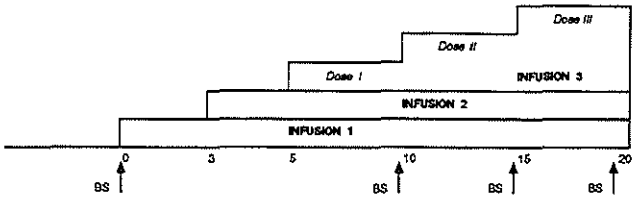
Abstract—To assess the importance for vasoconstriction of in situ angiotensin (Ang) II generation, as opposed to Ang II delivery via the circulation, we determined forearm vasoconstriction in response to Ang I (0.1 to 10 ng · kg^{-1} · min^{-1}) and Ang II (0.1 to 5 ng · kg^{-1} · min^{-1}) in 14 normotensive male volunteers (age 18 to 67 years). Changes in forearm blood flow (FBF) were registered with venous occlusion plethysmography. Arterial and venous blood samples were collected under steady-state conditions to quantify forearm fractional Ang I-to-II conversion. Ang I and II exerted the same maximal effect (mean±SEM 71±4% and 75±4% decrease in FBF, respectively), with similar potencies (mean EC_{50} [range] 5.6 [0.30 to 12.0] nmol/L for Ang I and 3.6 [0.37 to 7.1] nmol/L for Ang II). Forearm fractional Ang I-to-II conversion was 36% (range 18% to 57%). The angiotensin-converting enzyme (ACE) inhibitor enalaprilat (80 ng · kg^{-1} · min^{-1}) inhibited the contractile effects of Ang I and reduced fractional conversion to 1% (0.1% to 8%), thereby excluding a role for Ang I-to-II converting enzymes other than ACE (eg, chymase). The Ang II type 1 receptor antagonist losartan (3 mg · kg^{-1} · min^{-1}) inhibited the vasoconstrictor effects of Ang II. In conclusion, the similar potencies of Ang I and II in the forearm, combined with the fact that only one third of arterially delivered Ang I is converted to Ang II, suggest that in situ–generated Ang II is more important for vasoconstriction than circulating Ang II. Local Ang II generation in the forearm depends on ACE exclusively and results in vasoconstriction via Ang II type 1 receptors. (***Hypertension.*** **2000;35:764-768.**)

Key Words: angiotensin ■ angiotensin-converting enzyme inhibitors ■ receptors, angiotensin II ■ blood flow

Circulating angiotensin (Ang) I is converted to Ang II in many vascular beds.[1–5] The functional importance of this locally generated Ang II compared with arterially delivered Ang II is currently unknown. Organ bath experiments in which the contractile responses of isolated human or porcine coronary arteries were recorded have shown that Ang I and Ang II display similar vasoconstrictor potencies,[6,7] despite the fact that the levels of Ang II in the bath fluid during exposure to Ang I are <1% of those during exposure to Ang II.[6] These in vitro experiments therefore suggest that locally generated rather than circulating Ang II mediates vasoconstriction. A study of the local generation of Ang II and its vasoconstrictor effects in perfused rat hindquarters, in which the venous Ang II levels after the infusion of renin were compared with those after the infusion of Ang I or II, also indicated that vasoconstriction was caused by local Ang II rather than by Ang II in the perfusion buffer.[8]

Two enzymes have been reported to contribute to Ang I-to-II conversion: ACE and chymase. ACE is present both in circulating blood plasma and on the membrane of vascular endothelial cells, whereas chymase is located in the adventitia, in the cytosol of mast cells.[9,10] Although the results of in vitro studies in isolated human vessels[6,7] and tissue homogenates[11,12] support the contribution of chymase to Ang I-to-II conversion, in vivo studies do not support this view, because ACE inhibition suppressed Ang I-to-II conversion in the human and porcine coronary vascular beds by >90%.[3,13] However, coronary Ang I-to-II conversion in these latter studies was quantified with systemic or intracoronary infusions of ^{125}I-labeled Ang I, an approach that does not allow the detection of Ang II generation with chymase in the adventitia if such generation does not result in Ang II overflow into the blood compartment. Moreover, contractile effects were not quantified in these studies.

It was the aim of the present study to compare the in vivo potencies of Ang I and II to assess the functional importance of locally generated Ang II. Ang I and Ang II were infused into the brachial artery, and forearm vasoconstriction was recorded under steady state conditions. Forearm Ang I-to-II conversion was quantified with measurment of the venous Ang I and II levels at steady state. Infusions were made in the presence and absence of the ACE inhibitor enalaprilat and the



Experiment	INFUSION 1	INFUSION 2	INFUSION 3		
1	-	SNP	Angl 0.1	Angl 1	Angl 10
2	enal 80	SNP	Angl 0.5	Angl 5	Angl 50
3	-	SNP	Angll 0.1	Angll 0.5	Angll 5
4	los3000	SNP	Angll 0.1	Angll 0.5	Angll 5

Figure 1. Schematic presentation of infusion experiments. Top, Intra-arterial infusion protocol. In experiments 2 and 4, continuous infusion 2 was started 3 minutes after start of continuous infusion 1. In all experiments, infusion 3 was started 5 minutes after start of infusion 1; infusion 3 consisted of 3 dose steps that lasted 5 minutes each. Blood sampling (BS) occurred under baseline conditions, before start of infusions, and at end of each dose step of infusion 3, when a steady state had been reached. Bottom, infusion rates in ng · kg^{-1} · min^{-1}. SNP indicates sodium nitroprusside; Ang, angiotensin; enal, enalaprilat; and los, losartan.

Ang II type 1 (AT_1) receptor antagonist losartan to investigate (1) whether enzymes other than ACE contribute to the local generation of Ang II and (2) whether Ang II mediates vascular effects through receptors other than the AT_1 receptor.

Methods

Subjects

Fourteen white male volunteers (mean age 39 years, range 18 to 67 years; mean weight 83 kg, range 64 to 107 kg) were recruited via advertisement after the Medical Ethics Committee of the Leiden University Medical Center approved the protocol of the study. All participants gave their informed consent. Medical history, physical examination, and routine laboratory tests did not reveal any abnormalities. All subjects were on a normal-sodium diet (≈180 mmol/d), and none of them received medication. Subjects did not smoke, and they refrained from the consumption of alcohol or caffeine-containing substances for ≥12 hours before the experiment.

Experimental Set-Up

Each experiment was performed with the subject in the supine position in a quiet room at a constant temperature of 22° to 24°C. Forearm and hand volumes were measured with water displacement. One-lead ECG was monitored continuously. After local anesthesia with 1% lidocaine, the brachial artery of the nondominant arm was cannulated. The cannula (1.0×45 mm) was connected to a Statham P23Id pressure transducer (Gould Inc). Drugs were infused into the brachial artery with Harvard Apparatus volumetric precision pumps (model 22). Both forearms were instrumented with mercury-in-Silastic strain gauges, which were connected to a Hokanson EC-2 plethysmograph. Both upper arms were connected to a Hokanson E-10 rapid cuff inflator. For the measurement of forearm blood flow (FBF), R wave–triggered cuff inflation (at 40 mm Hg) for venous occlusion plethysmography was controlled with a personal computer.[14] FBF was measured 4 times per minute, and the final 6 measurements at the end of each dose step, when a steady state had been reached,[15] were used for further analysis. During each infusion experiment, the hands were continuously excluded from the circulation with the inflation of small wrist cuffs to a minimum of 40 mm Hg above systolic blood pressure. Heart rate from the ECG, intra-arterial blood pressure, and left and right FBF values were recorded on a polygraph (Gould Inc) and on a personal computer with an analog-to-digital converter (model DT 2801; Data Translation Inc).

Study Protocol

The infusion studies were started ≥60 minutes after the cannulation of the brachial artery. Between the various infusion experiments, the wrist cuffs were deflated, and sufficient time (minimum of 45 minutes) was taken to allow the subjects to recover from hand ischemia and to allow FBF to return to baseline levels. The protocol is summarized in Figure 1. Baseline arterial and venous blood samples were taken before the start of the infusions. Steady-state venous blood samples were obtained at the end of each Ang infusion. Sodium nitroprusside was used to predilate the vascular bed of the forearm to ≈5 mL · 100 mL^{-1} · min^{-1} because measurements of vasoconstrictor effects are more accurate when flow levels remain at >1 mL · 100 mL^{-1} · min^{-1}.[16]

Blood Sampling

Blood for Ang measurements was rapidly drawn with a plastic syringe containing the following inhibitors (0.25 mL inhibitor solution in 5 mL blood): 6.25 mmol/L disodium EDTA, 1.25 mmol/L 1,10-phenanthroline, and 0.01 mmol/L concentration of the renin inhibitor remikiren (final concentrations in blood). The blood was transferred into prechilled polystyrene tubes and centrifuged at 3000*g* for 10 minutes at 4°C. Plasma was stored at −70°C.

Measurement of Ang I and II

Baseline arterial and venous Ang I and II concentrations were measured with radioimmunoassay, after SepPak extraction and high-performance liquid chromatographic separation, as described previously.[2,3] The high Ang concentrations in the venous samples collected under steady state conditions at the end of each infusion were measured without prior high-performance liquid chromatographic separation.[6]

Data Analysis

Data were normally distributed and are expressed as mean±SEM. The Ang-induced effects are expressed as percentage change in FBF of the infused forearm. The percentage change was calculated relative to the values measured at baseline (ie, at the beginning of infusion 3) (Figure 1). The steady state arterial Ang plasma concentrations (in pmol/L) during the infusions were calculated as follows: $[Ang]_{art,\ steady\ state} = IR \times BW \times 10^5 / [(1-Ht) \times FBF \times FAV \times MW] + [Ang]_{art,\ baseline}$, where IR is Ang I or II infusion rate (in ng · kg^{-1} · min^{-1}), BW is body weight (in kg), Ht is hematocrit, FAV is forearm volume, MW is molecular weight of Ang I or II, and $[Ang]_{art,\ baseline}$ is arterial Ang I or II concentration at baseline.

Fractional conversion and degradation of Ang I (ie, the percentage of arterially delivered Ang I that is converted to Ang II or degraded into other metabolites) and fractional degradation of Ang II (ie, the

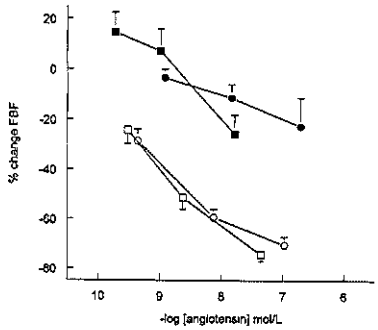


Figure 2. Percent change of forearm blood flow (FBF) in response to Ang I without (○) or with (●) enalaprilat and to Ang II without (□) or with (■) losartan. Data are mean±SEM of 14 experiments.

percentage of arterially delivered Ang II that is degraded) in the forearm were calculated as described previously.[2,3]

EC_{50} values (ie, the arterial Ang I or II concentration at which 50% of the maximal effect is achieved) were calculated from the arterial plasma concentrations and the corresponding FBF values with 4-parameter logistic regression analysis (InPlot 2.0; GraphPAD Software).[15,17]

Student's *t* test and ANOVA for repeated measures were used for statistical evaluation. Values of $P<0.05$ were considered statistically significant.

Results

Baseline arterial Ang I and II levels (6.9±0.6 and 2.7±0.2 fmol/mL, respectively) were comparable to baseline venous Ang I and II levels (10.8±2.3 and 3.0±0.2 fmol/mL, respectively). Baseline Ang levels were unrelated to age or weight.

FBF did not change in the noninfused control arm during the infusions, nor did the Ang infusions affect heart rate and blood pressure (data not shown).

Ang I and II reduced FBF by a maximum of 71±4% and 75±4%, respectively, with comparable potencies (EC_{50} 5.6±1.0 and 3.6±0.5 nmol/L, respectively; P=NS) (Figure 2). Enalaprilat virtually completely blocked the constrictor effects of Ang I. Losartan blocked the vasoconstrictor effects of Ang II; in fact, a tendency for a vasodilator effect (P=NS) was observed at the 2 lowest doses of Ang II in the presence of this drug.

Fractional Ang I conversion was similar at all Ang I doses and was reduced to very low values in the presence of enalaprilat (Table). Fractional Ang I and II degradations were higher at the high doses than at the low doses of these peptides, most likely because of the reduced FBF at these high doses. In support of this assumption, FBF correlated negatively with fractional Ang I and II degradation (fractional Ang I degradation=$-0.06\times$FBF$+0.69$ [$r=0.56$, $P<0.05$] and fractional Ang II degradation=$-0.06\times$FBF$+0.97$ [$r=0.76$, $P<0.01$]). The relationship between FBF and Ang degradation was unaltered with enalaprilat and losartan (data not shown).

Discussion

The results of the present study provide in vivo evidence in humans that in situ–generated Ang II is more important for vasoconstriction than circulating Ang II. This conclusion is based on 2 findings. First, Ang I and Ang II induced forearm vasoconstriction with similar potencies, despite the fact that only one third of Ang I was converted to Ang II in the forearm circulation. Second, the venous Ang II levels at the highest Ang I infusion rate in the presence of enalaprilat were comparable to the venous Ang II levels at a 50-fold lower

Venous Angiotensin Levels, Fractional Ang I Conversion, and Fractional Ang I and II Degradation During Infusion of Ang I or II With or Without Concomitant Infusion of ACE Inhibitor Enalaprilat (80 ng · kg^{-1} · min^{-1}) or AT_1 Receptor Antagonist Losartan (3000 ng · kg^{-1} · min^{-1})

	Infusion Rate, ng · kg^{-1} · min^{-1}					
	Ang I			Ang I+Enalaprilat		
Parameter	0.1	1.0	10	0.5	5.0	50
Ang I, pmol/L	43±6	304±56	1691±334	583±108	6583±1029	46 161±6243
Ang II, pmol/L	70±5	544±96	5307±1037	29±5	103±30	662±193
Fractional Ang I conversion, %	41±3	32±3	34±2	2±1†	1±1†	1±0†
Fractional Ang I degradation, %	48±2	62±2*	64±2*	38±7	48±8	68±5*
	Ang II			Ang II+Losartan		
	0.1	0.5	5.0	0.1	0.5	5.0
Ang II, pmol/L	97±10	458±66	4283±627	97±7	443±62	3743±498
Fractional Ang II degradation, %	64±4	78±3*	88±3*	48±4	58±4	76±3*

Data are mean±SEM (n=14).
*$P<0.01$ vs lowest infusion rate.
†$P<0.001$ vs without enalaprilat.

Ang I infusion rate without enalaprilat, yet vasoconstriction was observed only in the latter case. These results confirm previous in vitro observations of the importance of local Ang II in rats and humans.[6,8] They are also in agreement with recent studies that show that despite the fact that ACE is present in the plasma of tissue ACE knockout mice, these animals display the same hemodynamic and vascular abnormalities as mice that are completely ACE deficient.[18,19] The potencies obtained for Ang I and II in the present study were in the nanomolar range, which is in agreement with the known affinity of AT receptors for Ang II.[20,21] It is therefore unlikely that vasoconstriction during Ang I infusion was mediated by Ang I.

In contrast with findings in isolated human blood vessels,[6,7,22] we did not obtain evidence for chymase-dependent vasoconstriction in the human forearm. The ACE inhibitor enalaprilat not only blocked forearm Ang I-to-II conversion by >95% but also almost completely inhibited Ang I–induced vasoconstriction. It is unlikely that the absence of a chymase-mediated effect in the present study is due to the inability of arterially infused Ang I to reach vascular chymase (ie, to diffuse into the adventitia[10]). It has been previously demonstrated that circulating Ang I and Ang II both rapidly diffuse into the interstitial space.[23,24] Furthermore, studies in which the chymase-specific substrate [Pro^{11},D-Ala^{12}]Ang I was administered intravenously to marmosets or hamsters showed clear dose-dependent pressor effects of this peptide that could not be blocked with an ACE inhibitor.[25,26] The discrepancy between in vitro and in vivo studies with regard to the importance of chymase might be due to the presence of an endogenous chymase inhibitor, α_1-antitrypsin, in interstitial fluid.[27] However, such in vivo chymase inhibition is not in agreement with the vasoconstrictor effects obtained with [Pro^{11},D-Ala^{12}]Ang I.[25,26] Moreover, α_1-antitrypsin appeared to inhibit chymase in tissue homogenates only,[27,28] not in intact preparations.[6,28] A more likely explanation therefore is disruption of mast cells during tissue storage or preparation, which will result in chymase concentrations in vitro that are far above those in vivo.

The forearm Ang I-to-II conversion rate obtained here is in agreement with previous studies in which forearm conversion was calculated during the infusion of ^{125}I-labeled Ang I.[2] In those studies, the levels of ^{125}I-Ang II that were obtained at steady state were too low to induce vasoconstriction. Remarkably, despite the clear dose-dependent vasoconstriction that occurred in the present study, fractional forearm Ang I-to-II conversion remained constant at all FBF values. In contrast, fractional forearm Ang I and II degradation correlated inversely with FBF. This latter finding is not surprising, because at lower flow rates, more time is available for metabolism. The fact that Ang I-to-II conversion was not related to FBF suggests that (1) it is a highly efficient process with a maximal result even at high flow rates and (2) conversion most likely precedes degradation (ie, that ACE might be located predominantly in the arterioles).

In the present study, losartan, a competitive AT$_1$ receptor antagonist,[29] fully prevented vasoconstriction at the 2 lowest Ang II doses and in large part (>70%) inhibited vasoconstriction at the highest dose of Ang II. These data are in agreement with the contention that Ang II induces vasoconstriction in the human forearm through the activation of AT$_1$ receptors.

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The travelling

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Renin-angiotensin system components in cardiac cells

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Cultured neonatal rat cardiac myocytes and fibroblasts do not synthesize renin or angiotensinogen: evidence for stretch-induced cardiomyocyte hypertrophy independent of angiotensin II.

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Cultured neonatal rat cardiac myocytes and fibroblasts do not synthesize renin or angiotensinogen: evidence for stretch-induced cardiomyocyte hypertrophy independent of angiotensin II

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Abstract

Objective: The hypertrophic response of cardiomyocytes exposed to mechanical stretch is assumed to depend on the release of angiotensin (Ang) II from these cells. Here we studied the synthesis of renin–angiotensin system (RAS) components by cardiac cells under basal conditions and after stretch. **Methods:** Myocytes and fibroblasts were isolated by enzymatic dissociation from hearts of 1–3-day-old Wistar rat strain pups, grown for 1 day in serum-supplemented medium and then cultured in a chemically defined, serum-free medium. Medium and cell lysate were collected 5 days later or after exposure of the cells to cyclic stretch for 24 h. Prorenin, renin and angiotensinogen were measured by enzyme-kinetic assay; Ang I and Ang II were measured by radioimmunoassay after SepPak extraction and HPLC separation. **Results:** Prorenin, but none of the other RAS components, could be detected in the medium of both cell types. However, its levels were low and the Ang I-generating activity corresponding with these low prorenin levels could not be inhibited by the specific rat renin inhibitor CH-732, suggesting that it was most likely due to bovine and/or horse prorenin sequestered from the serum-containing medium to which the cells had been exposed prior to the serum-free period. When incubated with Ang I, both myocytes and fibroblasts generated Ang II in a captopril-inhibitable manner. Myocyte and fibroblast cell lysates did not contain prorenin, renin, angiotensinogen, Ang I or Ang II in detectable quantities. Stretch increased myocyte protein synthesis by 20%, but was not accompanied by Ang II release into the medium. **Conclusion:** Cardiac myocytes and fibroblasts do not synthesize renin, prorenin or angiotensinogen in concentrations that are detectable or, if not detectable, high enough to result in Ang II concentrations of physiological relevance. These cells do synthesize ACE, thereby allowing the synthesis of Ang II at cardiac tissue sites when renin and angiotensinogen are provided via the circulation. Ang II is not a prerequisite to observe a hypertrophic response of cardiomyocytes following stretch.

Keywords: Angiotensin; ACE inhibitor; Myocytes; Renin–angiotensin system; Stretch

1. Introduction

The existence of a local renin–angiotensin system (RAS) in the heart, often invoked to explain the beneficial effects of ACE inhibitors in heart failure [1,2], is still a controversial issue. The presence of RAS components in cardiac tissue [3–5] cannot be taken as direct evidence for local production of these components. One or more components may have been sequestered from the circulation. For instance, circulating renin may bind to cardiac cell receptors and to renin binding proteins in the heart [6–8], and circulating Ang II is known to accumulate in cardiac tissue via AT_1 receptor-mediated endocytosis [9]. The levels of renin and angiotensinogen mRNA in the

heart are low or undetectable [10–13], thereby suggesting that the presence of these components in cardiac tissue may indeed depend on uptake rather than local production. The uncertainties concerning local synthesis arising from tissue measurements can be avoided when measurements are made in cells cultured in the absence of serum. The use of serum-free culture medium is necessary to exclude the uptake of RAS components present in serum.

Most so-called renin-expressing extrarenal cells produce prorenin rather than renin. These cells do not store prorenin and secrete it in a constitutive manner [14–17]. With regard to cardiomyocytes, both Dzau and Re [18] and Dostal et al. [19] reported on the presence of renin in these cells. No distinction between renin and prorenin was made, nor did these authors determine (pro)renin release into the culture medium. Constitutive secretion of angiotensinogen has also been described [20–22]. The single report on the synthesis of angiotensinogen by cardiac cells focuses on its presence in these cells [19]. ACE, a cell membrane-bound enzyme, has been demonstrated in cardiac cells by enzyme-kinetic and immunohistochemical methods [23–26].

According to several investigations, serum-deprived cardiac cells release angiotensins into the culture medium. The Ang I and II levels in the medium, however, show huge variations, from <10 fmol/ml to >1000 fmol/ml [23,26–29]. Ang II in the medium increased 100-fold after the induction of mechanical stretch [27], possibly by release from intracellular storage sites [23,27], and this cell-derived Ang II may play a role in the stretch-induced hypertrophic response of cardiomyocytes [26–29].

It was the aim of the present study to investigate the synthesis of RAS components by neonatal rat cardiomyocytes and fibroblasts by measuring renin, prorenin, angiotensinogen, Ang I and Ang II in the medium and cell lysate of serum-deprived cells with the help of well-established biochemical techniques. Cellular ACE activity was investigated by quantifying Ang I-to-II conversion by intact cells in the presence and absence of captopril. Stretch-induced release of angiotensin II and its role in cellular hypertrophy were examined in cardiomyocytes exposed to cyclic, circular stretch for 24 h. For comparison, studies were also performed in cells cultured in the presence of serum.

2. Materials and methods

2.1. Reagents

Fetal calf serum, horse serum, penicillin and streptomycin were purchased from Boehringer Mannheim (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM) and Medium 199 were from Gibco, Life Technologies (Middlesex, UK). Trypsin (type III) and captopril were from Sigma (St. Louis, MO, USA). Methanol and *ortho*-phosphoric acid (both analytical grade) were from Merck (Darmstadt, Germany). Ang I was obtained from Bachem (Bubendorf, Switzerland). [^{3}H]-leucine was from Amersham (Buckinghamshire, UK). The rat renin inhibitor CH-732 was a kind gift of Dr. M. Szelke, Ferring Research Institute, (Southampton, UK) [30]. Rat renin was prepared from rat kidneys as described before [31]. Human recombinant prorenin was a gift of Dr. W. Fischli (Hoffmann-La Roche, Basel, Switzerland). Angiotensinogen was prepared from plasma of nephrectomized rats [31].

2.2. Cell culture

All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Primary cultures of neonatal ventricular cardiomyocytes and fibroblasts were prepared from 1–3-day-old Wistar strain rats as described before [32]. Briefly, ventricles from newborn 1–3-day-old Wistar rats were minced, and cells were isolated by eight subsequent trypsinization steps at 30°C. Non-cardiomyocytes were separated from the cardiomyocytes by differential preplating. Cardiomyocytes were seeded in 20-cm^2 culture dishes (Falcon, Becton & Dickinson, Plymouth, UK) at $1.5 \cdot 10^5$ cells/cm^2, giving a confluent monolayer of spontaneously contracting cells after 24 h. The preplated cells (fibroblast fraction) were passaged after 4 days, using a 0.02% trypsin–0.05% EDTA solution, in 20-cm^2 culture dishes at $0.75 \cdot 10^5$ cells/cm^2. The cells were maintained at 37°C and 5% CO_2–95% air in 5 ml culture medium consisting of DMEM and Medium 199 (4:1), supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. After incubation for 24 h, cells were either serum-deprived or maintained in serum-supplemented culture medium for 5 days.

2.3. Collection of medium and cells for the measurement of RAS components

Cardiomyocyte- and fibroblast-conditioned culture medium (5 ml) was collected for the measurement of RAS components after the cells had been maintained with or without serum for 5 days. The RAS component content of unconditioned medium, i.e. medium that had not been in contact with either cardiomyocytes or fibroblasts, was also studied. Medium for the measurement of prorenin, renin and angiotensinogen was frozen at −70°C without the addition of inhibitors. Medium for the measurement of Ang I and II was mixed with 250 μl angiotensinase inhibitor solution (containing 125 mmol/l disodium EDTA and 25 mmol/l 1,10-phenanthroline) and frozen at −70°C.

To measure RAS components in the cells, each well was washed three times with 6 ml ice-cold phosphate buffered

saline (PBS; 140 mmol/l NaCl, 2.6 mmol/l KCl, 1.4 mmol/l KH_2PO_4, 8.1 mmol/l Na_2HPO_4, pH 7.4). After washing, cells used for the measurement of prorenin, renin and angiotensinogen were lysed in 0.5 ml ice-cold PBS containing 0.2% Triton X-100, and the cell lysates were quickly frozen on dry ice. Cells used for the measurement of Ang I and II were scraped with a rubber policeman in a volume of 0.5 ml ice-cold PBS. The cell–PBS mixture was centrifuged at 1000 *g* at 4°C for 1 min, after which the pellet was homogenized in 0.5 ml 0.1 mol/l HCl–80% ethanol using a hand-operated douncer. The ethanol was evaporated under vacuum rotation at 4°C using a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, USA). The concentrated homogenates were dissolved in 0.5 ml 1% *ortho*-phosphoric acid and applied to SepPak columns (see Section 2.6).

2.4. Angiotensin I-to-II conversion by ACE

To determine whether 5-day-old cardiomyocytes and fibroblasts contain ACE, Ang I-to-II conversion by these cells was studied in the presence or absence of captopril (final concentration in the medium: 0.5 μmol/ml). Ang I was added to the medium (final concentration: 1 pmol/ml), and 150 μl samples were obtained over a period of 40 min (from cells cultured in the presence of serum) or 120 min (from cells cultured in the absence of serum). The samples were rapidly mixed with 10 μl angiotensinase inhibitor solution and frozen at −70°C. No corrections were made for the small volume changes (approximately 3% per sample) occurring as a consequence of fluid sampling. Ang I-to-II conversion was also studied in unconditioned medium.

2.5. Angiotensin II generation during stretch of cardiomyocytes

To study the generation of Ang II during prolonged stretch and its contribution to the increased protein synthesis occurring under these conditions, cardiomyocytes were subjected to cyclic, circular stretch for 24 h. Following isolation (see Section 2.2) the cells were seeded in flexible-bottomed 6-well culture plates (type I collagen-coated, 5 cm^2/well; Flexcell, Hillsborough, NC, USA). They were maintained at 37°C and 5% CO_2–95% air in 1 ml culture medium consisting of DMEM and Medium 199 (4:1), supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. After 24 h the medium was replaced by DMEM and Medium 199 (4:1), supplemented with 4% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. The stretch experiment was performed 48 h later under serum-free conditions. The cells were preincubated for 30 min with 1.5 ml serum-free medium. The 6-well plates were then placed on a Flexcell Strain Unit (FX-2000, Flexcell), and the cells were stretched at 30 cycles per minute (1 s strain, 1 s relaxation) at 20% elongation for 24 h. Control cells, grown on non-flexible-bottomed culture plates, were studied in parallel. For comparison, control cells cultured on non-flexible-bottomed culture plates were also incubated for 24 h with endothelin-1 (ET-1; final concentration in the medium: 10^{-8} mol/l), an agent known to induce protein synthesis in cardiomyocytes [32,33].

The effect of stretch on protein synthesis was evaluated by adding [^{3}H]-leucine to the medium (final concentration: 0.5 μCi/ml) of control-, stretched- and ET-1-treated cells 2 h before the end of the 24 h study period. After 24 h, incorporated [^{3}H]-leucine was determined as described before [33]. Total cellular protein was measured after 24 h in control-, stretched- and ET-1-treated cells that had not been incubated with [^{3}H]-leucine, using the Bradford assay [34]. Cellular hyperplasia was investigated by measuring optical density after incubation of the cells for 2 h at 37°C with (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), an agent which is converted to the colored product formazan via intact mitochondria. A change in optical density correlates directly with a change in cell number [35].

The effect of stretch on Ang I and II synthesis was studied by collecting 0.15 ml samples from the culture medium of each well of two 6-well plates after 1, 2, 6, and 24 h. The twelve 0.15 ml samples obtained at each time point were added together and mixed with 0.1 ml angiotensinase inhibitor solution (final volume 1.9 ml). All samples were stored at −70°C.

2.6. Biochemical measurements

2.6.1. Renin and prorenin

Renin was quantified in duplicate by measuring Ang I generation at pH 7.4 during incubation at 37°C for 2–4 h with a saturating concentration of rat angiotensinogen in the presence angiotensinase-, ACE- and serine protease-inhibitors [4,7]. Two different inhibitor solutions were used, one with and one without the rat renin inhibitor CH-732 (final concentration in the incubation mixture: 5 μmol/l). Inhibition of rat kidney renin is >95% at this concentration (Fig. 1). Ang I was measured with a sensitive radioimmunoassay [36]. The lowest renin level that could be detected was 1.0 fmol Ang I/min per ml medium and 0.5 fmol Ang I/min per 10^6 cells.

Prorenin was first converted into renin by proteolytic activation and then also measured with the above assay. Based upon our experience with the activation of prorenin in tissues [4,7], two different activation procedures were tested, i.e. acidification only or acidification followed by treatment with plasmin at neutral pH. Medium or cell lysate were acidified by dialysis at 4°C for 48 h against 0.05 mol/l glycine buffer, pH 3.3, containing 0.001 mol/l disodium EDTA and 0.095 mol/l NaCl. This was followed by either 1) dialysis at 4°C for 24 h against 0.1 mol/l phosphate buffer, pH 7.4, containing 0.001 mol/l disodium

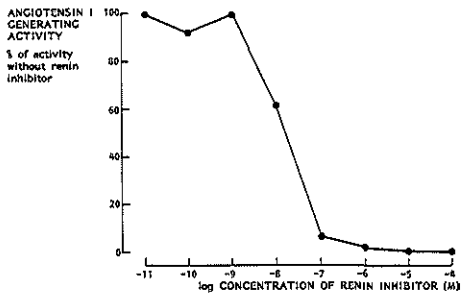

Fig. 1. Dose-dependent inhibition of rat kidney renin by increasing concentrations of the rat renin inhibitor CH-732.

EDTA and 0.075 mol/l NaCl, or 2) quick adjustment of pH to 7.4 with 1 mol/l NaOH and the subsequent addition of 0.1 volume of a solution of human plasmin (final concentration: 0.5 casein units/ml) in 0.15 mol/l NaCl and incubation at 4°C for 48 h. Acid-treatment followed by restoration of pH to 7.4 and treatment with plasmin led to virtually complete activation of prorenin, as was demonstrated by the >90% conversion and recovery of human recombinant prorenin that had been added to the samples before the activation procedure (n=3). Acid-treatment followed by restoration of pH to 7.4 without subsequent plasmin treatment led to less complete activation of prorenin; the recovery of added prorenin, measured as renin, was 45–55% (n=3). All samples were therefore activated by the combined acid-and-plasmin method.

2.6.2. Angiotensinogen

The concentration of angiotensinogen was determined as the maximum quantity of Ang I that was generated during incubation for 1 h at 37°C and pH 7.4 with rat kidney renin in the presence of a mixture of angiotensinase-, ACE- and serine protease-inhibitors [4,31]. The lowest level of angiotensinogen that could be measured was 0.1 pmol per ml medium and 0.05 pmol/10^6 cells.

2.6.3. Angiotensin I and II

The Ang I and II concentrations in medium collected during the measurement of ACE activity were measured directly with sensitive radioimmunoassays [36]. Measurements were made in 50 µl medium. The lowest measurable Ang I concentration was 15 fmol/ml, and the lowest measurable Ang II concentration was 10 fmol/ml.

In all other samples (medium and cell homogenates) Ang I and II were measured by radioimmunoassay, after SepPak extraction and reversed-phase high-performance liquid chromotography (HPLC) separation [36]. ^{125}I-labeled Ang I was added to the samples before SepPak extraction, to determine the recovery of Ang I and II. The recovery was better than 90%, and the Ang I and II results were not corrected for incomplete recovery. The lower limit of detection for Ang I and Ang II in the culture medium were 0.2 and 0.1 fmol/ml, respectively. In cell homogenates, it was 0.3 and 0.2 fmol/10^6 cells.

2.7. Calculations

Ang I is eliminated by conversion to Ang II by ACE, and by breakdown into small biologically inactive peptides by various other enzymes. The latter process is referred to as degradation of Ang I. The first order rate constants for Ang I degradation (k_1) and conversion (k_2) were calculated as described before [36]. The percent contribution of conversion to the total metabolism of Ang I is defined as follows:

Contribution of conversion to metabolism (%) =

$$[k_2/(k_1 + k_2)] \times 100\%.$$

2.8. Statistical analysis

Results are expressed as mean±S.D. One-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (Student's t-test for paired observations, with Bonferroni correction) was used for comparison between groups. Values of $P<0.05$ were considered significant.

3. Results

3.1. Renin, prorenin and angiotensinogen

Renin and prorenin were detectable in unconditioned fetal calf serum- and horse serum-supplemented medium (Fig. 2). The Ang I-generating activity corresponding with these renin and prorenin levels was not inhibited by the rat renin inhibitor CH-732 (5 µmol/l), which suggests that CH-732, at this concentration, does not inhibit bovine or horse renin. Following 5 days of incubation with either cardiomyocytes or fibroblasts, the levels of renin and prorenin in serum-supplemented medium were unchanged.

Renin was undetectable in cardiomyocyte- and fibroblast-conditioned, serum-deprived medium. Low levels of prorenin were present in the cardiac cell-conditioned, serum-free media, but they were not inhibited by CH-732, indicating that the Ang I generation we measured after in vitro prorenin activation was not caused by rat renin. Both renin and prorenin were undetectable in cell lysates of serum-deprived cardiomyocytes and fibroblasts.

Low levels of angiotensinogen were detected in unconditioned, serum-supplemented medium, which did not change after 5 days of incubation with cardiac cells (Fig. 2). These levels therefore most likely represent bovine and horse angiotensinogen. Angiotensinogen was undetectable in cardiomyocyte- and fibroblast-conditioned serum-free medium and in the lysates of these cells.

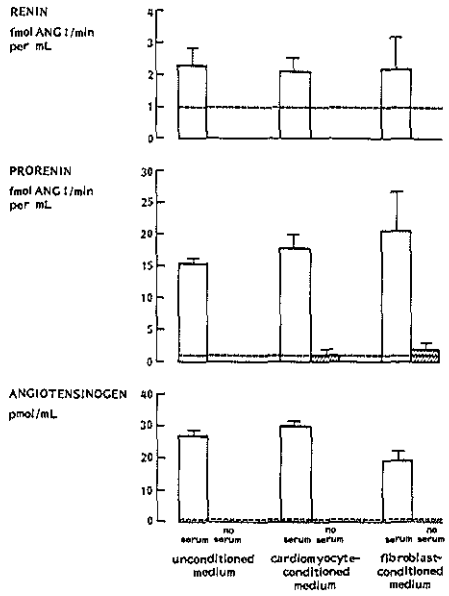

Fig. 2. Renin, prorenin, and angiotensinogen levels in unconditioned medium ($n=3$), cardiomyocyte-conditioned medium ($n=5$), and fibroblast-conditioned medium ($n=5$) with (open bars) or without (hatched bars) serum. Addition of the specific rat renin inhibitor CH-732 (5 μmol/l) did not affect the outcome of the renin and prorenin measurements (data not shown). Data are mean±S.D.. The dotted line represents the limit of detection. Renin and angiotensinogen were below the detection limit in the cell-conditioned serum-free media.

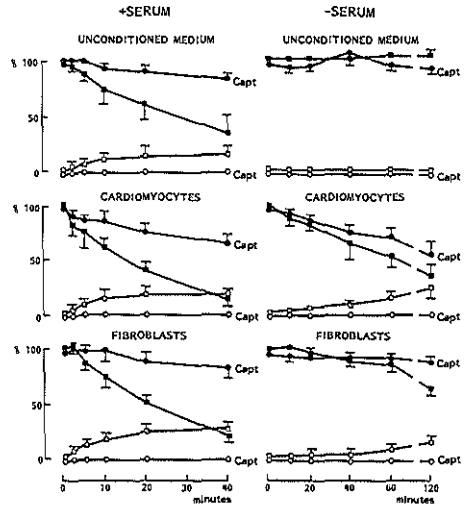

Fig. 3. Metabolism of angiotensin I added to unconditioned medium (top panels, $n=4$), 5-day-old cardiomyocyte cultures (middle panels, $n=6$) and 5-day-old fibroblast cultures (bottom panels, $n=3$) with (left panels) or without (right panels) serum. Data, which are expressed as a percentage of the angiotensin I levels in the medium at $t=0$, are mean±S.D.; where no S.D. is given, it was smaller than the symbol. Closed symbols represent Ang I, open symbols represent Ang II. CAPT, experiment in the presence of captopril (0.5 μmol/ml).

3.2. Angiotensin I-to-II conversion by ACE

Ang I added to unconditioned serum-supplemented medium was rapidly metabolized, Ang II being a major metabolite (Fig. 3). Captopril prevented the formation of Ang II completely. On the basis of the difference in Ang I metabolism with and without captopril it could be calculated that more than 90% of the Ang I metabolism in unconditioned serum-supplemented medium was due to ACE-dependent Ang I-to-II conversion (Table 1). During incubation with unconditioned serum-deprived medium, no significant Ang I metabolism could be demonstrated over a period of 2 h.

Ang I metabolism in cardiomyocyte and fibroblast cell cultures that had been maintained for 5 days in the presence of serum tended to be more rapid than Ang I metabolism in unconditioned serum-supplemented medium (Fig. 3). Ang II was again a major metabolite, and captopril prevented its formation completely. In both cell cultures, approximately 80% of Ang I metabolism was due to ACE-dependent Ang I-to-II conversion (Table 1).

Ang I added to serum-deprived cardiomyocyte and fibroblast cell cultures was also converted to Ang II, and captopril inhibited the formation of Ang II completely (Fig. 3). This indicates that, in the absence of serum, cardiomyocytes and fibroblasts contain detectable ACE activity. Approximately 60–70% of the Ang I metabolism was due to ACE (Table 1).

3.3. Angiotensin I and II

Low levels of Ang I and II were present in unconditioned serum-supplemented medium (Table 2). These levels remained low or decreased to levels below the detection limit after 5 days of incubation with cardiomyocytes or fibroblasts. The cellular levels of Ang I and II at that time were also close to or below the detection limit of our assays (Table 2). Ang I and II were undetectable in cardiomyocyte- and fibroblast-conditioned serum-deprived medium and could also not be demonstrated in cell homogenates of cardiomyocytes and fibroblasts that had been serum-deprived for 5 days (Table 2).

Ang I and II were also undetectable in medium collected from cells that had been serum-deprived for 1 h ($n=3$, data not shown). This excludes the possibility that Ang I and II are released by the cells at the start of the 5-day period and subsequently metabolized during further incubation.

Table 1
Half-lives ($t_{1/2}$) and rstorder rate constants for degradation (k_1) and conversion (k_2) of angiotensin I added to unconditioned culture medium, 5-day-old cardiomyocyte cultures or 5-day-old broblast cultures in the absence or presence of 0.5 μmol/ml captopril[a]

	Unconditioned medium ($n=4$)	Cardiomyocytes ($n=6$)	Fibroblasts ($n=3$)
With serum			
$t_{1/2}$ without captopril (h)	0.63±0.35	0.27±0.03	0.30±0.02
$t_{1/2}$ with captopril (h)	4.35±0.90*	1.53±0.37*	2.22±1.08*
k_1 (h^{-1})	0.17±0.03	0.62±0.16	0.46±0.15
k_2 (h^{-1})	1.69±0.77	2.13±0.22	1.92±0.09
Conversion (%)	93±10	79±4	81±6
Without serum			
$t_{1/2}$ without captopril (h)	No metabolism	1.42±0.33	3.22±0.10
$t_{1/2}$ with captopril (h)	No metabolism	4.15±1.52*	14.0±1.97*
k_1 (h^{-1})	–	0.31±0.09	0.05±0.01§
k_2 (h^{-1})	–	0.42±0.17§	0.17±0.01§
Conversion (%)	–	56±9	76±4

[a]The culture medium was either serum-deprived or contained 5% fetal calf serum and 5% horse serum. The percentage of metabolism due to angiotensin I-to-II conversion by ACE is given for each condition.Values are mean±S.D. *$P<0.01$ without captopril vs. with captopril. §$P<0.05$ without serum vs. with serum.

3.4. Angiotensin II generation during stretch of cardiomyocytes

Cyclic, circular stretch of cardiomyocytes for 24 h led to the expected increase in protein synthesis rate and total cellular protein, although the effects were modest as compared to those observed after 24 h of exposure to ET-1 (Table 3). Using the MTT assay, no change in cell number was observed following stretch ($n=3$, data not shown). This indicates that stretch induced cellular hypertrophy rather than cellular hyperplasia. Ang I in the medium of control cells and cells exposed to stretch was close to the detection limit at 1 h after the start of the experiment (<0.2– 0.7 fmol/ml and <0.2– 0.8 fmol/ml, respectively; range of 3 experiments) and decreased to undetectable levels during prolongation of the experiment. Ang II was undetectable at all time points, both in the medium of control cells and in the medium of stretched cells. Exposure to ET-1 also did not result in Ang I or Ang II release (Table 3).

4. Discussion

This study indicates that cultured neonatal rat cardiomyocytes and broblasts do not synthesize renin,

Table 3
[^{3}H]-Leucine incorporation, total cellular protein, and the angiotensin I and II levels in the medium of untreated cardiomyocytes (control), cardiomyocytes exposed to cyclic stretch for 24 h (stretch) and cardiomyocytes incubated with 10^{-8} mol/l endothelin-1 (ET-1) for 24 h[a]

	n	Control	Stretch	ET-1
Incorporated [^{3}H]-leucine (dpm/well)	19	5992±215	7300±288*	9479±397*
Total cellular protein (mg/well)	19	0.15±0.01	0.17±0.01*	0.18±0.01*
Ang I (fmol/ml medium)	3	<0.2	<0.2	<0.2
Ang II (fmol/ml medium)	3	<0.1	<0.1	<0.1

[a]Ang, angiotensin. Values are mean±S.D. *$P<0.05$ vs. control. Angiotensin levels in the medium were below the detection limit.

Table 2
Angiotensin I and II levels in unconditioned medium ($n=3$), and in conditioned medium ($n=5$) and cell lysates ($n=5$) of 5-day-old cardiomyocytes and 5-day-old broblasts cultured in the presence or absence of serum[a]

	Medium			Cells	
	Unconditioned (fmol/ml)	Cardiomyocyte-conditioned (fmol/ml)	Fibroblast-conditioned (fmol/ml)	Cardiomyocytes (fmol/10^6 cells)	Fibroblasts (fmol/10 cells)[b]
With serum					
Ang I	1.3±0.3	2.1±1.2	<0.6±0.4	<0.5±0.2	<0.3
Ang II	0.4±0.3	0.9±0.4	<0.1	<0.2	<0.2
Without serum					
Ang I	<0.2	<0.7±0.3	<0.6±0.3	<0.3	<0.3
Ang II	<0.1	<0.1	<0.1	<0.2	<0.2

[a]Ang, angiotensin. Values are mean±S.D. If one or more values was below the detection limit, this is denoted as <mean±S.D. If all values were below the detection limit this is denoted as less than detection limit.

prorenin, or angiotensinogen in concentrations that are detectable or, if not detectable, high enough to result in Ang I or Ang II concentrations of physiological relevance. Both types of cardiac cells do appear to synthesize ACE, and thus are capable of converting Ang I into Ang II. Evidence for the presence of other enzymes capable of converting Ang I into Ang II (e.g., chymase [37]) was not obtained.

All RAS components are present in serum-containing medium, and will therefore be detected when measurements are made in the medium of cells cultured in the presence of serum. We used the specific rat renin inhibitor CH-732 [30,38] to distinguish Ang I generation by rat renin from Ang I generation by other enzymes, such as bovine renin and/or horse renin. The latter two are present in the fetal calf serum and horse serum applied in the present study to obtain cell adherence and confluency prior to serum-deprivation, and both renins are known to react with rat angiotensinogen [17,39].

No CH-732 inhibitable Ang I-generating activity could be detected in the medium of cells maintained in the presence of serum, whereas Ang I generation in medium of serum-deprived cells was below the detection limit. A possible explanation for this lack of renin release from rat cardiac cells might be that extrarenal cells release prorenin rather than renin [14–17]. However, although the Ang I-generating activity of medium obtained from cells cultured with serum increased nearly 10-fold following prorenin activation, it could again not be inhibited by CH-732. Moreover, the levels of prorenin measured in serum-supplemented conditioned medium did not differ from those in serum-supplemented unconditioned medium. Thus, the increase in Ang I-generating activity following activation is most likely due to the activation of bovine and/or horse prorenin.

Interestingly, medium of cells cultured in the absence of serum also contained low levels of prorenin. None of the Ang I-generating activity corresponding with these prorenin levels could be inhibited by CH-732, nor did the prorenin levels differ between cardiomyocytes and fibroblasts. Most likely therefore, this prorenin represents bovine and/or horse prorenin trapped or bound by the cells during their incubation in the presence of serum and released back into the medium during incubation under serum-deprived conditions. In support of this assumption, we have recently shown that neonatal rat cardiac cells, during incubation with prorenin, are capable of binding and internalizing prorenin, and that membrane-bound, non-internalized prorenin is released back into the medium when the cells are subsequently incubated with fresh medium without prorenin [8]. Uptake of renin and/or prorenin might also explain the presence of renin in rat cardiac cells described by others [18,19]. The lack of Ang I-generating activity in lysates of serum-deprived cells in the present study may be due to metabolism of (pro)renin following internalization.

The low levels of angiotensinogen present in unconditioned serum-supplemented medium (corresponding to $<5\%$ of the normal plasma angiotensinogen levels in the rat) did not change during incubation with cardiac cells, nor did the cells release angiotensinogen into the medium when incubated under serum-free conditions. All other cells described to synthesize angiotensinogen [20–22] release this substrate into the medium, without storing it intracellularly. In vivo, angiotensinogen also appears to be limited to the extracellular fluid compartment [4,5,31]. Therefore, our data do not support synthesis and/or release of angiotensinogen by neonatal rat cardiomyocytes or fibroblasts.

In view of the absence of (pro)renin and angiotensinogen synthesis by cardiac cells, it is not surprising that Ang I and II were below the detection limit in medium samples obtained from cardiomyocytes and fibroblasts incubated in the absence of serum. This finding contrasts with data obtained by others [23,26–29], who found angiotensin levels ranging from <10 to >1000 fmol/ml in medium of serum-deprived cardiomyocytes and fibroblasts. Part of this discrepancy may be due to the fact that angiotensins in other studies were measured directly by radioimmunoassay, an approach which may result in an overestimation of the true angiotensin levels [40]. Furthermore, it must be kept in mind that, in view of the levels measured in cardiac tissue in vivo (Ang I, $\approx$5 fmol/g; Ang II, $\approx$20 fmol/g) [3,4,41,42] even levels of 5–10 fmol per ml medium are very high, since in most studies only 1 to 4 million cells are incubated with a few milliliters of medium.

Sadoshima et al. [27] found the Ang II concentration in the medium of serum-deprived neonatal rat cardiomyocytes to increase nearly 100-fold upon stretch. This Ang II, which is assumed to be responsible for the hypertrophic response of cardiomyocytes after the induction of stretch [26–28], appeared to originate from intracellular storage sites. Since neonatal rat cardiomyocytes do not synthesize renin and angiotensinogen in detectable quantities, the Ang II in these storage sites may be derived, via AT_1-receptor mediated endocytosis [9], from the serum-containing medium used to culture the cells prior to stretch. We studied intracellular storage and stretch-induced release of Ang II by measuring angiotensin levels in the cells after 5 days of incubation with serum and in the serum-free medium at various time points after the initiation of stretch. Ang II was below the detection limit under all conditions. Yamazaki et al. reported that the concentration of exogenous Ang II needed to exert a similar hypertrophic response as stretch is $\approx 10^{-8}$ mol/l [28]. In our cells, such concentrations of Ang II were found to induce near-maximal hypertrophic effects [33]. Although in the present study Ang II was undetectable (i.e., $<10^{-13}$ mol/l), the well-known increase in cellular protein synthesis did occur in response to stretch. It appears therefore that the stretch-induced hypertrophic response of car-

diomyocytes observed here was independent of Ang II. In support of this conclusion, we could not inhibit the stretch-induced hypertrophic response in cardiomyocytes with the AT_1 receptor antagonist losartan (unpublished observations). Similarly, others observed hypertrophic responses in stretched cardiomyocytes obtained from angiotensinogen- or AT_1 receptor-deficient mice, and in isolated, buffer-perfused rat or guinea pig hearts (which do not contain renin [31]) exposed to increased systolic load [43–46].

The absence of Ang II release following stretch in the present study does not correspond with several earlier studies showing an upregulation, at the expression level, of renin [47], angiotensinogen [27,29,47–50], and ACE [47,49]. Although differences in experimental conditions (presence/absence of serum, static/cyclic stretch) may have played a role, a more likely explanation for this discrepancy is that the increases in expression may not have been translated to the protein level. Indeed, despite the increases in ACE mRNA that have been described after stretch [47,49], Miyata et al. [26] were unable to observe an increase in ACE activity in stretched cells. Possibly, the low mRNA levels of RAS components in cardiac cells, which in most cases can be detected only after>25 cycles of PCR, are the result of illegitimate transcription [51] and/or do not play a physiological role [13].

Taken together, our data, obtained in neonatal rat cardiac cells, do not support the concept of cardiac angiotensin generation independent of kidney and liver. At present it is not known to what degree results obtained in neonatal cells can be extrapolated to adult cells [29]. Lack of renin synthesis by cardiac cells does not necessarily implicate that angiotensins are not generated locally in the heart. In fact, we have recently shown in pigs that the majority of cardiac Ang I and Ang II is synthesized at tissue sites by renal renin [4,42]. Possibly therefore, renin and/or prorenin need to be taken up from the circulation in order to generate angiotensins locally in the heart [8]. Angiotensinogen diffuses freely from the blood into the interstitial fluid compartment, and may react with renin or activated prorenin on or in the cardiac cells [31]. ACE appears to be the only RAS component involved cardiac Ang II generation that does not have to be sequestered from the circulation.

Acknowledgements

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Prorenin uptake by cardiac cells

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High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin.

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High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin

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Saris, Jasper J., Frans H. M. Derkx, René J. A. De Bruin, Dick H. W. Dekkers, Jos M. J. Lamers, Pramod R. Saxena, Maarten A. D. H. Schalekamp, and A. H. Jan Danser. High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin. *Am J Physiol Heart Circ Physiol* 280: H1706–H1715, 2001.—Mannose-6-phosphate (man-6-P)/insulin-like growth factor-II (man-6-P/IgF-II) receptors are involved in the activation of recombinant human prorenin by cardiomyocytes. To investigate the kinetics of this process, the nature of activation, the existence of other prorenin receptors, and binding of native prorenin, neonatal rat cardiomyocytes were incubated with recombinant, renal, or amniotic fluid prorenin with or without man-6-P. Intact and activated prorenin were measured in cell lysates with prosegment- and renin-specific antibodies, respectively. The dissociation constant (K_d) and maximum number of binding sites (B_{max}) for prorenin binding to man-6-P/IGF-II receptors were 0.6 ± 0.1 nM and 3,840 ± 510 receptors/myocyte, respectively. The capacity for prorenin internalization was greater than 10 times B_{max}. Levels of internalized intact prorenin decreased rapidly (half-life = 5 ± 3 min) indicating proteolytic prosegment removal. Prorenin subdivision into man-6-P-free and man-6-P-containing fractions revealed that only the latter was bound. Cells also bound and activated renal but not amniotic fluid prorenin. We concluded that cardiomyocytes display high-affinity binding of renal but not extrarenal prorenin exclusively via man-6-P/IGF-II receptors. Binding precedes internalization and proteolytic activation to renin thereby supporting the concept of cardiac angiotensin formation by renal prorenin.

local renin-angiotensin system; cardiomyocytes; fibroblasts; heart; kidney

THE BENEFICIAL CARDIAC EFFECTS of angiotensin-converting enzyme (ACE) inhibitors in subjects with heart failure are usually attributed to interference of these drugs with ANG II generation at cardiac tissue sites. Although initially it was thought that this generation depends on the de novo synthesis of renin in the heart, it is now well established that such synthesis does not occur either under normal circumstances (13, 24, 34, 35, 40, 56) or under pathological conditions (14, 31, 50). Therefore, the heart must sequester renin from the circulation to synthesize ANG II locally. Renin may diffuse into the interstitial space (18, 28) or bind to renin receptors and/or renin-binding proteins (8, 46, 53). Because renin in blood is predominantly present in the form of its inactive precursor prorenin (11), it is also conceivable that the heart sequesters prorenin instead of renin. This prorenin must then be activated locally. In support of this concept we recently demonstrated that endothelial cells and cardiac myocytes and fibroblasts, which do not synthesize (pro)renin (1, 59), are capable of binding recombinant human prorenin to cell-surface mannose-6-phosphate (man-6-P)/insulin-like growth factor-II (man-6-P/IGF-II) receptors (1, 58). Binding precedes rapid internalization and appearance of renin-specific enzymatic activity. Furthermore, the receptors also bound and internalized recombinant human renin. It is well established that recombinant human prorenin produced in Chinese hamster ovary (CHO) cells contains the man-6-P signal that is required to bind to man-6-P/IGF-II receptors (2, 25). This is not a unique property because many other prohormones [e.g., latent transforming growth factor-β (19), procathepsin D (29), and proliferin (39)] also carry the man-6-P recognition marker, and these prohormones (like prorenin) are activated after binding and internalization via man-6-P/IGF-II receptors.

At present it is unlikely that the man-6-P/IGF-II receptor is the only renin- and prorenin-binding receptor, because excess man-6-P did not block prorenin binding to microsomal membrane fractions prepared from various rat tissues (53). In addition, it is not known whether intracellular prorenin activation occurs proteolytically or nonproteolytically. Proteolytic activation involves the actual removal of the prosegment by any of the known prorenin-renin convertases [e.g., cathepsin B (45), kallikrein (37), and prohormone convertases (51)]. Nonproteolytic activation implies transient unfolding of the prosegment so that it no longer folds over the enzymatic cleft, thereby allowing

prorenin to cleave angiotensinogen. In vitro, acid pH or cold storage favor the latter type of activation (21). Moreover, a recent study in mice has shown that nonproteolytically activated prorenin is capable of generating ANG I at tissue sites in vivo (43).

The aim of the present study was to investigate in neonatal rat cardiomyocytes and fibroblasts *1*) the kinetics of man-6-P/IGF-II receptor-dependent prorenin binding [dissociation constant (K_d), maximum number of binding sites (B_{max})] and activation; *2*) the possibility of prorenin binding independent of the man-6-P/IGF-II receptor; *3*) the nature of the prorenin activation (proteolytic or nonproteolytic) and, in case of proteolytic activation, the nature of the prorenin-activating enzyme; and *4*) possible differences between binding of recombinant human prorenin and binding of native human prorenin from renal and extrarenal sources.

METHODS

Cell culture. All experiments were performed according to the regulations of the Animal Care Committee of Erasmus University Rotterdam, Rotterdam, The Netherlands, and in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society.

Primary cultures of rat neonatal cardiac cells were prepared as previously described (57). Briefly, ventricles of Wistar strain rat pups (age 1–3 days) were minced and cells were dispersed by eight subsequent trypsinization steps. Nonmyocytes were separated from myocytes by differential preplating. Myocytes were seeded in noncoated 12-well plates (Corning Costar Europe; Badhoevedorp, The Netherlands) yielding a confluent monolayer of spontaneously beating cells at 1.5×10^5 cells/cm^2 after 24 h. The preplated cells (fibroblast fraction) were passaged after 4 days to noncoated 12-well plates yielding a confluent monolayer of 0.75×10^5 cells/cm^2 after 2 days. The cells were maintained for 72 h in a humidified incubator at 37°C with 5% CO_2 in air and 1.5 ml of growth medium consisting of a 4:1 (vol/vol) ratio of DMEM (GIBCO Life Technologies; Breda, The Netherlands) and medium 199 (GIBCO) supplemented with 5% FCS (Roche Diagnostics; Almere, The Netherlands), 5% horse serum (Sigma-Aldrich; Zwijndrecht, The Netherlands), 100 U/ml of penicillin (Roche), and 100 mg/ml of streptomycin (Roche). The incubations with prorenin (see *Incubation of cells with prorenin at 4 or 37°C*) were carried out under serum-free conditions. Before the start of each experiment, cells were washed with 1 ml of warm (37°C) PBS consisting of (in mM) 140 NaCl, 2.6 KCl, 1.4 KH_2PO_4, and 8.1 Na_2HPO_4 (pH 7.4). The cells were then preincubated either at 4 or 37°C for 30 min with 0.4 ml of incubation medium consisting of a 4:1 (vol/vol) ratio of DMEM and medium 199 supplemented with 1% (wt/vol) BSA (Sigma-Aldrich).

Prorenin preparation. Recombinant human prorenin was a kind gift of Dr. S. Mathews (Hoffmann-LaRoche; Basel, Switzerland). It was secreted by CHO cells transfected with a vector containing human prorenin cDNA. To remove traces of renin, the prorenin was partially purified by Cibacron blue Sepharose affinity chromatography (Amersham Pharmacia Biotech; Roosendaal, The Netherlands). The intrinsic renin activity of the prorenin preparation before proteolytic activation was <2% of the activity after complete proteolytic activation when the prorenin preparation contained ~2×10^5 U/l (4 μM) renin.

Man-6-P receptor affinity chromatography. To separate prorenin into fractions that do or do not contain the man-6-P signal, recombinant human prorenin was applied to a 0.5-ml bovine liver man-6-P/IGF-II receptor column (kindly provided by Dr. S. Kornfeld, St. Louis, MO). The column was equilibrated with column buffer containing 50 mM imidazole at pH 6.5, 150 mM NaCl, 5 mM Na-β-glycerophosphate, 0.1% (wt/vol) BSA, 6 μM antipain, 8 μM leupeptin, 6 μM pepstatin A, 7 μM chymostatin (all from Sigma-Aldrich), and 10 kallikrein inhibitory U/ml of aprotinin (Bayer; Mijdrecht, The Netherlands) (26). All manipulations were performed at 4°C. After the application of recombinant human prorenin (500 units in 0.1 ml of column buffer), the column was washed with column buffer ("column runthrough"). Subsequently, 10 mM man-6-P was added to the column buffer and man-6-P-containing prorenin was eluted. Prorenin was measured in 0.5-ml fractions. Fractions corresponding to column runthrough (i.e., man-6-P-free prorenin) and man-6-P-containing prorenin were separately pooled, concentrated, and adjusted to contain PBS (pH 7.4) by Centricon C-30 ultrafiltration (Amicon Bioseparations; Bedford, MA).

Incubation of cells with prorenin at 4 or 37°C. After preincubation at 4 or 37°C for 30 min under serum-free conditions (see *Cell culture*), experiments were started by replacing the incubation medium by 4 or 37°C incubation medium containing either recombinant human prorenin (final concentration 3–1,000 U/l), man-6-P-free recombinant human prorenin (10 U/l), or man-6-P-containing recombinant human prorenin (10 U/l). Incubations at 37°C were also performed with pools of human plasma and human amniotic fluid diluted to a 1:3 ratio with incubation medium. Plasma was obtained from subjects with renal artery stenosis (one man and two women, age 41–66 yr). Amniotic fluid was obtained from three women (age 19–38 yr) after natural delivery. All incubations at 4 or 37°C lasted 4 h and were performed in both the presence and absence of 10 mM man-6-P to determine man-6-P/IGF-II receptor-specific prorenin binding (58). To investigate the intracellular presence of man-6-P/IGF-II receptors in myocytes, incubations with recombinant human prorenin (1,000 U/l) at 4°C were also performed after prior permeabilization of the cells with PBS containing 0.2% saponin (Merck; Amsterdam, The Netherlands) (52). Finally, to determine what proteases are responsible for prorenin activation, incubations at 37°C were performed in the presence of the following five protease inhibitors: 0.04 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF, Calbiochem; LaJolla, CA); 0.1 mM leupeptin; 0.14 mM L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E64, Sigma-Aldrich); 1.0 mM 1,10-phenanthroline (Merck); or 0.1 mM pepstatin A.

At the end of the incubation period the culture medium was removed. Each well was washed three times with 1 ml of ice-cold PBS. Prorenin was not detectable in the last PBS wash. Cells were then lysed in 0.2 ml of ice-cold PBS containing 0.2% Triton X-100 (Merck), and the cell lysates were quickly frozen on dry ice. Cell lysates were stored at −70°C until assays for total and cell-activated prorenin were performed.

To determine whether prorenin had been internalized, the acid-wash method was used (58). At low pH surface-bound prorenin dissociates from the cells; internalized prorenin, however, is acid resistant. Briefly, after the cells had been washed three times with ice-cold PBS, cells were incubated at 4°C with 0.4 ml of an acid solution containing 50 mM glycine and 150 mM NaCl at pH 3.0. After 10 min the acid solution was removed, the cells were washed and lysed as described above, and the cell lysates were stored at −70°C until being assayed.

Incubation of cells at 4°C followed by incubation at 37°C. To study the kinetics of prorenin activation in more detail, cells cultured in six-well plates (Corning Costar) were loaded with 1 ml of recombinant human prorenin-containing incubation medium (final concentration 100 U/l) for 2 h at 4°C. After this period, free prorenin was removed by washing the cells three times with 3 ml of ice-cold PBS. After the last wash, 1 ml of fresh incubation medium without prorenin at 37°C was added, and the cells were incubated at 37°C. The incubation was terminated after 15, 30, 60, 120, 180, or 240 min by removing the culture medium and subsequently washing the cells three times with 3 ml of ice-cold PBS. The cells were then lysed in 0.5 ml of ice-cold PBS containing 0.2% Triton X-100 as described above. Culture medium and cell lysate were stored at −70°C until assays for total prorenin, cell-activated prorenin, and intact propeptide-containing prorenin were performed.

Prorenin measurement. In the experiments with recombinant human prorenin in myocytes, cell-activated prorenin and total prorenin (i.e., cell-activated plus nonactivated prorenin) were measured by immunoradiometric assay (IRMA). The proteolytic activation of recombinant human prorenin by myocytes was monitored with an IRMA specific for intact prorenin, i.e., prorenin in which the propeptide was still bound to the renin part of the molecule. The IRMAs are not sensitive enough to measure the low levels of cell-activated and total recombinant human prorenin in fibroblasts. The prorenin measurements in these cells were therefore performed by enzyme-kinetic assay. The renin and prorenin measurements in the experiments with human plasma and human amniotic fluid were also performed by enzyme-kinetic assay.

Enzyme-kinetic assay. Cell-activated recombinant prorenin and native renin were measured by incubating a 100-μl sample for 3 h with a saturating amount of sheep renin substrate at 37°C and pH 7.4 in the presence of serine protease and angiotensinase inhibitors (58). The generated ANG I was quantified by RIA. Results were expressed as microunits per 1,000,000 cells or microunits per milliliter medium using plasmin-activated recombinant human prorenin as a reference. The lower limit of detection was 1 $\mu U/10^6$ cells or 1 μU/ml of medium. To measure total recombinant prorenin and native prorenin, the samples were first incubated for 48 h at 4°C with plasmin (0.5 caseinolytic U/ml, obtained from Chromogenix; Mölndal, Sweden). This preincubation with plasmin causes complete proteolytic activation of prorenin. The serine protease inhibitor aprotinin (final concentration 100 kallikrein-inhibiting U/ml) was added to the incubation medium of the ANG I-generating step to inactivate plasmin.

Immunoradiometric assays. The monoclonal antibodies (MAb) used in these assays were MAb R3–36–16 (Nichols Institute; Wychen, The Netherlands), which reacts equally well with activated and nonactivated prorenin (K_d = 0.6 pM) (30), MAb R1–20–5 (Nichols Institute), which recognizes the active site of renin (K_d = 250 nM) (60), and MAb F258–37-B1 (a kind gift of Dr. S. Mathews, Hoffmann-LaRoche), which is directed against the COOH-terminal part (P20-P43) of the propeptide (Fig. 1) and does not react (<0.1%) with renin (F. H. M. Derkx, unpublished observation). MAbs R1–20–5 and F258–37-B1 do not react (<0.1%) with intact inactive prorenin. However, they do react with prorenin after the treatment of prorenin with the renin inhibitor remikiren (0.1 mM; a kind gift of Dr. W. Fischli, Hoffmann-LaRoche; Basel, Switzerland) for 48 h at 4°C (16). 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. This nonproteolytic conformational change not only allows subsequent recognition of the active site by MAb R1–20–5, it also causes the propeptide to move to the surface of the molecule so that it can react with MAb F258–37-B1. In the IRMA for cell-activated and total prorenin, 200 μl of untreated and remikiren-treated sample (diluted to a 1:9 ratio in heat-inactivated sheep serum, obtained from Biotrading; Mijdrecht, The Netherlands), respectively, were incubated for 6 h at 37°C with biotinylated MAb R3–36–16, ^{125}I-labeled R1–20–5 (250,000 counts/min), and an avidin-coated bead as described (15). In the IRMA for intact propeptide-containing prorenin, 250 μl of remikiren-treated sample (diluted to a 1:4 ratio in heat-inactivated sheep serum) were incubated for 6 h at 37°C with an avidin-coated bead to which 1.6 μg of biotinylated MAb F258–37-B1 had been bound (1). The bead was then washed three times with 2 ml of PBS containing 0.1% (wt/vol) BSA and subsequently incubated with 100 μl (250,000 counts/min) of ^{125}I-labeled MAb R1–20–5 and 200 μl of heat-inactivated sheep serum containing 0.1 mM remikiren for 24 h at room temperature. After the 6-h and 24-h incubation periods, the beads in both IRMAs were washed three times with 2 ml of PBS containing 0.01% (vol/vol) Triton X-100, and bound radioactivity was measured in a gamma-counter. The results of these assays were expressed as microunits per 1,000,000 cells using intact recombinant human prorenin as a reference. The lower limit of detection was 5 $\mu U/10^6$ cells.

Statistical analysis. Results are expressed as means ± SE. Data were compared using Student's *t*-test for paired observations or ANOVA. A value of $P < 0.05$ was considered to be significant. Binding data were analyzed by nonlinear regression analysis using the GraphPad Prism (version 3) computer program (GraphPad; San Diego, CA).

RESULTS

Incubation of myocytes and fibroblasts with recombinant human prorenin at 4 or 37°C. Myocytes (Fig. 2) and fibroblasts (Fig. 3) bound recombinant human pro-

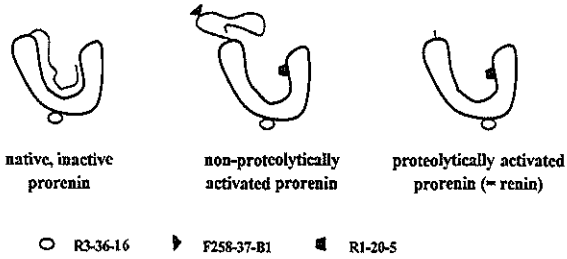

Fig. 1. Prorenin isoforms and recognition sites of the monoclonal antibodies used in the present study.

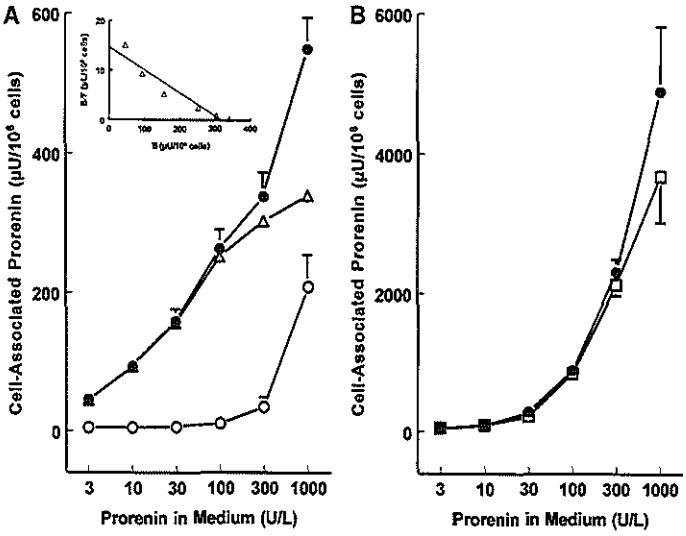

Fig. 2. *A*: levels of cell-associated total prorenin after incubation of myocytes with recombinant human prorenin at 4°C for 4 h in the absence (●) or presence (○) of 10 mM mannose-6-phosphate (man-6-P). Man-6-P/insulin-like growth factor-II (IGF-II) receptor-specific binding (△) was taken as the difference between levels of cell-associated prorenin with and without man-6-P. Data are means ± SE; n = 8. Dissociation constant (K_d) and maximum number of binding sites (B_{max}) were calculated from a plot according to Scatchard (*inset*; B/F represents the ratio of bound to free prorenin). *B*: levels of cell-associated total (●) and cell-activated (□) prorenin after incubation of myocytes with recombinant human prorenin at 37°C for 4 h. Data are means ± SE; n = 7.

renin in a concentration-dependent manner at both 4 and 37°C. Cell-associated prorenin at 37°C but not at 4°C was acid resistant (data not shown), indicating that prorenin internalization occurred at 37°C only. For a given prorenin concentration in the medium, the level of cell-associated total prorenin after 4 h of incubation was 10–15 times higher at 37°C than at 4°C.

Binding of recombinant human prorenin to man-6-P/IGF-II receptors: K_d and B_{max}. Man-6-P significantly reduced recombinant human prorenin binding in both myocytes (Fig. 2*A*) and fibroblasts (Fig. 3*A*). The reduction was much smaller in fibroblasts than in myocytes, suggesting that fibroblasts may contain a second prorenin binding site that cannot be blocked by man-6-P. However, because the levels of cell-associated prorenin after 4 h of incubation in the presence of man-6-P were similar in myocytes and fibroblasts, a more likely explanation is that non-man-6-P/IGF-II receptor-mediated prorenin binding represents nonspecific binding and that the man-6-P-induced reduction in prorenin binding is smaller in fibroblasts because these cells contain less cell-surface man-6-P/IGF-II receptors. Indeed, Scatchard analysis revealed that binding of prorenin to man-6-P/IGF-II receptors occurred with simi-

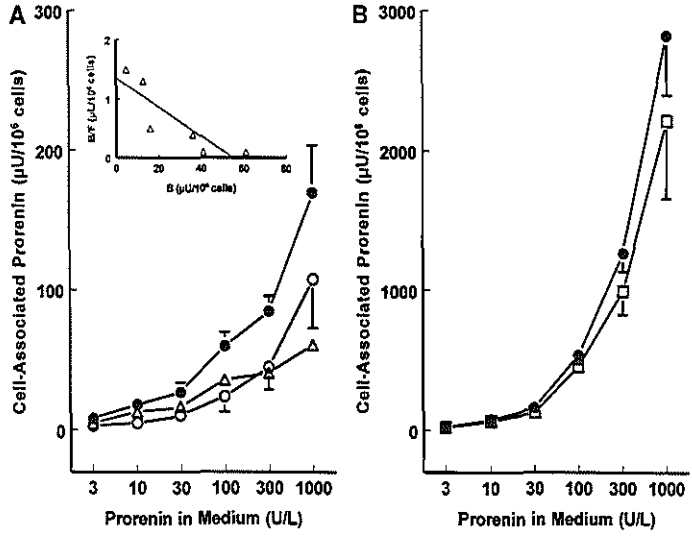

Fig. 3. *A*: levels of cell-associated total prorenin after incubation of fibroblasts with recombinant human prorenin at 4°C for 4 h in the absence (●) or presence (○) of 10 mM man-6-P. Man-6-P/IGF-II-receptor-specific binding (△) was taken as the difference between levels of cell-associated prorenin with and without man-6-P. Data are means ± SE; n = 4. K_d and B_{max} were calculated from a plot according to Scatchard (*inset*). *B*: levels of cell-associated total (●) and cell-activated (□) prorenin after incubation of fibroblasts with recombinant human prorenin at 37°C for 4 h. Data are means ± SE; n = 4.

lar affinity in both myocytes (K_d = 0.6 ± 0.1 nM; n = 8) and fibroblasts (K_d = 0.8 ± 0.2 nM; n = 4) and that B_{max} was 3,840 ± 510 sites/myocyte and 650 ± 150 sites/fibroblast. Prior permeabilization of myocytes with saponin increased man-6-P/IGF-II receptor-dependent prorenin binding 7.7 ± 0.4-fold (n = 4), indicating that >85% of the man-6-P/IGF-II receptors is located intracellularly. Recycling of these intracellular receptors to the cell membrane most likely explains why the levels of cell-associated prorenin at 37°C are much higher than at 4°C.

Does prorenin binding occur independently of man-6-P/IGF-II receptors? To investigate whether prorenin binding occurs independently of man-6-P/IGF-II receptors, recombinant human prorenin was separated into man-6-P-free and man-6-P-containing fractions with the help of a bovine man-6-P/IGF-II receptor-affinity column (Fig. 4). The amount of recombinant human prorenin (38 ± 1%; n = 3) that was not bound by this column (which did not contain the man-6-P signal) resembled the amount of prorenin (38 ± 2%) that eluted only after the addition of man-6-P to the elution buffer (which did contain the man-6-P signal). The remaining prorenin eluted shortly after the first runthrough peak (before the addition of man-6-P) and was not investigated further.

Incubation of myocytes and fibroblasts with man-6-P-containing prorenin resulted in cellular prorenin levels that were ≈1.5–2.5 times higher than observed after incubation with nonfractionated prorenin (Fig. 5). Assuming that only man-6-P/IGF-II receptors are involved in prorenin binding, this is exactly what one would predict when exposing cardiac cells to a prorenin solution in which either all or only ≈40% of the prorenin molecules carry the man-6-P signal.

Incubation with man-6-P-free prorenin resulted in cellular prorenin levels that were equal to or lower than the levels observed after incubation with nonfractionated prorenin in the presence of man-6-P (Fig. 5). Because the latter level was similar for myocytes and fibroblasts (Figs. 2A and 3A), this level most likely represents nonspecific prorenin binding. Thus our findings do not provide evidence for prorenin binding to receptors other than the man-6-P/IGF-II receptor.

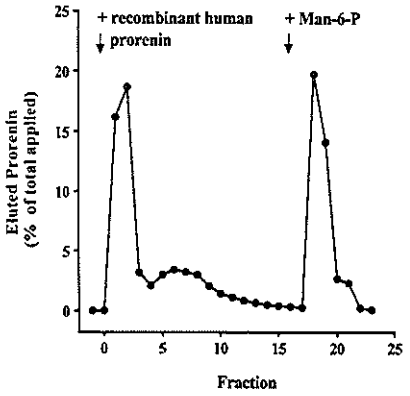

Fig. 4. Bovine liver man-6-P/IGF-II receptor-affinity chromatography of recombinant human prorenin. Representative example is shown. After application of prorenin (500 units), the column was washed with column buffer (see METHODS). Man-6-P-containing prorenin was eluted with 10 mM man-6-P in column buffer.

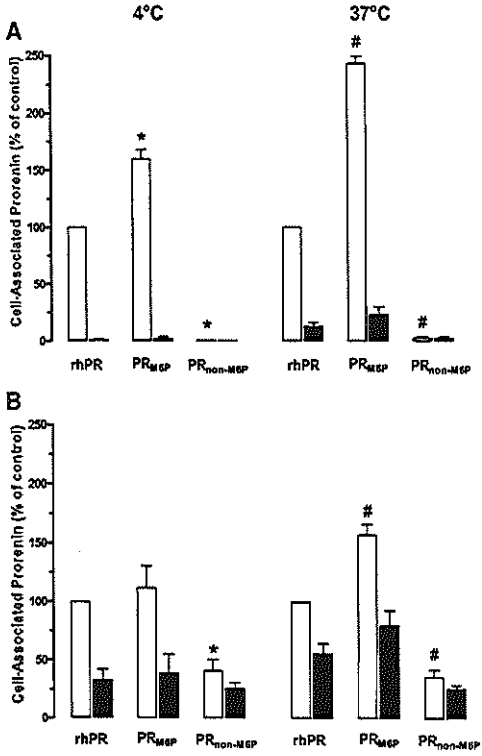

Fig. 5. Levels of cell-associated total prorenin after incubation of myocytes (*A*) or fibroblasts (*B*) with nonfractionated recombinant human prorenin (rhPR, 10 U/l; control), man-6-P-containing recombinant human prorenin (PR$_{M6P}$, 10 U/l), or man-6-P-free recombinant human prorenin (PR$_{non-M6P}$, 10 U/l) at 4 or 37°C for 4 h in the absence (open bars) or presence (filled bars) of 10 mM man-6-P. Data are means ± SE (n = 3) expressed as a percentage of the cellular levels of control. *P < 0.05 vs. control at 4°C; #P < 0.05 vs. control at 37°C.

Activation of recombinant human prorenin: effect of protease inhibitors. Activation of recombinant human prorenin was detectable at 37°C only (Figs. 2*B* and 3*B*). Saturation of the activation process did not occur because the percentage of cell-associated prorenin that was activated was similar at all concentrations of prorenin to which the cells were exposed [ranging from 88 ± 15% at 3 U/l to 78 ± 8% at 1,000 U/l in myocytes (n = 7) and from 83 ± 9% at 3 U/l to 75 ± 9% at 1,000 U/l in fibroblasts (n = 4)]. The serine protease inhibitor AEBSF partially blocked the activation of prorenin in myocytes but had no effect in fibroblasts (Table 1). None of the other protease inhibitors that were tested

Table 1. *Effect of protease inhibitors on cellular levels of total (i.e., cell-activated and nonactivated) prorenin and cell-activated prorenin after incubating myocytes and fibroblasts for 4 h at 37°C with 100 U/l recombinant human prorenin*

		Myocytes			Fibroblasts		
Protease Inhibitor	Type Protease Inhibited	*n*	Total prorenin, % of Control	Activated prorenin, % of Total prorenin	*n*	Total prorenin, % of Control	Activated prorenin, % of Total prorenin
None		7	100 ± 18	75 ± 6	5	100 ± 24	77 ± 6
Pepstatin A	Aspartic	4	109 ± 12	87 ± 3	4	115 ± 10	87 ± 7
Leupeptin	Serine and cysteine	5	143 ± 5*	85 ± 8	4	179 ± 18†	85 ± 6
AEBSF	Serine	5	118 ± 17	52 ± 4*	4	73 ± 6	71 ± 10
E64	Cysteine	5	136 ± 10	70 ± 11	3	136 ± 15	82 ± 12
1,10-Phenanthroline	Metallo	5	111 ± 17	69 ± 9	4	72 ± 20	64 ± 6

Data are means ± SE; *n*, no. of myocytes or fibroblasts. AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride; E64, L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine. *$P < 0.05$ vs. control; †$P < 0.01$ vs. control.

blocked prorenin activation in either myocytes or fibroblasts. The cysteine protease inhibitor E64 and the mixed serine-cysteine protease inhibitor leupeptin increased the level of cell-associated total prorenin by 40–50% in myocytes and by 40–80% in fibroblasts, thereby indicating that cysteine proteases contribute to (pro)renin degradation in cardiac cells.

Proteolytic or nonproteolytic activation of prorenin in myocytes. After 2 h of incubation at 4°C with 100 U/l prorenin and repeated washing with ice-cold PBS, the level of cell-associated total prorenin was 350 ± 45 μU/10^6 cells (n = 7). Acid wash confirmed that at that time, all cell-associated prorenin was located on the cell surface. Immediately after the temperature was raised to 37°C, the level of cell-associated intact (i.e., prosegment-containing) prorenin started to decrease. The decrease followed a biphasic pattern (Fig. 6). The first phase [half-life ($t_{½}$) = 5 ± 3 min] corresponds with the release of cell-surface-bound intact prorenin into the medium and did not differ from the first phase that was observed for total prorenin ($t_{½}$ = 4 ± 1 min). This phase is determined by the rapidity of the internalization process. The second phase represents the proteolytic removal of the prosegment from internalized prorenin ($t_{½}$ = 21 ± 4 min), because a rise in the cellular levels of activated prorenin was simultaneously observed. These levels reached a maximum after 60 min and then started to decrease, with a $t_{½}$ (67 ± 8 min) similar to that of the second phase of total prorenin ($t_{½}$ = 74 ± 5 min). Release of activated prorenin into the medium could not be demonstrated during the 6-h observation period (data not shown). Taken together, these findings suggest that prorenin, after its internalization, is rapidly activated by proteolytic cleavage of the prosegment and that activated prorenin is subsequently metabolized by degrading enzymes without being released into the medium.

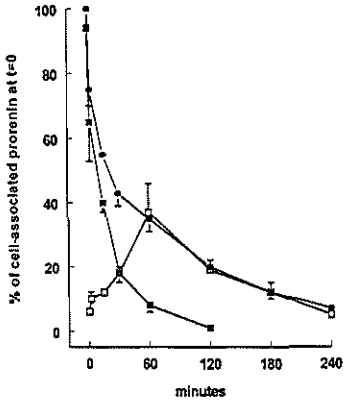

Fig. 6. Time course of internalization and proteolytic activation of prorenin by myocytes. Cells were preincubated with recombinant human prorenin (100 U/l) for 2 h at 4°C and were washed with ice-cold PBS. Fresh culture medium free of prorenin was added and the incubation was continued at 37°C. Data are means ± SE (n = 7) expressed as a percentage of the cellular levels of prorenin at the end of the 2-h preincubation period (t = 0). Total prorenin (●), cell-activated prorenin (□), and intact prosegment-containing prorenin (■) are shown.

Incubation of myocytes with native human (pro)renin at 37°C. To verify man-6-P/IGF-II receptor-dependent binding, internalization, and activation of native human prorenin of renal and nonrenal origin, myocytes were incubated at 37°C during 4 h with human plasma or human amniotic fluid (diluted to a 1:3 ratio with incubation medium) in the presence or absence of man-6-P. Expressed as a percentage of the sum of renin and prorenin, plasma and amniotic fluid contained 79 ± 10% and 94 ± 3% prorenin, respectively (Fig. 7). Incubation with plasma resulted in man-6-P/IGF-II receptor-mediated (pro)renin uptake by myocytes. After incubation with plasma, myocytes contained predominantly (>75%) renin (Fig. 7). Because man-6-P/IGF-II receptors bind and internalize man-6-P-containing renin and prorenin equally well (58), this is not due to selective uptake of plasma renin. The increased renin-to-prorenin ratio in cell lysates therefore suggests that internalized plasma prorenin, like internalized recombinant human prorenin, is activated to renin by myocytes. The cellular (pro)renin levels after incubation with amniotic fluid were close to the detection limit and did not differ with or without man-6-P (Fig. 7). Thus amniotic fluid does not contain prorenin that carries the man-6-P signal.

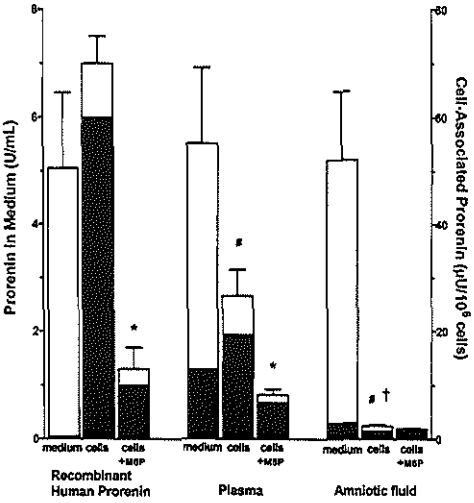

Fig. 7. Levels of total prorenin in medium and cell lysate after incubation of myocytes with recombinant human prorenin, human plasma (diluted to 1:3 ratio in incubation medium), or human amniotic fluid (diluted to 1:3 ratio in incubation medium) at 37°C for 4 h in the absence or presence of 10 mM man-6-P. Solid region represents activated prorenin (i.e., renin). Data are means ± SE (n = 4). *P < 0.05 vs. without man-6-P; #P < 0.05 vs. recombinant human prorenin; †P < 0.01 vs. plasma.

DISCUSSION

The data of the present study show that man-6-P-containing prorenin binds with high affinity to man-6-P/IGF-II receptors on neonatal rat myocytes and fibroblasts. Binding is followed by internalization and subsequent proteolytic activation to renin, possibly by a serine protease. Internalization is greatly enhanced by receptor recycling. Obtained with recombinant human prorenin, these results could be fully reproduced with native human prorenin of renal origin (i.e., prorenin in human plasma) but not with native human prorenin of extrarenal origin (i.e., prorenin in human amniotic fluid), suggesting that local angiotensin production by nonrenin-producing cells such as myocytes and fibroblasts (59) depends on renin of renal origin.

Evidence for prorenin binding to receptors other than the man-6-P/IGF-II receptor was not obtained. First, man-6-P significantly reduced prorenin binding in myocytes and fibroblasts. Although the reduction was more modest in fibroblasts, prorenin binding in the presence of man-6-P (i.e., "nonspecific" prorenin binding) was similar in myocytes and fibroblasts and did not differ from prorenin binding to human umbilical vein endothelial cells in the presence of man-6-P (data not shown). These findings may point to the existence of a second unidentified prorenin receptor. However, in view of the similarity of the non-man-6-P/IGF-II receptor-mediated prorenin binding in cardiac and endothelial cells, a more likely explanation is that binding in the presence of man-6-P represents nonspecific binding. Second, when exposing cardiac cells to a recombinant human prorenin fraction in which all molecules contain the man-6-P signal (as opposed to only 40% of the molecules in the nonfractionated recombinant human prorenin preparation), binding and internalization increased 1.5- to 2.5-fold. This is within the range expected when increasing the level of man-6-P-containing prorenin ≈2.5-fold and also indicates that the true K_d is ≈2.5-fold lower than the values reported here. The increases were somewhat smaller in fibroblasts, which is most likely due to the fact that man-6-P/IGF-II receptor-specific prorenin binding in these cells was comparable to prorenin binding not mediated via man-6-P/IGF-II receptors (i.e., nonspecific prorenin binding). A low number of cell-surface man-6-P/IGF-II receptors also explains why the decrease in prorenin binding in the presence of excess man-6-P was more modest in fibroblasts than in myocytes.

Binding of prorenin to man-6-P/IGF-II receptors occurred with high affinity (K_d < 1 nM), which suggests that prorenin occupies both man-6-P binding sites of this receptor (22, 38). In both fibroblasts and myocytes, the levels of cell-associated prorenin increased 10–15-fold when the cells were incubated with prorenin at 37°C instead of 4°C. This can be explained on the basis of the internalization and continuous recycling of man-6-P/IGF-II receptors among the cell surface and intracellular compartments (e.g., Golgi and endosomes) that are known to occur at 37°C but not at 4°C (10). In myocytes, >85% of the man-6-P/IGF-II receptors were found to be located in the cells. Internalization of the receptor-prorenin complex appeared to occur rapidly with a half-life of <5 min and was followed by activation of prorenin. The rapid decline in the levels of prorenin still containing the carboxy terminal part of its prosegment [as measured with monoclonal antibody F258–37-B1 (Fig. 6)] confirms that prorenin activation was a proteolytic process and that cleavage occurred in the correct manner. The cells did not release proteolytically activated prorenin (i.e., renin) into the medium. Instead, renin was degraded intracellularly with a half-life of ~1 h. This raises the possibility that the man-6-P/IGF-II receptor is involved in (pro)renin clearance. Alternatively and perhaps more likely in view of the rapid activation process, cell-activated prorenin may contribute to intracellular angiotensin generation before its destruction. Several studies have provided evidence for intracellular angiotensin generation (17, 32, 42), and ANG II is known to activate intracellular AT_1 receptors in the cytosol and nucleus (23, 27). In the absence of local renin synthesis, such intracellular angiotensin generation will depend on the uptake of circulating renin or prorenin. Moreover, because in previous studies we were unable to demonstrate angiotensinogen synthesis in neonatal rat cardiac myocytes and fibroblasts (59), it seems that renin substrate also has to be internalized to allow intracellular angiotensin generation in the neonatal heart. This may be different in the adult heart or under pathological conditions (40). In addition, age as well as pathological conditions may affect the density of cardiac man-6-P/

IGF-II receptors and/or give rise to the appearance of alternative (pro)renin receptors or uptake mechanisms (4, 8, 46, 48, 53).

At present it cannot be concluded what enzymes are responsible for the intracellular prorenin activation. With the exception of the serine protease inhibitor AEBSF, none of the protease inhibitors used in this study exerted an inhibitory effect on prorenin activation. This is not due to the inability of these blockers to reach the proper intracellular compartment because others have demonstrated efficacy in the same setup (7, 44). Moreover, the mixed serine-cysteine protease inhibitor leupeptin and the cysteine protease inhibitor E64 increased the levels of cell-associated prorenin by >40%, indicating that cysteine proteases contribute to (pro)renin degradation and that exogenous inhibitors apparently are capable of reaching the intracellular sites where degradation occurs. Possible candidates for the prorenin-activating enzyme are kallikrein (37), prohormone convertases (51), and cathepsin B (45), although the latter seems unlikely in view of the absence of an inhibitory effect of E64. These enzymes have been demonstrated in the heart (6, 41, 49, 55). Furthermore, Baba and colleagues (3) described a serine protease (mol mass 26 kDa) capable of activating prorenin in rat adrenal explant cultures. The pH optimum for prorenin activation by this enzyme was 6.5. The rapid activation of prorenin in the present study, which is indicative for early endosomal (i.e., at pH 6.5) removal of the prosegment, as well as the inhibitory effects of AEBSF on prorenin activation in myocytes, is in agreement with the existence of a similar serine protease in the heart.

Finally, our results are not limited to recombinant human prorenin but also apply to native human plasma prorenin. Plasma prorenin is predominantly of renal origin (11), although extrarenal prorenin sources such as the eye (12), ovary (20), placenta (33), and testis (54) are also known to contribute to circulating levels of prorenin. When incubated at 37°C for 4 h with plasma containing prorenin (and low levels of renin), myocytes were found to contain predominantly renin. It is unlikely that this is due to selective uptake of plasma renin because man-6-P/IGF-II receptors do not make a distinction between man-6-P-containing renin and prorenin (58). Therefore, these findings suggest that plasma prorenin, like recombinant prorenin, is activated intracellularly to renin. In absolute terms, the levels of cell-associated renin and prorenin after incubation with plasma were two to three times lower than after incubation with equal amounts of recombinant prorenin (Fig. 7). This may have at least two explanations. First, the percentage of plasma prorenin carrying the man-6-P signal may be lower than the percentage of recombinant human prorenin carrying this signal. Second, the high (in the nanomolar range) levels of soluble man-6-P/IGF-II receptors that have been reported in plasma (9) may affect prorenin binding to cellular man-6-P/IGF-II receptors, especially because the density of the latter (expressed per million cells) is in the femtomole range. Studies reporting on the presence of high-molecular-weight forms of prorenin in plasma (5, 47) are in agreement with the contention that plasma prorenin is in part bound to soluble man-6-P/IGF-II receptors. Further experiments are required to resolve this issue. Interestingly, prorenin present in human amniotic fluid did not bind to myocytes, which suggests that this prorenin does not contain the man-6-P signal. Amniotic fluid prorenin is derived primarily from the placental chorion laeve (33) (i.e., is synthesized extrarenally), and its isoelectric focusing pattern differs from that of plasma prorenin (36). The levels of soluble man-6-P/IGF-II receptors in amniotic fluid are ~100 times lower than in plasma (9). Whether prorenin from other extrarenal sources also lacks the man-6-P signal is currently unknown, but if so, this would imply that only prorenin of renal origin is meant to be taken up by the heart and thus that cardiac angiotensin generation is regulated by the kidney and not by other (pro)renin-producing tissues. Consequently, the release of large amounts of prorenin from extrarenal sources [e.g., during pregnancy (33)] would not necessarily result in increased cardiac angiotensin generation.

In conclusion, our data support the concept of cardiac angiotensin generation by renal (pro)renin. Prorenin is sequestered from the circulation by cardiac cells through binding to man-6-P/IGF-II receptors. Binding occurs with high affinity and is limited to prorenin containing the man-6-P signal. After binding, the prorenin-man-6-P/IGF-II receptor complex is internalized and prorenin is activated to renin, possibly by a serine protease in an early endosomal compartment. The receptor then returns to the cell surface to repeat the binding and internalization process. Activated prorenin may participate in intracellular ANG I production before its destruction by cysteine proteases in lysosomes.

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Prorenin uptake and angiotensin generation by endothelial cells

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Prorenin Accumulation and Activation in Human Endothelial Cells

Importance of Mannose 6-Phosphate Receptors

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Abstract—ACE inhibitors improve endothelial dysfunction, possibly by blocking endothelial angiotensin production. Prorenin, through its binding and activation by endothelial mannose 6-phosphate (M6P) receptors, may contribute to this production. Here, we investigated this possibility as well as prorenin activation kinetics, the nature of the prorenin-activating enzyme, and M6P receptor–independent prorenin binding. Human umbilical vein endothelial cells (HUVECs) were incubated with wild-type prorenin, K/A-2 prorenin (in which Lys42 is mutated to Ala, thereby preventing cleavage by known proteases), M6P-free prorenin, and nonglycosylated prorenin, with or without M6P, protease inhibitors, or angiotensinogen. HUVECs bound only M6P-containing prorenin (K_d 0.9±0.1 nmol/L, maximum number of binding sites [B_{max}] 1010±50 receptors/cell). At 37°C, because of M6P receptor recycling, the amount of prorenin internalized via M6P receptors was >25 times B_{max}. Inside the cells, wild-type and K/A-2 prorenin were proteolytically activated to renin. Renin was subsequently degraded. Protease inhibitors interfered with the latter but not with prorenin activation, thereby indicating that the activating enzyme is different from any of the known prorenin-activating enzymes. Incubation with angiotensinogen did not lead to endothelial angiotensin generation, inasmuch as HUVECs were unable to internalize angiotensinogen. Most likely, therefore, in the absence of angiotensinogen synthesis or endocytosis, M6P receptor–mediated prorenin internalization by endothelial cells represents prorenin clearance. (***Arterioscler Thromb Vasc Biol.* 2001;21:911-916.**)

Key Words: human umbilical vein endothelial cells ■ mannose 6-phosphate receptors ■ prorenin ■ renin ■ angiotensin

Vascular angiotensin II (Ang II), through stimulation of Ang II type 1 (AT_1) receptors, induces the generation of superoxide anions, thereby resulting in endothelial dysfunction.[1] Blockade of this process, with ACE inhibitors or AT_1 receptor antagonists, reverses endothelial dysfunction in human atherosclerosis.[2] At present, it is still being debated whether vascular Ang II generation depends on locally synthesized or circulating kidney-derived renin. In support of the latter, angiotensins cannot be demonstrated in the perfusate of vascular preparations obtained from nephrectomized animals, unless renin is added to the perfusion buffer.[3] Because renin in circulating blood plasma is predominantly present in the form of its inactive precursor, prorenin,[4] it is also conceivable that kidney-derived prorenin, after its local activation, contributes to vascular angiotensin generation.

Renin and/or prorenin may enter the vascular wall through binding to (pro)renin receptors. Indeed, we have recently demonstrated that human umbilical vein endothelial cells (HUVECs) bind renin and prorenin to cell surface mannose 6-phosphate (M6P) receptors.[5] Binding was followed by internalization and proteolytic activation of prorenin. The latter process is not unique, inasmuch as M6P receptors are known to be involved in the activation of several other prohormones carrying the M6P recognition marker, such as thyroglobulin.[6] Moreover, M6P receptor–mediated prorenin activation also occurs in cardiac cells.[7] The enzyme responsible for prorenin activation is currently not known. Possible candidates include cathepsin B, glandular kallikreins, and members of the prohormone convertase family.[8–10] Furthermore, receptors other than the M6P receptor may also contribute to (pro)renin binding.[11,12]

It was the aim of the present study to investigate the kinetics of prorenin binding and activation in HUVECs, the nature of the prorenin-activating enzyme in these cells, endothelial prorenin binding independent of M6P receptors, and whether endothelial prorenin binding and activation result in angiotensin generation.

Methods

Human Prorenin Preparations

Recombinant human prorenin (wild-type prorenin), produced in Chinese hamster ovary cells transfected with a vector containing human prorenin cDNA, was kindly provided by Dr S. Mathews (Hoffmann-LaRoche, Basel, Switzerland).[5] To remove traces of renin, it was partially purified by Cibacron blue Sepharose affinity chromatography (Pharmacia).[5,7] Wild-type prorenin was stored at −80°C in aliquots containing ≈2×10^5 U/L (4 μmol/L) in 0.1% BSA. It was also separated into an M6P-containing and an M6P-free fraction by use of a bovine M6P receptor affinity column, provided by Dr S. Kornfeld (Washington University School of Medicine, St. Louis, Mo).[13] In short, 1 U recombinant wild-type prorenin was applied to a 0.5-mL bovine M6P receptor affinity column. The column was washed with column buffer,[13] and M6P-containing prorenin was eluted by adding 10 mmol/L M6P to the column buffer. Fractions corresponding to the column run-through material (ie, M6P-free prorenin) and to M6P-eluted material (ie, M6P-containing prorenin) were separately pooled and stored at −80°C in aliquots containing ≈100 U/L.

K/A-2 prorenin, ie, prorenin that cannot be cleaved by known proteases, was produced in GH4 cells transfected with a vector containing human prorenin cDNA in which Lys42 is mutated to Ala.[14] Nonglycosylated prorenin was produced in GH4 cells transfected with a vector containing human prorenin cDNA in which Asn at positions 5 and 75 of renin is mutated to Ser.[15] The K/A-2 and nonglycosylated prorenin mutants were stored at −80°C in aliquots containing ≈700 and 30 U/L, respectively, in DMEM with 5% FCS.

Cell Culture

HUVECs were isolated from umbilical cords, cultured to confluence, trypsinized, and stored in liquid nitrogen as described earlier.[5] For an experiment, an aliquot of HUVECs (passages 2 to 5) was thawed. The cells were cultured to confluence in a 75-cm^2 tissue culture flask coated with fibronectin (10 μg/cm^2) in modified medium 199 containing 10% newborn calf serum, 10% human serum, 150 μg/mL endothelial cell growth factor,[5] 15 U/mL heparin, 50 U/mL penicillin, and 5 μg/mL streptomycin at 37°C in a humid 5% CO_2 atmosphere. Cells were trypsinized and seeded in 12-well plates. Studies were carried out ≈1 day after confluence (corresponding to a density of 4×10^4 cells/cm^2) had been reached. Before the start of each experiment, the cells were washed 3 times with 2 mL warm (37°C) PBS (140 mmol/L NaCl, 2.6 mmol/L KCl, 1.4 mmol/L KH_2PO_4, and 8.1 mmol/L Na_2HPO_4, pH 7.4). The cells were then preincubated either at 37°C or 4°C for 30 minutes with 0.5 mL incubation medium consisting of modified medium 199 without newborn calf serum or human serum but supplemented with 1% human serum albumin.

Incubation With Prorenin at 4°C or 37°C

After preincubation at 37°C or 4°C for 30 minutes, experiments were started by replacing the incubation medium by incubation medium of 37°C or 4°C containing wild-type prorenin (final concentration 3 to 300 U/L), M6P-containing wild-type prorenin (10 U/L), M6P-free wild-type prorenin (10 U/L), K/A-2 prorenin (100 U/L), or nonglycosylated prorenin (10 U/L) with or without 10 mmol/L M6P. Cells were then incubated at 37°C or 4°C for maximally 4 hours. To investigate the nature of the prorenin-renin–converting enzyme, incubations at 37°C with wild-type prorenin (100 U/L) were also performed in the presence of the serine protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 0.5 mmol/L, Calbiochem), aprotinin (0.001 mmol/L, Hoffmann-LaRoche), and chymostatin (0.1 mmol/L, Hoffmann-LaRoche), the cysteine protease inhibitor L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E-64, 0.1 mmol/L, Sigma Chemical Co), the mixed serine/cysteine protease inhibitor leupeptin (0.1 mmol/L, Sigma), the metalloprotease inhibitors EDTA (1 mmol/L), phenanthroline (1 mmol/L, Merck), and phosphoramidon (0.5 mmol/L, Sigma), and the aspartic protease inhibitor pepstatin A (0.1 mmol/L, Sigma). At the end of the incubation period, the culture medium was removed. The cells were washed 3 times with 2 mL ice-cold PBS and lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100. Subsequently, the cell lysate was frozen on dry ice. Cell lysates were stored at −80°C.

To distinguish cell surface–bound from internalized prorenin, the acid-wash method was used.[7] Cells were then lysed as described above.

Incubation With Prorenin at 4°C Followed by Incubation at 37°C

The kinetics of prorenin internalization and activation were studied by incubating HUVECs, which were cultured in 6-well plates, with 1 mL wild-type prorenin-containing incubation medium (final concentration 100 U/L) for 2 hours at 4°C. Thereafter, the cells were washed 3 times with 3 mL ice-cold PBS and further incubated at 37°C with prorenin-free incubation medium. The incubation was terminated after various times (ranging from 10 to 360 minutes) by washing the cells 3 times with 3 mL ice-cold PBS. The cells were then lysed in 0.5 mL ice-cold PBS containing 0.2% Triton X-100 as described above. Cell lysates were stored at −80°C.

Angiotensin Generation During Incubation With Prorenin at 37°C

To study prorenin-induced endothelial angiotensin generation, HUVECs, which were cultured in 6-well plates, were incubated at 37°C for 4 hours with 1 mL incubation medium containing 10 U/L wild-type prorenin and/or 150 nmol/L human angiotensinogen (Sigma). HUVECs incubated without prorenin or angiotensinogen served as the control. After 4 hours of incubation, the medium was rapidly mixed with 50 μL inhibitor solution (containing 0.1 mmol/L remikiren, 200 mmol/L disodium EDTA, and 0.2 mmol/L lisinopril) and frozen at −70°C. Cells used for the measurement of angiotensinogen were washed 3 times with 3 mL ice-cold PBS and lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100 as described above. Cells used for the measurement of angiotensin I (Ang I) and Ang II were scraped with a rubber policeman in a volume of 0.5 mL ice-cold PBS. The cell-PBS mixture was centrifuged at 1000*g* at 4°C for 1 minute, after which the pellet was homogenized in 0.5 mL 0.1 mol/L HCl/80% ethanol by using a hand-operated Dounce homogenizer. ^{125}I-Ang I was added to the samples before the homogenization procedure to determine angiotensin recovery. Ethanol was evaporated under vacuum rotation at 4°C by using a Speed Vac Concentrator. The concentrated homogenates were dissolved in 0.5 mL 1% orthophosphoric acid and applied to Sep-Pak columns (see below).

In view of the partial catalytic activity of prorenin,[16] Ang I was also measured in incubation medium containing 10 U/L wild-type prorenin and/or 150 nmol/L angiotensinogen that had been incubated without HUVECs for 4 hours at 37°C.

Finally, HUVEC-mediated Ang I–Ang II conversion and Ang I degradation was studied by incubating HUVECs for up to 4 hours at 37°C with 1 nmol/L Ang I in the presence or absence of 10 μmol/L quinaprilat. Samples (0.1 mL) for the measurement of Ang I and II were taken at 0, 0.5, 1, 2, and 4 hours, rapidly mixed with 10 μL inhibitor solution, and frozen at −70°C.

Biochemical Measurements

Total prorenin (ie, cell-activated plus nonactivated) and cell-activated prorenin in the cell lysates obtained from the 37°C experiments were measured by immunoradiometric assay (IRMA).[17] Proteolytic activation of prorenin in these experiments was verified with an IRMA specific for intact prorenin, ie, prorenin in which the C-terminal part of the prosegment is still attached to the renin part of the molecule.[5] The results of these IRMAs are expressed as microunits per 10^6 cells with intact recombinant human prorenin used as a reference. The lower limit of detection was 5 μU/10^6 cells. The IRMAs are not sensitive enough to measure the low levels of prorenin that were present in the cell lysates at 4°C. Therefore, the prorenin measurements in these experiments were performed by enzyme-kinetic assay.[7] The results of this assay are expressed as microunits per 10^6 cells with plasmin-activated recombinant human prorenin used as a reference. The lower limit of detection was 1 μU/10^6 cells.

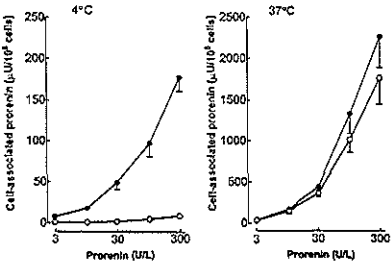

Figure 1. Cell-associated prorenin after incubation of HUVECs with wild-type prorenin at 4°C (left) or 37°C (right) for 4 hours. The open and closed circles represent the cellular levels of cell-activated and total (cell-activated plus nonactivated) prorenin, respectively. Data are mean±SEM (n=6).

The concentration of angiotensinogen in the cell lysates was determined as the maximum quantity of Ang I that was generated during incubation at 37°C and pH 7.4 with excess recombinant human renin in the presence of a mixture of angiotensinase, ACE, and serine protease inhibitors.[17-19] The lowest level that could be measured was 20 fmol/10^6 cells.

Ang I and II levels in medium and in cell homogenates were measured by radioimmunoassay after Sep-Pak extraction and reversed-phase high-performance liquid chromatography separation.[18,19] Recovery was better than 65%, and results were corrected for incomplete recovery. The lower limits of detection for Ang I and II were 2 and 1 fmol per milliliter medium or per 10^6 cells, respectively.

Statistical Analysis

All data are expressed as mean±SEM. Differences between the cellular prorenin levels at 37°C and 4°C and between the cellular levels of total prorenin and cell-activated prorenin in the presence or absence of protease inhibitors were evaluated for statistical significance by ANOVA. Statistical significance was accepted at $P<0.05$.

Results

Incubation With Prorenin at 4°C or 37°C

HUVECs bound wild-type prorenin in a concentration-dependent manner at 4°C and at 37°C (Figure 1). Cell-associated prorenin at 37°C, but not at 4°C, was acid resistant (data not shown), indicating that prorenin internalization occurred at 37°C only. For a given prorenin concentration in the medium, the level of cell-associated prorenin after 4 hours of incubation was 10 times higher at 37°C than at 4°C. Prorenin activation was detectable at 37°C only. Saturation of the activation process did not occur, inasmuch as the percentage of cell-associated prorenin that was activated was similar at all concentrations of prorenin to which the cells were exposed (ranging from 82±5% at 3 U/L to 76±6% at 300 U/L, n=6; P=NS). None of the protease inhibitors that were tested (n=5 for each inhibitor) blocked the activation of prorenin (ranging from 68±5% [with AEBSF] to 81±9% [with EDTA]), although some inhibitors affected the amount of cell-associated total prorenin. E64, leupeptin, and pepstatin A increased the levels of cell-associated total prorenin to 135±9%, 138±11%, and 126±7% of control ($P<0.05$ versus control), respectively, thereby indicating that cysteine and aspartic proteases contribute to (pro)renin degradation in endothelial cells. AEBSF and phenanthroline reduced the levels of cell-associated total prorenin to 49±7% and 39±7% of control, respectively ($P<0.05$). This was not due to interference with the internalization process, because the percentage of cell-associated prorenin that was acid resistant was similar in the presence and absence of these inhibitors (data not shown). Aprotinin (96±9% of control), chymostatin (119±6%), EDTA (85±8%), and phosphoramidon (94±9%) did not affect the levels of cell-associated total prorenin.

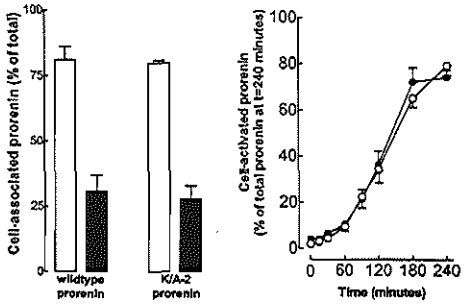

Figure 2. Left, Cellular levels of cell-activated (open bars) and intact prosegment-containing (closed bars) prorenin after incubation of HUVECs for 4 hours at 37°C with wild-type or K/A-2 prorenin. Levels (mean±SEM, n=4) are expressed as a percentage of the cellular levels of total (cell-activated plus nonactivated) prorenin after 4 hours of incubation. Right, Time-dependent increase in the cellular levels of activated prorenin during incubation of HUVECs at 37°C with wild-type prorenin (open circles) or K/A-2 prorenin (closed circles). Levels (mean±SEM, n=4) are expressed as a percentage of the cellular levels of total prorenin after 4 hours of incubation.

K/A-2 prorenin was bound and internalized by HUVECs to the same degree as wild-type prorenin (Figure 2, left). Moreover, after 4 hours of incubation with K/A-2 prorenin at 37°C, 80±2% of cell-associated total prorenin was in the activated form. The activation was due to proteolytic cleavage of the prosegment, inasmuch as <30% of cell-associated total prorenin still contained the C-terminal part of the prosegment. Furthermore, the rate of prorenin activation over the 4-hour incubation period was identical for wild-type and K/A-2 prorenin (Figure 2, right). Taken together, therefore, HUVECs activated K/A-2 prorenin in a manner indistinguishable from the activation of wild-type prorenin, thereby supporting the idea that activation was not mediated by any of the known prorenin-renin convertases.

M6P significantly inhibited native prorenin binding at 37°C and at 4°C (Figure 3). In the presence of M6P, prorenin binding was observed only at prorenin concentrations >10 U/L, and the levels of cell-associated prorenin were identical at 37°C and at 4°C. Scatchard analysis of the results obtained at 4°C revealed that M6P receptor–specific binding of prorenin occurred with high affinity (K_d 0.9±0.1 nmol/L). The number of prorenin-binding M6P receptors on the cell surface (B_{max}) was 1010±50 sites per cell.

Binding studies after the separation of wild-type prorenin into an M6P-free and an M6P-containing fraction, with the help of a bovine M6P receptor affinity column, revealed that only M6P-containing prorenin and not M6P-free prorenin was bound by HUVECs (Figure 4). In agreement with this finding, the cells did not bind or internalize nonglycosylated prorenin (n=4, data not shown).

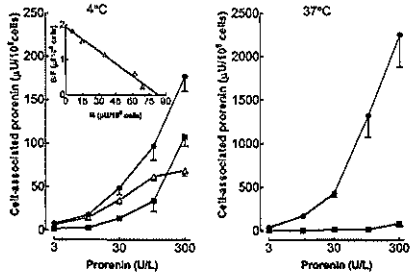

Figure 3. Cell-associated prorenin after incubation of HUVECs with wild-type prorenin in the absence (closed circles) or presence (closed squares) of 10 mmol/L M6P at 4°C (left) or 37°C (right) for 4 hours. M6P receptor–specific binding (open triangles) was taken as the difference between levels of cell-associated prorenin with and without M6P. Data are mean±SEM (n=6). B/F indicates bound-to-free ratio; B, bound prorenin. K_d and B_{max} were calculated from a plot according to Scatchard (insert).

Incubation With Prorenin at 4°C Followed by Incubation at 37°C

After 2 hours of incubation at 4°C with 100 U/L wild-type prorenin, followed by repeated washing with ice-cold PBS, the level of cell-associated total prorenin was 197±26 μU/10⁶ cells (n=5). Acid wash confirmed that all cell-associated prorenin at that time was located on the cell surface. Immediately after elevating the temperature to 37°C, the level of cell-associated total prorenin started to decrease. The decrease followed a biphasic pattern (Figure 5). The rapid phase (half-time [t1/2] 7±1 minutes) corresponds with the release of cell-associated prorenin into the medium, whereas the slow phase (t1/2 405±72 minutes) represents intracellular degradation after internalization. As soon as prorenin release into the medium no longer occurred (ie, at the time all remaining cell surface–bound prorenin had been internalized, after ≈40 minutes), the cellular levels of activated prorenin started to rise rapidly, reaching a maximum after 60 minutes. Thereafter, these levels decreased with a half-life (t1/2 305±33 minutes) similar to that of the degradation of cell-associated total prorenin. Taken together, these findings suggest that activation precedes degradation and that the half-lives of both processes differ 40- to 60-fold.

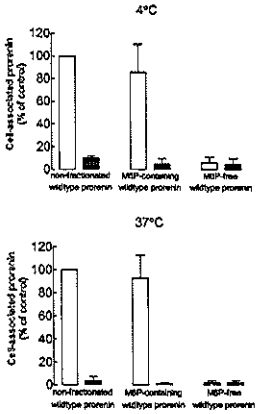

Figure 4. Cell-associated prorenin after incubation of HUVECs with nonfractionated wild-type prorenin (10 U/L), M6P-containing wild-type prorenin (10 U/L), and M6P-free wild-type prorenin (10 U/L) at 4°C (top) or 37°C (bottom) for 4 hours in the absence (open bars) or presence (closed bars) of 10 mmol/L M6P. Data (mean±SEM, n=5) are expressed as a percentage of the cellular levels of control (16±9 μU/10⁶ cells and 158±51 μU/10⁶ cells at 4°C and 37°C, respectively).

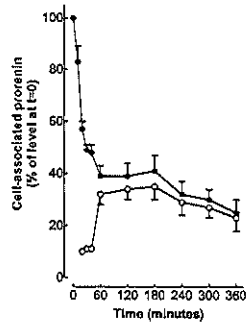

Figure 5. Time course of internalization and cellular activation of wild-type prorenin by HUVECs. Cells were preincubated with prorenin (100 U/L) for 2 hours at 4°C. They were then washed with ice-cold PBS, fresh culture medium free of prorenin was added, and the incubation was continued at 37°C. The cellular level of total prorenin (cell-activated plus nonactivated) at the end of the preincubation at 4°C (0 minutes) was 197±26 μU/10⁶ cells (100%). The open and closed circles represent the cellular levels of cell-activated and total prorenin, respectively. Data are mean±SEM (n=5).

Angiotensin Generation During Incubation With Prorenin at 37°C

HUVECs that had been incubated under serum-free conditions for 4 hours in the absence of prorenin and angiotensinogen did not contain detectable levels of angiotensinogen, Ang I, or Ang II (n=4), nor could these renin-angiotensin system components be demonstrated in the medium (n=4) of these cells. Cellular angiotensinogen and angiotensin levels remained undetectable after a 4-hour incubation with 10 U/L wild-type prorenin and/or 150 nmol/L angiotensinogen (n=4 for each condition). Medium of HUVECs that had been incubated for 4 hours with 10 U/L wild-type prorenin plus 150 nmol/L angiotensinogen contained 569±85 pmol/L Ang I and 125±26 pmol/L Ang II (n=4). A 4-hour incubation of medium containing the same amount of wild-type prorenin and angiotensinogen in the absence of HUVECs also resulted in the appearance of Ang I (2520±130 pmol/L, n=4) but not Ang II. Medium containing either prorenin or angiotensinogen, after incubation with or without HUVECs, did not contain angiotensins (n=4 for each condition).

HUVECs metabolized Ang I (t1/2 1.0±0.1 hour, n=4), and this resulted in the appearance of Ang II in the medium, which reached a peak level (191±24 pmol/L) after 2 hours. Quinaprilat fully prevented the generation of Ang II. On the basis of the half-life of Ang I in the presence of the ACE inhibitor (t1/2 1.7±0.2 hours, n=4; $P<0.01$ versus control),

calculations[19] revealed that 42±7% of the Ang I metabolism by HUVECs was due to conversion by ACE.

Discussion

With the use of prorenin mutants as well as wild-type prorenin, the present study shows that endothelial internalization and activation of prorenin are mediated exclusively via M6P receptors. We have demonstrated earlier that these receptors also bind and internalize renin, in a manner indistinguishable from that of prorenin.[5] Thus, despite evidence of the presence of other M6P-independent (pro)renin receptors in vascular preparations,[11] our data do not support the idea that such receptors are localized on human endothelial cells. M6P receptor–dependent (pro)renin binding to endothelial cells also provides an explanation for the selective endothelial staining of renin in human arteries.[20]

At present, 2 different M6P receptors have been identified: the cation-independent type (also known as insulin-like growth factor II [IGFII] receptor) and the cation-dependent type. These receptors contain 2 M6P binding sites and 1 M6P binding site, respectively.[21] The high-affinity binding of prorenin in the present study resembles the high-affinity binding of diphosphorylated oligosaccharides that occupy 2 M6P binding sites.[22] Most likely, therefore, the endothelial prorenin-binding receptor is the cation-independent M6P receptor. In this respect, prorenin resembles other M6P-carrying prohormones that are internalized and activated after binding to cell surface cation-independent M6P receptors.[6]

Internalized prorenin was rapidly activated to renin, and the activation occurred proteolytically, as evidenced by the use of an antibody directed against the C-terminal part of the prosegment. Inhibitors of known proteases did not prevent cleavage, although some inhibitors interfered with the subsequent renin degradation by cysteine and aspartic proteases. The latter shows that protease inhibitors do enter endothelial cells and thus confirms that their lack of effect on prorenin activation is not due to their inability to get into the cells. Unexpectedly, K/A-2 prorenin, a prorenin mutant that is not cleaved in vitro in isolated cells or in vivo in rat pituitary glands,[10,14,23] was also cleaved to renin. Remarkably, its activation occurred as rapidly as that of wild-type prorenin, although in vitro we found that plasmin activated K/A-2 prorenin 4 to 5 times as slowly as it did wild-type prorenin (authors' unpublished data, 2000). Taken together, therefore, endothelial cells contain an as-yet-unidentified protease that is capable of cleaving prorenin at or near its natural cleavage site. The rapidity of this process after internalization suggests that this enzyme is located in endosomes.

Activated intracellular prorenin may contribute to endothelial angiotensin generation. This would require the simultaneous uptake or endothelial synthesis of angiotensinogen. The demonstration of Ang II in the cytoplasm of rat endothelial cells[24] and of the release of intracellularly generated Ang II from bovine endothelial cells[25] supports this concept. However, we were unable to demonstrate intracellular endothelial angiotensin generation during the incubation of HUVECs with prorenin and angiotensinogen. Although Ang I and II could be detected in the medium during these experiments, it is important to note that Ang I generation also occurred during the incubation of prorenin and angiotensinogen in the absence of HUVECs and that the addition of Ang I to HUVECs resulted in the immediate appearance of Ang II in the medium. Taken together, therefore, the presence of Ang I and II in the medium of cells incubated with prorenin and angiotensinogen most likely reflects the partial catalytic activity of prorenin that is due to the temporal unfolding of its prosegment[16] rather than endothelial activation of prorenin. On the basis of the Ang I level measured after 4 hours of prorenin plus angiotensinogen incubation in the absence of HUVECs, it can be estimated that <2% of prorenin is catalytically active; ie, it exists in an "open" form.

The absence of intracellular angiotensin generation, despite the prorenin internalization (and subsequent activation) that occurred at 37°C, is most likely due to the fact that HUVECs did not sequester angiotensinogen. Although this does not necessarily apply to all endothelial cells in the human body, it suggests that intraendothelial angiotensin generation will occur only in endothelial cells that synthesize angiotensinogen. Evidence for the latter is currently not available.

In the absence of intracellular angiotensin generation, the high vascular levels of Ang II[26] can be explained only on the basis of AT_1 receptor–mediated internalization of Ang II after its extracellular generation.[27,28] Such extracellular angiotensin generation most likely involves interstitial renin[29] or renin bound to the surface of vascular cells via receptors other than the M6P receptor.[11,12,30]

Finally, in view of the intracellular degradation of activated prorenin, it is conceivable that M6P receptors function as clearance receptors of (pro)renin and that prosegment cleavage is a first step toward intracellular destruction. For instance, binding and internalization of IGFII to M6P/IGFII receptors results in the lysosomal degradation of this ligand.[6] An argument against this concept is the large difference in half-life between prorenin activation and degradation. This difference would leave activated prorenin ample time to contribute to intracellular angiotensin generation. However, even if M6P receptors serve as clearance receptors for (pro)renin, this would still allow these receptors to affect vascular angiotensin generation.

In conclusion, prorenin internalization by HUVECs is mediated exclusively via high-affinity M6P receptors and is greatly enhanced by receptor recycling. Internalized prorenin is rapidly activated to renin by a protease that is different from any of the known prorenin-activating enzymes. Activation is followed by degradation and/or, if angiotensinogen is present, may result in intracellular angiotensin generation. Both possibilities support the regulation of vascular angiotensin generation by M6P receptors.

Acknowledgment

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Native human prorenin uptake by cardiac cells

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Cardiomyocytes bind and activate native human prorenin: role of soluble mannose 6-phosphate receptors.

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Cardiomyocytes Bind and Activate Native Human Prorenin

Role of Soluble Mannose 6-Phosphate Receptors

Jasper J. Saris, Frans H.M. Derkx, Jos M.J. Lamers, Pramod R. Saxena, Maarten A.D.H. Schalekamp, A.H. Jan Danser

Abstract—Cardiomyocytes bind, internalize, and activate recombinant human prorenin through mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptors. To investigate whether this also applies to native human prorenin, neonatal rat myocytes were incubated for 4 hours at 37°C with various prorenin-containing human body fluids. Uptake and activation by M6P/IGFII receptors were observed for plasma prorenin from subjects with renal artery stenosis and/or hypertension and for follicular fluid prorenin. The total amount of cellular renin and prorenin (expressed as percentage of the levels of renin and prorenin in the medium) after 4 hours of incubation was 4 to 10 times lower than after incubation with recombinant human prorenin. Although plasma contains alkaline phosphatases capable of inactivating the M6P label as well as soluble M6P/IGFII receptors that block prorenin binding in a competitive manner and proteins (eg, insulin, IGFII) that increase the number of cell-surface M6P/IGFII receptors, these factors were not responsible for the modest uptake of native human prorenin. Uptake did not occur during incubation of myocytes with plasma prorenin from anephric subjects or with amniotic fluid prorenin, and this was not due to the presence of excessively high levels of M6P/IGFII receptors and/or phosphatase activity in these fluids. In conclusion, myocytes are capable of binding, internalizing, and activating native human prorenin of renal and ovarian origin through M6P/IGFII receptors. Differences in prorenin glycosylation and/or phosphorylation as well as the concentration of soluble M6P/IGFII receptors and growth factors affecting cell-surface M6P/IGFII receptor density determine the amount of prorenin entering the heart and thus cardiac angiotensin II production. (***Hypertension.*** **2001;37[part 2]:710-715.)**

Key Words: myocytes ■ receptors, angiotensin II ■ insulin growth factor ■ renin

The beneficial effects of ACE inhibitors on postinfarct remodeling and in subjects with heart failure are generally attributed to interference with cardiac angiotensin (Ang) II production.[1] Initially, it was thought that the renin required for cardiac Ang II generation is synthesized de novo in the heart. However, it is now well established that cardiac renin is largely if not completely derived from the circulation, both under normal and pathological conditions.[2–5] Circulating renin and/or its inactive precursor, prorenin, diffuse into the cardiac interstitial space[5,6] or bind to (pro)renin receptors.[7,8] We recently reported that one of these receptors is identical to the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor.[9,10] M6P/IGFII receptors not only have binding domains for M6P-containing ligands such as renin and prorenin but also for IGFII and retinoic acid.[11,12] Interestingly, prorenin binding to M6P/IGFII receptors is followed by rapid internalization and activation.[9,10] This is not a unique property because it also applies to other M6P-containing prohormones (eg, latent transforming growth factor-β).[13]

In our studies on M6P/IGFII receptor–mediated prorenin binding, we made use of recombinant human prorenin. This prorenin may differ from native human renal prorenin with regard to its glycosylation and/or phosphorylation.[14–16] Similar differences exist between prorenin of renal and extrarenal origin.[16,17] In fact, the absence of the M6P label on extrarenal prorenin could explain why Ang II is virtually undetectable in the heart after a bilateral nephrectomy[2,18] despite the fact that prorenin, unlike renin, is still present in circulating blood of nephrectomized subjects, sometimes at levels as high as those in normal individuals.[19–21]

In this study, we set out to investigate whether cardiomyocytes bind, internalize, and activate native human prorenin of renal and extrarenal origin through M6P/IGFII receptors, taking into consideration the fact that plasma and other prorenin-containing human body fluids (eg, amniotic fluid) contain factors that may interfere with prorenin binding, such as soluble M6P/IGFII receptors, phosphatases, and proteins that increase the number of cell-surface M6P/IGFII receptors (eg, insulin and IGFII).[11,22]

Methods

Cell Culture

All experiments were performed according to the regulations of the Animal Care Committee of Erasmus University Rotterdam (The

Netherlands), in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society.

Primary cultures of rat neonatal cardiac cells were prepared as described before.[9] Briefly, ventricles of newborn 1- to 3-day-old Wistar strain rat pups were minced, and cells were dispersed by trypsinization. Myocytes were separated from nonmyocytes by differential preplating and seeded in noncoated 12-well plates (Corning Costar), giving a confluent monolayer of spontaneously beating cells at 1.5×10^5 cells/cm^2 after 24 hours. Cells were maintained at 37°C in a humidified 5% CO_2 incubator in 1.5 mL medium consisting of DMEM and Medium 199 (4:1) (Gibco Life Technologies) and supplemented with 100 U penicillin/mL (Roche), 100 mg streptomycin/mL (Roche), 5% fetal calf serum (Roche), and 5% horse serum (Sigma). Before the start of each experiment, cells were washed 3 times with 1 mL warm (37°C) PBS (consisting of 140 mmol/L NaCl, 2.6 mmol/L KCl, 1.4 mmol/L KH_2PO_4, and 8.1 mmol/L Na_2HPO_4, pH 7.4). They were then preincubated at 4°C or 37°C for 30 minutes with 0.4 mL medium supplemented with 1% (wt/vol) BSA (Sigma).

At 4°C, prorenin binds to cell-surface receptors without being internalized, whereas at 37°C, prorenin binding M6P/IGFII receptors is followed by internalization and intracellular activation to renin.[9,10] Moreover, at the latter temperature, M6P/IGFII receptors continuously recycle between the cell surface and intracellular compartments.[11]

Binding and Activation of Native Prorenin

To study binding and activation of native human prorenin, cells were incubated at 37°C with 0.4 mL incubation medium containing 30% (vol/vol) blood plasma, amniotic fluid, or follicular fluid. Plasma was obtained from 3 subjects with renal artery stenosis (1 man, 2 women; age, 41 to 66 years), 3 subjects with essential hypertension treated with the ACE inhibitor captopril (2 men, 1 woman; age, 51 to 67 years), and 4 anephric subjects (1 man, 3 women; age, 33 to 61 years) who had been anephric for 1 to 11 years. Amniotic fluid was obtained from 3 women (age, 19 to 38 years) after natural delivery. Follicular fluid was obtained from 3 women (age, 30 to 39) during an in vitro fertilization program. All incubations lasted 4 hours and were performed with and without 10 mmol/L M6P to determine M6P/IGFII receptor–specific binding. For comparison, incubations were also performed with recombinant human prorenin (a kind gift of Dr S. Mathews, Hoffmann-LaRoche) diluted in incubation medium to a concentration comparable to the lowest concentration in the native prorenin samples. At the end of the incubation period, the medium was removed. Each well was washed 3 times with 1 mL ice-cold PBS. Cells were then lysed in 0.2 mL ice-cold PBS containing 0.2% Triton X-100 (Merck), and the cell lysates were quickly frozen on dry ice. Media and cell lysates were stored at −70°C.

Recombinant Prorenin Binding and Internalization in the Presence of Plasma or Amniotic Fluid

To study whether the soluble M6P/IGFII receptors and growth factors that are present in plasma and amniotic fluid affect prorenin binding and internalization by myocytes, cells were incubated at 4°C or 37°C with 0.4 mL incubation medium containing 100 mU/mL recombinant human prorenin in the presence of 0%, 1%, 3%, 10%, or 30% (vol/vol) plasma (obtained from 6 healthy men; age, 26 to 64 years) or amniotic fluid (obtained from 3 women, see above). On the basis of the levels of endogenous renin plus prorenin in plasma and amniotic fluid (240 and 5200 μU/mL, respectively), it can be estimated that the addition of plasma or amniotic fluid to medium containing 100 mU/mL recombinant human prorenin marginally (<2%) affected the levels of immunoreactive total renin. For comparison, incubations were also performed with plasma that had been incubated at 56°C for 1 hour to denature soluble M6P/IGFII receptors. Incubations lasted 4 hours, and media and cell lysates were collected and stored as described above.

Effect of Preincubation of Myocytes With Plasma on Recombinant Prorenin Binding

To study whether incubation with plasma affects the number of cell-surface M6P/IGFII receptors, cells were preincubated at 37°C for maximally 2 hours with 0%, 3%, or 30% (vol/vol) plasma (obtained from 6 healthy men, see above) or plasma that had been incubated at 56°C for 1 hour. The cells were then washed 3 times with 1 mL ice-cold PBS and incubated at 4°C for 4 hours with 100 mU/mL recombinant human prorenin. Thereafter, media and cell lysates were collected and stored as described above.

Effect of Preincubation of Recombinant Prorenin With Plasma or Amniotic Fluid

To study whether plasma or amniotic fluid contains phosphatase activity toward the M6P label, 1 U recombinant human prorenin was incubated for 24 hours at 4°C or 37°C in 1 mL 100 mmol/L HEPES buffer (pH 7.4, Sigma), 0.5 mL HEPES buffer+0.5 mL plasma (obtained from 6 healthy men, see above), or 0.5 mL HEPES buffer+0.5 mL amniotic fluid (obtained from 3 women, see above) in the presence or absence of the phosphatase-inhibitors imidazole (25 mmol/L, final concentration) (Sigma), Na β-glycerophosphate (5 mmol/L) (Sigma), and Na *o*-vanadate (2 mmol/L) (BDH Chemicals). Next, the pretreated recombinant human prorenin (diluted in incubation medium to a final concentration of 100 mU/mL) was incubated with myocytes for 4 hours at 4°C. Media and cell lysates were collected and stored as described above.

Renin and Prorenin Measurements

In the experiments with recombinant human prorenin, cell-activated prorenin (ie, renin) and total prorenin (ie, cell-activated plus nonactivated prorenin) were measured by immunoradiometric assay, as described before.[10,23] The results of this assay are expressed as milliunits per million cells or per milliliter of medium, with recombinant human prorenin used as a reference. The lower limit of detection was 5 μU per million cells or per milliliter of medium. The immunoradiometric assay is not sensitive enough to allow the detection of the cellular renin and prorenin levels in the experiments with plasma, amniotic fluid, and follicular fluid. The renin and prorenin measurements in these experiments were therefore performed by enzyme-kinetic assay.[9] The results of this assay are expressed as milliunits per million cells or per milliliter of medium, with plasmin-activated recombinant human prorenin used as a reference. The lower limit of detection was 1 μU per million cells or per milliliter of medium.

Statistical Analysis

Results are expressed as mean±SEM. Data were compared by means of a Student's *t* test for paired observations or ANOVA. A value of $P<0.05$ was considered to be significant.

Results

Binding and Activation of Native Prorenin

All samples of human origin (plasma, amniotic fluid, and follicular fluid) contained predominantly prorenin. As a result, the level of prorenin (expressed as a percentage of the sum of renin and prorenin) in the media containing these samples was ≥80% (Figure 1, top).

Incubation at 37°C with plasma from subjects with renal artery stenosis or plasma from hypertensive subjects treated with captopril, as well as incubation with follicular fluid, resulted in uptake of renin and prorenin by myocytes (Figure 1, bottom). The uptake of plasma and follicular (pro)renin was M6P/IGFII receptor mediated; it was abolished when M6P was added to the medium. However, the amount of (pro)renin present in the cells after 4 hours of incubation with plasma or follicular fluid, expressed as a percentage of the

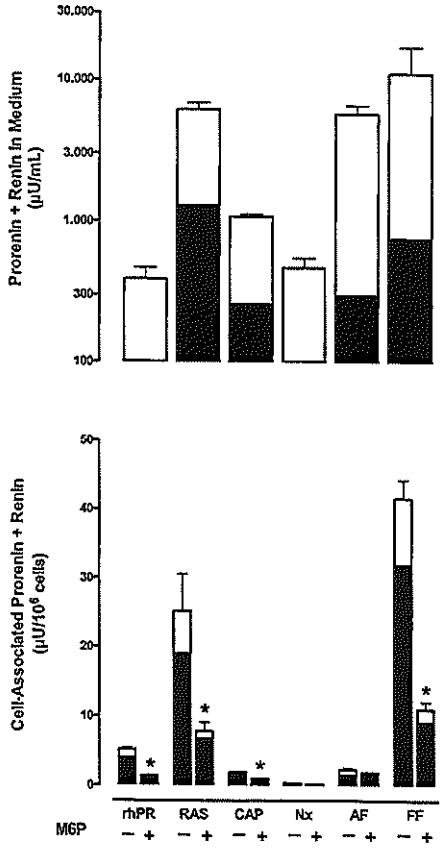

Figure 1. Binding and activation of native and recombinant human prorenin by myocytes. Top, Renin (black bars) and prorenin (white bars) levels in medium. Bottom, Levels of cell-associated renin (black bars) and prorenin (white bars) after 4 hours of incubation at 37°C in presence or absence of 10 mmol/L M6P. rhPR indicates recombinant human prorenin; RAS, plasma from subjects with renal artery stenosis; CAP, plasma from hypertensive subjects treated with captopril; Nx, plasma from nephrectomized subjects; AF, amniotic fluid; and FF, follicular fluid. Data are mean±SEM of 4 experiments. *$P<0.05$ vs without M6P.

renin and prorenin levels in the medium, was 4 to 10 times lower than after incubation of the cells with recombinant human prorenin (Figure 1).

At the end of the incubation period, the cells contained predominantly (>75%) renin, indicating that the internalized prorenin had been activated.

Incubation with plasma from nephrectomized subjects, or with amniotic fluid, did not result in M6P/IGFII receptor–mediated accumulation of renin or prorenin, despite the fact that the prorenin levels in the medium containing these samples were comparable to or much higher than the levels of recombinant human prorenin that did result in cellular accumulation of prorenin (Figure 1).

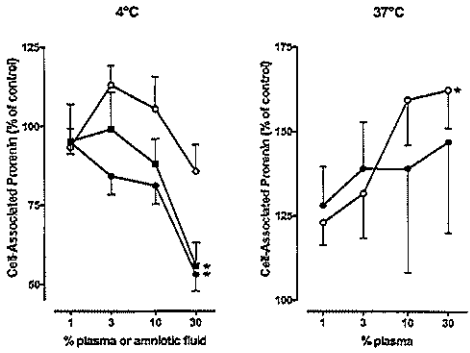

Figure 2. Cellular prorenin levels after incubation of myocytes for 4 hours with 100 mU/mL recombinant human prorenin in presence of noninactivated plasma (●), heat-inactivated plasma (○), or amniotic fluid (■) at 4°C (left) or 37°C (right). Levels (mean±SEM; n=6 to 8) are expressed as percentage of levels measured in absence of plasma or amniotic fluid. *$P<0.05$ vs 100%.

Recombinant Prorenin Binding and Internalization in the Presence of Plasma or Amniotic Fluid

Plasma from healthy men as well as amniotic fluid inhibited recombinant human prorenin binding at 4°C in a concentration-dependent manner (Figure 2, left). Heat inactivation, which denatures soluble M6P/IGFII receptors, abolished this effect. Similar data were obtained with plasma from anephric subjects (n=3, data not shown). Remarkably, at 37°C, heat-inactivated plasma from healthy men enhanced recombinant human prorenin uptake in a concentration-dependent manner (Figure 2, right). This effect was not observed during incubation with noninactivated plasma at 37°C. Activation of recombinant human prorenin by myocytes (after 4 hours of incubation at 37°C, 85±5% of total cell-associated prorenin was activated, n=6) was not affected by coincubation with noninactivated or heat-inactivated plasma (data not shown).

Effect of Preincubation of Myocytes With Plasma on Recombinant Prorenin Binding

A 30-minute preincubation of myocytes with plasma of healthy men increased recombinant human prorenin binding by ≈100% (Figure 3, left). This effect was diminished on longer preincubation with plasma and occurred in a concentration-dependent manner (Figure 3, right). Preincubation with heat-inactivated plasma yielded similar results.

Effect of Preincubation of Recombinant Prorenin With Plasma or Amniotic Fluid

Preincubation of recombinant human prorenin with plasma or amniotic fluid, with or without phosphatase inhibitors, did not affect its binding to myocytes (Figure 4).

Discussion

This study shows that neonatal rat cardiomyocytes bind native human prorenin through M6P/IGFII receptors. Binding is limited to prorenin of renal and follicular origin and is

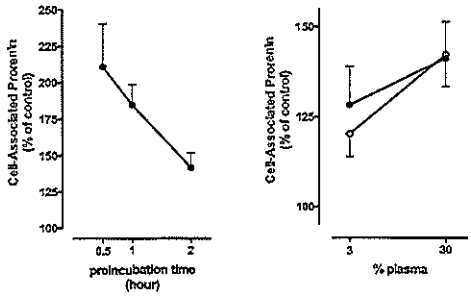

Figure 3. Cellular prorenin levels after incubation of myocytes for 4 hours with 100 mU/mL recombinant human prorenin after preincubation of cells with 30% plasma for 0.5, 1, or 2 hours (left) or with 3% or 30% noninactivated (●) or heat-inactivated (○) plasma for 2 hours (right). Levels (mean±SEM; n=6) are expressed as percentage of levels measured without preincubation of cells.

followed at 37°C by internalization and activation to renin. Preincubation of myocytes with plasma at 37°C increases the number of cell-surface M6P/IGFII receptors, thereby enhancing subsequent prorenin binding, whereas coincubation with soluble M6P/IGFII receptors containing human body fluids reduces prorenin binding to myocytes.

In previous studies, we demonstrated that myocytes bind M6P-containing recombinant human renin and prorenin exclusively through M6P/IGFII receptors.[24] No evidence was obtained for the presence of other (pro)renin receptors on myocytes. Our current data, showing M6P/IGFII receptor-mediated binding and activation of plasma prorenin, support the concept of circulating, kidney-derived prorenin contributing to cardiac Ang II production. Our inability to demonstrate uptake of circulating prorenin of anephric subjects suggests that extrarenally produced prorenin lacks the M6P signal. This might explain why cardiac tissue levels of Ang II in anephric animals are close to or below the detection limit,[2,18] despite the continuous presence of prorenin in the

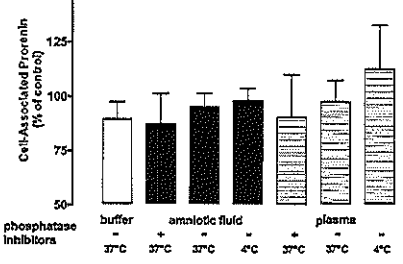

Figure 4. Cellular prorenin levels after incubation of myocytes for 4 hours at 4°C with 100 mU/mL recombinant human prorenin after preincubation of recombinant human prorenin for 24 hours at 4°C or 37°C with HEPES buffer, plasma, or amniotic fluid in presence or absence of phosphatase inhibitors. Levels (mean±SEM; n=3 to 6) are expressed as percentage of levels obtained with nonpreincubated recombinant human prorenin.

circulation of anephrics.[19-21] Glycosylation differences between prorenin of renal and extrarenal origin are in full agreement with the isoelectric heterogeneity of prorenin in human body fluids.[16,17,25]

However, not all extrarenal prorenin lacks the M6P signal; we did observe M6P/IGFII receptor–mediated binding and activation of ovary-derived prorenin. Ovarian prorenin is produced and secreted by the mature follicle and by the corpus luteum[26] and is largely responsible for the rise in plasma prorenin that normally occurs during pregnancy.[26,27] The function of ovarian prorenin in plasma is currently unknown. On the basis of our data, it appears that ovarian prorenin may participate in cardiac and vascular Ang II production. Chorionic prorenin, in contrast with ovarian prorenin, does not enter the circulation in significant amounts,[27] nor did we observe M6P/IGFII receptor–mediated uptake of this prorenin by myocytes. The latter is not due to the presence of phosphatase activity in amniotic fluid (Figure 4). Moreover, in agreement with our findings, the isoelectric focusing profile of chorionic prorenin is different from that of renal prorenin.[17]

All prorenin-containing human body fluid samples that were applied in the present study also contained small amounts of renin (<20% of total renin). After 4 hours of incubation at 37°C with plasma or ovarian prorenin, the myocytes, however, were found to contain predominantly (>75%) renin. Because M6P/IGFII receptors do not make a distinction between M6P-containing renin or prorenin,[9,10] the high cellular levels of renin cannot be explained on the basis of selective uptake of renin. A more likely explanation is therefore that native human prorenin, like recombinant human prorenin, is proteolytically activated after its binding to M6P/IGFII receptors. Such activation occurs intracellularly, as demonstrated previously with the acid-wash method.[10]

M6P/IGFII receptors recycle between the cell surface and intracellular compartments, and the majority of the cellular M6P/IGFII receptors is located intracellularly.[11] Because of this continuous recycling, the cellular prorenin levels are higher after incubation at 37°C than after incubation at 4°C and increase proportionally with the levels of prorenin in the medium.[24] In the present study, the cellular levels of native renin+prorenin (expressed as a percentage of the renin+prorenin levels in the medium), measured after 4 hours of incubation at 37°C with plasma, were several times lower than after incubation with recombinant human prorenin. This may have several reasons. First, the percentage of plasma (pro)renin molecules containing the M6P signal may be lower than the percentage of recombinant human prorenin molecules (≈40%)[24] carrying this signal. If so, this is not due to phosphatase activity in plasma (Figure 4).

Second, plasma contains high levels (≈3.5 nmol/L)[22] of soluble M6P/IGFII receptors, which, through competition, will prevent M6P-containing prorenin from binding to cellular M6P/IGFII receptors. Under the conditions of our incubation experiments (0.6 million cells exposed to 0.4 mL medium containing 30% plasma), the number of soluble receptors in the medium will exceed the number of cell-surface receptors (≈4000/cell)[24] by a factor of 100. Indeed, in agreement with the presence of soluble M6P/IGFII receptors,

we observed that coincubation of recombinant human prorenin with 30% plasma at 4°C decreased recombinant human prorenin binding to myocytes by ≈50%. This effect disappeared after heat inactivation of soluble plasma M6P/IGFII receptors. Similar data were obtained with amniotic fluid and plasma of anephric subjects, thereby indicating that the absence of prorenin binding during incubation with these fluids is not due to the presence of exceptionally high soluble M6P/IGFII receptors in anephric plasma or amniotic fluid. In fact, the levels of soluble M6P/IGFII receptors in amniotic fluid are lower than in plasma.[22] No data are currently available on the presence of these receptors in follicular fluid. The function of soluble M6P/IGFII receptors is not yet known but may involve transport of IGFII.[22] Interestingly, the levels of soluble M6P/IGFII receptors are highest in circulating blood plasma of pregnant women and diabetics, two groups of subjects with high plasma prorenin levels.[23,28]

Finally, several growth factors in plasma, including insulin and IGFII, decrease the rate of M6P/IGFII receptor internalization through induction of receptor dephosphorylation, thereby increasing the steady-state cell-surface M6P/IGFII receptor number.[11] Indeed, preincubation of myocytes with plasma at 37°C enhanced binding of recombinant human prorenin by the cells during subsequent incubation at 4°C. The increase in cell-surface receptor number occurred rapidly and appeared to diminish on longer incubation with plasma.

Coincubation of cells with recombinant human prorenin and heat-inactivated plasma at 37°C also resulted in higher levels of cell-associated prorenin than incubation with recombinant human prorenin alone. This confirms that heat inactivation at 56°C does not result in the destruction of growth factors. Enhanced prorenin binding was not observed when coincubating noninactivated plasma with recombinant human prorenin, demonstrating that the growth factor–induced upregulation of cell-surface M6P/IGFII receptors may compensate for the decrease in prorenin binding caused by the presence of soluble M6P/IGFII receptors. Taken together, therefore, the most likely explanation for the 4- to 10-fold-lower uptake of plasma and follicular fluid prorenin as compared with recombinant human prorenin is a difference in glycosylation and/or phosphorylation between native and recombinant prorenin.

Conclusions

Myocytes bind and activate native human prorenin through M6P/IGFII receptors. This process depends on the presence of the M6P signal on prorenin and is affected by the presence of soluble M6P/IGFII receptors and growth factors in human body fluids. These data show the complexity of cardiac prorenin uptake, which, eventually, determines the degree of Ang II generation in the heart.

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The travelling

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Does prorenin induce cellular effects?

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Prorenin-Induced Myocyte Proliferation: No Role for Intracellular Angiotensin II.

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Prorenin-Induced Myocyte Proliferation

No Role for Intracellular Angiotensin II

Jasper J. Saris, Mark M.E.D. van den Eijnden, Jos M.J. Lamers, Pramod R. Saxena, Maarten A.D.H. Schalekamp, A.H. Jan Danser

Abstract—Cardiomyocytes bind, internalize, and activate prorenin, the inactive precursor of renin, via a mannose 6-phosphate receptor (M6PR)–dependent mechanism. M6PRs couple directly to G-proteins. To investigate whether prorenin binding to cardiomyocytes elicits a response, and if so, whether this response depends on angiotensin (Ang) II, we incubated neonatal rat cardiomyocytes with 2 nmol/L prorenin and/or 150 nmol/L angiotensinogen, with or without 10 mmol/L M6P, 1 μmol/L eprosartan, or 1 μmol/L PD123319 to block M6P and AT_1 and AT_2 receptors, respectively. Protein and DNA synthesis were studied by quantifying [^{3}H]-leucine and [^{3}H]-thymidine incorporation. For comparison, studies with 100 nmol/L Ang II were also performed. Neither prorenin alone, nor angiotensinogen alone, affected protein or DNA synthesis. Prorenin plus angiotensinogen increased [^{3}H]-leucine incorporation (+21±5%, mean±SEM, $P<0.01$), [^{3}H]-thymidine incorporation (+29±6%, $P<0.01$), and total cellular protein (+14±3%, $P<0.01$), whereas Ang II increased DNA synthesis only (+34±7%, $P<0.01$). Eprosartan, but not PD123319 or M6P, blocked the effects of prorenin plus angiotensinogen as well as the effects of Ang II. Medium Ang II levels during prorenin and angiotensinogen incubation were <1 nmol/L. In conclusion, prorenin binding to M6PRs on cardiomyocytes per se does not result in enhanced protein or DNA synthesis. However, through Ang II generation, prorenin is capable of inducing myocyte hypertrophy and proliferation. Because this generation occurs independently of M6PRs, it most likely depends on the catalytic activity of intact prorenin in the medium (because of temporal prosegment unfolding) rather than its intracellular activation. Taken together, our results do not support the concept of Ang II generation in cardiomyocytes following intracellular prorenin activation. (***Hypertension*. 2002;39[part 2]:573-577.**)

Key Words: myocytes ■ renin ■ insulin growth factor ■ receptors, angiotensin

Cardiac angiotensin synthesis depends on renin of renal origin, both under normal and pathological conditions.[1–4] Circulating kidney-derived renin diffuses into the cardiac interstitial space,[5,6] and/or may bind to renin receptors.[7–9] In addition, the heart may sequester prorenin, the precursor of renin, from the circulation. Prorenin could contribute to angiotensin generation at cardiac tissue sites, either because of its inherent catalytic activity, which is the consequence of temporal prosegment unfolding,[10,11] or following its local conversion to renin. In support of this concept, we have recently demonstrated that cardiac myocytes and fibroblasts are capable of binding and internalizing recombinant human renin and prorenin via mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptors, and that prorenin following its internalization is rapidly activated to renin by proteolytic cleavage of the prosegment.[12,13]

Recombinant as well as native human renin and prorenin contain the M6P signal that is required to bind to M6P/IGFII receptors.[14–16] These receptors also contain binding domains for IGFII and retinoic acid,[17,18] and binding of the latter agonists to M6P/IGFII receptors results in second messenger activation and growth inhibition, respectively.[19,20] Moreover, proliferin, which, similar to (pro)renin, binds to M6P/IGFII receptors via its M6P group,[21] has been reported to induce endothelial cell chemotaxis via these receptors in a G-protein- and mitogen-activated protein kinase-dependent manner.[22] In this respect, it is of interest to note that renin binding to mesangial cells resulted in enhanced ^{3}H-thymidine incorporation and plasminogen-activator inhibitor-1 (PAI-1) release, without intermediate angiotensin generation.[9]

M6P/IGFII receptor-mediated accumulation of renin and activated prorenin in cardiac cells may result in intracellular angiotensin generation, and such intracellular angiotensin synthesis could underlie the stretch-mediated release of angiotensin II (Ang II) that has been demonstrated in myocytes.[23,24] However, to allow intracellular Ang II synthesis, the intracellular presence of angiotensinogen and ACE is also required, and in previous studies we were unable to detect angiotensinogen synthesis by cardiomyocytes.[25]

In the present study, we set out to investigate whether prorenin binding, internalization, and activation by neonatal rat cardiomyocytes results in a cellular response, either directly (without intermediate Ang II generation), via binding to M6P/IGFII receptors, or indirectly, via the generation of Ang II and subsequent AT receptor activation. We measured protein and DNA synthesis following incubation of cells with recombinant human prorenin with or without angiotensinogen. Experiments were repeated in the presence of M6P, eprosartan, and PD123319, to antagonize M6P/IGFII -, AT_1 -, and AT_2 receptors, respectively. For comparison, we also studied the effect of Ang II in the presence and absence of these antagonists. Finally, we investigated Ang I and II generation during incubation of myocytes with prorenin and angiotensinogen, taking into consideration that prorenin itself displays catalytic activity (ie, without prosegment cleavage).

Methods

Cell Culture

All experiments were performed according to the regulations of the Animal Care Committee of the Erasmus University Medical Center Rotterdam, in accordance with the "Guiding Principles" of the American Physiological Society.

Primary cultures of neonatal Wistar rat (Harlan) cardiomyocytes were prepared as previously described.[12,13] Cells were seeded in noncoated 24-well plates (Corning Costar), giving a confluent monolayer of spontaneously beating myocytes at 1.5×10^5 cells/cm^2 after a 24-hour incubation in 1.5 mL medium (consisting of DMEM and Medium 199 [4:1], supplemented with 5% fetal calf serum [Life Technologies], 5% horse serum [Sigma], 100 U/mL penicillin, and 100 mg/mL streptomycin [Roche]). Thereafter, cells were incubated for 48 hours in medium supplemented with 5% horse serum and for 24 hours in serum-free medium. Before the start of each experiment, cells were rinsed 3 times with 1 mL warm (37°C) phosphate-buffered saline. Next, myocytes were incubated for 24 hours at 37°C with 250 μL serum-free medium, supplemented with 1% bovine serum albumin (BSA), and containing 100 U/L (≈2 nmol/L) recombinant human prorenin (a kind gift of Dr S. Mathews, Hoffmann-LaRoche, Basel, Switzerland) and/or 150 nmol/L human angiotensinogen (Sigma) in the presence or absence of 10 mmol/L M6P, 1 μmol/L eprosartan, or 1 μmol/L PD123319. For comparison, experiments with 100 nmol/L Ang II (Bachem) were also performed. Cells incubated without prorenin, angiotensinogen, or Ang II served as control.

Protein and DNA Synthesis

Protein and DNA synthesis rates were determined in triplicate by quantifying [^{3}H]-leucine and [^{3}H]-thymidine incorporation during the last 6 hours of the above 24-hour incubation period in the presence of Ang II or prorenin and/or angiotensinogen.[26] Total cellular protein and DNA were quantified after solubilization as described previously using BSA and salmon sperm as standard, respectively.[26]

Angiotensin Generation

To measure angiotensin generation, myocytes were cultured in 6-well plates, and incubated at 37°C for 4 hours with 1 mL medium containing 10 or 100 U/L recombinant human prorenin and/or 150 nmol/L angiotensinogen. Cells incubated without prorenin or angiotensinogen served as control. After 1 hour and 4 hours of incubation, 75 μL medium was rapidly mixed with 6 μL inhibitor solution[27] and frozen at −70°C. Cells were collected after 4 hours (when the cellular levels of activated prorenin are maximal[12,13]) as described before.[27] Ang I and Ang II levels in medium were measured by radioimmunoassay (detection limit 40 and 20 fmol/mL, respectively).[25,27] 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 0.4 and 0.2 fmol/10^6 cells).[25,27] For comparison, cellular angiotensin levels following a 4-hour incubation with 100 nmol/L Ang II were also measured.

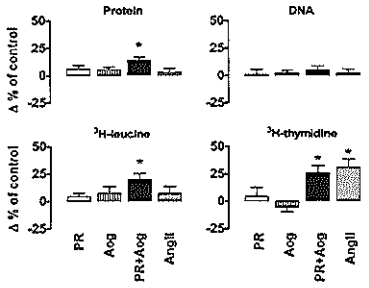

Figure 1. Effect of 2 nmol/L prorenin, 150 nmol/L angiotensinogen, 2 nmol/L prorenin plus 150 nmol/L angiotensinogen, and 100 nmol/L angiotensin (Ang) II on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes. Data are expressed as percentage change from control (mean±SEM of 8–16 experiments). PR indicates prorenin; Aog, angiotensinogen. *P<0.01 versus control.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was by ANOVA, followed by post-hoc evaluation according to Dunnett where appropriate. Statistical significance was accepted at P<0.05.

Results

Protein and DNA Synthesis

The total cellular protein and DNA contents of myocytes, following a 24-hour incubation with vehicle, were 147±14 and 8.3±1.0 μg/well (n=15–16), respectively. Incorporation of ^{3}H-leucine and ^{3}H-thymidine during the last 6 hours of the 24-hour incubation period amounted to 27901±3707 and 57403±6262 dpm/well (n=16), respectively. None of the receptor blockers affected protein or DNA content or incorporation of ^{3}H-leucine and ^{3}H-thymidine (n=8 for each blocker, data not shown). Prorenin alone and angiotensinogen alone were without effect (Figure 1). Prorenin (2 nmol/L) combined with angiotensinogen increased total cellular protein (P<0.01), ^{3}H-leucine incorporation (P<0.01), and ^{3}H-thymidine incorporation (P<0.01), whereas Ang II increased the latter (P<0.01) only. The effects of prorenin combined with angiotensinogen were not observed at a prorenin concentration of 0.2 nmol/L (n=10, data not shown). All effects of prorenin combined with angiotensinogen as well as the effect of Ang II on ^{3}H-thymidine incorporation were blocked by eprosartan but not PD123319 or M6P (Figures 2 and 3). PD123319 tended to enhance the effects of Ang II on total cellular protein, but the difference was not significant (Figure 3).

Angiotensin Generation

Ang I and Ang II were undetectable in medium or cells under control conditions and following incubations with either prorenin alone or angiotensinogen alone. At 1 hour after the addition of 2 nmol/L prorenin combined with 150 nmol/L

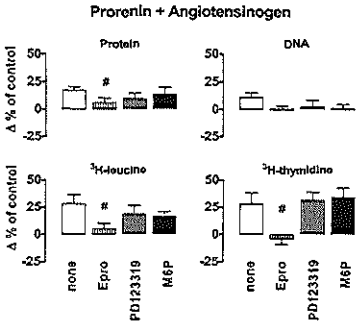

Figure 2. Effect of 2 nmol/L prorenin plus 150 nmol/L angiotensinogen on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes in the presence of vehicle (none), the AT_1 receptor antagonist eprosartan (Epro; 1 μmol/L), the AT_2 receptor antagonist PD123319 (1 μmol/L), or the M6P/IGFII receptor antagonist M6P (10 mmol/L). Data are expressed as percentage change from control (mean±SEM of 8 experiments). #P<0.05 versus none.

angiotensinogen to the cells. Ang I and Ang II levels in the medium were 4278±207 and 372±23 pmol/L (n=3), and after 4 hours these levels amounted to 4704±462 and 795±102 pmol/L. When using 0.2 nmol/L prorenin in combination with 150 nmol/L angiotensinogen, the Ang I and Ang II levels in the medium were 656±161 and 85±14 pmol/L after 1 hour (n=3), and 876±43 and 129±18 pmol/L after 4 hours. Cellular Ang I levels measured after 4 hours of incubation with prorenin and angiotensinogen or 100 nmol/L Ang II were below the detection limit (n=3 for each condition). Cellular Ang II levels were also undetectable following a 4-hour incubation with 0.2 nmol/L prorenin combined with 150 nmol/L angiotensinogen. However, at a 10-fold higher prorenin concentration, as well as following a 4-hour incubation with 100 nmol/L Ang II, cellular Ang II levels amounted to 1.3±0.5 and 1.9±0.3 fmol/10^6 cells, respectively (n=3 for each condition). These levels represent <0.5% of the levels in the medium.

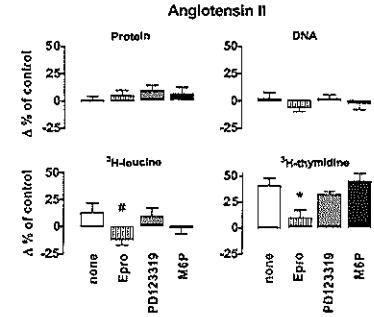

Figure 3. Effect of 100 nmol/L Ang II on total cellular protein content, total cellular DNA content, [^{3}H]-leucine incorporation, and [^{3}H]-thymidine incorporation in cardiomyocytes in the presence of vehicle (none), the AT_1 receptor antagonist eprosartan (Epro; 1 μmol/L), the AT_2 receptor antagonist PD123319 (1 μmol/L), or the M6P/IGFII receptor antagonist M6P (10 mmol/L). Data are expressed as percentage change from control (mean±SEM of 8 experiments). #P<0.05, *P<0.01 versus none.

Discussion

Prorenin binding to M6P/IGFII receptors located on the cell surface of neonatal rat cardiomyocytes does not result in enhanced protein or DNA synthesis. Enhanced protein and DNA synthesis were observed however when exposing the cells to prorenin combined with angiotensinogen, suggesting that these effects depend on angiotensin generation. Because prorenin binding to M6P/IGFII receptors is followed by internalization and rapid intracellular activation to renin, without subsequent release of renin to the medium,[12,13] we reasoned that intracellular angiotensin synthesis might underlie these findings. Such intracellular angiotensin generation will not occur in the absence of prorenin, because neonatal rat cardiomyocytes do not synthesize renin in detectable amounts.[25] However, the addition of 10 mmol/L M6P to the medium, which fully blocks prorenin internalization and activation,[12,13] did not block the prorenin plus angiotensinogen-induced effects on DNA and protein synthesis. Remarkably, blockade was observed with the AT_1 receptor antagonist eprosartan, while the AT_2 receptor antagonist PD123319, like M6P, was without effect. In view of the virtual lack of internalization of receptor antagonists,[28] the most likely explanation for these findings is that the inherent catalytic activity of prorenin, caused by temporal unfolding of the prosegment, results in extracellular Ang II generation and subsequent AT_1 receptor stimulation. In support of this concept, Ang I and Ang II could be detected in nanomolar concentrations in the medium during incubation of the cells with 2 nmol/L prorenin and 150 nmol/L angiotensinogen.

The lack of effect of prorenin binding per se was unexpected, because several groups have reported that binding of M6P-containing glycoproteins to M6P/IGFII receptors results in a cellular response in a G-protein-dependent manner, eg, chemotaxis or increased c-fos expression.[19,22] Moreover, binding of renin to human mesangial cells was found to enhance ^{3}H-thymidine incorporation and PAI-1 release, independent of angiotensin generation.[9] The receptor mediating the latter effect has not yet been identified and is not necessarily the M6P/IGFII receptor.[9] Furthermore, the IGFII analog [Leu^{27}]IGFII, which binds to the M6P/IGFII receptor with equal affinity as IGFII, induced chemotaxis but not DNA synthesis.[29,30] Therefore, taken together, it is still very well possible that prorenin binding to M6P/IGFII receptors elicits other cellular responses than protein and DNA synthesis.

The addition of human angiotensinogen to the medium did not result in protein or DNA synthesis. This suggests that neonatal rat cardiomyocytes, like mouse cardiomyocytes,[31] do not posses enzymes (eg, cathepsins) capable of cleaving human angiotensinogen into Ang I and des-angiotensinogen. Ang I generation only occurred when combining human prorenin with human angiotensinogen, and this generation was limited to the extracellular compartment. The absence of intracellular Ang I, despite the proteolytic cleavage of prorenin to renin in myocytes, is in agreement with previous studies showing neither binding of angiotensinogen to cardiac

and vascular membrane fractions,[1,8] nor angiotensinogen internalization.[27] Apparently, the intracellular presence of Ang II in myocytes must be explained on the basis of AT_1 receptor-mediated endocytosis,[32–34] rather than intracellular Ang II generation. In support of this contention, Ang II was also detected in cell lysates following a 4-hour incubation with 100 nmol/L Ang II. Furthermore, the low cellular Ang II levels during incubation with prorenin plus angiotensinogen (<0.5% of the levels in the medium) also argue against synthesis and/or storage of Ang II in myocytes.[23]

In the present study, extracellular Ang I generation occurred in a prorenin concentration-dependent manner. At the highest prorenin concentration tested (2 nmol/L), the Ang I levels in the medium reached a steady state within 4 hours. The levels were in the order of 5 nmol/L, which is within the range expected based on the concept that <2% of prorenin is catalytically active.[27] We previously reported that, in the absence of serum, Ang I–II conversion by ACE on myocytes is responsible for approximately 50% of Ang I metabolism by neonatal rat cardiomyocytes, and that the Ang I half life under these conditions is 1 hour.[25] The Ang II half life is much longer,[26] and this may explain why the medium Ang II levels in the present study continued to rise between 1 and 4 hours. Importantly however, the Ang II levels in the medium after 4 hours of prorenin plus angiotensinogen incubation were less than 1 nmol/L, and it is unlikely, in view of the steady-state Ang I levels, that these levels would have become much higher on longer incubation. Yet, despite these relatively low Ang II levels, the effects of prorenin combined with angiotensinogen on protein and DNA synthesis were equal to or stronger than those of 100 nmol/L Ang II. There are several explanations for this apparent discrepancy. First, Ang I–II conversion by ACE may occur in close proximity of AT_1 receptors and may thus result in higher Ang II levels in the microenvironment of these receptors than in the medium.[35] Second, long-term exposure to low levels of Ang II (as a consequence of continuous Ang II generation) might be more efficient to induce cellular responses than short-term exposure to high levels of Ang II, for instance because the latter results in rapid downregulation of AT_1 receptors.[36] Finally, because neonatal rat cardiomyocytes possess both AT_1 and AT_2 receptors, Ang II may also stimulate AT_2 receptors, and this could counteract the AT_1 receptor-induced effects.[26,37] It is possible that ACE-dependent local Ang II generation predominantly leads to AT_1 receptor activation, because ACE is located in close proximity of AT_1 receptors,[38] whereas exogenous Ang II results in equal AT_1 and AT_2 receptor activation. In agreement with this concept, as in our previous study,[26] the Ang II-mediated effect on total cellular protein increased in the presence of PD123319 (Figure 3).

The effects of locally generated and exogenous Ang II on protein and DNA synthesis rate were of modest proportion and exceeded those on total cellular protein and DNA content, suggesting that they may have been counterbalanced, at least in part, by protein and DNA degradation.

In conclusion, the partial catalytic activity of prorenin is responsible for the enhanced protein and DNA synthesis observed in cardiomyocytes during their incubation with prorenin and angiotensinogen. We found no evidence for intracellular angiotensin generation in these cells, nor did prorenin binding to M6P/IGFII receptors per se result in cell proliferation.

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On the way home

(9)

Summary and concluding remarks

Summary and concluding remarks

Chapter 1 & 2- Introduction

The renin-angiotensin system (RAS) was originally seen as an endocrine system regulating blood pressure and Na^+ excretion. According to this concept, its active endproduct, angiotensin II, is generated in the circulation by the enzymes renin (released from the kidneys) and angiotensin-converting enzyme (ACE) from the liver-derived substrate angiotensinogen. Angiotensin II activates angiotensin II (AT) receptors, resulting in vascular contraction and renal Na^+ and H_2O retention.
To explain the success of ACE inhibitors and AT receptor blockers in the treatment of cardiovascular diseases the concept of local or tissue RAS was developed, separate from the circulating RAS.[1,2] This concept implies *in situ* generation of angiotensin II, rather than delivery of circulating angiotensin II to tissues.[3]
At first it was thought that the renin and angiotensinogen required for such *in situ* angiotensin II generation are synthesized *de novo* at tissue sites. Although this may be the case in some organs, in the heart the mRNA levels for renin and angiotensinogen are low or undetectable.[2,4] Moreover, renin activity disappeared from the heart following a bilateral nephrectomy and the perfusate of isolated perfused hearts does not contain renin or angiotensinogen.[5-7] Both proteins, however, can be detected in the heart under normal as well as pathological conditions.[8-11] Presumably therefore, most of cardiac renin and angiotensinogen, if not all, is taken up from the circulation. As the levels in the circulation of prorenin, the inactive precursor of renin, are much higher than the levels of renin, it is also conceivable that prorenin is sequestered and, following activation, contributes to cardiac angiotensin formation. Inhibition of cardiac uptake and/or activation of kidney-derived prorenin might reduce local angiotensin II generation in the heart.
It was the aim of the present thesis to study the functional importance of local angiotensin II generation and the possible role of (pro)renin in this process.

Chapter 3

To assess the importance of *in situ* angiotensin II formation in the arterial wall, as opposed to angiotensin II delivery via the circulation, we determined forearm vasoconstriction in response to infusion of angiotensin I or angiotensin II in 14 normotensive male volunteers.

Both angiotensins reduced forearm blood flow by maximally 70-75% with equal potency (4 nmol/L). Forearm fractional angiotensin I-II conversion was 36% (range 18% to 57%). The ACE inhibitor enalaprilat inhibited the contractile effects of angiotensin I and reduced fractional conversion to 1% (range 0.1% to 8%), thereby excluding a role for converting enzymes other than ACE. Blockade of AT_1 receptors with losartan inhibited the vasoconstrictor effect of angiotensin II. In conclusion, the equal potencies of angiotensin I and angiotensin II, combined with the limited conversion of angiotensin I, suggests that *in situ* generation of angiotensin II is more important for vasoconstriction than circulating angiotensin II.

Chapter 4

To study angiotensin II synthesis by cardiac cells, independently of renal renin and liver derived angiotensinogen, we measured renin, prorenin, angiotensinogen, ACE, angiotensin I and angiotensin II in cell lysates and conditioned media of neonatal rat cardiac myocytes and fibroblasts cultured under serum-free conditions for 5 days. Measurements were also made in the unconditioned horse serum- and fetal calf serum-containing medium used for isolation and adherence of the cells, and in the conditioned serum-free medium of myocytes exposed to cyclic stretch, since stretch-induced hypertrophy of myocytes is believed to depend on the release of angiotensin II by these cells.[12-15]

All RAS components were detectable in unconditioned serum-containing medium. In serum-free conditioned medium low levels of prorenin, but none of the other RAS components, could be detected. The angiotensin I-generating activity corresponding with these low prorenin levels was not of rat origin, as the activity could not be inhibited by the rat renin-specific inhibitor CH-732. Most likely therefore, this prorenin is bovine and/or horse prorenin that is sequestered from the serum-containing medium used for the isolation and adherence of the cells, prior to the serum-free incubation period.

Both myocytes and fibroblasts converted angiotensin I to angiotensin II and this was completely inhibited by captopril. Renin, prorenin, angiotensinogen, angiotensin I or angiotensin II were not detected in the lysates of serum-deprived myocytes or fibroblasts. The application of stretch to myocytes under serum-free conditions increased protein synthesis by 20%, but this was not accompanied by angiotensin II secretion.
In conclusion, cultured cardiac myocytes and fibroblasts do not synthesize (pro)renin and angiotensinogen in amounts that are high enough to result in angiotensin II concentrations of physiological relevance. Myocytes and fibroblasts do express ACE on the cell surface, thereby allowing the synthesis of angiotensin II at cardiac tissue sites when renin and angiotensinogen, or angiotensin I, are provided from the circulation. ACE is the only angiotensin I-converting enzyme on cardiac cells. Angiotensin II release is not a prerequisite for the hypertrophic response, following stretch, of cardiomyocytes.

Chapter 5 and 6

Earlier reports have suggested that mannose 6-phosphate/insulin-like growth factor II (IGFII) receptors are involved in the binding and activation of recombinant human prorenin by cardiac and endothelial cells.[16,17] To investigate the kinetics of this process, the nature of activation, and the existence of other prorenin receptors, neonatal rat cardiac myocytes and fibroblasts or human umbilical vein endothelial cells (HUVECs) were incubated with recombinant wild-type prorenin, K/A-2 prorenin (in which Lys^{42} is mutated to Ala^{42}, thereby preventing cleavage by known proteases), mannose 6-phosphate-free prorenin and non-glycosylated prorenin, with or without mannose 6-phosphate, protease inhibitors or angiotensinogen. Intact and activated prorenin were measured in cell lysates with prosegment- and renin-specific antibodies, respectively. Cardiac cells, as well as HUVECs, only bound mannose 6-phosphate-containing prorenin. The dissociation constant (K_d) for prorenin binding to mannose 6-phosphate/IGFII receptors was approximately 1 nmol/L for either cell type. The maximum number of binding sites (B_{max}) was highest in cardiomyocytes. At 37°C, due to mannose 6-phosphate/IGFII receptor recycling, the amount of prorenin internalized via mannose 6-phosphate/IGFII receptors was >10 times B_{max}. The levels of internalized intact prorenin decreased rapidly, indicating proteolytic prosegment

removal. Renin was subsequently degraded. Protease inhibitors added to the culture medium marginally affected prorenin activation in cardiomyocytes, and were without effect on prorenin activation in fibroblasts and HUVECs. Moreover, the proteolytic activation of K/A-2 prorenin in HUVECs was indistinguishable from wild-type prorenin activation. These data indicate that the prorenin-activating enzyme most likely is different from any of the previously known prorenin-activating enzymes. Incubation of HUVECs with prorenin and angiotensinogen did neither led to internalization of angiotensinogen, nor, to intracellular angiotensin generation. In conclusion, cardiac myocytes, cardiac fibroblasts and endothelial cells display high affinity binding of prorenin exclusively via mannose 6-phosphate/IGFII receptors. Binding is followed by internalization and proteolytic activation to renin. Although this process may underlie intracellular angiotensin synthesis, our data in HUVECs do not support this concept, and suggest that prorenin internalization represents prorenin clearance.

Chapter 7

Since recombinant human prorenin may differ from native human prorenin with regard to its glycosylation and/or phosphorylation,[18,19] we repeated the studies regarding (pro)renin binding to mannose 6-phoshate/IGFII receptors with cardiac cells using various prorenin-containing body fluids.

Uptake and activation via mannose 6-phosphate/IGFII receptors were observed for plasma prorenin from subjects with renal artery stenosis and/or hypertension, and for ovarian follicular fluid prorenin. The total amount of cellular renin and prorenin (expressed as percentage of the levels of renin and prorenin in the medium) after 4 hours of incubation was 4-10 times lower than after incubation with recombinant human prorenin. Although plasma contains alkaline phosphatases capable of inactivating the mannose 6-phosphate label, as well as soluble mannose 6-phosphate/IGFII receptors that block prorenin binding in a competitive manner, and proteins (e.g., insulin, IGFII) that increase the number of cell surface mannose 6-phosphate/IGFII receptors, these factors were not responsible for the modest uptake of native human prorenin. Uptake did not occur during incubation of myocytes with plasma prorenin from anephric subjects or with amniotic fluid prorenin, and this was not due to the presence of excessively high levels of mannose 6-phosphate/IGFII receptors and/or phosphatase activity in these fluids. In conclusion, myocytes are

capable of binding, internalizing and activating native human prorenin of renal and ovarian origin via mannose 6-phosphate/IGFII receptors. Differences in prorenin glycosylation and/or phosphorylation, as well as the concentration of soluble mannose 6-phosphate/IGFII receptors and growth factors affecting the cell surface density of mannose 6-phosphate/IGFII receptors, may determine the amount of blood-derived prorenin in the heart, and, thereby influence cardiac angiotensin II production.

Chapter 8

Mannose 6-phoshate/IGFII receptors on the cell surface couple to intracellular second messenger systems, and renin binding to renal glomerular mesangial cells increases ^{3}H-thymidine incorporation directly (i.e., independent of angiotensin II formation).[20-22] Therefore, to investigate whether prorenin binding to cardiac myocytes elicits a response, and if so, whether this response depends on intra- or extracellular angiotensin II generation (Figure 1), we incubated neonatal rat cardiomyocytes with 2 nmol/L prorenin and/or 150 nmol/L angiotensinogen, with or without mannose 6-phosphate, eprosartan or PD123319 to block mannose 6-phosphate/IGFII-, AT_1- and AT_2-receptors, respectively. Protein and DNA synthesis were studied by quantifying ^{3}H-leucine and ^{3}H-thymidine incorporation. For comparison, studies with 100 nmol/L angiotensin II were also performed.

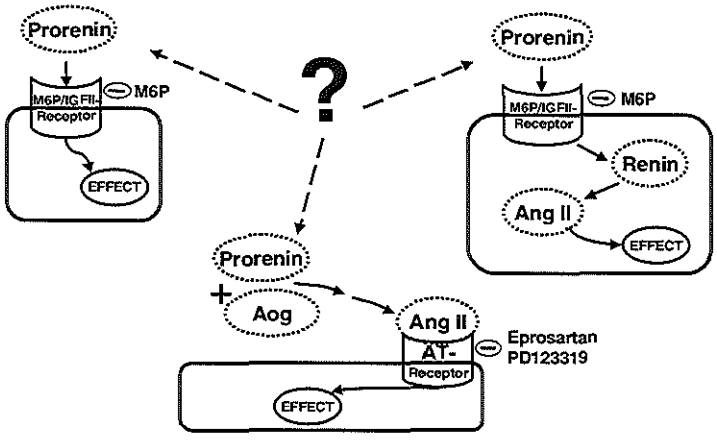

Figure 1: Mechanisms for prorenin-induced effects on myocytes. Binding to mannose 6-phosphate/-IGFII receptors directly confers a cellular effect (top left), or following intracellular activation and angiotensin II formation (top right). Alternatively, angiotensin II is generated extracellularly independent of prorenin binding, as a consequence of the partial activity of prorenin (bottom).

Neither prorenin alone, nor angiotensinogen alone affected protein or DNA synthesis. Prorenin plus angiotensinogen increased ^{3}H-leucine incorporation by ≈20%, ^{3}H-thymidine incorporation by ≈30% and total cellular protein by ≈15%, whereas angiotensin II increased DNA synthesis only (by ≈35%). Eprosartan, but not PD123319 or mannose 6-phosphate, blocked the effects of prorenin plus angiotensinogen as well as the effects of angiotensin II. Angiotensin II levels in the medium during prorenin and angiotensinogen incubation were very low (< 1 nmol/L) with respect to its levels (100 nmol/L) in incubations in which angiotensin II was added for comparison.

In conclusion, prorenin binding to mannose 6-phoshate/IGFII receptors on cardiomyocytes per se does not result in enhanced protein or DNA synthesis. However, through angiotensin II generation, prorenin is capable of inducing myocyte hypertrophy and proliferation. Since this generation occurs independently of mannose 6-phoshate/IGFII receptors, it most likely depends on the catalytic activity of intact prorenin in the medium (due to temporal prosegment unfolding) rather than its intracellular activation. Local generation of angiotensin II results in more efficient AT_1 receptor activation and/or second messenger generation. Taken together, the results described in this chapter do not support the concept of angiotensin II generation in cardiomyocytes following intracellular prorenin activation.

Concluding Remarks

Local angiotensin II generation and receptor-dependent prorenin binding

The experiments described in this thesis focus on the cardiac generation of RAS components and the possible contribution of prorenin to angiotensin II generation in the heart.

Despite many reports in the literature on the generation of angiotensin II by cultured cardiac cells,[12-15] we were unable to show that these cells are capable of synthesizing (pro)renin or angiotensinogen. Our data are in agreement with the concept that ACE and AT receptors are present on cardiac cells, and that angiotensin II mediates its hypertrophic effect via these receptors. Thus, either renin is not involved in the generation of angiotensin II by cultured cardiac cells, or the

renin (and angiotensinogen) required for angiotensin II synthesis are derived from the media used to culture the cells. A role for enzymes other than renin in the generation of angiotensins in the heart seems unlikely in view of the absence of angiotensin II-mediated effects following angiotensinogen application to cardiomyocytes. Cardiac cells may sequester circulating (pro)renin via binding to cell surface mannose 6-phoshate/IGFII receptors. However, non-mannose 6-phoshate/IGFII receptor (pro)renin binding sites (or receptors) have also been demonstrated in membrane preparations from various rat tissues[23,24] and in human glomerular mesangial cells.[22] The gene encoding the latter receptor has recently been cloned and characterized.[25] Expression of the receptor in a SV40-immortalized human mesangial cell line revealed high-affinity renin-binding properties with concurrent more efficacious angiotensin I formation. Renin binding to this receptor within minutes also led to an increase in phosphorylation of the cytosolic serine/threonine kinases ERK1 and ERK2, which are both involved in intracellular signal transduction.[25] Localization studies with antibodies against several domains of the receptor revealed that its expression is restricted to vascular smooth muscle cells and mesangial cells. It appeared to be absent in endothelial and cardiac cells.[25] These results agree with ours as prorenin binding sites other than mannose 6-phoshate/IGFII receptors could not be demonstrated in rat cardiac myocytes and fibroblasts, or human umbilical cord endothelial cells.

Binding of mannose 6-phoshate-labeled prorenin to cardiac and endothelial cells is perhaps somewhat unexpected, since the mannose 6-phoshate label classically confers lysosomal targeting of proteins that are either generated de novo in the endoplasmatic reticulum or endocytosed from the cell surface. In this regard, mannose 6-phosphate labeling of prorenin might be due to evolutionary conservation, since prorenin's homologue, the aspartic protease cathepsin D, is normally present in lysosomes only. Prorenin also contains signal motifs for storage and activation in secretory vesicles, and the presence of multiple signal motifs might explain why part of prorenin, including mannose 6-phosphate-labeled prorenin, 'escapes' into the circulation.[26-28] Furthermore, the mannose 6-phosphate label has additional functions, as in some cases (e.g., latent-TGFβ[29] and leukemia inhibitory

factor[30]) mannose 6-phosphate is required to dock a protein on the cell surface, prior to its internalization and transportion to lysosomes. Another example of the versatility of the mannose 6-phosphate label is its role in the sorting and secretion of the apoptosis-inducing enzymes granzyme A and B by cytotoxic T cells, and in the subsequent internalization of these enzymes by target cells.[31,32] Finally, prorenin of non-renal origin does not always contain the mannose 6-phosphate signal.[18,19]

Irrespective of (pro)renin binding to mannose 6-phosphate/IFGII receptors, the similar potencies of angiotensin I and angiotensin II in the human forearm, despite the fact that only about one-third of angiotensin I was converted to angiotensin II, and the comparable effects of prorenin plus angiotensinogen versus angiotensin II on myocyte proliferation, despite the 100-fold lower angiotensin II levels that were present in the medium during prorenin plus angiotensinogen incubation, show that local angiotensin II generation is of greater importance than angiotensin II delivery via the circulation. The lack of effect of mannose 6-phosphate during prorenin plus angiotensinogen incubations excludes a role of mannose 6-phoshate/IGFII receptors in the local angiotensin I generation on the cell surface. Since we were unable to demonstrate significant cellular uptake of angiotensinogen our data also argue against the possibility of intracellular angiotensin II generation. Possibly, therefore, (pro)renin binding to mannose 6-phoshate/IGFII receptors represents (pro)renin clearance, rather than the initial step leading to (intracellular) angiotensin II generation.

Local transcription of renin and angiotensinogen: physiologically relevant?

Regardless the conflicting data documenting the presence or absence of cardiac renin and/or angiotensinogen synthesis (i.e., transcription followed by translation), the majority of renin and angiotensinogen required for cardiac angiotensin I synthesis is derived from the circulation, both under normal and pathological conditions.

In our experimental setup cardiac gene transcription of renin and angiotensinogen, if occurring, did not result in detectable levels of the mature proteins. The low renin transcription levels that have been detected in cardiac tissue could, however, represent a recently described renin splice variant (dubbed: renin-1A).[33] This renin

splice-variant, originally discovered in adrenal tissue, lacks the secretion signal motif encoding sequence and one-third of the prosegment. Theoretically, therefore, it should give rise to a protein product that remains intracellularly and displays renin activity.[33,34] Indeed, *in vitro* generated renin-1A protein products are translocated into adrenal mitochondria, when incubated with a cell-free mitochondria preparation, in accordance with previous described immunohistochemical evidence for its presence in adrenal mitochondria.[34] Unfortunately, no attempts have been presented so far to unravel the capacity of renin-1A to generate angiotensin I intracellularly (or to perform alternative functions).
As far as angiotensinogen is concerned, there is currently no evidence for a naturally occurring angiotensinogen variant lacking the signal peptide that normally leads to secretion.

Future study options:

The results described in this thesis belong to the first characterizing the binding and activation of prorenin by extrarenal cells and investigating the involvement of the mannose 6-phosphate/IGFII receptor in this process. In addition, cardiac myocytes and fibroblasts were shown not to have (pro)renin- and angiotensinogen-generating capacity, thus emphasizing the importance of (pro)renin sequestration. Remarkably, however, (pro)renin binding to mannose 6-phosphate/IGFII receptors did not result in detectable angiotensin formation. The following future studies may help to clarify some of the remaining and newly raised questions.

Is the mannose 6-phosphate/IGFII receptor the only (pro)renin-binding receptor on the cell surface?
We have obtained compelling evidence that the mannose 6-phosphate/IGFII receptor is the only (pro)renin binding receptor on cardiomyocytes and -fibroblasts, isolated from neonatal rat hearts, and on endothelial cells, isolated from human umbilical cord veins. However, cardiac and endothelial cells isolated from adult tissue, as well as non-endothelial, non-cardiac cells, could express an additional (pro)renin binding site/receptor. Furthermore, pathological conditions might result in the expression of alternative (pro)renin binding sites. Therefore, it is worthwhile to

expand our binding studies with recombinant human prorenin to adult cardiac and endothelial cells and/or cells from other (pathological) tissues, in order to assess the presence or absence of binding sites for (pro)renin, other than mannose 6-phosphate/IGFII receptors.
One candidate is a recently cloned renin receptor, that is specifically expressed on the cell surface of vascular smooth muscle and mesangial cells.[25]

Does local angiotensin II generation occur in close proximity of AT_1 receptors?
Studies on the molar ratio and the exact location of ACE and AT receptors on the cell surface of myocytes (in other words their vicinity) might explain the observed efficient AT_1 receptor activation (i.e., occurring at much lower extracellular angiotensin II levels) in cardiomyocytes and the forearm by locally generated angiotensin II in comparison to exogenous angiotensin II. Enhanced AT_1 receptor activation was not dependent on mannose 6-phosphate/IGFII receptor-binding of prorenin. However, binding of (pro)renin to the recently cloned renin receptors[25] on vascular smooth muscle and glomerular mesangial cells might further enhance the efficiency of such a local angiotensin II-generating system (Figure 2).

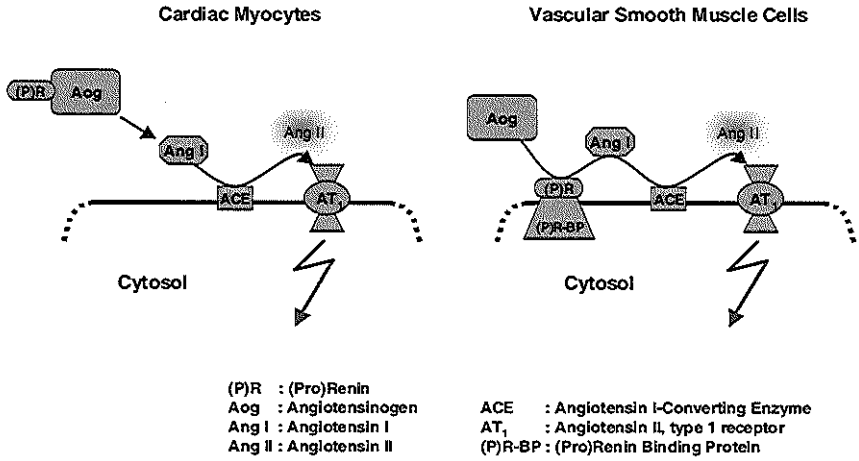

Figure 2: Efficient AT_1 receptor activation in the heart (left) following cell surface angiotensin II generation. (Pro)renin binding to (Pro)Renin Binding Proteins (i.e., the renin receptors that have been identified in vascular smooth muscle cells) might further enhance the efficiency (right).

Is prorenin binding to mannose 6-phosphate/IGFII receptors of functional importance?
Prorenin binding to mannose 6-phosphate/IGFII receptors did not result in angiotensin generation and is not required for angiotensin generation in the presence of angiotensinogen, suggesting that this receptor might be involved in local (pro)renin clearance, rather than local angiotensin generation. In this regard the role of soluble mannose 6-phosphate/IGFII receptors in the circulation is also of interest; such binding might play a role in (pro)renin transport.
Uptake of native prorenin from human plasma is relatively low compared to the uptake of recombinant prorenin. Explanations for this phenomenon include the presence of soluble mannose 6-phosphate/IGFII receptors, the presence of growth factors affecting the number of cell surface-expressed mannose 6-phosphate/IGFII receptors, and the possibility that a lower proportion of circulating (pro)renin is mannose 6-phosphate-labeled. The latter might be due to the fact that *in vivo* most mannose 6-phosphate labeled (pro)renin has already been removed from blood plasma by binding to mannose 6-phosphate/IGFII receptors, e.g. on the vascular endothelium. Establishing the degree of mannose 6-phosphate labeling of endogenous (pro)renin is essential to extend our understanding of the importance of (pro)renin binding to mannose 6-phosphate/IGFII receptors *in vivo*.

Furthermore, the possibility remains that a specific cellular effect induced directly upon prorenin binding to mannose 6-phosphate/IGFII receptors has been overlooked in the present experimental setup. Therefore, expanding the panel of cellular effects studied to discover mannose 6-phosphate-labeled (pro)renin-mediated mechanisms remains necessary. Proteomics and cDNA microarray might be of help with regard to this issue.
Establishing the cardiac (pro)renin levels, as well as the cardiac morphology and/or physiology of mice devoid of mannose 6-phosphate/IGFII receptors in the heart (due to cardiac-specific deletion of the mannose 6-phosphate/IGFII receptors using the Cre-LoxP system) might also shed light on the functional consequences of mannose 6-phosphate/IGFII receptor dependent (pro)renin binding in the heart. As (pro)renin measurements in cardiac tissue of mice are cumbersome, angiotensin levels could be determined instead in the hearts of such mice. Differences could be augmented by first generating double transgenics by back-crossing with mice overexpressing

either prorenin or renin.[35] Glycosylation and phosphorylation profiles should, however, be monitored when (pro)renin originates from non-renal tissue and/or (pro)renin overexpressing kidneys. Additionally, to circumvent compensatory mechanisms during development, introduction of an inducible overexpression system would be preferable.[36]

Do alternative renin and angiotensinogen gene-transcripts yield intracellular proteins?
Studies are required addressing whether the cardiac expression of the recently discovered renin splice-variant (renin-1A) actually results in measurable renin levels and/or a renin-1A -specific intracellular function.[33] A method would be to generate, via homologous recombination techniques, cell lines and/or mice expressing the renin-1A transcript with a small peptide tag (e.g. the triple HIS tag) and follow mitochondrial translocation and/or cellular uptake of renal (pro)renin by microscopical techniques. The peptide tag offers sensitive purification and/or imaging options. More importantly, tagging might facilitate discrimination between *in vivo* cardiac uptake of renal non-tagged (pro)renin and cardiac transcription/translation of the renin-1A mRNA protein product, and between endogenous production versus cellular uptake of serum components in the case of *in vitro* cell studies. Therefore, the tag should preferably be located 5-prime of the remaining prosegment, resulting in expression wherever the renin-1A mRNA splice-variant is generated or, when not feasible, reliable tissue-specific gene promotors should be introduced.

The existence of alternative splicing of the renin gene, either ignored or overlooked previously, implies the same possibility for angiotensinogen. A systematical analysis of alternative transcription by primer walking and RT-PCR of the angiotensinogen gene is therefore required.

"Transgenics to the rescue!?!"
The question whether and to which degree the heart contributes to its own levels of (pro)renin and angiotensinogen is still being debated, despite hundreds of publications on this issue, and is of importance for, among others, the following reasons. First, cardiac expression of renin and local activation of circulating prorenin

might represent new targets for interference with the local generation of angiotensin II. Second, speculative but intriguing, the postulated mitochondrial presence of the renin-1A isoform in heart tissue might coincide with a recently hypothesized cardiac aldosterone synthesis system.[37,38]
Problems regarding sensitivity and discussions regarding *ex vivo* experiments versus non-physiological conditions *in vivo* (e.g., bilateral nephrectomy) could perhaps be resolved by a set of experiments involving transgenic mice expressing a 3-prime tagged renin gene, generated by homologous recombination, followed by transplantation of the heart of such transgenic animals to wild type mice (Figure 3).

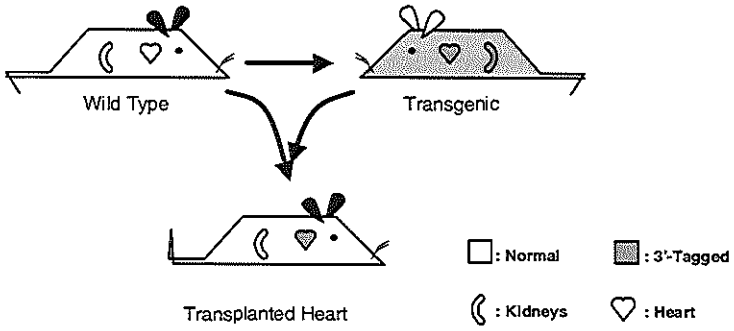

Figure 3: Use of transgenic mice and transplantation to differentiate the sources of cardiac renin (i.e., cardiac renin-1A synthesis versus uptake of circulating (pro)renin).

The insertion of a small protein-tag located 3-prime of renin promises improved sensitivity over earlier performed experiments, without interference with renin's angiotensin I-generating activity, and will allow, after transplantation, determination of *wild type* and tagged renin. The use of transplantation is necessary to unambiguously discriminate between endogenous cardiac renin synthesis and/or (pro)renin uptake by the heart and, as transplantation from a parental strain donor to a transgenic acceptor does not require immunosuppression, should affect the normal physiology of the animal to a limited degree only. It also circumvents the problems arising from differences in glycosylation and/or phosphorylation when using infusions of recombinant (pro)renin or when using mice overexpressing (pro)renin.
Transplantation of a normal heart to a transgenic animal will shed further light on the cardiac uptake of circulating (pro)renin and its translocation to specific cellular compartments (e.g., mitochondria) in the heart. Similar experiments could be

performed with mice transgenic for 3-prime tagged angiotensinogen or transgenic for both such renin and angiotensinogen genes. As this proposal is quite ambitious, due to the complex manipulations and the required expertise on the RAS system, the initiation of a concerted action between specialized groups in angiotensin II research is desirable.

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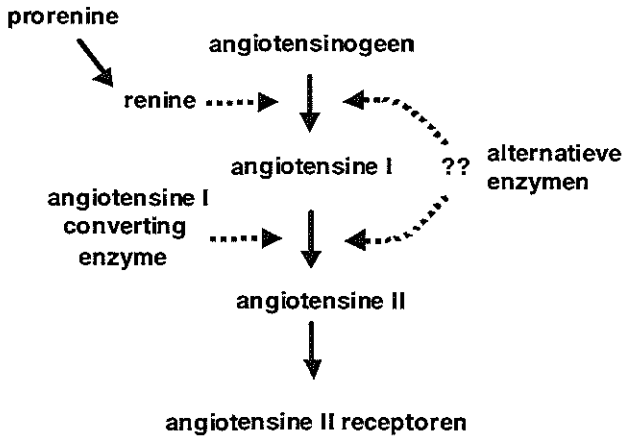
Before closing the doors

Nederlandse samenvatting

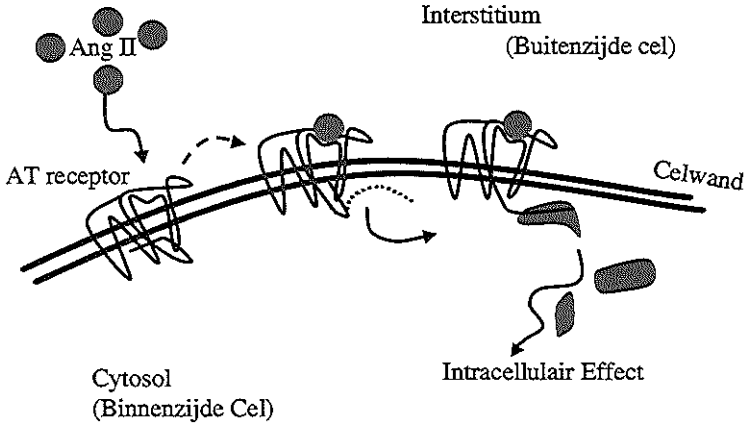
Nederlandse samenvatting

Introductie

Het "renine-angiotensine II systeem" (RAS) in de bloedvaten is van oudsher voorgesteld als een op afstand werkend hormonaal systeem, dat de "normale" bloeddruk en de water- en zouthuishouding van het lichaam regelt.
Het eindproduct van het RAS is het hormoon angiotensine II (zie figuur 1). Dit ontstaat in het bloed uit het eiwit angiotensinogeen (afkomstig uit de lever) door inwerking van de enzymen renine (afkomstig van de nieren) en ACE (het "angiotensine I-converterend enzym", aanwezig op de endotheelcellaag).
Door binding van angiotensine II moleculen aan speciale bindingsplaatsen (de AT-receptoren) op het oppervlak van cellen worden deze AT-receptoren "geactiveerd". Dit houdt in dat de vorm van de receptoren verandert (zie figuur 2), waardoor in de cel een signaal wordt afgegeven met een bepaald gevolg, dat specifiek is voor het type cel. Voorbeelden hiervan zijn "vasoconstrictie" (d.w.z. bloedvatvernauwing door samenknijpen van de spiercellen in de bloedvatwand), het vasthouden van water en zout in de nieren en "hypertrofie" (d.w.z. de vergroting van individuele hartspiercellen).



Figuur 1: Vorming van angiotensine II en binding aan "angiotensine II receptoren".



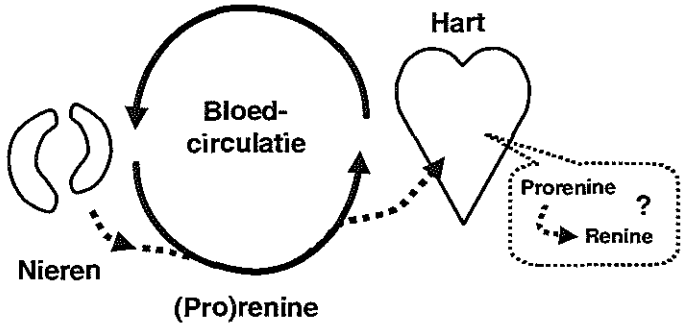
Figuur 2: Binding van angiotensine II (Ang II) aan een AT-receptor in de celwand (links) veroorzaakt een vervorming van deze AT-receptor (midden), waardoor aan de binnenzijde van de cel de "staart" van deze receptor zich openvouwt. Daardoor kunnen verschillende andere eiwitten in de cel zich binden aan de staart van de receptor (rechts) zodat een "effect", zoals samentrekking van een spiercel, kan optreden.

Het RAS is betrokken bij het ontstaan van hartfalen (verlies van pompfunctie) en hypertensie (te hoge bloeddruk).

Overmatige belasting van het hart kan leiden tot verlies van voldoende capaciteit om het bloed door het lichaam te pompen. Dit hartfalen kent vele oorzaken, bijvoorbeeld een hartinfarct (d.w.z. een deel van de hartspier sterft door zuurstoftekort) en hypertensie (bijvoorbeeld als gevolg van een te hoge weerstand in de bloedvaten). Een overmatig belaste hartspier zal in eerste instantie diverse compenserende mechanismen activeren. Dit leidt tot "hypertrofie" van de individuele hartspiercellen en vergroting van de spiermassa. Dit remodelleringsproces gaat vaak gepaard met "fibrose" (d.w.z. verstijving van de spierbundels). Blijft de oorzaak van de overbelasting bestaan, bijvoorbeeld de genoemde te hoge bloeddruk, dan zal de remodellering doorgaan. Dit laatste kan uiteindelijk resulteren in een te sterke verstijving van het hart en/of uitval van hartspiercellen met als gevolg een sterk verminderde pompcapaciteit van de hartspier.
Vaak is bij hartfalen ook het RAS geactiveerd en wordt extra angiotensine II gevormd.

Hoewel ingrijpen aan het begin van een cascade zoals het RAS (figuur 1), meestal de meest wenselijke stap is, worden remmers van renine (nog) niet gebruikt door patiënten. Middelen die de vorming van angiotensine II door ACE remmen of de binding ervan aan de AT-receptoren blokkeren (z.g. receptor antagonisten) worden wel in de klinische praktijk toegepast. Met name de ACE-remmers zijn succesvol bij de behandeling van hartfalen en hypertensie. Opmerkelijk is dat daarbij meer levens zijn gered dan verwacht op basis van de bereikte bloeddrukverlaging. Men neemt daarom aan dat angiotensine II vorming ook plaatsvindt in het hartweefsel zelf, naast die in de bloedbaan. In dit concept past ook dat lokale vorming van angiotensine II kan leiden tot efficiëntere activatie van de AT-receptoren op het celoppervlak dan AT-receptor activatie door angiotensine II afkomstig uit de bloedbaan.

Recent onderzoek in ons laboratorium heeft uitgewezen dat zelfs vrijwel al in het hart aanwezige angiotensine II daar gevormd is. Dit betekent echter niet dat alle eiwitten die nodig zijn voor cardiale angiotensine II vorming ook zelf daadwerkelijk in het hart gemaakt worden. En inderdaad, na verwijdering van de nieren zijn renine en angiotensine niet meer aantoonbaar in hartweefsel. Ook bleek het mRNA (de boodschappermoleculen, nodig voor de vorming van eiwitten) van de genen van renine en angiotensinogeen nauwelijks aantoonbaar. Het in het hart aanwezige renine wordt dus, waarschijnlijk, uit het bloed opgenomen en is zodoende afkomstig van de nieren. Renine komt in de bloedcirculatie ook voor in een inactieve pro-vorm, het z.g. prorenine. De prorenine concentraties zijn ongeveer 10x hoger dan die van renine. Het is niet onwaarschijnlijk dat ook dit prorenine door het hart wordt opgenomen. Het moet in het hart, na opname, nog wel worden omgezet in renine



Figuur 3: (Pro)renine in het hart is afkomstig van de nieren en wordt getransporteerd via de bloedbaan. Na opname zou prorenin geactiveerd moeten worden om angiotensine te kunnen vormen.

("activatie") om angiotensine I te kunnen vormen.
Remming van de (pro)renine-opname en/of de activatie van prorenine in het hart zou een aanvullende medicatie kunnen zijn op de bestaande, succesvolle toepassing van ACE-remmers en AT-receptor-blokkers, of een vervangende therapie voor die mensen die deze medicijnen slecht verdragen.

Het doel van het onderzoek in dit proefschrift was het verkrijgen van een beter inzicht in de lokale vorming van angiotensine II en de rol van (pro)renine in dit proces.

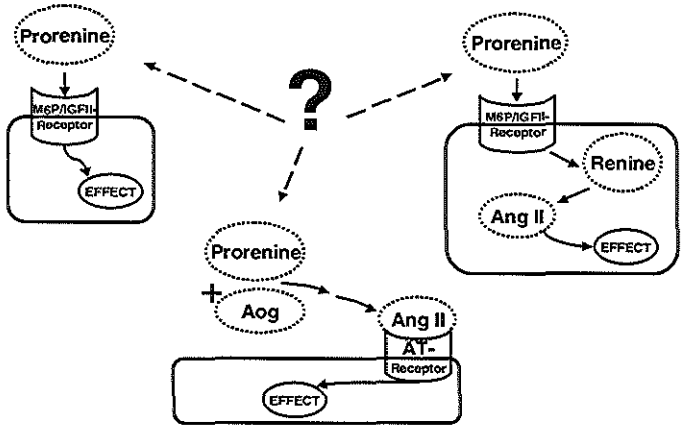
Resultaten van de studie:

Voorbereidende experimenten in ons laboratorium lieten zien dat hartspiercellen van ratten in staat zijn om renine en prorenine op te nemen, waarbij bovendien prorenine na opname werd geactiveerd (zie figuur 3).

In de huidige studie hebben we gebruik gemaakt van hartspiercellen van ratten en van endotheelcellen van mensen om de opname van (pro)renine beter uit te zoeken. Het belang van lokale angiotensine II vorming *in vivo* (d.w.z. in het echt) is onderzocht door de doorbloeding van de onderarm te bestuderen van gezonde vrijwilligers tijdens infusie van angiotensine I of II.

De resultaten laten zien dat hartspiercellen en endotheelcellen geen renine, prorenine of angiotensinogeen maken; wel hebben deze cellen ACE en AT-receptoren op het celoppervlak. Ze kunnen dus wel angiotensine I omzetten in angiotensine II. Dit angiotensine II zou vervolgens de AT-receptoren kunnen activeren.
Daarnaast werd duidelijk dat hartspiercellen en endotheelcellen prorenine efficiënt kunnen opnemen met behulp van speciale bindingseiwitten, de z.g. mannose-6-fosfaat/IGFII receptoren, op het celoppervlak. Alleen prorenine waaraan in de nieren het z.g. mannose-6-fosfaat molecuul was gekoppeld werd door de cellen gebonden. Na binding ging prorenine de cel in, waar het werd omgezet in renine. Het zo

gevormde renine bleef in de cellen. De binding en/of internalisatie van (pro)renine leidde niet tot (intra-) cellulaire effecten, zoals angiotensine II vorming in de cel of hypertrofie van de hartspiercellen. Tot onze verbazing bleek ook dat voor de lokale angiotensine II vorming door hartspiercellen, in aanwezigheid van angiotensinogeen, prorenine binding aan mannose-6-fosfaat/IGFII receptoren niet noodzakelijk was (zie figuur 4).



Figuur 4: Mogelijke mechanismen voor door prorenine veroorzaakte effecten op hartspiercellen. 1) Binding aan de mannose-6-fosfaat/IGFII bindingsplaatsen geeft een specifiek effect in de cel (links). 2) Na binding en opname wordt prorenine omgezet in renine. Dit zou, via de vorming van angiotensine II in de cel kunnen leiden tot een effect (rechts). 3) Angiotensine II wordt buiten de cel gevormd, waarna activatie van AT-receptoren op het celoppervlak leidt tot een effect in de cel (onder).

Tijdens de experimenten met hartspiercellen waarbij prorenine en angiotensinogeen aan de kweekvloeistof (d.w.z. de vloeistof boven de cellen) waren toegevoegd, werd maar weinig angiotensine II gevormd. Echter, het effect daarvan op de cellen was even sterk als dat van in veel hogere (±100x) concentraties toegevoegd. Dit kan verklaard worden met het idee dat de vorming van angiotensine II op het celoppervlak plaatsvindt, zodat alleen daar de concentratie van angiotensine II hoger is en niet in de gehele kweekvloeistof.
Bovenstaande effect, de plaatselijke vorming en efficiënte activatie van de AT-receptoren, kwam ook naar voren bij bestudering van de bloeddoorstroming van de onderarm bij vrijwilligers. Een infuus met angiotensine I leverde dezelfde mate van

vermindering van doorstroming als een infuus met dezelfde hoeveelheid angiotensine II, terwijl maar een klein deel van de aangeboden angiotensine I werd omgezet in angiotensine I. De voor de activatie van de AT-receptoren (en dus vernauwing van de bloedvatwand) van belang zijnde omzetting van angiotensine I naar angiotensine II verliep geheel via het ACE op de cellen van de vaatwand van het bloedvatbed van de onderarm.

Samenvattend:

1) Uit de experimenten betreffende de hartspiercellen van ratten en de bloeddoorstroming van de onderarm van vrijwilligers werd duidelijk dat lokale angiotensine II vorming efficiënter tot AT-receptor activatie leidt, dan van buitenaf aangeboden angiotensine II.
2) Prorenine bindt aan mannose-6-fosfaat/IGFII receptoren op hartspiercellen, gaat vervolgens de cel in en wordt daar dan omgezet in renine. Dit laatste leidt niet tot intracellulaire angiotensine II vorming. Mogelijk is de opname en omzetting in renine enkel de eerste stap van een intracellulair opruimingsproces. Dit opname- en afbraakproces zou dan een mechanisme kunnen zijn dat de concentratie van renine en prorenine buiten de cel reguleert.
3) De ontwikkeling van remmers van (pro)renine binding door het hart en/of activatie van prorenine (buiten de cellen) blijft een mogelijkheid voor toekomstig alternatief ingrijpen op de lokale angiotensine II vorming in het hart.

Dankwoord

Bij het bereiken van de kade blijkt deze maar smal en ligt er nog veel meer water achter. Maar het levert nu wel de tijd en ruimte om iedereen te bedanken die mij heeft geholpen om deze dijk te bereiken.

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Ik heb deze afgelopen jaren op drie afdelingen rondgefladderd, *Interne, Biochemie en Farmacologie* en heb ondersteuning van veel verschillende mensen ervaren. Enkele wil ik er uitlichten.

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Niet ieder van de velen is genoemd, maar de waardering is er wel.

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(Full papers)

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Curriculum Vitae

Jasper Joachim Saris werd op 6 oktober 1964 geboren te Rotterdam. Na het behalen van het HAVO-diploma aan de Maarten Luther te Rotterdam in 1982, behaalde hij in 1986 op het Van Leeuwenhoek Instituut te Delft zijn HLO-diploma, richting Procesmikrobiologie. Zijn opleiding vervolgde hij met het Scheikunde avond-programma aan de Universiteit Utrecht, alwaar hij het doctoraaldiploma behaalde in 1992. Daarnaast werkte hij, vanaf januari 1987 tot en met augustus 1997, in de groep van Prof. Dr. M.H. Breuning in de afdeling Anthropogenetica van de Universiteit Leiden op projecten gericht op de chromosomale mapping, klonering en karakterisering van de *polycystine* I en II genen, verantwoordelijk voor het onstaan de dominant overervende ziekte *polycysteuze cystenieren* (ADPKD). Sinds september 1997 is hij werkzaam bij de afdelingen Biochemie, Inwendige Geneeskunde en Farmacologie van de Erasmus Universiteit te Rotterdam onder de supervisie van de promotoren prof. Dr. M.A.D.H. Schalekamp en Prof. Dr. P.r. Saxena en co-promotor Dr. A.H.J. Danser. Daar verricht hij onderzoek, in het kader van hartfalen, aan het locale angiotensine II genererende systeem in het hart en met name de rol van (pro)renine daarin.

Zeg 't nou eens kort:

Het (pro)renine eiwit in het hart komt uit de nieren en verzorgt mogelijk de plaatselijke vorming van angiotensine. Te veel angiotensine in het hart kan leiden tot verlies van de hartfunctie. Het remmen van dit proces met reeds bestaande geneesmiddelen (met name de z.g. ACE-remmers en AT-receptor blokkers), is van levensbelang voor patiënten met hart- en vaatziekten. Dit proefschrift laat zien dat het mannose-6-fosfaat molecuul een rol speelt bij de opname van (pro)renine in het hart en beschrijft tevens hoe en waar de vorming van angiotensine in het hart plaatsvindt. Het werk kan gebruikt worden als basis voor de ontwikkeling van nieuwe geneesmiddelen die nog beter de vorming en effecten van angiotensine tegengaan. Verhindering van de opname van prorenine door het hart zou zo'n alternatieve behandelingsmethode kunnen zijn.