(PRO)RENIN UPTAKE AND PRORENIN ACTIVATION IN THE CARDIOVASCULAR SYSTEM

(PRO)RENINE OPNAME EN PRORENINE ACTIVATIE IN HET CARDIOVASCULAIRE SYSTEEM

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Voor Maarten en Femke

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CHAPTER 1

General Introduction

Circulating and local renin-angiotensin system(s)

Traditionally the renin-angiotensin system (RAS) has been viewed as a circulating system. Circulating renin, released by the kidneys, cleaves liver-derived angiotensinogen, which yields the inactive decapeptide angiotensin I (Ang (1-10) or Ang I) (Figure 1). Ang I is subsequently converted into an octapeptide, angiotensin II (Ang (1-8) or Ang II), by angiotensin-converting enzyme (ACE), an enzyme that is either located at the luminal side of the endothelium or circulating freely in plasma. Ang II is rapidly metabolized by so-called angiotensinases.

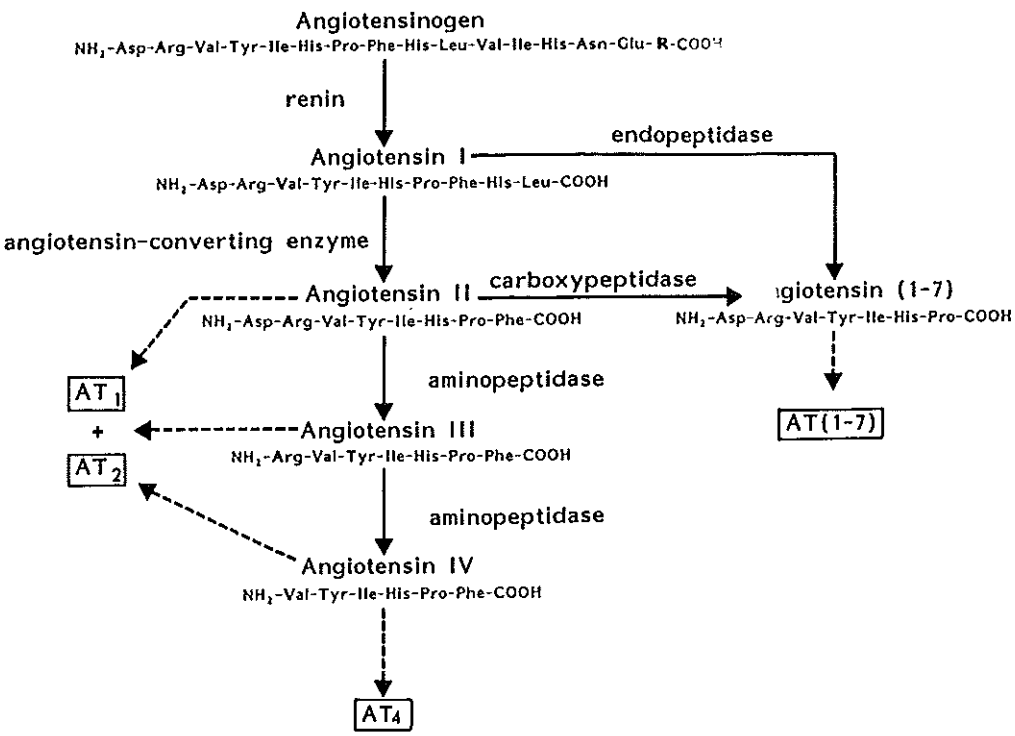

Figure 1. Generation of angiotensin I, II, III, and angiotensin (1-7) from angiotensinogen and activation of the various angiotensin receptors (AT) by angiotensin II and its metabolites. R in angiotensinogen consists of 437 amino acids.

Ang II is an important agent in the regulation of cardiovascular homeostasis. It was first recognized as a potent vasoconstrictor. Other functions that were subsequently described, include stimulation of renal sodium reabsorption and adrenal aldosterone synthesis,[1-3] induction of cellular growth and hypertrophy,[4-6] as well as facilitation of sympathetic neurotransmission.[7,8]

The actions of Ang II are initiated by binding to a receptor. Receptor binding is followed by an intracellular cascade of second messenger reactions, which eventually lead to a cellular response. At present, at least three different angiotensin receptors have been identified based upon different binding characteristics of non-peptide Ang II receptor antagonists: the AT_1, AT_2 and AT_4 receptor.[9] The AT_4 receptor preferentially binds a metabolite of Ang II, Ang (3-8) or Ang IV; its natural ligand may however be Leu-Val-Val-hemorphin 7 rather than Ang IV.[10,11] Furthermore, a binding site for another angiotensin metabolite, Ang (1-7), has been described; characterisation of this site has not yet been completed.[9] The AT_1 - and AT_2 receptor belong to the seven transmembrane domain receptor superfamily. The AT_1 receptor mediates all of the above described effects of Ang II. Recent investigations suggest that stimulation of the AT_2 receptor may counteract some of the effects mediated by the AT_1 receptor.[12-15] The AT_4 receptor appears to regulate memory acquisition, memory retrieval and neurite outgrowth.[16] The Ang (1-7) receptor is linked to vasodilatation, vasopressin release, and prostaglandin synthesis.[17]

Angiotensin binding sites have also been detected in the cytosol and nucleus of hepatocytes and vascular smooth muscle cells. The function of these intracellular receptors is presently unknown.[18,19]

The introduction of drugs interfering with the RAS (ACE inhibitors, renin inhibitors and AT_1 receptor antagonists) has greatly facilitated our understanding of the RAS. It is now well-established that, in addition to angiotensin generation in the circulation, angiotensins may also be synthesized at tissue sites.[20-23] Tissue angiotensin generation is often invoked to explain the long-term beneficial effects of RAS inhibitors in heart failure and hypertension.[24,25] The origin of the renin, angiotensinogen and ACE involved in tissue angiotensin production is still a matter of debate. Both local synthesis and uptake from the circulation have been proposed. Local synthesis would imply that tissue angiotensin generation may occur independently from the circulating RAS.

Uptake from plasma would imply a close relationship between the circulating RAS and the 'tissue RAS'. Most likely, under normal circumstances local synthesis of angiotensins in the heart and blood vessel wall depends on renin taken up from the circulation.[22,26,27] This may be different under pathological conditions.[28]

Effect of angiotensin II on second messengers

Binding of Ang II to the AT_1 receptor activates phospholipase C-β (PLC-β) via a G_q-protein-dependent mechanism. PLC-β hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular stores, and DAG activates protein kinase C (PKC). The increase in intracellular Ca^{2+} and the activation of PKC are both capable of inducing mitogen-activated protein (MAP) kinases and phospholipase D (PLD).
PLD hydrolyses phosphatidylcholine, and to a lesser extent other phospholipids, to form phosphatidic acid (PA), which either augments Ang II-induced MAP kinase activation or affects other undefined events leading to gene transcription.[4,29,30] MAP kinases are cytosolic serine/threonine kinases (e.g., $p44^{MAPK}$ (Erk1) and $p42^{MAPK}$ (Erk2)) that are involved in one or more signal transduction pathways linking events at the plasma membrane with events in the nucleus.[31]

Recent evidence suggests that the AT_1 receptor shares properties with cytokine receptors.[32] Similar to cytokines, Ang II induces tyrosine phosphorylation of intracellular kinases such as Jak2 and Tyk2, thereby leading to increased activity of these kinases. This increased activity results in the tyrosine phosphorylation of the Jak family substrates STAT1 and STAT2 ('Signal Transducers and Activators of Transcription'). The STAT proteins translocate to the nucleus, where they stimulate transcription of early growth response genes. Other tyrosine kinases activated by Ang II include the Src family of tyrosine kinases. PLC-γ is an excellent substrate for members of this family, and it has therefore been proposed that not only PLC-β but also PLC-γ is involved in Ang II-induced PIP_2 hydrolysis.[33]

The various signal transduction pathways that are activated by Ang II may subserve different cellular responses, e.g., the Jak/STAT pathway may trigger a stress response, while the MAPK cascade may favor mitogenesis or hypertrophy.

The actual cell response would be determined by the relative level of activation of these pathways and crosstalk among them.

The intracellular mechanisms that are activated by AT_2 receptors are not yet fully established. Studies in PC-12W pheochromocytoma cells and in N1E-115 murine neuroblastoma cells indicate that Ang II, via AT_2 receptors, can stimulate or inhibit protein-tyrosine phosphatases.[34,35] In addition, it has been shown that AT_2 receptors mediate activation of a specific tyrosine phosphatase (MAP kinase phosphatase 1), which leads to MAP kinase inhibition and the induction of apoptosis.[36,37]

Angiotensin II and the response of cardiomyocytes to mechanical stretch

According to several investigators mechanical stretch of rat cardiomyocytes kept under serum-free conditions causes release of Ang II from these cells.[38-42] However, the Ang II levels that have been reported vary widely, and not all investigators could detect Ang II in the culture medium after the induction of stretch. Ang II release into the medium by serum-deprived cardiomyocytes might suggest that these cells are capable of generating Ang II independently of circulating renin and angiotensinogen.[43,44]

Mechanical stretch of cardiomyocytes causes activation of multiple second messenger systems that are similar to growth factor-induced cell signalling systems. The mechanisms underlying the conversion of mechanical stretch into growth and subsequent cellular hypertrophy are presently unknown. One possibility is that mechanical stretch, via mechanosensing mechanisms, stimulates the production and/or secretion of growth factors such as Ang II and endothelin-1, which then induce the hypertrophic response. Alternatively, mechanosensors ('stretch receptors') may directly activate second messengers. Possible mechanosensors are *ion channels*, such as nonselective cation channels and K^+ channels.[45,46] Second, *integrins*, heterodimeric transmembrane receptors that couple components of the extracellular matrix with the actin cytoskeleton as well as the N-terminal domain of focal adhesion tyrosine kinase (FAK), might convert mechanical signals into intracellular second messenger signalling.[47,48] Thirdly, *tyrosine kinases* (e.g. Src family tyrosine kinases) have transmembrane segments, and membrane stretch may directly cause conformational changes of these tyrosine kinases, thereby activating them.[49]

At present is unclear to what degree the stretch-induced hypertrophic response depends on Ang II. Furthermore, the mechanism of Ang II production by serum-deprived cardiomyocytes remains to be elucidated: is Ang II generated intracellularly and subsequently stored in intracellular storage sites, in order to be released after the induction of stretch? In vivo, cardiac Ang II production independent of the circulating RAS seems unlikely, at least under normal circumstances, because of the low or undetectable levels of renin mRNA in the heart.[50,51] If the same applies to cardiomyocytes (i.e., lack of renin synthesis), either non-renin enzymes may be involved in the intracellular angiotensin generation, or the renin required for angiotensin synthesis is derived from the serum used to culture the cells.

Evidence for binding and/or uptake of circulating (pro)renin

Angiotensin release from the isolated perfused rat Langendorff heart or hindquarter (well-known models to study cardiac and vascular angiotensin production, respectively) depends on the addition of renin to the perfusion fluid.[52-55] The release of Ang I from the isolated heart during perfusion with renin was too high to be attributed to Ang I generation in either intravascular or interstitial fluid, indicating a role for vascular surface-bound renin.[52] Interestingly, angiotensin release continued after discontinuation of the renin perfusion, i.e., at a time when renin was no longer present in the perfusate.[54,55] Furthermore, the renin levels in cardiac tissue *in vivo* are too high to be explained by simple diffusion into the interstitial fluid, and renin is enriched in membrane fractions prepared from freshly obtained cardiac tissue.[22] Thirty hours after bilateral nephrectomy in pigs, renin is no longer detectable in cardiac tissue.[22] In the rat, aortic renin also disappeared after nephrectomy, with a longer half life than plasma renin.[56,57] Taken together, these findings suggest that renin is sequestered from the circulation by cardiac and vascular tissues, and that tissue-bound renin contributes to local angiotensin production.

The mechanism of renin uptake and/or binding is still unknown. It must be kept in mind that renin has an inactive precursor, prorenin. In humans, the circulating levels of prorenin are tenfold higher than those of renin. Under certain circumstances (e.g., diabetes and pregnancy) this difference may become even larger.[58-61]

It is therefore conceivable that not only renin, but also prorenin, is taken up by the tissues. Prorenin may either compete with renin for binding sites, thereby leading to an inhibition of local angiotensin generation, or prorenin may be converted locally into renin, thereby stimulating local angiotensin generation (Figure 2).[62] Studies in which prorenin was infused into animals provided no evidence for the release of activated prorenin into the circulation, indicating that if prorenin is activated locally, it remains in the tissue rather than being released back into the circulation.[63,64]

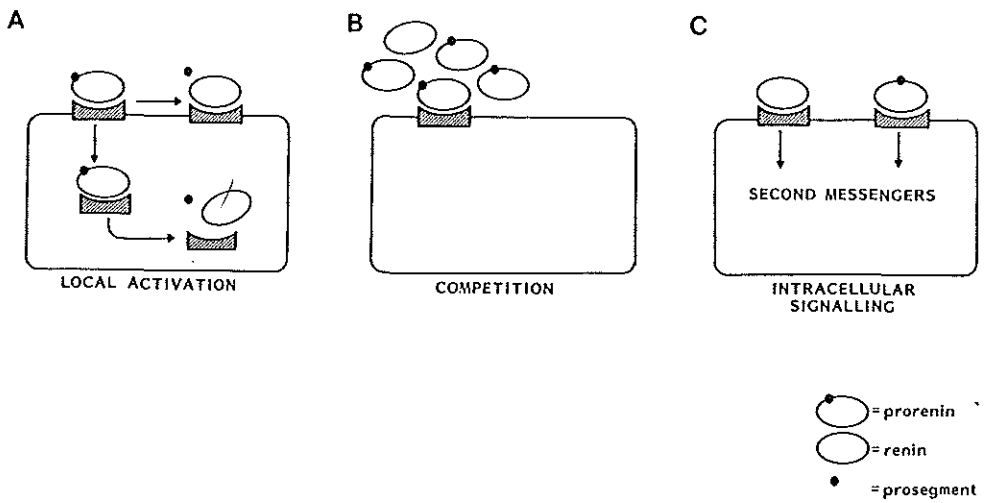

Figure 2. Prorenin binding to (pro)renin binding proteins/receptors may lead to prorenin activation (A), either extra- or intracellularly, thereby stimulating local angiotensin generation, or, when activation does not occur (B), it may competitively prevent renin from binding, thereby inhibiting local angiotensin generation. Finally, (pro)renin binding may directly activate second messengers (C), thereby inducing an intracellular response without angiotensins acting as intermediates.

At present, both (pro)renin binding proteins and receptors have been described. An intracellular renin-binding protein (RnBP, mol. wt. 40 kDa) was discovered in the early eighties in humans, rats and pigs.[65-67] Binding to this RnBP reduces the Ang I generating activity of renin by more than 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 in renin uptake.[68] Subsequently, using chemical cross-linking, two vascular RnBPs (mol. wt. 40 and 70 kDa, resp.) were identified by Campbell and colleagues in membranes isolated from rat mesenteric arteries or cultured rat aortic smooth muscle cells. 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.[69]

More recently, with the use of radiolabelled (pro)renin, high affinity renin binding sites/receptors ($K_d \approx 1$ nM) were demonstrated in human mesangial cells and in membranes prepared from rat tissues.[70,71] 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.[70] This contrasts with Campbell's findings. In human mesangial cells, renin binding led to the induction of DNA synthesis, most likely without Ang II playing a role as an intermediary between renin binding and the hypertrophic response.[71] Thus, a renin receptor may have been identified that directly transduces an intracellular signal (Figure 2).

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 5 or 6 forms with different isoelectric points have been described in rats and humans.[72-75] 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.[76] In studies on the uptake of ^{125}I-labelled recombinant human renin by the liver, Marks et al. were able to show that the receptor involved in the uptake process is the mannose receptor, present on sinusoidal endothelial and Kupffer cells.[77] This receptor (mol. wt. 175 kDa) binds glycoproteins bearing high mannose chains and recycles rapidly between the cell surface, where ligand binding occurs, and various acid intracellular compartments, where the ligand is discharged.[78] Following binding to the mannose receptor, renin was found to be rapidly delivered to lysosomes, where degradation occurred.[77]

Maximal inhibition of hepatic renin uptake by mannan (a specific inhibitor of the mannose receptor) was 70%, indicating that there may be additional mechanisms involved in the hepatic clearance of renin, e.g. binding to the asialoglycoprotein receptor or the mannose 6-phosphate receptor.[77]

Faust et al. demonstrated that renin, like cathepsin D, acquires phosphomannosyl residues during its biosynthesis that enable it to bind to the mannose 6-phosphate receptor.[79] According to Aeed and colleagues, the percentage of recombinant human (pro)renin carrying the mannose 6-phosphate signal is small, most likely < 10%.[80] If the uptake of renin by cardiovascular tissues involves the mannose 6-phosphate receptor, this low percentage may explain why less than 1% of intravenously infused ^{125}I-labelled renin was found to accumulate in these tissues.[81-83]

Mannose 6-phosphate receptors

Synthesis of lysosomal enzymes, secretory proteins, and plasma membrane proteins occurs in the rough endoplasmatic reticulum. Following glycosylation at selected asparagine residues, the proteins move by vesicular transport to the Golgi apparatus where they undergo a variety of post-translational modifications. Subsequently, they have to be segregated from one another for targeting to their final destinations.[84] A key step in the sorting process is the generation of phosphomannosyl residues on the lysosomal enzymes.

These residues serve as high affinity ligands for binding to mannose 6-phosphate receptors (MPRs) in the Golgi. The ligand-receptor complex exits the Golgi via a coated vesicle, and is delivered to a prelysosomal acidified compartment, where the ligand dissociates. The released lysosomal enzyme is packaged into a lysosome while the receptor either returns to the Golgi via a coated vesicle to repeat the process, or moves to the plasma membrane where it functions to internalize exogenous lysosomal enzymes.[84]

At present, two different MPRs have been identified (Figure 3): a large MPR (mol. wt. 300 kDa), which binds ligand independent of divalent cations (cation-independent or CI-MPR),[85] and a small MPR (mol. wt. 46 kDa), which requires divalent cations for optimal binding (cation-dependent or CD-MPR).[86]

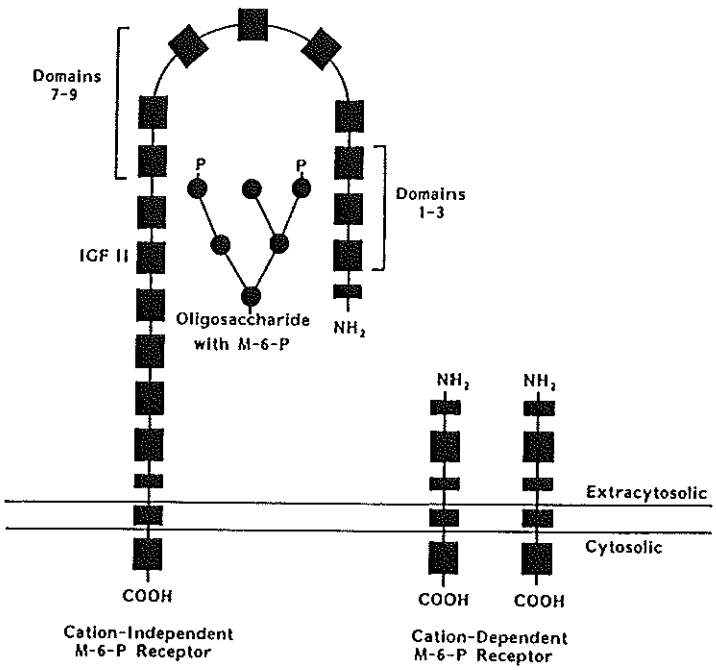

Figure 3. Schematic representation of the mannose 6-phosphate receptors. The two ligands of the cation-independent mannose 6-phosphate are depicted. Arginine residues at positions 435 and 1334 in domains 3 and 9, respectively, are essential for high-affinity binding of mannose 6-phosphate. Sequences in domain 11 are involved in binding IGFII. Modified from references 84 and 113.

Both receptors show similar binding specifities toward phosphorylated oligosaccharides.[85,86] In 1987, it was discovered that the CI-MPR and the insulin-like growth factor II receptor are the same protein.[87] Thus, this CI-MPR is now also known as the M-6-P/IGFII receptor. The receptor binds IGFII, which is non-glycosylated, and phosphomannosylated proteins at distinct sites.[88,89] Each ligand may however influence the binding of the other ligand to the receptor.[90,91] The M-6-P/IGFII receptor binds one mole of IGFII,[88] and has two M-6-P binding sites, allowing the binding of 2 moles of mannose 6-phosphate or one mole of a diphosphorylated oligosaccharide per monomer. Diphosphorylated oligosaccharides are bound with much higher affinity ($\approx 10^{-9}$ M) than mannose 6-phosphate ($\approx 10^{-5}$ M).[85]

The CD-MPR binds one mole of the monovalent ligand mannose 6-phosphate and 0.5 mole of a diphosphorylated high-mannose oligosaccharide per monomeric subunit (with affinities of $\approx 10^{-5}$ M and $\approx 10^{-7}$ M, respectively).[86]

The M-6-P/IGFII receptor consists of a large extracellular domain, containing 15 repeat regions, and a small cytoplasmic domain (Figure 3). The extracytoplasmic domain of the CD-MPR is similar to each of the repeating units of the extracellular domain of the M-6-P/IGFII receptor, suggesting that the two receptors may be derived from a common ancestor.[92] The M-6-P/IGFII receptor exists as a monomer,[93] whereas the CD-MPR can exist as a monomer, dimer or tetramer.[86] MPRs cycle constitutively among the Golgi, endosomes, and the plasma membrane. The majority (90%) of the M-6-P/IGFII receptors is located in a late endosomal/prelysosomal compartment, with the rest being distributed over the plasma membrane, early endosomes, and the Golgi.[94] Extracellular lysosomal enzymes which bind to the cell surface M-6-P/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.[95-97] Endocytosis of lysosomal enzymes via the CD-MPR is of limited importance because of its poor binding of ligand at the cell surface.[98]

Binding and internalization of IGFII to the M-6-P/IGFII receptor results in the lysosomal degradation of this ligand.[99] In addition, IGFII mediates growth-stimulatory responses via this receptor.[100] Thus, its function with regard to IGFII may both be clearance and coupling to second messengers. Furthermore, the M-6-P/IGFII receptor is involved in the clearance and activation of other hormones carrying the mannose 6-phosphate recognition marker, such as thyroglobulin,[101] proliferin,[102] and latent transforming growth factor-β (TGF-β).[103] Binding of latent TGF-β results in cleavage of this prohormone into its active form, which is potent growth inhibitor.

Kang et al. recently demonstrated that the M-6-P/IGFII receptor also binds retinoic acid (RA) with high affinity at a site that is distinct from those for mannose 6-phosphate and IGFII.[104] RA is an important regulator of cell growth in embryonic development and oncogenesis. Binding of RA upregulates the endocytotic functions of the M-6-P/IGFII receptor. Taken together, these findings suggest that the M-6-P/IGFII receptor may play a critical role in the regulation of cell growth.

Indeed, recent findings from transgenic animal experiments, as well as human studies, have shown that the M-6-P/IGFII receptor is essential for normal fetal development[105-107] and has anti-cancer activity.[108-110] The levels of the receptor are highest in fetal tissues, and decline rapidly after birth.[111,112]

Aim of the thesis

It was the aim of the present study to investigate uptake mechanism(s) of renin and/or prorenin in the cardiovascular system. In addition, we studied whether prorenin, once bound and/or internalized, is activated to renin. Finally, the role of angiotensin II in the hypertrophic response of cardiac cells was investigated.

Most studies described in this thesis have been performed with cultured, serum-deprived cells, in order to avoid the problems arising from studies in whole tissues, which may contain both locally synthesized RAS components and components that are taken up from the circulation.

First, we verified the synthesis of RAS components by neonatal rat cardiac myocytes and fibroblasts, as well as the release of Ang II by cardiomyocytes after the induction of stretch (Chapter 2). We then studied the effects of exogenous Ang II on protein and DNA synthesis in unstimulated and endothelin-1-stimulated neonatal rat cardiac cells (Chapter 3). Subsequently, the binding and internalization of recombinant human renin and prorenin, as well as the activation of prorenin by neonatal rat cardiac cells (Chapter 4) and human umbilical vein endothelial cells (Chapter 5) were investigated, with special emphasis on the role of the M-6-P/IGFII receptor in this process. Finally, we compared the cardiac tissue levels of RAS components in heart donors who died of non-cardiovascular disorders and subjects with dilated cardiomyopathy undergoing cardiac transplantation (Chapter 6), in order to answer the question whether (pro)renin is synthesized in cardiac tissue under pathological conditions.

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CHAPTER 2

Cultured Neonatal Rat Cardiac Myocytes And Fibroblasts Do Not Synthesize Renin or Angiotensinogen. Evidence for Stretch-Induced Cardiomyocyte Hypertrophy Independent of Angiotensin II.

Abstract

Demonstration of renin-angiotensin system (RAS) components in cardiac tissue cannot be taken as definite evidence for synthesis of these components in the heart, due to interference with plasma-derived RAS components. To avoid the problems arising from ex-vivo measurements, we therefore measured renin, prorenin, angiotensinogen, ACE, angiotensin (Ang) I and Ang II in the medium and cell lysate of neonatal rat cardiac myocytes and fibroblasts, cultured for 5 days in a chemically defined, serum-free medium. Measurements were also made in the fetal calf serum- and horse serum-containing medium which had been used to obtain cell adherence and confluency prior to the serum-free period, and in medium of serum-deprived cardiomyocytes exposed to cyclic stretch (20% elongation at 30 cycles per min for 24 hours). Prorenin (after its in vitro activation to renin), renin and angiotensinogen were measured by enzyme-kinetic assay; Ang I and Ang II were measured by radioimmunoassay after SepPak extraction and HPLC separation. All RAS components were detectable in unconditioned serum-supplemented medium. Prorenin, but none of the other RAS components, could be detected in medium of serum-deprived cells. 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 were exposed prior to the serum-free period. When incubated with Ang I under serum-free conditions, both cardiomyocytes and fibroblasts generated Ang II in a captopril-inhibitable manner. Lysates of serum-deprived cells did not contain renin, prorenin, angiotensinogen, Ang I or Ang II in detectable quantities. Stretch increased protein synthesis by 20% and was not accompanied by angiotensin release into the medium. Taken together, our results suggest that 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 cardiac cells following stretch.

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 with regard to local synthesis which arise 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 focusses 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 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 stretch for 24 hours. For comparison, studies were also performed in cells cultured in the presence of serum.

Materials and Methods

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 Chemical Co., 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]

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 *Guiding Principles in the Care and Use of Animals* approved by the American Physiological Society.

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 & Company, Plymouth, UK) at 1.5 x 10^5 cells/cm^2, giving a confluent monolayer of spontaneously contracting cells after 24 hours. 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 x 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 hours, cells were either serum-deprived or maintained in serum-supplemented culture medium for 5 days.

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. the 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 1,000 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 below under 'Biochemical measurements').

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.

Angiotensin generation during cyclic stretch of cardiomyocytes

To study the generation of Ang II during prolonged cyclic stretch and its contribution to the increased protein synthesis occurring under these conditions, cardiomyocytes were subjected to cyclic stretch for 24 hours. Following isolation (see above under 'Cell culture') the cells were seeded in flexible-bottomed 6-well culture plates (type I collagen-coated, 5 cm^2/well; Flexcell Int. Corp., 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 hours 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 hours 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 Int. Corp.), and the cells were stretched at 30 cycles per minute (1 sec strain, 1 sec relaxation) at 20% elongation for 24 hours. 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 hours 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 two hours before the end of the 24 hour-study period. After 24 hours, the medium was discarded and the cells were fixed overnight with 500 μL 10% trichloric acid (TCA). Cells were rinsed three times with 10% TCA to remove unincorporated label and then lysed during 6 hours with 500 μL 1 M NaOH. The lysate was transferred to scintillation vials. After neutralization with 500 μL 1 M HCl, 5 mL scintillation fluid (Instagel, Packard, Meriden, CT, USA) was added, and samples were counted for 5 min in a β-counter. Total cellular protein was measured after 24 hours in control-, stretched-, and ET-1-treated cells that had not been incubated with [^{3}H]-leucine. The cells were washed twice with 500 μL ice-cold PBS, dissolved in 250 μL 1 mol/L NaOH, and kept overnight at 4°C. Subsequently, the cell lysates were collected and protein was measured by the Bradford assay,[34] using bovine serum albumin in 1 M NaOH as a standard.

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 hours under either control or stretch conditions. 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.

Biochemical measurements

Renin and prorenin. Renin was quantified in duplicate by measuring Ang I generation at pH 7.4 during incubation at 37°C with a saturating concentration of rat angiotensinogen in the presence angiotensinase-, ACE-, and serine protease-inhibitors.[4,7] The incubation mixture of the renin assay consisted of 1) 100 μL sample, 2) 100 μL of 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, 3) 200 μL angiotensinogen, and 4) 14 μL of an inhibitor solution. Two different inhibitor solutions had been used, one with the rat renin inhibitor CH-732 and one without renin inhibitor. Both solutions contained phenylmethylsulfonyl fluoride (0.0024 mol/L), disodium EDTA (0.005 mol/L), 8-hydroxyquinoline sulphate (0.0034 mol/L) and aprotinin (100 kallikrein-inhibiting units per mL) (final concentrations in the incubation mixture). The renin inhibitor CH-732 was used in a final concentration of 5 μmol/L.

Inhibition of rat kidney renin is > 95% at this concentration (Figure 1). Incubation time was 2 or 4 hours and Ang I generation was linear during this period. Ang I was measured with a sensitive radioimmunoassay.[35] 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.

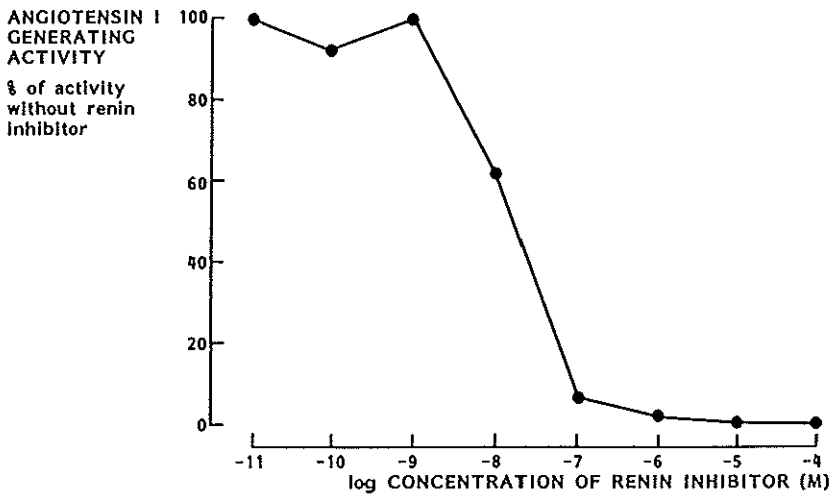

Figure 1. Dose-dependent inhibition of rat kidney renin by increasing concentration of the rat renin inhibitor CH-732.

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 hours 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 hours against 0.1 mol/L phosphate buffer, pH 7.4, containing 0.001 mol/L disodium 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 hours. 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.

Angiotensinogen. The concentration of angiotensinogen was determined as the maximum quantity of Ang I that was generated during incubation 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 incubation mixture of the angiotensinogen assay consisted of 1) 250 µL sample, 2) 50 µL rat kidney renin (diluted 1/50 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl), and 3) 14 µL inhibitor solution without rat renin inhibitor (see above under 'Renin and Prorenin'). Incubation time was 1 hour, and the conditions of the assay were chosen in such a way that Ang I formation was completed within 1 hour. The lowest level of angiotensinogen that could be measured was 0.1 pmol per mL medium and 0.05 pmol/10^6 cells.

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.[35] 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 separation.[35] ^{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.

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 constant for Ang I elimination (k_{el}) is taken to be equal to the sum of the first order rate constants for degradation (k_1) and conversion (k_2).[35]

k_{el} can be calculated as follows:

$$k_{el} = -1/t \times \ln([\text{Ang I}]_t / [\text{Ang I}]_0),$$

in which $[\text{Ang I}]_t$ is the concentration of Ang I at time t and $[\text{Ang I}]_0$ is the concentration of Ang I at t=0 (immediately after the addition of Ang I). In the presence of captopril, k_2=0, so that $k_{el,\ \text{with captopril}} = k_1$. In the absence of captopril $k_{el,\ \text{without captopril}} = k_1 + k_2$. The difference between $k_{el,\ \text{with captopril}}$ and $k_{el,\ \text{without captopril}}$ is equal to k_2.

The percent contribution of conversion to the total metabolism of Ang I is defined as follows: contribution of conversion to metabolism (%) = $[k_2 / (k_1 + k_2)] \times 100\%$.

Results

Renin, prorenin and angiotensinogen

Renin and prorenin were detectable in unconditioned fetal calf serum- and horse serum-supplemented medium (Figure 2). The Ang I-generating activity corresponding with these renin and prorenin levels was not inhibited by the renin inhibitor CH-732 (5 µmol/L), which is specific for rat renin. Following 5 days of incubation with either cardiomyocytes or fibroblasts, the levels of renin and prorenin in serum-supplemented medium were unchanged.

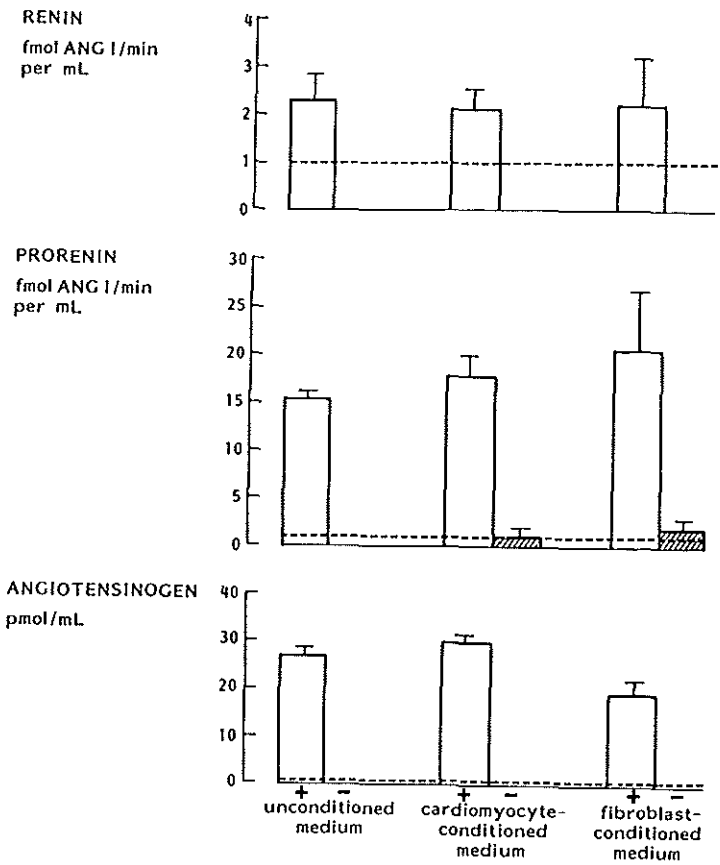

Figure 2. Renin, prorenin, and angiotensinogen levels in unconditioned medium (n=3), cardiomyocyte-conditioned medium (n=5), and fibroblast-conditioned medium (n=5) with (+, open bars) or without (-, hatched bars) serum. Addition of the specific rat renin inhibitor CH-732 (5 μmol/L) did not affect the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

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 (Figure 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.

Angiotensin I-to-II conversion by ACE

Ang I added to unconditioned serum-supplemented medium was rapidly metabolized, Ang II being a major metabolite (Figure 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 hours.

Ang I metabolism in cardiomyocyte - and fibroblasts 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 (Figure 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 (Figure 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).

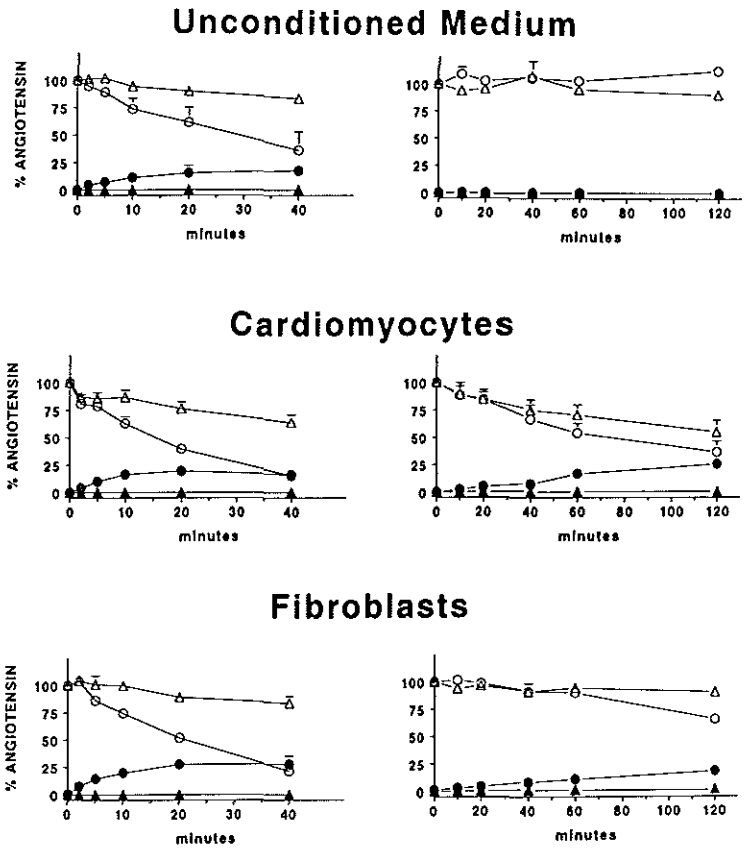

Figure 3. Metabolism of angiotensin I added to unconditioned medium (top panels, n=4), 5-day old cardiomyocyte cultures (middle panels, n=6) and 5-day old fibroblast cultures (bottom panels, n=3) with (left panels) or without (right panels) serum. Data are means ± SD. Open symbols represent Ang I, closed symbols represent Ang II. Circles, experiments without captopril, triangles, experiments with captopril.

Table 1. First order rate constants for degradation (k_1) and conversion (k_2) of angiotensin I added to unconditioned serum-supplemented medium, 5-day old serum-deprived cardiomyocyte cultures and 5-day old serum-deprived fibroblast cultures. The percentage of metabolism due to angiotensin I-to-II conversion is given for each condition.

	unconditioned serum-supplemented medium	cardiomyocyte-conditioned medium	fibroblast-conditioned medium
n	4	6	3
k_1 (h^{-1})	0.17 ± 0.03	0.31 ± 0.09	0.05 ± 0.01
k_2 (h^{-1})	1.69 ± 0.77	0.42 ± 0.17	0.17 ± 0.01
% conversion	92.9 ± 10.3	55.8 ± 9.4	76.1 ± 3.6

Values are means ± SD.

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).

Table 2. Angiotensin I and II levels in unconditioned serum-supplemented medium, cardiomyocyte-conditioned serum-free medium, fibroblast-conditioned serum-free medium, and in cell lysates of serum-deprived cardiomyocytes and fibroblasts.

MEDIUM	Ang I fmol/mL	Ang II fmol/mL
unconditioned serum-supplemented	1.3 ± 0.3	0.4 ± 0.3
cardiomyocyte-conditioned serum-free	<0.7 ± 0.3	< 0.1
fibroblast-conditioned serum-free	<0.6±0.3	< 0.1
CELL LYSATES	Ang I fmol/10^6 cells	Ang II fmol/10^6 cells
serum-deprived cardiomyocytes	< 0.3	< 0.2
serum-deprived fibroblasts	< 0.3	< 0.2

Ang is angiotensin. Values are means ± SD. If one or more values was below the detection limit, this is denoted as < mean ± SD. If all values were below the detection limit this is denoted as < detection limit.

Angiotensin generation during cyclic stretch of cardiomyocytes

Cyclic stretch of cardiomyocytes for 24 hours 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 hours of exposure to ET-1 (Table 3). Ang I in the medium of control cells and cells exposed to stretch was close to the detection limit at 1 hour after the start of the experiment (<0.2-0.7 fmol/mL and <0.2-0.8 fmol/mL, respectively; n=3 in both cases) 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.

Table 3. [^{3}H]-Leucine incorporation and total cellular protein in untreated cardiomyocytes (control), cardiomyocytes exposed to cyclic stretch for 24 hours (stretch) and cardiomyocytes incubated with endothelin-1 (ET-1) for 24 hours.

		control	stretch	ET-1
incorporated [^{3}H]-leucine	dpm/well	5992 ± 215	7300 ± 288*	9479 ± 397*
total cellular protein	mg/well	0.15 ± 0.01	0.17± 0.01*	0.18 ± 0.01*

Values are means ± SD. *P <0.05 vs. control.

Discussion

This study indicates that cultured neonatal rat cardiomyocytes and fibroblasts do not synthesize renin, 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 cardiac cells do appear to synthesize ACE, and thus are capable of converting Ang I into Ang II.

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 investigated medium obtained from cells maintained in the absence of serum as well as medium of cells kept in the presence of serum. The specific rat renin inhibitor CH-732[30,36] was used 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.[17,37] 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] The receptor involved in the binding process is the mannose 6-phosphate receptor, which is identical with the insulin-like growth factor II receptor.

Uptake of renin and/or prorenin might also explain the immunoreactivity for renin described by Dostal et al.[19] in neonatal rat cardiomyocytes and fibroblasts. These cells were serum-deprived for 5 days after a 48-hour incubation period in the presence of 10% newborn calf serum. Similarly, the Ang I generating activity detected in freshly isolated adult rat cardiomyocytes[18] might be attributed to renin sequestered from the circulation.

In the present study we were unable to demonstrate Ang I generating activity in cells that had been serum-deprived for 5 days. Possibly the intracellular metabolism of renin and prorenin following internalization was too rapid to allow detection of Ang I generating activity after 5 days of incubation under serum-free conditions.

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 the synthesis of angiotensinogen by neonatal rat cardiomyocytes or fibroblasts.

In view of the absence of renin, prorenin or 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 were sometimes measured directly by radioimmunoassay without prior SepPak extraction and/or HPLC separation. Furthermore, it must be kept in mind that, in view of the levels measured in cardiac tissue in vivo (Ang I, ≈ 5 fmol/g; Ang II, ≈ 20 fmol/g),[3,4,39,40] 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 mL 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 (from 5 to 450 pmol/L), whereas the Ang I level in the medium (5 pmol/L) did not change. The increase in Ang II, which occurred within 10-30 min after stretch had been initiated, was not affected by captopril. These data were interpreted as evidence for release of intracellularly stored Ang II; this Ang II is assumed to be responsible for the hypertrophic response of cardiomyocytes after the induction of stretch.[26-29] Immunoelectron microscopy confirmed the existence of secretory granule-like structures containing Ang II in ventricular cardiomyocytes prior to stretch (concentration ≈ 5 fmol/10^6 cells) in the study by Sadoshima and colleagues.[27] In contrast, Dostal[23] localized intracellular Ang I and II in the perinuclear region of neonatal rat serum-deprived cardiomyocytes and fibroblasts.

According to this latter study, the levels of Ang I and II in both cardiomyocytes and fibroblasts were approximately 25 pmol and 5 pmol per 10^6 cells (or ≈ 5000 and ≈ 500 times the levels measured by others per g heart tissue), respectively.

Without RAS component synthesis by cardiac cells, what might be the source of intracellular Ang II? Since Ang II is synthesized in the medium during the incubation of the cells in the presence of serum, it might be taken up by the cells, for instance via AT_1-receptor mediated endocytosis.[9] Release could then occur after the application of stretch. We studied this possibility by measuring the angiotensin levels in the cells after 5 days of incubation with serum and in the medium at various time points after the initiation of cyclic stretch. Ang I and II were 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 $\approx 10^{-8}$ M.[28] Although in our study the Ang II concentrations in the medium were $< 10^{-13}$ M, the well-known increase in cellular protein synthesis did occur in response to stretch. It appears therefore that the stretch-induced hypertrophic response of cardiomyocytes in the present study is independent of Ang II. In support of this assumption, we could not inhibit the stretch-induced hypertrophic response in cardiomyocytes with the AT_1 receptor antagonist losartan (unpublished observations). Similarly, others observed Ang II-independent hypertrophic responses in isolated perfused hearts (which do not contain renin[31]) and angiotensinogen-deficient cardiomyocytes.[41-43]

Taken together, our data do not support the concept of cardiac angiotensin generation independent of the kidney and liver. This does not mean that angiotensins are not generated locally in the heart. In fact, in a previous study in pigs we have shown that the majority of cardiac Ang I and II is synthesized at tissue sites, and not derived from the circulation.[40] Most likely, renin and/or prorenin need to be taken up from the circulation in order to generate angiotensins locally in the heart. Mannose 6-phosphate receptors, present on cardiomyocytes and fibroblasts, might be involved in the uptake process of renin and prorenin.[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.

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CHAPTER 3

Angiotensin II-mediated Growth and Antigrowth Effects in Cultured Neonatal Rat Cardiac Myocytes and Fibroblasts.

Abstract

Angiotensin II (Ang II) stimulates cardiovascular growth and remodeling via AT_1 receptors. Recent experiments have shown that Ang II may also exert antiproliferative effects via AT_2 receptors. We studied the effects of Ang II on protein and DNA content and synthesis rate in unstimulated and endothelin-1 (ET-1)-stimulated neonatal rat cardiomyocytes and fibroblasts, isolated from 1-3-day-old Wistar strain pups. Total protein and total DNA, as well as [^{3}H]leucine and [^{3}H]thymidine incorporation were measured following incubation with either vehicle, Ang II, ET-1 or Ang II + ET-1, both in the presence or absence of the AT_1 receptor blocker losartan or the AT_2 receptor blocker PD123319. In myocytes, ET-1 increased total protein (+38% relative to control) as well as [^{3}H]leucine (+66%) and [^{3}H]thymidine (+77%) incorporation. Ang II did not affect any of these parameters, nor did it influence the ET-1-induced responses. However, in the presence of PD123319 Ang II stimulated [^{3}H]leucine (+24%) and [^{3}H]thymidine (+30%) incorporation. In fibroblasts, ET-1 and Ang II did not significantly affect total DNA and [^{3}H]thymidine incorporation. Ang II tended to increase total protein in these cells, an effect which was significant only in the presence of PD123319 (+17%). Ang II stimulated [^{3}H]leucine incorporation (+24%) in fibroblasts. This effect was absent with losartan and enhanced in the presence of PD123319. These data demonstrate that AT_1 receptor-mediated proliferative effects of Ang II in neonatal cardiac cells may become apparent only when its AT_2 receptor-mediated antigrowth effects are blocked. The net growth effect of Ang II therefore depends on the cellular AT_1/AT_2 receptor ratio. Ang II does not appear to interfere with ET-1-induced effects.

Introduction

Angiotensin (Ang)II, formed from Ang I by angiotensin-converting enzyme (ACE), is a potent vasoconstrictor with growth-promoting properties. Its effects are mediated by specific receptors located on the cell surface. So far, at least two receptors for Ang II have been characterized: AT_1 and AT_2, selectively blocked by losartan and PD123319, respectively.[1] Both receptor types have been identified, in varying proportions, in membranes prepared from cardiac tissue.[2-4] The density of Ang II receptors appears to be regulated developmentally, the number of receptors reaching a maximum shortly after birth, and decreasing to lower values thereafter.[2,5,6] Receptor binding studies in rat isolated cardiac cells support the findings on developmental changes in Ang II receptor density. AT_2, but not AT_1, receptors decrease to low numbers after birth, both in cardiomyocytes and cardiac fibroblasts, whereas adult cardiac cells appear to have AT_1 receptors only.[7-9]

Ang II has been reported to induce hypertrophy of neonatal rat cardiomyocytes and hyperplasia of neonatal rat cardiac fibroblasts.[10-13] However, these growth-stimulatory effects of Ang II vary widely and could not always be confirmed.[14,15] Both responses are mediated via the AT_1 receptor subtype.[10,11] Stimulation of this G-protein coupled receptor results in activation of phospholipase C, production of inositolphosphates and a rise in intracellular calcium.[16,17] The function of the cardiac AT_2 receptor is not yet known. Its presence in the fetal and neonatal heart suggests that it may be involved in cardiac growth and development. The AT_2 receptor is coupled to an inhibitory G-protein, and stimulation of this receptor leads to inhibition of protein-tyrosine phosphatase.[18,19] In rat vascular smooth muscle cells transfected with the AT_2 receptor, as well as in coronary endothelial cells expressing low numbers of AT_2 receptors, AT_2 receptor stimulation resulted in anti-growth effects.[20,21]

It is likely that the previously described effects of Ang II on neonatal rat cardiac cell growth are the net effect of both stimulatory (AT_1-mediated) and inhibitory (AT_2-mediated) responses. It was the aim of the present study to investigate the stimulatory and inhibitory effects of Ang II on protein and DNA synthesis in neonatal rat cardiomyocytes and cardiac fibroblasts.

This was done under basal conditions and after stimulation of the cells with endothelin-1 (ET-1), because inhibitory effects of Ang II might be detectable after stimulation only. We also studied the early intracellular activation of phospholipase C by Ang II and ET-1 by measuring inositolphosphate accumulation. Cells were isolated from cardiac ventricles of 1-3-day-old rats, because at that age the heart shows the highest density of AT_1 and AT_2 receptors.[2,5,6] ET-1 was chosen as a stimulus in view of its known strong effects on protein and DNA synthesis in cardiac cells, which are mediated via the same intracellular second messenger pathways as described for Ang II.[13,22,23] In addition, endogenous ET-1 has been reported to contribute to the Ang II-induced effects in an autocrine/paracrine fashion, both in cardiomyocytes and cardiac fibroblasts.[13,22]

Materials and Methods

Reagents

Trypsin (type III) was from Sigma Chemical Co, St. Louis, MO, USA. Fetal calf serum, horse serum, endothelin-1 (ET-1), 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. Ang II was obtained from Bachem, Bubendorf, Switzerland. 24-well tissue plates were obtained from Costar, Cambridge, MA, USA. *myo*-[2-^{3}H]inositol, [^{3}H]leucine and [^{3}H]thymidine were from Amersham, Buckinghamshire, UK and Dowex AG 1-X8 (200-400 Mesh, formiate form) was from Bio-Rad Laboratories, Richmond, CA, USA. Phenanthroline and sodium-azide were from Merck, Darmstadt, Germany. Instagel was obtained from Packard, Meriden, CT, USA. The AT_1 receptor antagonist losartan (Dup 753) was a gift of dr R.D. Smith, DuPont-Merck, Wilmington, DE, USA. The AT_2 receptor antagonist PD123319 was kindly provided by dr D.G. Taylor, Parke-Davis, Ann Arbor, MI, USA.

Cell culture

Primary cultures of neonatal rat ventricular cardiomyocytes and fibroblasts were prepared as described before.[24] Briefly, ventricles from newborn 1-3-day-old Wistar rats were minced and cells were isolated by 8 subsequent trypsinization steps at 30°C. Non-cardiomyocytes were separated from the cardiomyocytes by differential preplating. Cardiomyocytes were seeded in 24-well plates at 1.5 to 1.75 x 10^5 cells/cm^2 giving a confluent monolayer of spontaneously beating cells after 24 hours The preplated cells (fibroblast fraction) were passaged after 4 days to 24-well dishes at 0.75 x 10^5 cells/cm^2. All cells were maintained at 37°C and 5% CO_2 - 95% air in complete 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 hours of culturing in complete medium, cells were further grown in serum-free medium. Experiments were performed after the cells had been serum-deprived for at least one day.

Determination of Ang II stability during incubations

To study the stability of Ang II during incubation, different amounts of Ang II (final concentration in the medium 10^{-6}, 10^{-7} and 10^{-8} M, n=3 for each concentration) were added to cardiomyocyte and fibroblast cultures. Samples of 200 µl were taken at 0, 0.25, 1, 2, 6 and 24 hours after the addition of Ang II. The samples were collected in vials, containing 1.25 mM phenanthroline and 6.25 mM disodium EDTA (final concentrations in the sample) and stored at -70°C. Within two days, Ang II was measured by radioimmunoassay as described before.[25] In short, samples were vacuum-dried, dissolved in 0.25 M Tris-buffer, pH 7.4, containing 0.35% bovine serum albumin and 0.02% sodium azide, and incubated with ^{125}I-Ang II and Ang II antiserum at 4°C for 24 hours. Separation of bound and free Ang II was achieved by charcoal adsorption, and both the supernatant and the pellet were counted in a γ-counter. The Ang II antiserum crossreacted with Ang III (55%), Ang (3-8) (73%) and Ang (4-8) (100%), but not (<0.2%) with Ang (1-7).[26]

Measurement of water-soluble inositolphosphates

Cardiomyocytes and fibroblasts were labelled with 2 µCi *myo*-[2-^{3}H]inositol/ml for 24 hours in medium without serum.[24] The cells were then washed and preincubated for 15 min with incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 0.44 mM NaH_2PO_4; 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 0.2% glucose; 20 mM HEPES; pH 7.4) in the presence of vehicle (medium), losartan (concentration range from 10^{-8} M to 10^{-5} M) or PD123319 (10^{-6} M) at 37°C and aerated with 5% CO_2 - 95% air. To stimulate the cells, vehicle (i.e., incubation medium) or agonist (Ang II or ET-1; concentration range 10^{-9} to 10^{-7} M) was added to the incubation buffer without buffer change, in the presence of 10 mM LiCl to inhibit inositolphosphatase activity. Incubations were terminated after 30 min. The cells were then rapidly washed with ice-cold buffer followed by two successive extractions with 250 µl ice-cold 4% (w/v) $HClO_4$, after which lipids were extracted with 2 x 250 µl ice-cold methanol:HCl (100:1, v/v). The perchloric acid extract, containing the water-soluble intracellular products, was rapidly neutralized by adding a solution of 2 M KOH and 1 M K_2CO_3. The water-soluble inositolphosphates ($InsP_n$) in the perchloric acid extract were then quantified by chromatography on Dowex AG 1-X8 as originally described by Berridge *et al.*[27] Amounts of $InsP_n$ are expressed as percentage of the total cellular amount of [2-^{3}H] inositol-labelled products, defined as the sum of water-soluble inositol-containing products and the inositol-containing lipids. To each sample 5 ml scintillation fluid (Instagel) was added, and samples were counted for 5 min in a β-counter.

Determination of total cellular protein and DNA

Cells were pre-incubated with either vehicle, losartan (10^{-6} M) or PD123319 (10^{-6} M) for 30 min. Next, vehicle, Ang II (10^{-8} M), ET-1 (10^{-8} M) or Ang II + ET-1 (both 10^{-8} M) were added to the cell cultures for 48 hours. After this incubation period the cells were washed twice with 500 µl ice-cold PBS. In order to dissolve cellular protein and DNA, 250 µl 1 M NaOH was added to each well. This was followed by an overnight incubation at 4°C, after which the cellysates were collected. Protein was measured by the Bradford assay, using bovine serum albumin in 1 M NaOH as standard.[28] DNA was measured fluorimetrically with 4',6-diamidino-2-phenylindole-dihydrochloride, using salmon sperm DNA in 1 M NaOH as a standard.[29]

[^{3}H]Leucine incorporation

Protein synthesis rate was determined by quantitating [^{3}H]leucine incorporation.[22] Briefly, cardiomyocytes or fibroblasts were pre-incubated for 0.5 hour in 500 µl serum-free medium with 1 µCi [^{3}H]leucine/ml, in the presence of vehicle, losartan (10^{-6} M) or PD123319 (10^{-6} M). After this period, either vehicle, Ang II (10^{-8} M), ET-1 (10^{-8} M), or Ang II + ET-1 (both 10^{-8} M) were added and the incubation was continued for 24 hours. Subsequently, the medium was discarded and cells were fixed overnight with 500 µl 10% trichloric acid (TCA). Cells were rinsed three times with 10% TCA to remove unincorporated label and then lysed during 6 hours with 500 µl 1 M NaOH. The lysate was transferred to scintillation vials. After neutralisation with 500 µl 1 M HCl, 5 ml scintillation fluid (Instagel) was added, and samples were counted for 5 min in a β-counter.

[^{3}H]Thymidine incorporation

DNA synthesis rate was determined by quantitating [^{3}H]thymidine incorporation.[30] Briefly, cardiomyocytes or fibroblasts were pre-incubated for 0.5 hour in 500 µl serum-free medium in the presence of vehicle, losartan (10^{-6} M) or PD123319 (10^{-6} M). After this period either vehicle, Ang II (10^{-8} M), ET-1 (10^{-8} M), or Ang II + ET-1 (both 10^{-8} M) were added. After 18 hours of incubation, [^{3}H]thymidine (1 µCi/ml) was added for 6 hours Subsequently, the medium was discarded and cells were fixed overnight with 500 µl 10% TCA. To remove unincorporated label, cells were rinsed three times with 10% TCA and then lysed during 6 hours with 500 µl 1 M NaOH. The lysate was transferred to scintillation vials. After neutralisation with 500 µl 1 M HCl, 5 ml scintillation fluid was added, and samples were counted for 5 min in a β-counter.

Statistics

The data are reported as percentage change relative to control values. Data obtained in experiments in the presence of losartan or PD123319 are reported as percentage change relative to control with losartan or PD123319. Data are given as mean±SEM of at least 5 experiments (each measured in quadruplicate).

One-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (Student's *t* test, with Bonferroni correction) was used for comparison between groups. Values of $P < 0.05$ were considered significant.

Results

Ang II stability during incubations

Ang II, when added to cardiomyocytes or fibroblasts in a concentration of 10^{-8} M, was slowly metabolized with a half-life of 6.9±0.7 and 13.3±1.7 h (n=3) respectively. The half life was not different at concentrations of Ang II that were 10 or 100 times higher (data not shown), indicating that Ang II metabolism at these concentrations followed first-order kinetics. Based on these data, we decided to add Ang II only once at the beginning of each experiment.

Formation of water-soluble inositolphosphates

The ability of ET-1 and Ang II to evoke acute production of $InsP_n$ was tested in both cardiomyocytes and fibroblasts. $InsP_n$ accumulation was stimulated dose-dependently by ET-1 in cardiomyocytes, and by Ang II in fibroblasts (Figure 1). For both ET-1 and Ang II the lowest concentration at which near-maximal effects occurred was 10^{-8} M. Ang II had only marginal effects on $InsP_n$ accumulation in cardiomyocytes, while ET-1 was ineffective in fibroblasts. The modest effects of Ang II in cardiomyocytes might be explained by the low angiotensin receptor density of these cells.[8]

Complete blockade of the Ang II-induced effects in fibroblasts was obtained with losartan in concentrations of 10^{-6} M and higher, whereas PD123319 at such concentrations had no effect (Figure 2). This indicates that the Ang II-induced effects on phospholipase C activation were mediated via AT_1 receptors. When Ang II was added 48 h after the addition of losartan (10^{-6} M), the AT_1 receptor blocker still effectively blocked the Ang II-induced response (n=2; data not shown). Losartan (10^{-6} M) nor PD123319 (10^{-6} M) affected the ET-1-induced effects on $InsP_n$ accumulation in cardiomyocytes (n=2; data not shown).

Based on these findings, it was decided to use Ang II and ET-1 in a concentration of 10^{-8} M and losartan in a concentration of 10^{-6} M in all further experiments. PD123319 was also used in a concentration of 10^{-6} M, based upon data obtained by others.[10,21,31] Furthermore, both receptor antagonists were added only once at the beginning of each experiment in all further studies.

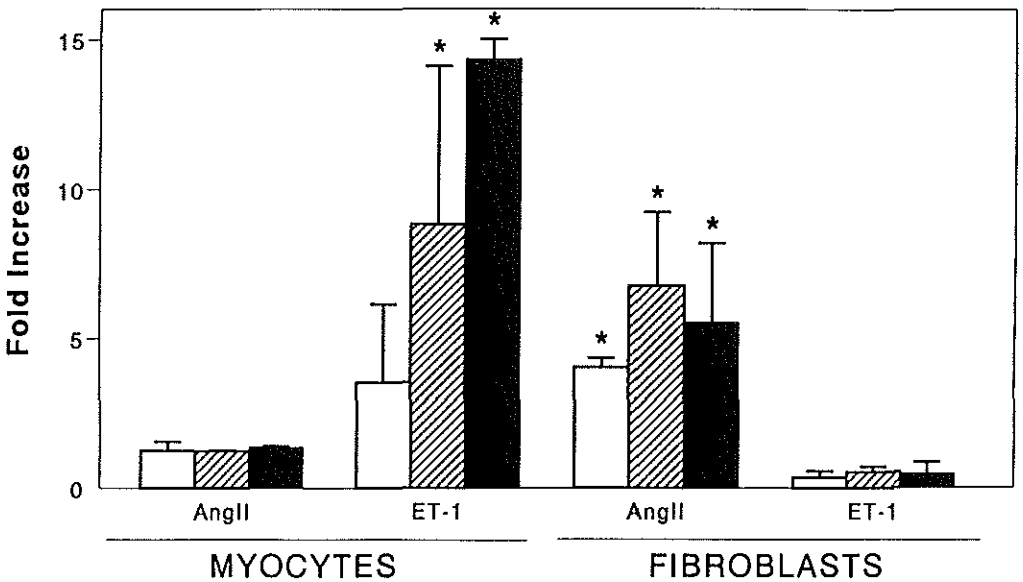

Figure 1. Effect of angiotensin II (Ang II) and endothelin-1 (ET-1) on inositolphosphate accumulation in cardiomyocytes and cardiac fibroblasts. The agonists were added in a concentration of 10^{-9} M (open bars), 10^{-8} M (hatched bars) or 10^{-7} M (black bars). The increase in inositolphosphate accumulation is expressed as fold increase relative to control. Data are mean±SEM of 3 experiments each (a * denotes that the increase was larger than 1-fold in all three experiments).

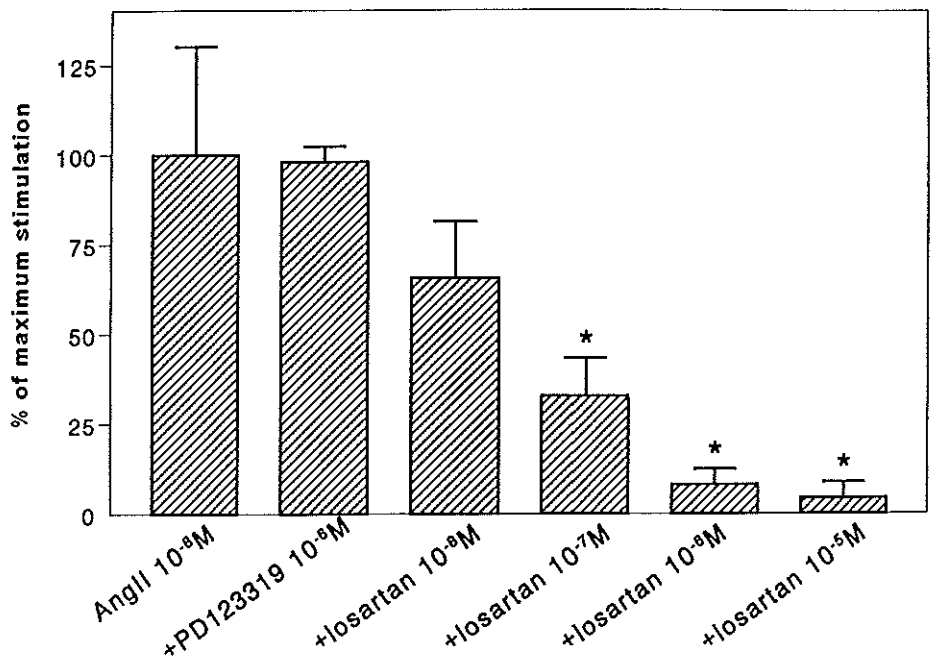

Figure 2. Effects of losartan and PD123319 on the inositolphosphate accumulation induced by angiotensin II (Ang II, 10^{-8} M) in cardiac fibroblasts. Losartan significantly (*, $P < 0.05$) inhibited the response at concentrations of 10^{-7} M and higher, whereas PD123319 had no effect at a dose of 10^{-6} M. Data are mean±SEM of 4 experiments each.

Total cellular protein and [^{3}H]leucine incorporation

The total protein contents of cardiomyocyte and fibroblast cultures, incubated for 48 hours with vehicle, were 86±18 and 42±8 µg/well, respectively (n=6). Neither losartan nor PD123319 affected total cellular protein in both cell types (data not shown). Ang II, added alone or in the presence of losartan, also did not affect total cellular protein (Figures 3A and 4A). Ang II, added in the presence of PD123319, tended to increase total cellular protein in cardiomyocytes, but the difference relative to control was not significant. In fibroblasts, Ang II added in the presence of PD123319 increased total cellular protein by 17±6% above control.

ET-1 increased total cellular protein in cardiomyocytes by 38±6% and had no effect in fibroblasts. The ET-1-induced effects in cardiomyocytes were not affected by Ang II, losartan, PD123319, or a combination of these drugs (Figure 3A). In fibroblasts, the effect of Ang II+ET-1 in the presence of PD123319 was similar to that observed with Ang II and PD123319 alone, suggesting that ET-1 did not interfere with the Ang II-mediated effects (Figure 4A).

[^{3}H]leucine incorporation by cardiomyocyte and fibroblast cultures (26 489±5706 and 10 801±3175 d/min/well, respectively, after 24 hours of incubation in the presence of vehicle) was not affected by losartan or PD123319 (data not shown). Ang II increased [^{3}H]leucine incorporation in fibroblasts by 24±5% above control (Figure 4B). Losartan, but not PD123319, inhibited this Ang II-mediated effect in fibroblasts. In cardiomyocytes, Ang II alone or in the presence of losartan did not affect [^{3}H]leucine incorporation, whereas in the presence of PD123319, Ang II increased [^{3}H]leucine incorporation by 24±10% above control (Figure 3B).

Incubation of cardiomyocytes with ET-1 led to an increase in [^{3}H]leucine incorporation of 66±11% above control, an effect which was not influenced by Ang II, losartan, PD123319, or a combination of these drugs (Figure 3B). In fibroblasts, ET-1 caused a small, non-significant increase in [^{3}H]leucine incorporation. The effect of Ang II+ET-1 in these cells equalled the sum of the effects of Ang II and ET-1 alone, and could not be blocked or enhanced with losartan or PD123319 (Figure 4B).

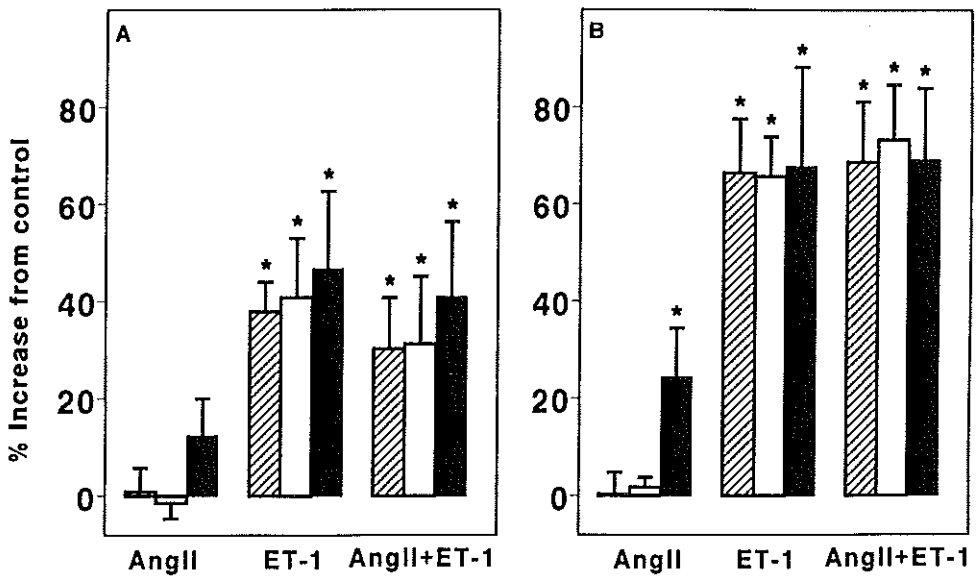

Figure 3. Effect of angiotensin II (Ang II, 10^{-8} M), endothelin-1 (ET-1, 10^{-8} M) or angiotensin II + endothelin-1 (both 10^{-8} M) on total cellular protein content (panel A) and [^{3}H]leucine incorporation (panel B) in cardiomyocytes in the presence of vehicle (hatched bars), losartan (10^{-6} M) (open bars) or PD123319 (10^{-6} M) (black bars). Data are expressed as percentage increase from control (mean±SEM of 5-7 experiments; *, $P < 0.05$ vs. control).

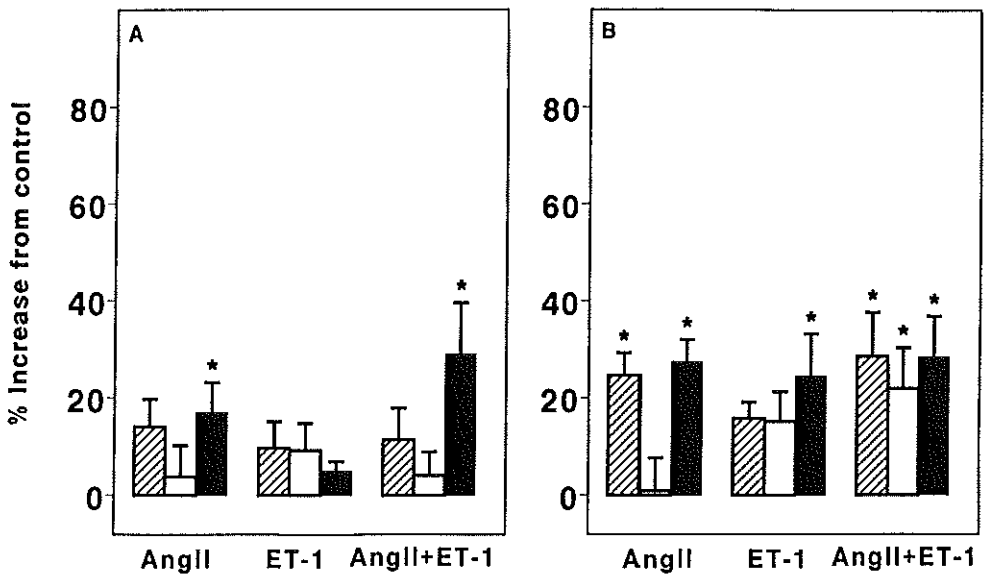

Figure 4. Effect of angiotensin II (Ang II, 10^{-8} M), endothelin-1 (ET-1, 10^{-8} M) or angiotensin II + endothelin-1 (both 10^{-8} M) on total cellular protein content (panel A) and [^{3}H]leucine incorporation (panel B) in cardiac fibroblasts in the presence of vehicle (hatched bars), losartan (10^{-6} M) (open bars) or PD123319 (10^{-6} M) (black bars). Data are expressed as percentage increase from control (mean±SEM of 5-7 experiments; *, $P < 0.05$ vs. control).

Total cellular DNA and [^{3}H]thymidine incorporation

Total cellular DNA was 2.5±0.2 and 1.0±0.2 μg/well in cardiomyocytes and fibroblasts, respectively, after 48 hours of incubation with vehicle. Losartan, PD123319, Ang II or ET-1 or a combination of these drugs did not affect total cellular DNA significantly in either cardiomyocytes or fibroblasts (Figures 5A and 6A).

[^{3}H]thymidine incorporation by cardiomyocyte and fibroblast cultures (24 966±5396 and 16 519±4912 d/min/well, respectively, after 24 hours of incubation in the presence of vehicle) was not affected by losartan or PD123319 (data not shown). Ang II caused a modest (non-significant) increase in [^{3}H]thymidine incorporation in cardiomyocytes, which was absent in the presence of losartan and enhanced to 30±6% ($P < 0.05$) above control with PD123319 (Figure 5B). Ang II had no effect on [^{3}H]thymidine incorporation in fibroblasts, either alone, or in the presence of losartan, PD123319 or ET-1 (Figure 6B). ET-1 increased [^{3}H]thymidine incorporation in cardiomyocytes by 77±21%, an effect which was not influenced by Ang II, losartan, PD123319, or a combination of these drugs (Figure 5B). ET-1 had no effect on [^{3}H]thymidine incorporation in fibroblasts under all circumstances tested (Figure 6B).

Protein/DNA ratio

No significant changes in protein/DNA ratio were observed under all conditions tested in our experiments. Changes in total cellular protein content and protein synthesis rate were paralleled by similar or smaller changes in total cellular DNA content and DNA synthesis rate (Figures 3-6), indicating that cellular hypertrophy, if occurring at all, was of modest proportion.

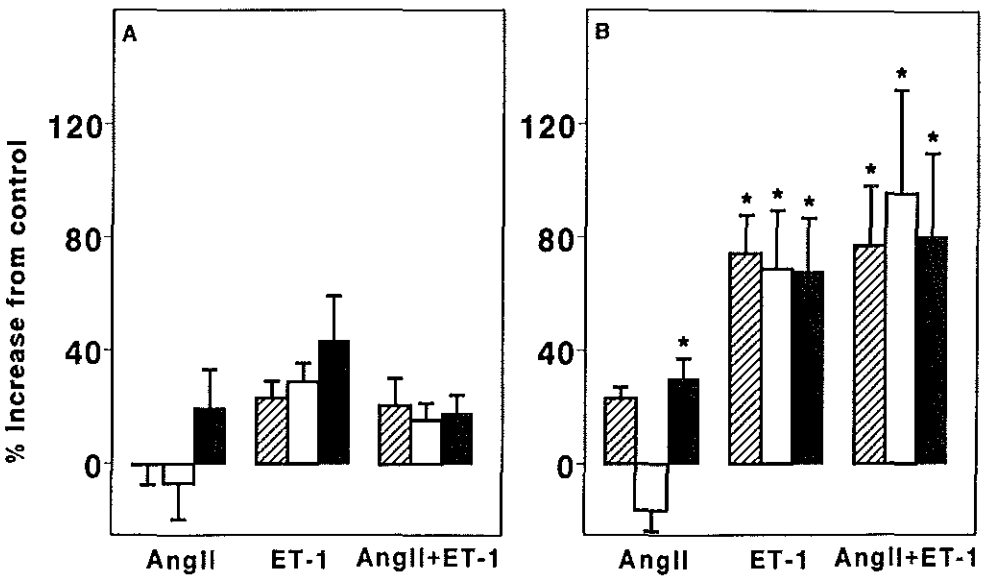

Figure 5. Effect of angiotensin II (Ang II, 10^{-8} M), endothelin-1 (ET-1, 10^{-8} M) or angiotensin II + endothelin-1 (both 10^{-8} M) on total cellular DNA content (panel A) and [^{3}H]thymidine incorporation (panel B) in cardiomyocytes in the presence of vehicle (hatched bars), losartan (10^{-6} M) (open bars) or PD123319 (10^{-6} M) (black bars). Data are expressed as percentage increase from control (mean±SEM of 5-7 experiments; *, $P < 0.05$ vs. control).

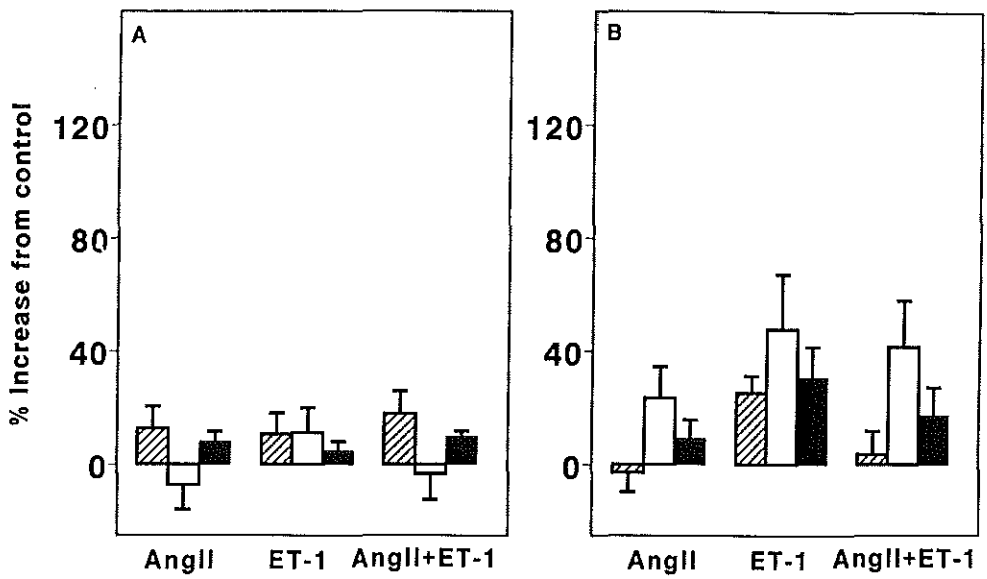

Figure 6. Effect of angiotensin II (Ang II, 10^{-8} M), endothelin-1 (ET-1, 10^{-8} M) or angiotensin II + endothelin-1 (both 10^{-8} M) on total cellular DNA content (panel A) and [^{3}H]thymidine incorporation (panel B) in cardiac fibroblasts in the presence of vehicle (hatched bars), losartan (10^{-6} M) (open bars) or PD123319 (10^{-6} M) (black bars). Data are expressed as percentage increase from control (mean±SEM of 5-7 experiments; *, $P < 0.05$ vs. control).

Table 1. Summary of the effects of angiotensin II (Ang II, 10^{-8} M), endothelin-1 (ET-1, 10^{-8} M), or Ang II + ET-1 (both 10^{-8} M) on total cellular protein content, [^{3}H]leucine incorporation, total cellular DNA content and [^{3}H]thymidine incorporation in cardiomyocytes (M) and cardiac fibroblasts (F). Experiments were performed without AT receptor blockade (control), with AT_1 receptor blockade (losartan) or with AT_2 receptor blockade (PD123319).

	total cellular protein content		[^{3}H]leucine incorporation		total cellular DNA content		[^{3}H]thymidine incorporation	
	M	F	M	F	M	F	M	F
Ang II								
control	0	0	0	+	0	0	0	0
losartan	0	0	0	0	0	0	0	0
PD123319	0	+	+	+	0	0	+	0
ET-1								
control	+	0	++	0	0	0	++	0
losartan	+	0	++	0	0	0	++	0
PD123319	+	0	++	+	0	0	++	0
Ang II+ET-1								
control	+	0	++	+	0	0	++	0
losartan	+	0	++	+	0	0	++	0
PD123319	+	+	++	+	0	0	++	0

0, no significant increase; +, modest increase (< 50%); ++, large increase (> 50%).

Discussion

The results obtained in this study, summarized in Table 1, demonstrate that Ang II, via AT_2 receptors, may antagonize its AT_1 receptor-mediated growth-stimulatory effects in cardiac cells. It appears therefore that the net growth effect of Ang II in these cells depends on the cellular AT_2/AT_1 receptor ratio. Ang II did not interfere with growth-stimulatory effects induced by ET-1.

We quantified the Ang II- and ET-1-induced effects on cell growth by measuring the total cellular content of protein and DNA as well as the synthesis rate of protein and DNA. This approach allowed us to study whether an increased cellular protein or DNA content was the consequence of enhanced synthesis and/or diminished degradation. Both neonatal rat cardiomyocytes and fibroblasts have the capacity to proliferate.[11,32] Cardiomyocytes lose the capacity to replicate DNA by week 3 of postnatal development.[33] It appeared that under all circumstances where an increase in DNA or protein content was observed, the synthesis rates of these components were also increased. This suggests that enhanced synthesis was the underlying cause of the increase in DNA or protein content. In fact, since the increases in synthesis rate were usually larger than the increase in content, enhanced rather than diminished degradation may have accompanied these increases in synthesis rate. We did not observe a significant increase in protein/DNA ratio, suggesting that hyperplasia rather than hypertrophy had occurred in our experiments, or that the degree of cellular hypertrophy, if occurring, was of modest proportion.

ET-1 potently stimulated protein and DNA synthesis in cardiomyocytes, but was ineffective with regard to these parameters in fibroblasts. The latter may be attributed to the much lower endothelin receptor number in rat cardiac fibroblasts as compared to rat cardiomyocytes.[34,35] Phospholipase C-induced formation of inositoltrisphosphate and diacylglycerol may underly the growth-stimulatory effects of ET-1.[36] Indeed, we found ET-1 to strongly enhance inositolphosphate formation in cardiomyocytes but not fibroblasts (Figure 1).

Ang II did not affect DNA synthesis in either myocytes or fibroblasts and stimulated protein synthesis rate in fibroblasts only. The latter effect is assumed to be AT_1 receptor-mediated and phospholipase C-dependent.[16]

Our data on Ang II-induced inositolphosphate formation are in agreement with such a mechanism. Ang II-induced stimulation of protein and DNA synthesis in neonatal rat cardiomyocytes and fibroblasts has been reported by others[10-13] but the results vary widely and could not always be confirmed.[14,15] Our findings may offer an explanation for these discrepancies. We only observed significant AT_1 receptor-mediated increases in the synthesis rate of both DNA and protein in cardiomyocytes and in the total protein content in fibroblasts in the presence of the selective AT_2 receptor antagonist PD123319. This suggests that the AT_2 receptor may normally inhibit the AT_1 receptor-mediated hypertrophic (fibroblasts) and hyperplastic (cardiomyocytes) effects.

It appears therefore that the balance between AT_1 and AT_2 receptors determines the net effect of Ang II. As the densities of these receptors rapidly change after birth, it is not unlikely that neonatal cells harvested at different times after birth may have different AT_1/AT_2 receptor ratios.[2,5-7] It is even possible that cells in the same culture have different receptor densities and that some cells have AT_1 receptors and others have AT_2 receptors. However, in view of the fact that most of the Ang II-mediated proliferative effects in our study could be demonstrated in the presence of PD123319 only, it seems more likely that both receptors are present on the same cell.

AT_2 receptor-mediated antigrowth effects have been demonstrated earlier in rat coronary endothelial cells.[21] In these cells Ang II inhibited the proliferative effects of bFGF by maximally 50% via AT_2 receptors. In the present study we could not demonstrate an inhibitory action of Ang II on the ET-1-induced proliferative effects in cardiomyocytes. The latter effects are believed to be mediated by ET_A receptors.[22,37] ET-1 was applied in our study because 1) it makes use of the same intracellular second messenger pathways as Ang II to induce proliferation,[37] and 2) it may be involved in the Ang II-induced proliferative effects.[13,22] Although our results do not argue against these findings, they do not support an AT_2 receptor-mediated inhibitory action on ET-1-mediated effects. It is possible that the AT_2 receptor-mediated effects in cardiac cells are relatively modest and could therefore not be detected. However, also under conditions where the ET-1-induced effects on protein synthesis were half maximal (ET-1 concentration 10^{-9} M), no inhibitory effect of Ang II via AT_2 receptor stimulation could be observed (unpublished observation). Thus, alternatively, there may be no crosstalk between AT_2 and ET_A receptors.

We used the antagonists losartan and PD123319 to block AT_1 and AT_2 receptors, respectively. Without the addition of Ang II or ET-1, these inhibitors had no effect on protein or DNA synthesis. It seems therefore that neonatal rat cardiomyocytes and fibroblasts, at least under the serum-free conditions as applied in the present study, do not synthesize Ang II in sufficient quantities to exert proliferative effects. This confirms previous findings on the absence of Ang II in the medium of cardiomyocytes.[38-40] The accelerated rate of protein synthesis known to occur following stretch of neonatal rat cardiomyocytes may be due to an autocrine induction of Ang II synthesis by these cells.[40,41]

How could the AT_2 receptor antagonize the AT_1 receptor-mediated effects? Studies in PC-12W pheochromocytoma cells and in N1E-115 murine neuroblastoma cells indicate that Ang II, via AT_2 receptors, can stimulate protein-tyrosine phosphatases.[42,43] This in turn will lead to inhibition of mitogen-activated protein (MAP) kinases.[44] MAP kinases, which are critical components in cellular processes such as growth and apoptosis,[45,46] are known to be activated by Ang II binding to AT_1 receptors. Huang *et al.* have recently demonstrated in rat brain neuronal cultures that Ang II has opposite actions on MAP kinases via AT_1 and AT_2 receptors.[31]
Such opposing actions may provide an intracellular basis for the modulatory effect of Ang II receptors on cell growth. The occurence of apoptosis, often studied by quantifying DNA fragmentation, could not be evaluated in the present study, because our method of DNA quantification does not allow us to draw conclusions on the presence of DNA fragments.

The capacity of an agonist to induce both proliferative and antiproliferative effects after stimulation of different receptor subtypes enables the cell to adapt to different physiological circumstances. This may not only be important during cardiac development, but also under pathological conditions. For instance, it has been described that the AT_2 receptor is re-expressed in rat hearts with left ventricular hypertrophy or after myocardial infarction.[47,48] Our data illustrate the possible consequences of such receptor changes.

While the preparation of our manuscript was at its final stage, the recent study by Booz and Baker came to our attention.[49] These authors demonstrated that Ang II (10^{-6} M) exerted a modest effect on protein synthesis in neonatal rat cardiomyocytes, which was markedly enhanced in the presence of an AT_2 receptor antagonist. Our study confirms these findings and addresses additionally the AT_2 receptor-mediated antigrowth effects in neonatal rat cardiac fibroblasts as well as in ET-1-stimulated cardiac cells.

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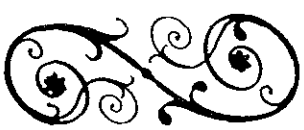
CHAPTER 4

Mannose 6-Phosphate Receptor-Mediated Internalization and Activation of Prorenin by Cardiac Cells.



Abstract

The binding and internalization of recombinant human renin and prorenin (2500 μU/mL), and the activation of prorenin were studied in neonatal rat cardiac myocytes and fibroblasts cultured in a chemically defined medium. Surface-bound and internalized enzyme were distinguished by the addition of mannose 6-phosphate to the medium, by incubating the cells both at 37°C and 4°C, and by the acid-wash method. Mannose 6-phosphate inhibited the binding of renin and prorenin to the myocyte cell surface in a dose-dependent manner. At 37°C, after incubation at 4°C for 2 hours, 60-70% of cell surface-bound renin or prorenin was internalized within 5 minutes. Intracellular prorenin was activated but extracellular prorenin was not. The half-time of activation at 37°C was 25 minutes. Ammonium chloride and monensin, which interfere with the normal trafficking and recycling of internalized receptors and ligands, inhibited the activation of prorenin. Results obtained with cardiac fibroblasts were comparable to those in the myocytes. This study is the first to show experimental evidence for the internalization and activation of prorenin in extrarenal cells by a mannose 6-phosphate receptor-dependent process. Our findings may have physiological significance in light of recent experimental data indicating that angiotensin I and II are produced at cardiac and other extrarenal tissue sites by the action of renal renin and that intracellular angotensin II can elicit important physiologic responses.

Introduction

A local renin-angiotensin system in the heart is often invoked to explain the long-term beneficial effects of angiotensin-converting enzyme inhibitor treatment on postinfarction cardiac remodelling and failure and in left ventricular hypertrophy.

Experimental studies of the isolated perfused rat heart have indeed demonstrated that the heart is capable of producing Ang I and Ang II.[1,2] However, attempts to provide evidence that the cardiac production of these peptides depends on in situ synthesized renin have failed so far. Rather, perfusion experiments in the isolated rat heart as well as studies of the effects of nephrectomy on the cardiac tissue levels of Ang I and II in the pig, indicate that the local production of these peptides depends on kidney-derived renin.[1-3]

Uptake of renin from the circulation by vascular tissues and by the heart has been reported and appears to contribute to blood pressure control.[3-5] Binding of rat renin to rat cardiac and other tissue membranes has also been reported, as has binding of human renin to human umbilical vein endothelial cells.[6-8] A recent study has demonstrated specific receptor binding of human renin to cultured human mesangial cells.[9]

Recombinant human renin synthesized by *Xenopus* oocytes and mouse L cells contains phosphomannosyl residues that are recognized by specific receptors (mannose 6-phosphate receptors), as does human prorenin synthesized by CHO cells.[10,11] Two different mannose 6-phosphate receptors have been identified; one is dependent on divalent cations, the other not. Both mannose 6-phosphate receptors function in the process of intracellular lysosomal enzyme sorting, and the cation-independent receptor is also capable of binding and internalizing extracellular lysosomal enzymes.[12,13]

The aim of the present study is to explore the possibility that cardiac myocytes and fibroblasts are capable of binding and internalizing renin and prorenin, and that these processes are mannose 6-phosphate receptor-mediated. We also adressed the possibility that receptor-mediated endocytosis of prorenin results in its activation.

Materials and Methods

Reagents

Trypsin (type III), bovine serum albumin, monensin, mannose 6-phosphate, mannose 1-phosphate and glucose 6-phosphate were from Sigma Chemical Co. Fetal calf serum, horse serum, penicillin and streptomycin were purchased from Boehringer-Mannheim. DMEM and Medium 199 were from Gibco. Six-well tissue plates were obtained from Costar.

Cell culture

All experiments were performed according to the regulations of the Animal Care Committee of Erasmus University, Rotterdam, Netherlands, in accordance with the *Guiding Principles in the Care and Use of Animals* approved by the American Physiological Society.

Primary cultures of neonatal rat ventricular cardiomyocytes and fibroblasts were prepared as described before.[14] Briefly, ventricles from newborn 1-3 day old Wistar rats were minced and cells were isolated by eight subsequent trypsinization steps at 30°C. Noncardiomyocytes were separated from the cardiomyocytes by differential preplating. Cardiomyocytes were seeded in 6-well plates at 1.5 x 10^6 cells/well giving a confluent monolayer of spontaneously beating cells after 24 hours. The preplated cells (fibroblast fraction) were passaged after 4 days to 6-well dishes at 0.75 x 10^6 cells/well. The cells were maintained in a humidified incubator at 37°C and 5% CO_2 in air, in growth medium consisting of DMEM and Medium 199 (4:1, v/v), supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin/mL and 100 µg streptomycin/mL for 72 hours. The incubations with renin and prorenin (see below) were carried out under serum-free conditions. Prior to the start of each experiment, cells were washed with 3 mL warm (37°C) 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). The cells were then preincubated either at 37°C or 4°C for 30 minutes with 0.9 mL incubation medium consisting of DMEM and Medium 199 (4:1, vol/vol), supplemented with 1% (wt/vol) bovine serum albumin.

Prorenin and renin

Recombinant human prorenin was a kind gift of dr W. Fischli (Hoffmann-LaRoche, Basel, Switzerland). It was produced in CHO cells transfected with a vector containing human prorenin cDNA. It was partially purified, to remove traces of renin, by Cibacron Blue Sepharose affinity chromatography.[15] The intrinsic renin activity of the prorenin preparation, prior to proteolytic activation, was less than 2% of the activity after complete proteolytic activation. After proteolytic activation the prorenin preparation contained approximately 8.5×10^6 μU/mL renin. Recombinant human renin was prepared by the proteolytic activation of the recombinant human prorenin with immobilized trypsin.[3,15] Sepharose-bound trypsin (final concentration 0.25 mg trypsin/mL) was added to the prorenin preparation and the mixture was kept at 4°C for 72 hours Trypsin was then removed by centrifugation. The prorenin and renin preparations were stored at -70°C.

Incubation of cells with renin or prorenin at 37 °C or 4 °C

After preincubation at 37°C or 4°C for 30 min in 0.9 mL serum-free medium (see above), experiments were started by the addition of 0.1 mL of a dilution of recombinant human renin or prorenin to the medium (final concentration in the wells approximately 2500 μU/mL). Cells were then incubated at 37°C or 4°C. Incubations were also performed in the presence of mannose 6-phosphate (0.01 μmol/mL to 50 μmol/mL), mannose 1-phosphate (1μmol/mL), glucose 6-phosphate (1 μmol/mL), ammonium chloride (50 μmol/mL) or monensin (0.01 μmol/mL).

At the end of the incubation period, the culture medium was removed and quickly frozen on dry ice. Each well was washed three times with 3 mL ice-cold PBS. Renin and prorenin were not detectable in the last PBS wash. Cells were then lysed in 0.5 mL ice-cold PBS containing 0.2% Triton X-100, and the cell lysates were quickly frozen on dry ice. Medium and cell lysate were stored at -70°C until assayed for renin and prorenin.

To determine the internalized renin or prorenin, the acid-wash method was used.[16] Briefly, after the cells had been washed three times with 3 mL ice-cold PBS, the cells were incubated at 4°C with 1 mL of an acid solution containing 50 mmol/L glycine and 150 mmol/L NaCl, pH 3.0.

After 10 minutes the acid solution was removed and the cells were washed three times with 3 mL ice-cold PBS. Cells were then lysed in 0.5 mL ice-cold PBS containing 0.2% Triton X-100, as described above.

To determine the prorenin that was bound to mannose 6-phosphate receptors on the cell surface, prorenin was measured in the culture medium before and after its displacement from the receptor by mannose 6-phosphate. For this purpose the cells were pre-incubated at 37°C or 4°C with prorenin (2500 μU/mL) for 2 hours. The cells were then washed three times with 3 mL ice-cold PBS. After the last wash 1 mL of fresh incubation medium, free of serum and renin and prorenin, containing mannose 6-phosphate (final concentration 10 μmol/mL) was added, and the incubations were continued at the same temperature as during the preincubation. After this second incubation period, the cells were washed and lysed as described above.

Incubation of cells at 4 °C followed by incubation at 37 °C

To study the kinetics of renin and prorenin internalization and prorenin activation in more detail, cells were incubated with renin or prorenin (final concentration approximately 2500 μU/mL) for two hours at 4°C. After this period, free renin and prorenin were removed by washing the cells three times with 3 mL ice-cold PBS. After the last wash 1 mL of fresh incubation medium at 37°C without renin and prorenin was added, and the cells were incubated at 37°C. The incubations were terminated by washing the cells with ice-cold PBS. The cells were then lysed as described above. Medium and cell lysate were assayed for renin and prorenin. Internalized renin and prorenin were determined with the acid-wash method as described above.

Renin and prorenin measurements

Measurements of renin were made by the enzyme-kinetic assay.[3,15] Briefly, 100 μL sample was incubated for 3 hours with saturating amounts of sheep renin substrate at 37°C and pH 7.4 in the presence of serine protease and angiotensinase inhibitors. The generated Ang I was quantitated by radioimmunoassay. Results were expressed as μU/mL using the international WHO human kidney renin standard, lot 68/356 (WHO International Laboratory for Biological Standards and Control, Potter Bar, Hertfordshire, UK), as a reference.

The Ang I-generating activity we measured in cells that had been exposed to renin in the culture medium represents 'true' renin; the specific inhibitor remikiren,[17] at a concentration of 10^{-8} mol/mL, caused complete inhibition of the Ang I-generating activity of the lysates from these cells.

Prorenin is the inactive biosynthetic precursor of renin. The prorenin-to-renin conversion in vivo is a proteolytic process by which the propeptide is cleaved from the renin part of the molecule. In vitro, prorenin can be activated both nonproteolytically (e.g., by exposure to low pH) and proteolytically by various enzymes. The Ang I-generating activity we measured in the lysates of cells that had been exposed to inactive prorenin in the culture medium represents prorenin that was activated by the cells. This cell-activated prorenin may or may not be identical with renin. We also measured 'total' prorenin, i.e. prorenin that was activated by the cells plus prorenin that was not activated by the cells. For these measurements the samples were incubated for 48 hours at 4°C with plasmin (0.5 casein units per mL) before the incubation with sheep renin substrate.[3] The preincubation with plasmin caused complete proteolytic activation of prorenin. Plasmin from the activation step was inactivated by the serine protease inhibitor aprotinin (final concentration 100 kallikrein-inhibiting units/mL) that had been added to the incubation medium of the Ang I-generating step.

Prorenin that could be measured without preincubation of the samples with plasmin is referred to as "cell-activated" prorenin. Prorenin that was measured in samples that had been pre-incubated with plasmin is referred to as "total" prorenin. Mannose 6-phosphate, ammonium chloride and monensin, in the concentrations we used, had no influence on the renin and prorenin assays.

Statistics

Data were compared using Student's t-test for unpaired observations. A value of $P<0.05$ was considered to be significant.

Results

Figure 1 (top) and figure 2 (top) show the amounts of cell-associated renin and prorenin after 4 hours of incubation of cardiac myocytes at either 4°C or 37°C with renin or prorenin respectively. At 4°C the cells bound both renin and prorenin but internalization occurred only at 37°C. The cell-associated renin or prorenin after 4 hours of incubation amounted to about 5% of the total quantity in the culture medium both at 4°C and 37°C. After incubation at 37°C most of the cell-associated prorenin was resistant to the acid-wash and was in an activated form, whereas at 4°C prorenin was washed away by acid and remained inactive.

Figure 3 shows the increase in cell-associated prorenin over time in the myocytes at 37°C. Cell-associated prorenin reached a maximum in 3-4 hours. It was 120±37 $\mu U/1.5x10^6$ cells (mean±SD, n=9) at 4 hours of incubation, and more than 90% was in an activated form at that time. When, at 2 hours of incubation at 37°C, the prorenin-containing medium was renewed and the cells were incubated for another 2 hours at 37°C, the cell-associated prorenin rose to levels well above the maximum obtained without renewal of the medium, and again more than 90% was in an activated form. The cell-associated prorenin at 4 hours of incubation, 2 hours after renewal of the medium, was 160±41% of the level at 4 hours of incubation without renewal of the medium (n=5, $P<0.05$ for difference from 100%).

When the incubations at 37°C were carried out in the presence of mannose 6-phosphate (10 µmol/mL), cell-associated prorenin at 4 hours of incubation was 24±8 $\mu U/1.5x10^6$ cells (n=5, $P<0.01$ for difference from incubations in the absence of mannose 6-phosphate). Figure 4 shows the effect of different concentrations of mannose 6-phosphate on the levels of cell-associated prorenin at 37°C and 4°C; 50% inhibition was reached at a mannose 6-phosphate concentration in the order of 0.1 µmol/mL. Mannose 1-phosphate (n=3) and glucose 6-phosphate (n=3) at a concentration of 1 µmol/mL were without effect (data not shown).

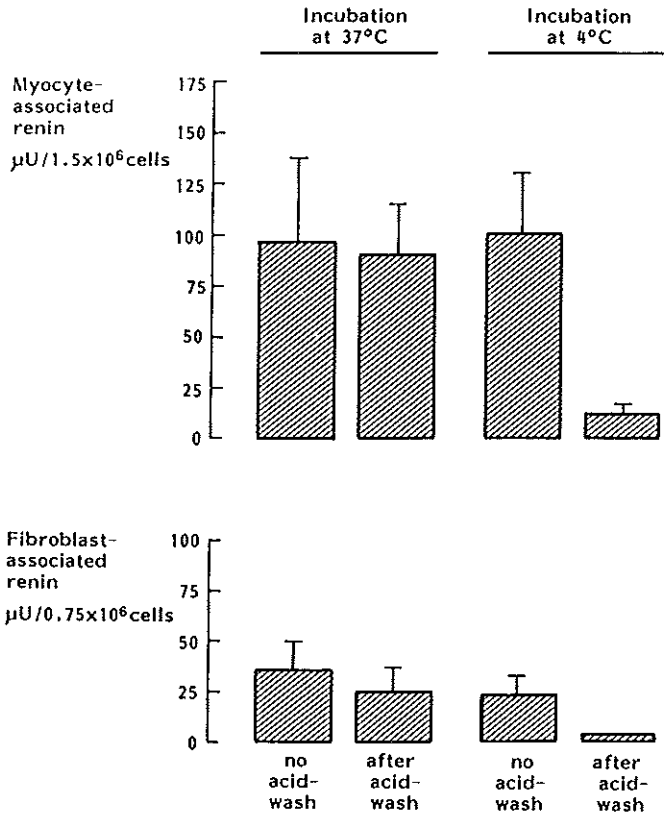


Figure 1. Cell-associated renin after 4 hours of incubation of cardiac myocytes and fibroblasts with renin. The concentration of renin in the culture medium was 2500 µU/mL, and the incubations were performed at 37°C or 4°C. The acid-wash method was used to determine the acid-resistant internalized renin. Data are means±SD, n=6-9.

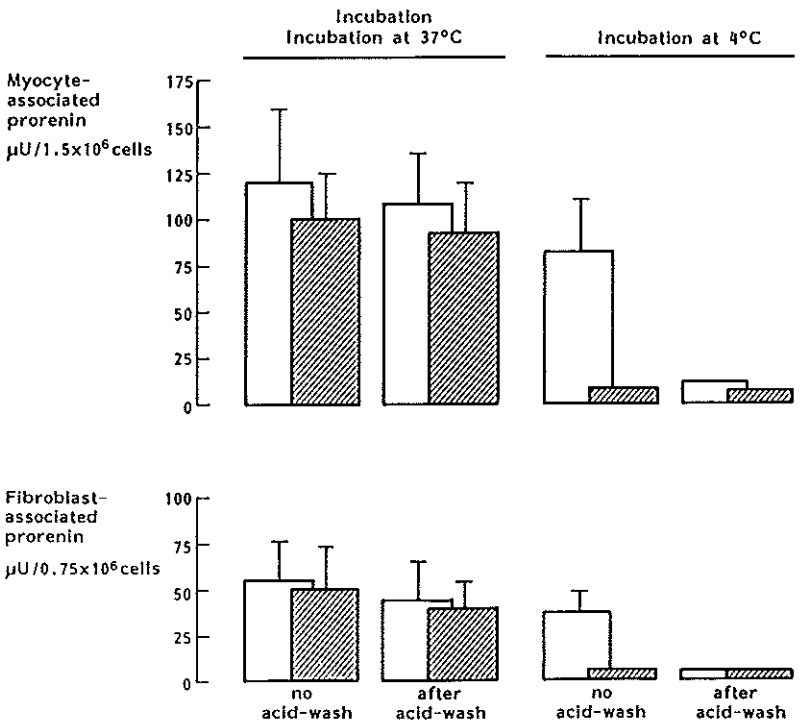


Figure 2. Cell-associated prorenin after 4 hours of incubation of cardiac myocytes and fibroblasts with inactive prorenin. The concentration of prorenin in the culture medium was 2500 μU/mL, and the incubations were performed at 37°C or 4°C. The acid-wash method was used to determine the acid-resistant internalized prorenin. The hatched and open bars represent the cellular levels of cell-activated prorenin and total (cell-activated plus non-activated) prorenin respectively. Data are means±SD, n=6-9.

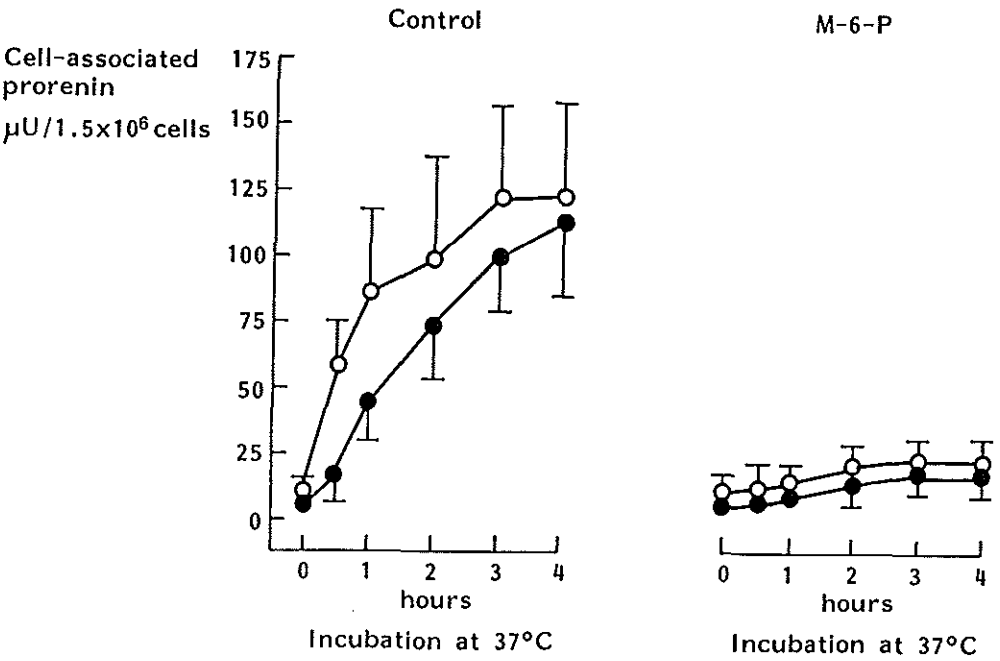


Figure 3. Increase in cell-associated prorenin over time during incubation of cardiac myocytes at 37°C with inactive prorenin (2500 μU/mL) in the absence or presence of mannose 6-phosphate (10 μmol/mL) in the culture medium. The closed and open circles represent the cellular levels of cell-activated prorenin and total (cell-activated plus non-activated) prorenin respectively. Data are means±SD, n=5.

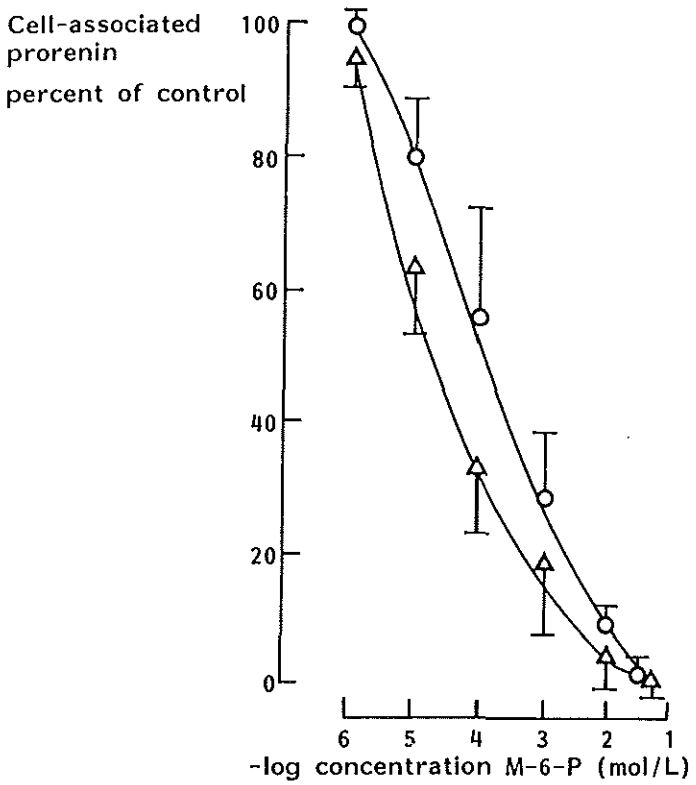


Figure 4. Dose-dependent decrease in cell-associated total (cell-activated plus non-activated) prorenin by mannose 6-phosphate. Cardiac myocytes were pre-incubated with mannose 6-phosphate for 30 minutes at 37°C or 4°C. Then inactive prorenin (final concentration 2500 μU/mL) was added to the culture medium and the incubation was continued for 4 hours at the same temperature as during the preincubation. The concentration of mannose 6-phosphate in the medium is here expressed as moles per liter. Open circles and triangles represent the cellular levels of total (cell-activated plus non-activated) prorenin after incubation at 37°C and 4°C respectively. After incubation at 37°C 90% of the cell-associated prorenin was in the cell-activated form, whereas after incubation at 4°C 10% was in the cell-activated form. Data are means±SD, n=3.

Figure 5 shows the effect of mannose 6-phosphate on myocytes that had been incubated for 2 hours with prorenin at either 4°C or 37°C. After incubation with prorenin, the cells were washed with ice-cold PBS and incubated at 4°C or 37°C respectively for 2 hours in serum-free medium to which mannose 6-phosphate (final concentration 10 μmol/mL), but not prorenin, had been added. After preincubation with prorenin at 4°C, mannose 6-phosphate displaced the cell-associated prorenin into the medium. No displacement by mannose 6-phosphate was observed after preincubation with prorenin at 37°C. Mannose 6-phosphate had similar effects after preincubation with renin (data not shown). These observations are in agreement with the results obtained with the acid-wash method. The two sets of results indicate that at 4°C the cell-associated renin and prorenin remain surface-bound, whereas at 37°C most of it appears to be internalized.

The marked reduction in cell-associated prorenin in response to mannose 6-phosphate was not seen when, instead of mannose 6-phosphate, ammonium chloride (50 μmol/mL) or monensin (0.01 μmol/mL) had been added to the medium (Figure 6). Both ammonium chloride and monensin, however, inhibited the cellular activation of prorenin. In the control incubations the fraction of cell-associated prorenin that was in an activated form rose from 30% after 30 minutes to 90% after 4 hours. This was reduced to 10% and 35% respectively by ammonium chloride and to 5% and 10% by monensin (Figure 7, left panel).

Figure 8 and figure 9 show the results of experiments in which the myocytes were pre-incubated with either renin or prorenin at 4°C for 2 hours and then incubated at 37°C without renin or prorenin. At 37°C, after the incubation at 4°C, approximately 70% of cell-surface-bound renin and 60% of cell surface-bound prorenin were internalized within 5 minutes, and most of the remainder was released into the medium. Results for renin and prorenin were not significantly different. Intracellular prorenin was activated but most, if not all, extracellular prorenin remained inactive. The half-time of intracellular prorenin activation was approximately 25 minutes. There was no evidence that prorenin, once internalized, was released by the cells back into the medium.

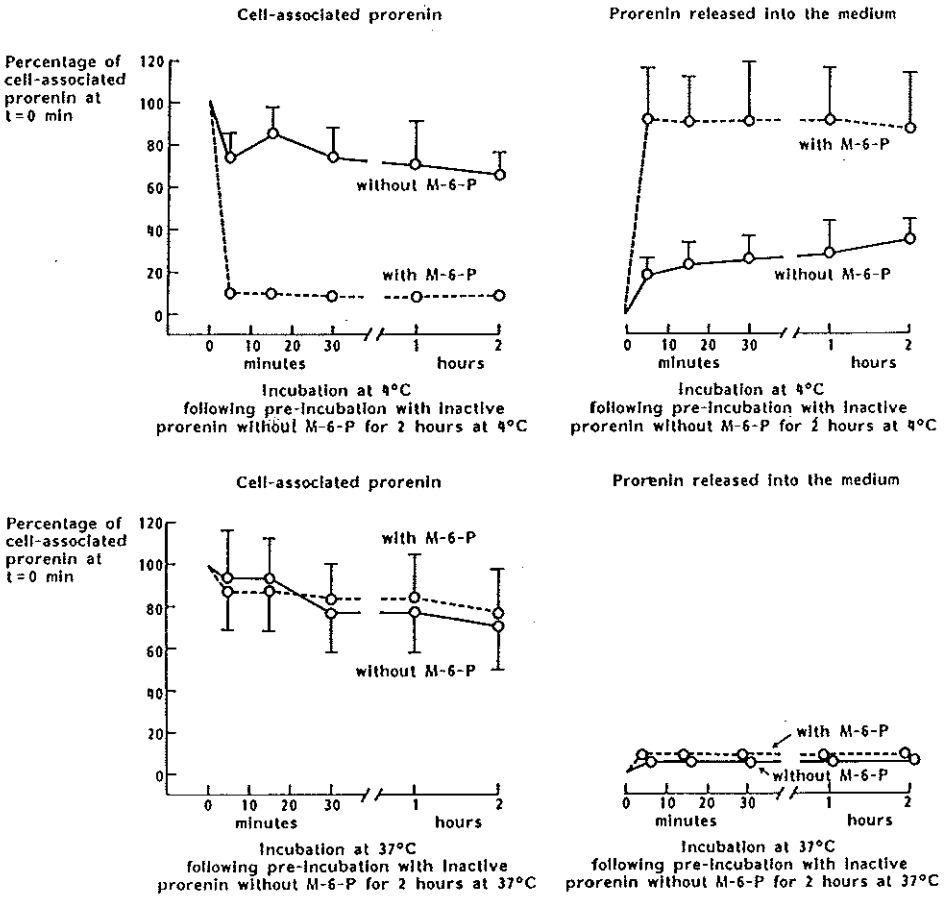


Figure 5. Displacement of cell-associated total (cell-activated plus non-activated) prorenin into the culture medium by mannose 6-phosphate. Cardiac myocytes were pre-incubated with inactive prorenin (2500 μU/mL) in the absence of mannose 6-phosphate for 2 hours at 37°C or 4°C. The cells were then washed with ice-cold phosphate-buffered saline, fresh medium free of renin or prorenin was added, and the incubation was continued at the same temperature as during the preincubation, in the presence or absence of mannose 6-phosphate (final concentration 10 μU/mL). The cellular levels of total (cell-activated plus non-activated) prorenin were 60±21 μU/1.5x10^6 cells at the end of the preincubation at 37°C, and 75±24 μU/1.5x10^6 cells at the end of the preincubation at 4°C. Data are means±SD, n=4.

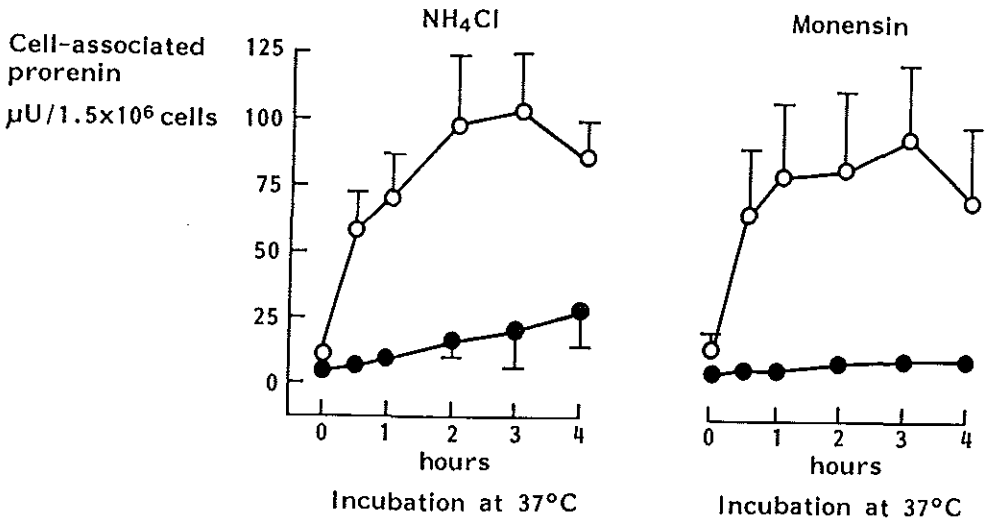


Figure 6. Effects of ammonium chloride and monensin on the cellular activation of cell-associated prorenin. Cardiac myocytes were pre-incubated with ammonium chloride (50 μmol/mL) or monensin (0.01 μmol/mL) for 30 minutes at 37°C. Then inactive prorenin (final concentration 2500 μU/mL) was added to the culture medium and the incubation was continued. The closed and open circles represent the cellular levels of cell-activated prorenin and total (cell-activated plus non-activated) prorenin respectively. Data are means±SD, n=4.

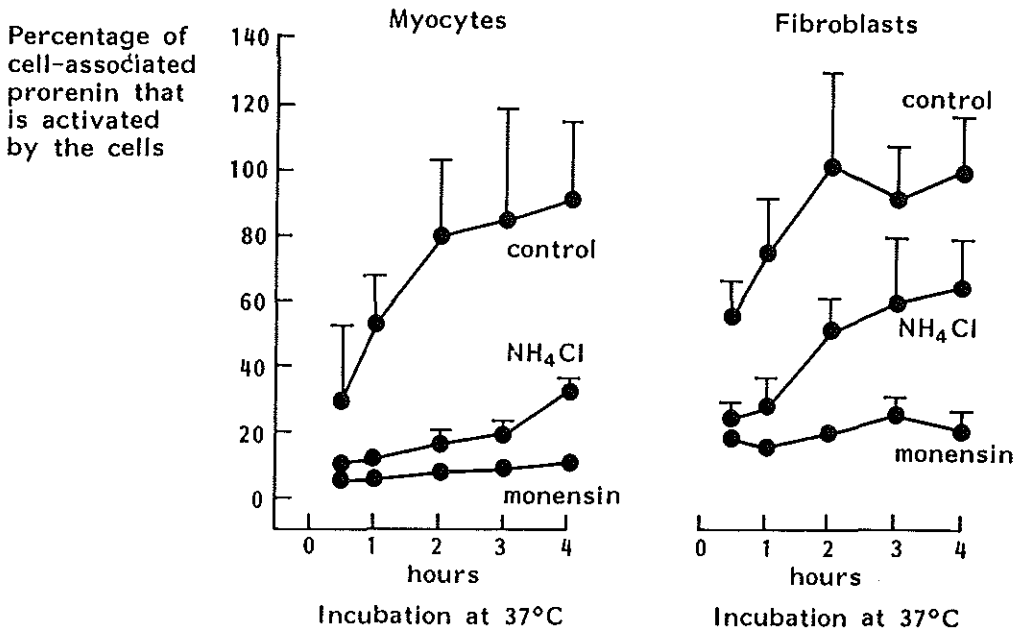


Figure 7. Comparison of the effects of ammonium chloride and monensin on the activation of cell-associated prorenin by cardiac myocytes and cardiac fibroblasts. The cells were pre-incubated with ammonium chloride (50 μmol/mL) or monensin (0.01 μmol/mL) for 30 minutes at 37°C. Then inactive prorenin (final concentration 2500 μU/mL) was added to the culture medium and the incubation was continued. Data are means±SD, n=4 to 6.

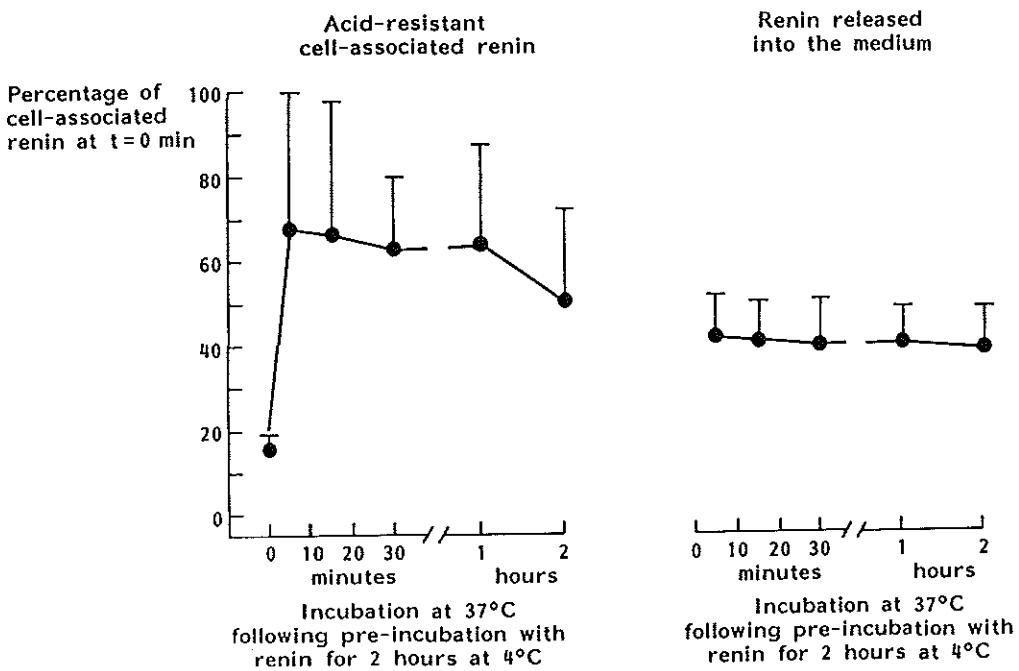


Figure 8. Time course of the internalization of renin by cardiac myocytes. The cells were pre-incubated with renin (2500 μU/mL) for 2 hours at 4°C. They were then washed with ice-cold phosphate-buffered saline, fresh culture medium free of renin or prorenin was added, and the incubation was continued at 37°C instead of 4°C. The cellular level of renin at the end of the preincubation at 4°C was 55±16 $\mu U/10^6$ cells. At the times indicated, the cells were washed with acid to remove the non-acid-resistant renin. Data are means±SD, n=5.

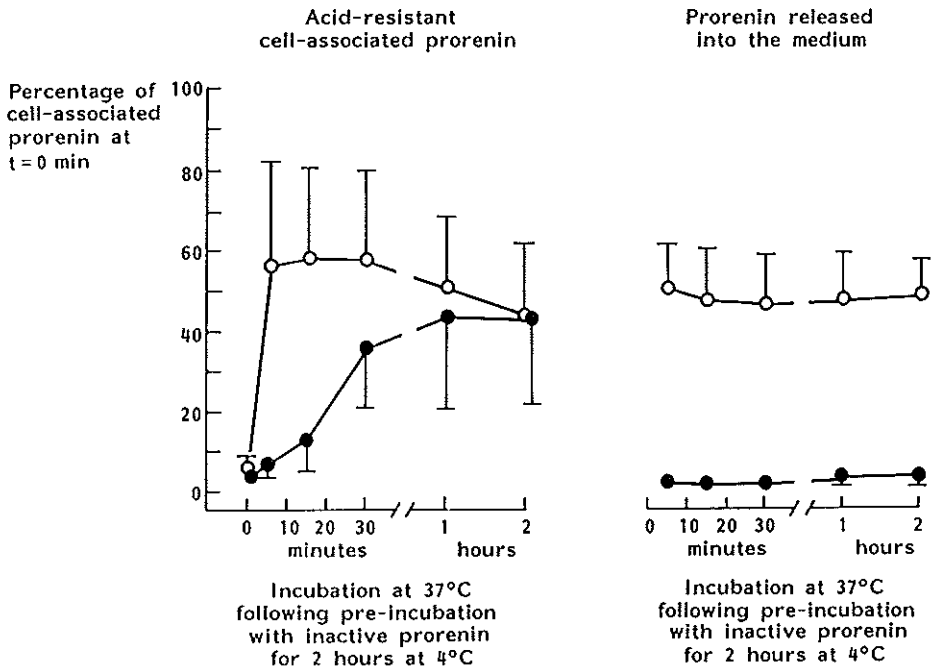


Figure 9. Time course of the internalization and cellular activation of prorenin. Cardiac myocytes were pre-incubated with inactive prorenin (2500 μU/mL) for 2 hours at 4°C. They were then washed with ice-cold phosphate-buffered saline, fresh culture medium free of renin or prorenin was added, and the incubation was continued at 37°C instead of 4°C. The cellular level of total (cell-activated plus non-activated) prorenin at the end of the preincubation at 4°C was 78±22 μU/10^6 cells. At the times indicated, the cells were washed with acid to remove the non-acid-resistant prorenin. The closed and open circles represent the levels of cell-activated prorenin and total (cell-activated plus non-activated) prorenin respectively. Data are means±SD, n=5.

Experiments identical with those performed in the cardiac myocytes, as shown in figure 1 (top), figure 2 (top) and figure 7 (left) were carried out with the use of cardiac fibroblasts. Results of the two sets of experiments were very similar (Figure 1 bottom, figure 2 bottom and figure 7 right). The kinetics of internalization and activation of prorenin by cardiac fibroblasts at 37°C, after preincubation at 4°C for 2 hours, were also similar to those observed in the myocytes (results not shown). At 37°C, after the incubation at 4°C, 42±19% of the cell surface-bound prorenin was internalized within 5 minutes (n=5), and the half-time of intracellular prorenin activation was approximately 25 minutes, as it was in the myocytes. Release of internalized prorenin from the fibroblasts back into the culture medium could not be detected, which also corresponds with our observations in the myocytes. As in the myocytes, the binding of renin and prorenin to the fibroblasts was inhibited by mannose 6-phosphate.

Discussion

This study indicates that renin and prorenin are internalized by cardiac myocytes and fibroblasts and that prorenin, after its internalization, is rapidly activated. At 4°C both renin and prorenin bound to the cell surface and at 37°C both were rapidly internalized. It appears therefore that neither the catalytic domain of the enzyme nor the propeptide domain are essential for the processes of binding and internalization. There was no indication that renin and prorenin, once internalized, were released into the culture medium. The acid-wash method that we used to distinguish between surface-bound and internalized renin or prorenin has been validated for a number of peptide hormone receptors and their ligands including lysosomal enzymes carrying the mannose 6-phosphate signal. Exposure to low pH causes rapid dissociation of these enzymes from cell surface mannose 6-phosphate receptors.[12] To check whether the acid-wash method is also applicable to the cell-binding of prorenin, the cells were incubated with prorenin at 4°C. At this low temperature the internalization process is known to be effectively inhibited. We found that, after incubation with prorenin for 2 hours, the cell-associated prorenin was completely removed from the cells by the acid-wash. In contrast, when, after incubation with prorenin at 4°C, the cells were incubated at 37°C with fresh medium without prorenin, nearly all cell-associated prorenin became resistant to the acid-wash within 5 minutes (the first measurement point). Thus, it appears that the acid-treatment of the cells did not affect the non-surface bound, ie, internalized, prorenin. The cell surface-binding of renin and prorenin at 4°C and its internalization at 37°C were confirmed by our observations on the effects of mannose 6-phosphate. After incubation with prorenin for 2 hours at 4°C, the cell-associated prorenin could be displaced into the medium by the addition of mannose 6-phosphate to the medium, whereas no such displacement was observed after incubation with prorenin for 2 hours at 37°C. The results were similar when the cells were incubated with renin instead of prorenin.

Our results strongly suggest that the internalization is a mannose 6-phosphate receptor-dependent process. The inhibition of prorenin internalization by mannose 6-phosphate was specific and saturable, with an IC_{50} in the order of 0.1μmol/mL. This corresponds with other mannose 6-phosphate receptor-mediated responses.[18,19]

Our finding that most of the cell surface-bound renin and prorenin after preincubation with these enzymes at 4°C was internalized within 5 minutes of incubation at 37°C, is also in agreement with published studies of other ligands that bind to cell surface mannose 6-phosphate receptors via the mannose 6-phosphate signal.[20]

Approximately 5% of the renin or prorenin from the culture medium was bound and internalized by the cardiac cells. This low percentage may be related to the fact that only a small percentage of the renin and prorenin molecules carries the mannose 6-phosphate signal.[11, 21] That this, rather than the number of cell receptors, is the rate-limiting factor is also suggested by our experiments in which the prorenin-containing culture medium was renewed after 2 hours of incubation at 37°C, at a time when the amount of internalized prorenin was at 85% of its maximum. Renewal of the medium in these experiments caused a rise of intracellular prorenin to levels well above the maximum that was reached without renewal of the medium. These results have to be confirmed by binding studies, using radiolabeled prorenin.

The effects of mannose 6-phosphate we observed were different from the inhibitory effects of ammonium chloride and monensin. Mannose 6-phosphate in the incubation experiments with prorenin at 37°C reduced the cellular concentrations of both total and cell-activated prorenin to equally low levels, whereas ammonium chloride and monensin had a much greater effect on the cellular level of cell-activated prorenin than on the cellular level of non-activated prorenin. This supports the view that mannose 6-phosphate inhibits the binding of prorenin to cell surface mannose 6-phosphate receptors, whereas ammonium chloride and monensin primarily act on events that follow the binding to these receptors. Ammonium chloride and monensin are known to interfere with the normal intracellular trafficking and the recycling and lysosomal degradation of internalized receptors and ligands.[22]

This study provides the first evidence of intracellular activation of prorenin, derived from the extracellular fluid, by extrarenal cells. The evidence in our study that a precursor protein is converted into a biologically active agent by a mannose 6-phosphate receptor-mediated and endocytosis-dependent process is not an isolated finding. It has been demonstrated for a number of precursor proteins, including the aspartyl protease cathepsin D.[23] Cathepsin D is a lysosomal enzyme showing a high degree of structural homology with renin[24].

Transforming growth factor beta, which may modulate the growth-promoting actions of Ang II,[25] is another example. This factor is produced in a latent form by vascular endothelial and smooth muscle cells, and is converted into the active form in co-cultures of these cells by a mannose 6-phosphate receptor-dependent process.[19] The mannose 6-phosphate receptor that is involved in the endocytosis-dependent activation of both procathepsin and the latent form of transforming growth factor beta is of the cation-independent type, which is identical with the insulin-like growth factor II receptor.[19, 23] It seems logical to assume that this is also the receptor that mediates the internalization and activation of prorenin we observed in the present study.

Our findings may have physiological significance in light of the experiments by Swales' group (Thurston et al[4]) in rats more than 15 years ago. Their experiments showed that the slow decrease in blood pressure after nephrectomy followed the same time course as the decrease in vascular renin and contrasted with the rapid decrease in circulating renin.[4] In recent years a series of perfusion studies using the isolated rat Langendorff heart and isolated rat hindquarters have provided direct evidence for the local production of Ang I and II.[1, 2, 26] Angiotensin production in these studies was dependent on the presence of renin in the perfusion fluid.

Binding of renin to cell surface receptors may not only be the first step in endocytosis of the renin-receptor complex but may also transduce other membrane-associated or intracellular events. Receptor-mediated binding of human renin to human renal mesangial cells in culture has been reported to cause an increase in [^{3}H]thymidine incorporation and an increase in the concentration of plasminogen activator inhibitor-1 antigen in the conditioned medium.[9] The concentration of renin at which these responses were observed was in the order of 100,000 fmol/mL. This is much higher than the concentration used in our experiments, which was 2500 μU/mL or approximately 50 fmol/mL. Even a concentration of 50 fmol/mL is very high; the normal concentrations of renin and prorenin in human plasma are approximately 0.5 fmol/mL and 5 fmol/mL, respectively.[27] However, the lower in-vivo concentrations may be sufficient, because in the in-vivo situation the cells are continuously exposed to prorenin carrying the mannose 6-phosphate signal, whereas in vitro this prorenin disappears from the culture medium because of uptake by the cells.

Cellular binding and internalization of renin and prorenin and intracellular activation of prorenin may lead to local levels of enzyme activity that are higher than in the extracellular fluid. If angiotensinogen is internalized via bulk fluid endocytosis concurrently with the receptor-mediated endocytosis of renin and prorenin, a scenario is provided for intracellular Ang I generation. Ang II is known to stimulate plasminogen activator inhibitor-1 production by renal mesangial cells.[28] The reported increase in plasminogen activator inhibitor-1 antigen in response to the binding of renin to these cells[9] would therefore fit in the view that this is an Ang II-mediated effect. However, the question of whether Ang I and/or II are formed within the cells is highly controversial. It is possible that the reported responses of mesangial cells to the cellular binding of renin are independent of Ang I and II formation.

Our observations on the internalization of renin and prorenin and on the intracellular activation of prorenin warrant further studies addressing this issue. Internalized Ang II has a long half life compared with circulating Ang II.[29] It rapidly accumulates in cardiac and vascular muscle cell nuclei,[30] where it may bind to chromatin and may influence transcriptional processes that are related to the growth-promoting effects of Ang II.[31] Finally, recent experiments, in which Ang II was injected into cardiac myocytes and vascular smooth muscle cells, also favor the concept that intracellular Ang II may serve important functions.[32,33] Studies addressing the possibility of intracellular angiotensin generation may therefore hold promise.

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CHAPTER 5

Uptake and Proteolytic Activation of Prorenin by Cultured Human Endothelial Cells

Abstract

This study was performed to investigate the mechanisms of vascular uptake of prorenin and renin and to explore the possibility of vascular activation of prorenin. For this purpose human umbilical vein endothelial cells (HUVECs) were cultured in a chemically defined medium and incubated with recombinant human prorenin or renin in the presence or absence of putative inhibitors of renin internalization. Cell surface-bound and internalized prorenin or renin were separated by the acid-wash method and were quantitated by enzyme-kinetic assays. The activation of prorenin was also monitored by a direct immunoradiometric assay (IRMA) with the use of a monoclonal antibody directed against the -p24-Arg to -1p-Arg C-terminal propeptide sequence of prorenin. Prorenin and renin were internalized at 37°C in a dose-dependent manner; with 1000 μU prorenin/mL medium, the quantity of cell-associated prorenin after 3 hours of incubation was 9.3±1.0 μU/4x10^5 cells, and with 75,000 μU/mL medium it was 670±75 μU/4x10^5 cells (mean±SD; n=5). Results for renin were similar. Prorenin that had been treated with endoglycosidase H to remove N-linked oligosaccharides was not internalized. Addition of mannose 6-phosphate (M-6-P) to the medium caused a dose-dependent inhibition of renin and prorenin internalization. Fifty percent inhibition was observed at 70 μmol/L M-6-P, whereas mannose 1-phosphate, glucose 6-phosphate and α-methylmannoside at this concentration had no effect. Ammonium chloride (50 mmol/L) and monensin (10 μmol/L) also inhibited internalization. Prorenin was activated by HUVECs, and cell-activated prorenin was only found in the internalized fraction, whereas the surface-bound prorenin remained inactive. Thus, it appears that the activation of prorenin took place at the time of its internalization or thereafter. The results of the prorenin IRMA indicated that activation was associated with proteolytic cleavage of the propeptide. Our findings provide evidence for M-6-P receptor-dependent endocytosis of (pro)renin and proteolytic prorenin activation by vascular endothelial cells.

Introduction

The formation of angiotensin (Ang) II at the vascular endothelial surface, through conversion of blood Ang I, by plasma membrane-bound angiotensin-converting enzyme (ACE), is well established. Whether Ang I and renin are also produced by vascular tissue and how this may contribute to the Ang II-mediated actions of the renin-angiotensin system, however, is still a matter of debate.

Experiments in which the isolated Langendorff rat heart was perfused with renin and angiotensinogen, provide evidence for Ang I production in the tissue not only in the interstitial fluid but also at the surface of cells or within the cells.[1,2] Most of the locally produced tissue Ang I was released into the coronary circulation rather than the cardiac interstitial fluid, which suggests that the blood vessel wall was the main site of tissue Ang I production.

Uptake of renin from the circulation by vascular tissue has been demonstrated and there is experimental evidence that vascular, kidney-derived, renin, in conjunction with circulating renin, may serve to maintain blood pressure.[3,4] Binding of renin to vascular tissue membranes has also been reported[5] and, in intact human gastroepiploic arteries, renin appeared to be present in endothelial cells.[6]

Recently our group reported that cultured neonatal rat cardiomyocytes and fibroblasts are capable of binding and internalizing prorenin and renin and of activating prorenin after its internalization.[7] The recombinant human prorenin and renin that were used in these experiments, were produced in Chinese hamster ovary (CHO) cells transfected with a vector containing human prorenin cDNA. It is known that this prorenin, on the renin part of its molecule, carries the mannose 6-phosphate signal that is recognized by specific receptors,[8,9] and the observed binding and internalization of prorenin and renin by the cardiac cells and the subsequent activation of prorenin appeared to depend on mannose 6-phosphate receptors.[7]

In the present study, we used cultured human umbilical vein endothelial cells (HUVECs) to investigate the binding, internalization and activation of prorenin.

Native human prorenin, which has little or no intrinsic enzymatic activity, can be activated proteolytically by enzymatic cleavage of the propeptide, and non-proteolytically by a conformational change resulting in displacement of the propeptide from the cleft of the prorenin molecule, in which renin's active site is located.[10-12] The activation of prorenin by HUVECs was therefore investigated in two ways: 1) by measuring the increase in enzymatic activity, and 2) by monitoring the cleavage of the propeptide with an immunoradiometric assay (IRMA) using a monoclonal antibody directed against a C-terminal sequence of the propeptide.

Materials and Methods

Reagents

Mannose 6-phosphate, mannose 1-phosphate, glucose 6-phosphate, α-methylmannoside, trypsin, bovine serum albumin (BSA) and monensin were from Sigma Chemical Co, St. Louis, MO, USA. Human serum albumin (HSA) was purchased from Behring, Marburg, Germany. It was electrophoretically 100% pure. Human plasmin, 25 caseinolytic units per mg protein, was purchased from Kabi Vitrum, Mölndal, Sweden. Endoglycosidase H, 40 units per mg protein, and fetal calf serum were from Boehringer Mannheim, Mannheim, Germany. The serine protease inhibitor aprotinin (trasylol), 7000 kallikrein-inhibitory units per mg, was from Bayer, Leverkusen, Germany. The specific active site-directed renin inhibitor Ro 42,5892 (remikiren), molecular mass 727 Da, was kindly provided by dr W. Fischli, Hoffmann-LaRoche, Basel, Switzerland. The K_i value for the reaction with human renin is 0.3 nmol/L.[13] Monoclonal antibody (Mab) F258-37-B1 was a kind gift of dr S. Mathews, Hoffmann-La Roche, Basel, Switzerland. This antibody was raised against a synthetic peptide consisting of the -p24-Arg to -p1-Arg sequence of prorenin, i.e. the C-terminal half of the propeptide. It was purified by $(NH_4)_2SO_4$ precipitation followed by chromatography with diethylaminoethylcellulose.[14] Purity, as assessed by sodium dodecylsulphate-polyacrylamide gel electrophoresis was >98%. Mab F258-37-B1 was biotinylated.[15] ^{125}I-labeled Mab R1-20-5 (specific activity 360 kBq/μg) was purchased from Nichols Institute, Wychen, The Netherlands. This antibody reacts with renin as well as with non-proteolytically activated prorenin and proteolytically activated prorenin, and does not recognize native prorenin.[16,17]

Prorenin and renin preparations

Recombinant human prorenin, produced in CHO cells transfected with a vector containing human prorenin cDNA,[18,19] was kindly provided by dr W. Fischli, Hoffmann-La Roche, Basel, Switzerland. It was partially purified, to remove traces of renin, by Cibacron Blue Sepharose affinity chromatography.[10] The intrinsic renin activity of the prorenin preparation, prior to proteolytic activation, was approximately 2% of the activity after complete proteolytic activation. Recombinant human renin was prepared by conversion of recombinant human prorenin into renin by immobilized trypsin.[10,20] Sepharose-bound trypsin (final concentration 0.25 mg trypsin/mL) was added to the prorenin preparation and the mixture was kept at 4°C for 72 hours. Trypsin was then removed by centrifugation. After proteolytic activation the prorenin preparation contained approximately 8.5×10^6 μU/mL renin. The prorenin and renin preparations were stored in the presence of 0.1% BSA at -70°C.

Prorenin is a glycoprotein, the oligosaccharides being attached to the renin part of the molecule. In order to examine the role of oligosaccharides attached to human prorenin, 100 μL of the above prorenin preparation was incubated at 37°C for 24 hours with 80 μL of a solution containing 1 unit/mL endoglycosidase H and 25 mmol/L EDTA in 50 mmol/L phosphate buffer, pH 5.5. Endoglycosidase H-treated prorenin, after its conversion to renin by trypsin-Sepharose, had the same activity in the enzyme-kinetic assay as untreated prorenin.

Cell culture

HUVECs were isolated from freshly obtained umbilical cords as previously described.[21] Umbilical cords were obtained from the Department of Gynaecology and Obstetrics of the University Hospital Dijkzigt, Rotterdam, The Netherlands. Isolated HUVECs were cultured in 75 cm^2 tissue culture flasks coated with fibronectin, using growth medium consisting of modified Medium 199 (Flow Laboratories, Irvine, UK), supplemented with 5% fetal calf serum, 5% human serum, 18 μg/mL endothelial cell growth factor,[22] 15 U/mL heparin (LEO Pharmaceutical Products, Weesp, The Netherlands), 50 U/mL penicillin (Flow Laboratories), and 5 μg/mL streptomycin (ICN Biochemicals, Zoetermeer, The Netherlands) at 37°C and 5% CO_2 in air.

Confluent cell cultures were trypsinized with 1 mL of 0.1 mg/mL trypsin in phosphate-buffered saline (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; PBS) per culture flask. The cells were stored in liquid nitrogen in small aliquots supplemented with 5% (vol/vol) dimethyl sulfoxide.

For a typical experiment an aliquot of HUVECs was thawed, the cells were washed once with growth medium and subsequently cultured to confluence in a 75 cm^2 tissue culture flask coated with fibronectin. Cells were trypsinized and seeded in 6-well plates (Becton and Dickinson, Franklin Lakes, NJ, USA). For most experiments HUVECs of passage number 3 or 4 were used. Studies were carried out approximately one day after confluency (corresponding with a density of 4 x 10^5 cells/well) had been reached. Prior to the start of each experiment, cells were washed three times with 3 mL warm (37°C) PBS. The cells were then preincubated either at 37°C or 4°C for 30 minutes with 0.9 mL incubation medium consisting of modified Medium 199 without fetal calf serum or human serum, but supplemented with 1% (wt/vol) HSA.

Incubation of HUVECs with prorenin or renin

After preincubation at 37°C or 4°C for 30 min in 0.9 mL medium (see above), experiments were started by the addition of 0.1 mL of a solution of recombinant human prorenin or renin to the wells to give a final concentration in the medium of 2500 μU/mL. Cells were then incubated at 37°C or 4°C for up to 240 minutes. Incubations were also performed in the presence of mannose 6-phosphate (2 μmol/L to 10 mmol/L), mannose 1-phosphate (8 μmol/L to 8 mmol/L), glucose 6-phosphate (8 μmol/L to 8 mmol/L), α-methylmannoside (8 μmol/L to 8 mmol/L), ammonium chloride (50 mmol/L) or monensin (10 μmol/L). At the end of the incubation period, the culture medium was removed and quickly frozen on dry ice. Each well was washed three times with 3 mL ice-cold PBS. Renin and prorenin were not detectable in the last PBS wash. Cells were lysed in 0.5 mL ice-cold PBS containing 0.2% Triton X-100, and the cell lysate was quickly frozen on dry ice. Medium and cell lysate were stored at -70°C until assayed for prorenin or renin.

To study the internalization and activation in more detail, cells were incubated with prorenin (final concentration, 75,000 μU/mL) for 2 hours at 4°C. The cells were then washed three times with 3 mL ice-cold PBS.

After the last wash, 1 mL of fresh incubation medium without prorenin was added, and the cells were incubated at 37°C. The incubation was terminated after 5, 15, 30, 60 or 120 min by washing the cells 3 times with 3 mL ice-cold PBS. The cells were then lysed as described above. Medium and cell lysate were assayed for "total" prorenin (i.e., prorenin that was activated by the cells plus prorenin that was not activated by the cells), cell-activated prorenin, and intact propeptide-containing prorenin.

Internalized prorenin was separated from cell surface-bound prorenin by the acid-wash method.[23] At low pH surface-bound prorenin dissociates from the cells; internalized prorenin, however, is acid-resistant.[7] Briefly, after the cells had been washed three times with 3 mL ice-cold PBS, the cells were incubated at 4°C with 1 mL of an acid solution containing 50 mmol/L glycine and 150 mmol/L NaCl, pH 3.0. After 10 minutes the acid solution was removed and the cells were washed three times with 3 mL ice-cold PBS. The cells were then lysed as described above.

Enzyme-kinetic assays of prorenin and renin

Renin and cell-activated prorenin were measured by incubating 100 µL sample for 3 hours with a saturating amount of sheep renin substrate at 37°C and pH 7.4 in the presence of serine protease and angiotensinase inhibitors.[7,20] The generated Ang I was quantified by radioimmunoassay. Results were expressed as µU/mL using the international WHO human kidney renin standard, lot 68/356 (WHO International Laboratory for Biological Standards and Control, Potter Bar, Hertfordshire, UK), as a reference. The lower limit of detection was one µU per mL medium or per $4x10^5$ cells. To measure "total" prorenin (i.e., cell-activated plus nonactivated prorenin) with the enzyme-kinetic assay, the samples were incubated for 48 hours at 4°C with plasmin (0.5 caseinolytic units per mL). This preincubation with plasmin caused complete proteolytic activation of prorenin.[7,20] The serine protease inhibitor aprotinin (final concentration 100 kallikrein-inhibiting units/mL) was added to the incubation medium of the Ang I generating step in order to inactivate plasmin. Recovery of renin or prorenin added to the cells before cell lysis was better than 95% (n=3). Mannose 6-phosphate, mannose 1-phosphate, glucose 6-phosphate, α-methylmannoside, ammonium chloride, and monensin in the concentrations we used in the cell cultures (see above) did not affect Ang I generation.

Immunoradiometric assay of prorenin

In order to monitor the proteolytic activation of prorenin by HUVECs, we developed a sandwich IRMA specific for intact prorenin, i.e. prorenin in which the propeptide was still bound to the renin part of the molecule. The primary antibody of this assay, Mab F258-37-B1, which is directed against the C-terminal part of the propeptide, did not react with native inactive prorenin. However, it did react with prorenin after the treatment of native prorenin with the renin inhibitor remikiren (0.1 mmol/L) for 48 hours at 4°C. The renin inhibitor enters the enzymatic cleft in which the active site is located, thereby inducing a slow conformational change of the inactive ('closed') form of the prorenin molecule into the active ('open') form.[12] By this non-proteolytic conformational change, the propeptide moves to the surface of the molecule so that it can react with Mab F258-37-B1. In the IRMA, 200 µL of remikiren-treated sample was incubated for 6 hours at 37°C with an avidin-coated bead of 8 mm diameter to which 1.6 µg of biotinylated Mab F258-37-B1 had been bound. The bead was then washed three times with 2 mL PBS, and subsequently incubated with 100 µL (250,000 cpm) of the ^{125}I-labeled Mab R1-20-5 (secondary antibody) and 200 µL assay buffer of the Nichols renin-kit (Nichols Institute, Wychen, The Netherlands) for 24 hours at room temperature. After the 24 hour-incubation period, the bead was washed three times with 2 mL PBS, and bound radioactivity was measured in a gamma counter. The results of this assay were expressed as µU/mL using intact recombinant human prorenin as a reference. The lower limit of detection was 5 µU per mL medium or per $4x10^5$ cells.

Results

Figure 1 shows the results of incubating HUVECs at 37°C or 4°C after either prorenin (left panel) or renin (right panel) had been added to the culture medium, both at a concentration of 2500 µU/mL. At 37°C the level of cell-associated prorenin rose over time until a plateau was reached after 3 hours. With initial concentrations of prorenin in the medium ranging from 1000 µU/mL to 75,000 µU/mL, the maximum level of prorenin in the cell lysates was proportional to the concentration in the medium (results not shown).

It was 9.3±1.0 μU/4x10^5 cells at an initial concentration in the medium of 1000 μU/mL, and 670±75 μU/4x10^5 cells at a concentration in the medium of 75,000 μU/mL (mean±SD, n=5). These levels represent approximately 1% of the amounts added to the medium. At 4°C, less than 0.4% of prorenin added to the medium was bound to the cells (Figure 1, left panel). Similar results were obtained when renin was added to the medium (Figure 1, right panel).

Cell-associated prorenin was activated during culture at 37°C but not at 4°C (Figure 1, left panel). Prorenin in the medium remained inactive for the full 240-minute incubation period both at 37°C and at 4°C.

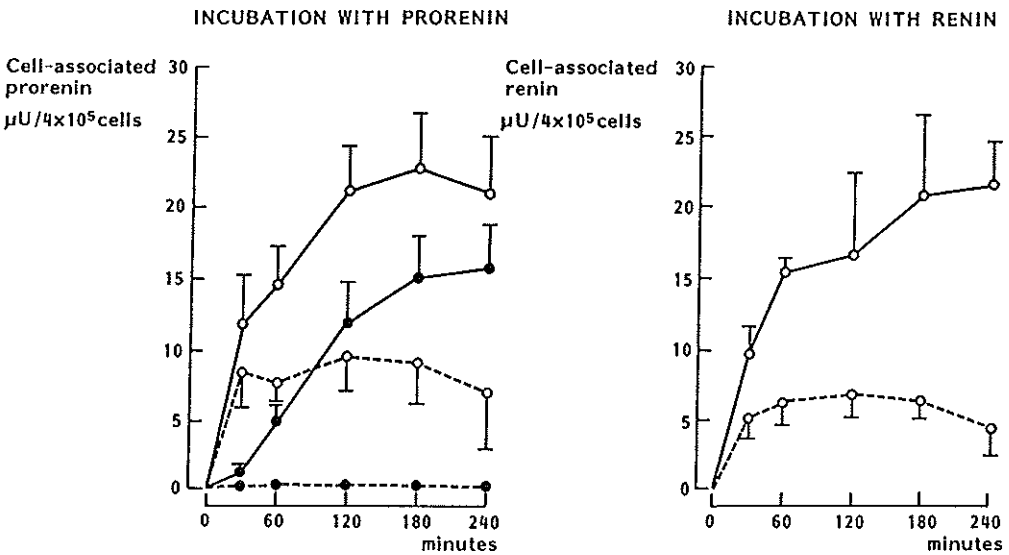

Figure 1. Increase in cell-associated prorenin (left panel) and renin (right panel) over time during incubation of HUVECs at 37°C (solid line) or 4°C (dotted line) with inactive prorenin or with renin (both 2500 μU/mL). The open circles in the left panel represent the cellular levels of total (cell-activated plus nonactivated) prorenin. The closed circles in the left panel represent the cellular levels of cell-activated prorenin. The open circles in the right panel represent the cellular levels of renin. Measurements were made by the enzyme-kinetic assay. Measurements at time zero were made in cells that had been separated from the medium immediately after the addition of prorenin or renin. Data are means and SD, n=5.

To investigate whether the results were influenced by the number of cell passages, primary HUVEC cultures and passages 1 to 5 were incubated with prorenin (initial concentration in the medium 2500 μU/mL) at 37°C. The concentration of prorenin in the cell lysates, expressed as μU/μg protein, in the passages 1 to 5 was similar to that in the primary culture (Figure 2). The fraction of cell-associated prorenin that was in an activated form was also similar.

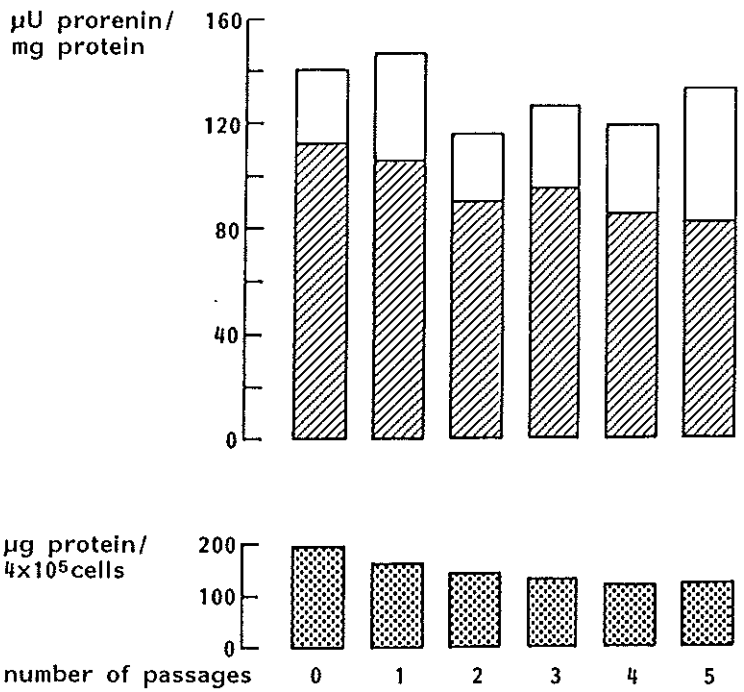

Figure 2. Upper panel. Cell-associated prorenin after 240 minutes of incubation of primary cultures of HUVECs and passages 1-5 with inactive prorenin (2500 μU/mL) at 37°C. Bars represent the levels of total (cell-activated plus nonactivated) prorenin. The hatched area represents the level of cell-activated prorenin. Measurements were made by the enzyme-kinetic assay. *Lower panel.* Cellular protein content of HUVECs. Data are means of two experiments.

Lysates of HUVECs that had been incubated with prorenin (initial concentration in the medium 2500 μU/mL) for 180 and 240 minutes at 37°C, were prepared with and without acid-pretreatment of the cells, in order to distinguish between internalized (acid-resistant) prorenin and cell surface-bound (acid-washable) prorenin. After incubation at 37°C, more than 90% of the cell-associated prorenin was acid-resistant both in the 180-minute and 240-minute cultures (n=5 in both cases, results not shown). When HUVECs were first incubated with prorenin at 4°C and then at 37°C in fresh medium to which no prorenin had been added, a large fraction of the cell-associated prorenin was released into the medium within 15 minutes (data not shown). The remainder (about 12%) was slowly internalized, with a half-time of internalization of approximately 15 minutes (Figure 3).

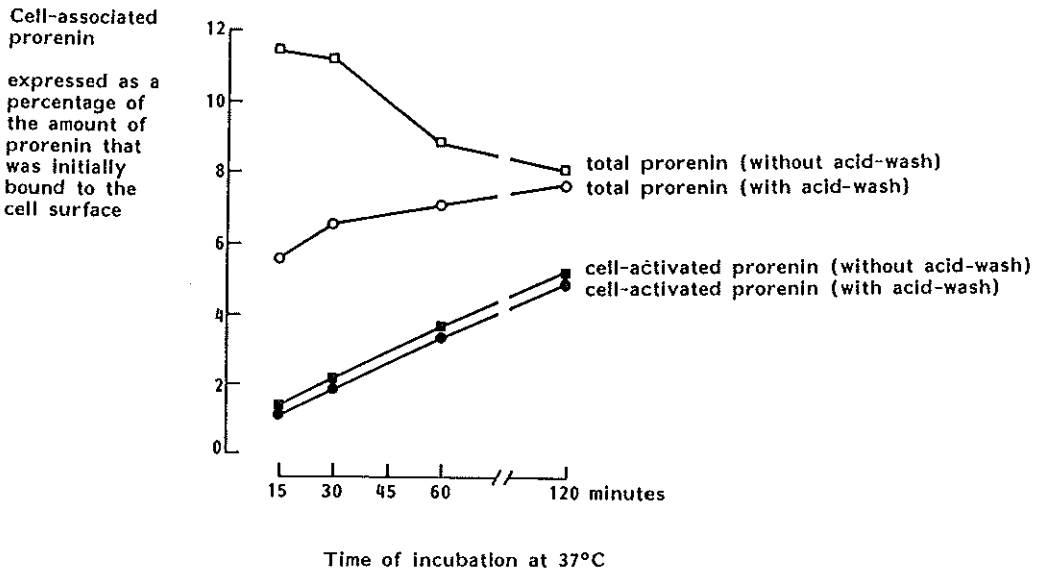

Figure 3. Time course of internalization and activation of prorenin by HUVECs. The cells were preincubated with inactive prorenin (75,000 μU/mL) for 120 minutes at 4°C. They were then washed with ice-cold PBS. Fresh culture medium free of prorenin was added, and the incubation was continued at 37°C. The cellular level of total (cell-activated plus nonactivated) prorenin at the end of the preincubation at 4°C was approximately 500 μU/4x10^5 cells (100%). The acid-wash method was used to separate surface-bound (nonacid-resistant) prorenin and internalized (acid-resistant) prorenin. Measurements were made by the enzyme-kinetic assay. Data are means of 3 experiments.

The percentage of cell-associated prorenin that was in the activated form rose over time, and all cell-activated prorenin appeared to be located in the cells, whereas all cell surface-bound prorenin was still in the inactive form (Figure 3). These results indicate that the activation process took place at the time of the internalization of prorenin or thereafter. There was no evidence for release of activated prorenin into the medium.

When HUVECs were incubated for 240 minutes at 37°C with prorenin (2500 μU/mL) that had been treated with endoglycosidase H, less than 0.2% of the prorenin was internalized by the cells (n=3, results not shown). Thus, it appears that N-linked oligosaccharides attached to prorenin are important for its uptake by HUVECs.

The question of whether the internalization of renin and prorenin could be mediated by mannose 6-phosphate receptors at the cell surface, was addressed by experiments, in which the cells were incubated at 37°C for 4 hours in a medium to which renin or prorenin had been added both at a final concentration of 2500 μU/mL, as well as mannose 6-phosphate in concentrations ranging from 2 μmol/L to 2 mmol/L. Figure 4 shows the results obtained with prorenin. As mentioned above, after culture at 37°C for 4 hours in the absence of mannose 6-phosphate, practically all cell-associated prorenin was resistant to the acid-wash, indicating that prorenin had been internalized by the cells. Mannose 6-phosphate had a dose-dependent inhibitory effect on prorenin internalization. Inhibition was maximally 85-90%, and the IC_{50} was approximately 70 μmol/L. Mannose 1-phosphate also inhibited prorenin internalization but only at concentrations of 2 mmol/L or higher. Glucose 6-phosphate and α-methylmannoside up to 8 mmol/L had no inhibitory effect. Similar results were obtained with renin (results not shown).

When HUVECs were incubated with prorenin in the presence of ammonium chloride (50 mmol/L) or monensin (10 μmol/L), the internalization of prorenin was markedly diminished (Figure 5). Moreover, practically all cell-associated prorenin remained inactive under these circumstances. This was also the case when mannose 6-phosphate (10 mmol/L) had been added to the culture medium.

A series of experiments was carried out to answer the question of whether the activation of prorenin by HUVECs was a proteolytic process. It is known that, in vitro, prorenin can also be activated by a non-proteolytic proces, by exposure to acid milieu.[10-12] The results, which are shown in figure 6, indicate that the activation of prorenin by HUVECs was associated with the proteolytic cleavage of the propeptide.

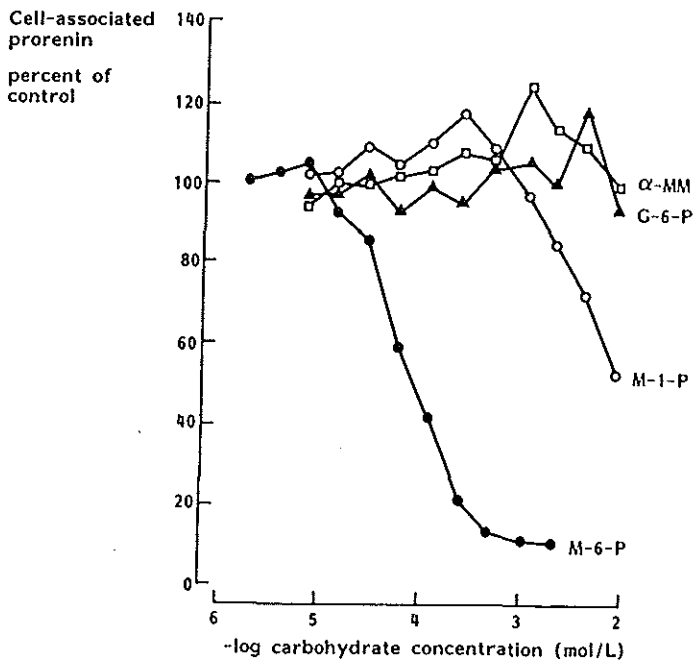

Figure 4. Cell-associated total (cell-activated plus nonactivated) prorenin after 240 minutes of incubation of HUVECs with inactive prorenin (2500 μU/mL) at 37°C in the presence of increasing concentrations of mannose 6-phosphate (M-6-P), mannose 1-phosphate (M-1-P), glucose 6-phosphate (G-6-P) or α-methylmannoside (α-MM). Measurements were made by the enzyme-kinetic assay. Values (means of 3 experiments) are expressed as a percentage of the values obtained under control conditions.

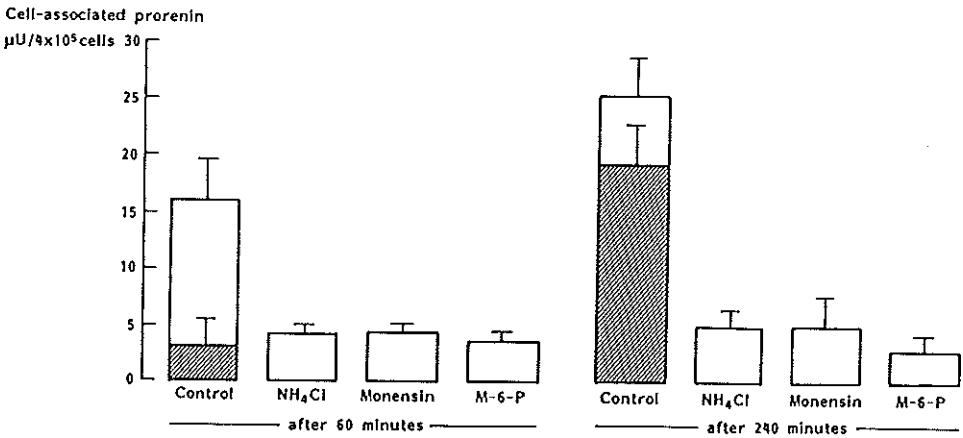

Figure 5. Cell-associated prorenin after 60 minutes and 240 minutes of incubation of HUVECs with inactive prorenin (2500 μU/mL) at 37°C in the presence or absence of ammonium chloride (50 mmol/L), monensin (10 μmol/L), and mannose 6-phosphate (10 mmol/L). Bars represent the levels of total (cell-activated plus nonactivated) prorenin. The hatched area represents the level of cell-activated prorenin. Cell-activated prorenin was not detectable in cells incubated in the presence of ammonium chloride, monensin or mannose 6-phosphate. Measurements were made by the enzyme-kinetic assay. Data are means and SD, n=4.

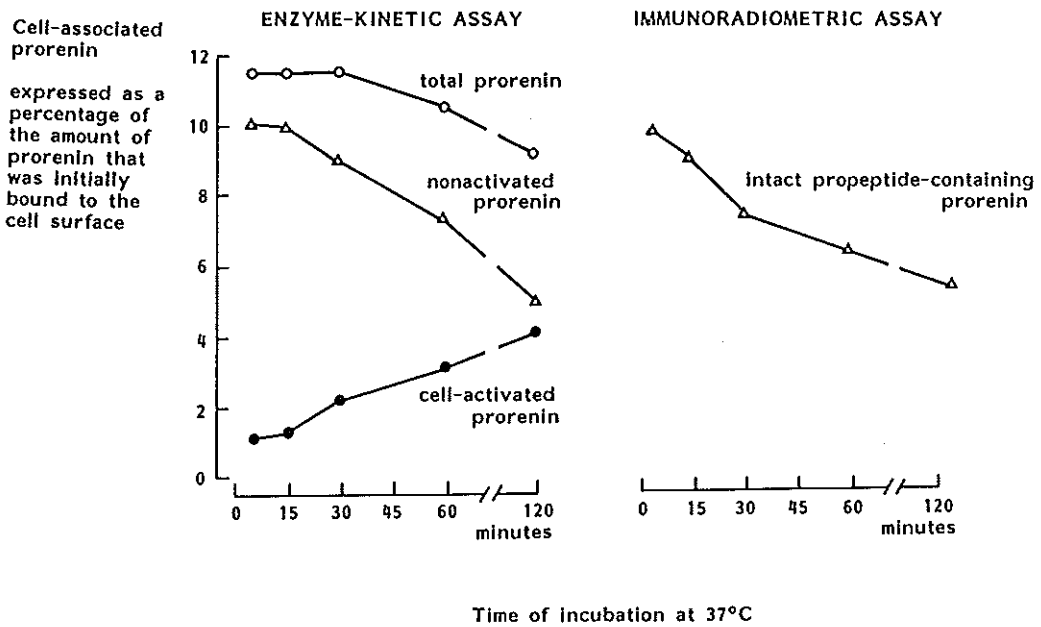

Figure 6. Time course of the proteolytic activation of prorenin. HUVECs were incubated with prorenin (75,000 μU/mL) for 120 minutes at 4°C. They were then washed with ice-cold PBS. Fresh incubation medium free of prorenin was added, and the incubation was continued at 37°C. The cellular level of total (cell-activated plus nonactivated) prorenin at the end of the preincubation at 4°C was approximately 500 μU/4x10^5 cells (100%). The left panel shows the cellular levels of total prorenin, nonactivated prorenin and cell-activated prorenin measured by the enzyme-kinetic assay. The right panel shows the cellular level of propeptide-containing prorenin measured by an IRMA specific for intact propeptide-containing prorenin. Changes in the level of propeptide-containing prorenin over time as measured by IRMA are similar to the changes of nonactivated prorenin as measured by the enzyme-kinetic assay, thereby demonstrating that activation of prorenin is associated with the proteolytic removal of the propeptide. Data are means of 3 experiments.

Discussion

Our results demonstrate that cultured HUVECs internalize recombinant human prorenin and renin and that these cells activate prorenin at the time of its internalization or thereafter. The internalization process appears to be mannose 6-phosphate receptor-dependent; inhibition with mannose 6-phosphate was specific and saturable. Two types of mannose 6-phosphate receptors have been identified, i.e. the cation-dependent and cation-independent types.[24,25] The cation-independent type receptor is identical with the insulin-like growth factor II receptor (IGFII/M-6-P receptor). Both types of receptor function in the process of intracellular lysosomal enzyme sorting. The IGFII/M-6-P receptor also mediates the endocytosis of extracellular lysosomal enzymes.[24,25] Because of this and because of the structural homology between renin and the lysosomal enzyme cathepsin D, we assume that it is the IGFII/M-6-P receptor that is involved in the internalization of prorenin and renin by HUVECs. The IC_{50} of the inhibition of (pro)renin internalization by mannose 6-phosphate was approximately 70 µmol/L, which is close to the IC_{50} of other IGFII/M-6-P receptor-mediated processes.[26,27] Mannose 6-phosphate, however, did not cause complete inhibition, which indicates that some (pro)renin was internalized by a mechanism independent of the M-6-P marker.

At concentrations of prorenin in the culture medium ranging from 1000 to 75,000 µU/mL, which is 4-300 times the normal concentration in human blood plasma, the maximum level of cell-associated prorenin, which was reached after 3 hours of incubation at 37°C, was proportional to the prorenin concentration in the medium; it was about 1% of the amount of prorenin added to the medium. Apparently, the fact that the cell lysates contained only a small fraction of the added prorenin, was not related to a high level of receptor occupancy. The recombinant human prorenin preparation we used had been produced by CHO cells.[18,19] It has been reported that only a small fraction of this prorenin contains the mannose 6-phosphate signal,[8,28] and this may explain why the amount of cell-associated prorenin was only 1% of the amount added to the cultures.

The internalization of prorenin by HUVECs was not only inhibited by mannose 6-phosphate but also by ammonium chloride and monensin, which are known to interfere with the recycling of internalized receptors to the cell surface.[29]

The low level of cell-associated prorenin in the presence of ammonium chloride and monensin may indicate that receptor recycling is an important factor determining prorenin uptake by HUVECs. Receptor recycling may also explain why during incubation with prorenin at 37°C, cell-associated prorenin rose to levels that were several times higher than during incubation at 4°C.

Prorenin, which has no enzymatic activity, can be reversibly activated in vitro by exposure to acid. In vivo, this might occur in the acid milieu of endosomes. Our results, however, show that the activation of prorenin by HUVECs was associated with proteolytic cleavage of the propeptide, most likely through the action of a cell-bound protease. It is unlikely that this activation has occurred as an artifact during preparation of the cell lysates, because the lysates of cells that had been incubated with prorenin at 4°C were free of activated prorenin.

A previous study of cultured neonatal rat cardiomyocytes and fibroblasts, using the same concentrations of (pro)renin as in the present study, also provided evidence for IGFII/M-6-P receptor-dependent endocytosis of (pro)renin and for the activation of cell-bound prorenin.[7] There are, however, some quantitative differences between the results obtained with HUVECs and the cardiac cells. At 4°C the cardiac cells bound a larger fraction of the (pro)renin that had been added to the culture medium than the HUVECs did, thereby indicating that the IGFII/M-6-P receptor density at the cell surface was higher in the cardiac cells. In addition, at 37°C, the internalization and activation of prorenin after its binding to the cell surface proceeded faster in the cardiac cells. When HUVECs were incubated for 2 hours with prorenin at 4°C and then incubated at 37°C in the absence of prorenin, less than 15% of the cell surface-bound prorenin was internalized, while the remainder was released into the medium. In similar experiments in cardiac cells, more than 50% was internalized. Despite such differences, the mechanisms involved in binding, internalization and activation of prorenin might basically be the same for HUVECs and cardiac cells.

Binding and internalization of lysosomal enzymes to the IGFII/M-6-P receptor results in intracellular degradation of these enzymes.[24,25] This receptor may therefore serve as a clearance receptor for (pro)renin.

In view of the IGFII/M-6-P receptor-dependent prorenin activation by vascular endothelial cells, as observed in our study, it is also possible that these cells contribute to local angiotensin I and II formation by the blood vessel wall. Early experiments by Swales' group demonstrated uptake of kidney-derived renin by the aortic wall in vivo.[4] These experiments also indicated that vascular kidney-derived can contribute to the maintenance of elevated blood pressure in two-kidney, one-clip hypertension.[3] Our results reported here might provide a mechanism for the vascular uptake of renin and prorenin. Our results are also relevant with respect to observations made in isolated rat hindquarters and the isolated rat Langendorff heart, which provided evidence for the release of locally produced angiotensins into the circulation during perfusion with renin.[1,2,30-32] Studies of the regional production of angiotensin I in various extrarenal vascular beds in pigs [33,34] and humans [35,36] also provided evidence for the release of locally produced angiotensin I into the circulation, over and above angiotensin I formation by the reaction of circulating renin with circulating angiotensinogen. Since, in humans, the level of prorenin in circulating blood is normally several times higher than the level of renin, and in view of our finding that prorenin is activated in vitro by vascular endothelial cells, it is conceivable that not only renin but also prorenin contributes to angiotensin generation by the blood vessel wall.

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CHAPTER 6

Prorenin, Renin, Angiotensinogen and Angiotensin-Converting Enzyme in Normal and Failing Human Hearts. Evidence for Renin-Binding.

Abstract

A local renin-angiotensin system in the heart is often invoked to explain the beneficial effects of ACE inhibitors in heart failure. The heart however produces little or no renin under normal conditions. In this study we compared the cardiac tissue levels of renin angiotensin system components in 10 potential heart donors who died of non-cardiac disorders and 10 subjects with dilated cardiomyopathy (DCM) who underwent cardiac transplantation. Cardiac levels of renin and prorenin in DCM patients were higher than in the donors. The cardiac and plasma levels of renin in DCM were positively correlated and extrapolation of the regression line to normal plasma levels yielded a tissue level close to that measured in the donor hearts. The cardiac tissue-to-plasma concentration (T/P) ratios for renin and prorenin were threefold the ratio for albumin, which indicates that the tissue levels were too high to be accounted for by admixture with blood and diffusion into the interstitial fluid. Cell membranes from porcine cardiac tissue bound porcine renin with high affinity. The T/P ratio for ACE, which is membrane-bound, was fivefold the ratio for albumin. Cardiac angiotensinogen was lower in DCM patients than in the donors and its T/P ratio was half that for albumin, which is compatible with substrate consumption by cardiac renin. These data in patients with heart failure support the concept of local angiotensin production in the heart by renin that is taken up from the circulation. Membrane-binding may be part of the uptake process.

Introduction

The RAS plays an important role in the regulation of blood pressure and salt and fluid homeostasis. The kidney releases both renin and its inactive precursor prorenin into the circulation. Liver-derived angiotensinogen is cleaved in the circulating blood by renin, to form Ang I, which is then converted by ACE, located on the luminal side of the vascular endothelium, into Ang II, a potent vasoconstrictor and stimulant of the release of aldosterone.

Agents that interfere with Ang II formation, the ACE inhibitors in particular, are now widely used for the treatment of hypertension and heart failure. Clinical studies indicate that the beneficial effects of ACE inhibitor treatment in heart failure and left ventricular hypertrophy are not solely determined by the effect of ACE inhibition on systemic arterial pressure.[1,2] It is, therefore, postulated that these beneficial responses are independent, at least partly, of the effects of ACE inhibition on the circulating RAS.

There is growing evidence to suggest that in cardiac tissue, Ang II is produced locally and does not originate from circulating Ang I.[3,4] However, whether the cardiac Ang I and II production depends on renin from the kidney, remains a matter of dispute. In normal cardiac tissue of mice and rats, renin mRNA levels are undetectable or extremely low.[5,6] In support of this finding is the fact that, after bilateral nephrectomy in the pig, cardiac renin, Ang I and Ang II decrease to levels at or below the detection limit[4]; the same is true for cardiac Ang I and II in the rat.[7] Renin expression, however, may be induced under pathological conditions. Renin mRNA can be detected in rat ventricle after myocardial infarction[8] and in rat atrium after low-sodium diet and treatment with the ACE inhibitor enalapril.[9]

Angiotensinogen and ACE mRNAs have been detected in normal cardiac tissue.[10-13] Angiotensinogen mRNA is increased during post-infarction ventricular remodeling in the rat[11], and ACE mRNA is increased during pressure overload-induced ventricular hypertrophy in the rat[12] and heart failure in humans.[13] Increased levels of ACE activity have been found in left ventricular aneurysms of patients after myocardial infarction.[14]

In the present study we measured the tissue levels of prorenin, renin, angiotensinogen and ACE in normal and failing human hearts. We compared the tissue levels of these RAS components with the levels in simultaneously obtained plasma, to address the possibility of cardiac angiotensin formation independent of kidney-derived renin. The possibility of sequestration of circulating renin by cardiac tissue through binding to cardiac cell membranes was investigated in renin-binding studies, with the use of cardiac membranes from freshly obtained porcine hearts.

Materials and Methods

Collection of cardiac tissue and blood samples

Left ventricular tissue was obtained from 10 subjects (9 men and 1 woman; age, 30 to 64 years) with end-stage DCM (origin: ischemic heart disease, 7; idiopathic, 2; Becker muscular dystrophy, 1) undergoing cardiac transplantation, and from 10 subjects (5 men and 5 women, age, 3 to 54 years) who had died from non-cardiac causes (cerebrovascular accident, 4; polytrauma, 4; brain tumor, n=2) <24 hours before the tissue arrived in the laboratory. The heart donors were not on cardiovascular medication. The medication of one subject with end-stage DCM could not be retraced. Of the remaining 9 subjects, 9 were receiving a diuretic; 8, an ACE inhibitor (captopril, 5 [average dose 56.25 mg/d]; enalapril, 3 [average dose 10 mg/d]; 6, a positive inotropic drug; 3, an antiarrhythmic drug; 2, isosorbide dinitrate 1, nifedipine; and 1, hydralazine.

The donor hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services Foundation/Eurotransplant Foundation) after removal of the aortic and pulmonary valves for homograft valve transplantation. The hearts had been taken out of the body immediately after circulatory arrest and maintained at 0° to 4°C in a sterile organ-protecting solution (University of Wisconsin-, EuroCollins-, or HTK-Bretschneider-solution).[15] After arrival in the laboratory, a 5 to 10-g piece of left ventricular free wall was dissected from the heart and stored at -70°C. Pieces of left ventricular free wall (3 to 5 g) taken from the failing hearts were frozen in liquid nitrogen and also stored at -70°C, immediately after the heart had been removed from the body.

Blood was obtained from 6 cardiac transplant recipients at the time of transplantation. Blood samples were collected into polystyrene tubes containing trisodium citrate (final concentration in blood, 0.013 mol/L), and centrifuged at 3000 *g* for 10 minutes at room temperature. Plasma was stored at -70°C and assayed within 1 month.

Frozen cardiac tissue (≈1 g) was rapidly minced into small pieces and homogenized (1:2 [w/vol]) in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, with a Polytron PT10/35 (Kinematica).[4] The cardiac tissue homogenates were stored at -20°C and assayed within 1 week.

Measurements of renin and prorenin

Renin in plasma was quantified by measuring Ang I generation at pH 7.4 during incubation at 37°C with a saturating concentration of sheep angiotensinogen in the presence of angiotensinase, ACE, and serine protease inhibitors (routine enzyme kinetic assay). Ang I generation under these conditions is linear for at ≥3 hours and the recovery of Ang I added to plasma before the incubation step at 37°C is 98%.[16]

Due to the presence of high angiotensinase activity in cardiac tissue homogenates, Ang I generation cannot be measured by the routine enzyme kinetic assay in these homogenates, despite the addition of angiotensinase inhibitors.[4] Thus, to reliably measure Ang I-generating activity in cardiac tissue homogenates, the angiotensinases must be removed (e.g., through an acidification step)[4,16] or the breakdown of Ang I must be prevented by rapidly binding it to an Ang I antibody (antibody-trapping enzyme kinetic assay).[4,17] Because acidification also leads to activation of prorenin[4,18], we used the antibody-trapping enzyme kinetic method to measure renin in the cardiac tissue homogenates. With this assay, we found Ang I-generation at 37°C to be linear for 30 minutes In addition, the recovery of [Ile5]-Ang I added to cardiac tissue homogenates before incubation at 37°C was >75% (n=4). Results of the routine enzyme kinetic and antibody-trapping assays show good agreement.[4]

Prorenin was first converted into renin (activation) and then measured with the routine enzyme kinetic assay. Prorenin in plasma was activated with Sepharose-bound trypsin.[16] Based on our experience with the activation of prorenin in tissues,[4,19] we tested two different procedures to convert cardiac prorenin into renin (i.e., acidification only or acidification followed by treatment with plasmin at neutral pH).

Tissue homogenate was acidified through dialysis at 4°C for 48 hours 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 (1) dialysis at 4°C for 24 hours against 0.1 mol/L phosphate buffer, pH 7.4, containing 0.001 mol/L disodium 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 0.2 vol of a solution of human plasmin (final concentration, 1 casein unit/mL) in 0.15 mol/L NaCl and incubation at 4°C for 48 hours.[4,18] The acidification step in the two procedures effectively removed the angiotensinase activity; the recovery of [Ile5]-Ang I added to cardiac tissue homogenate at pH 7.4, after acid-treatment or acid-and-plasmin treatment, was >95% in the routine enzyme kinetic assay (n=4).

Acid-treatment of cardiac tissue homogenate 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 (a gift of dr W. Fischli, Hoffmann-La Roche, Basel, Switzerland)[4] that was added to the homogenates before the activation step (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 65% to 78% (n=3). The cardiac tissue homogenates were therefore activated by the combined acid-and-plasmin method.

All samples were assessed in duplicate. Results are expressed as μU/mL or μU/g with the use of the international human kidney renin standard MRC 68/356 (Medical Research Council, National Institute of Biological Standards and Control, London, UK) as a reference. The normal range in plasma is 8 to 55 μU/mL for renin and 88 to 390 μU/mL for prorenin.[20]

Identification of cardiac Ang I-generating activity as renin.

Part of the Ang I-generating activity of cardiac tissue homogenates may be related to the presence of pseudorenin (e.g., cathepsin D).[21] To distinguish true renin from pseudorenin, we used the specific renin inhibitor remikiren, which has an IC_{50} value of 7×10^{-10} mol/L for human renin and 3.5×10^{-5} mol/L for bovine cathepsin D.[22]

Percent inhibition of Ang I-generating activity was determined at inhibitor concentrations ranging from 10^{-11} to 10^{-5} mol/L. The inhibition curves for cardiac tissue homogenates were compared with those for plasma and the human kidney renin standard MRC 68/356.

We also used monoclonal renin antibodies to identify Ang I-generating activity as 'true' renin. For this pupose, renin was measured with a sandwich immunoradiometric assay.[20] Monoclonal antibody R 3-36-16[23], which reacts equally well with renin and prorenin, was biotinylated and served as a primary antibody in the assay.[24] Monoclonal antibody R 1-20-5[25], which reacts with renin but not with prorenin, was labeled with ^{125}I (specific activity, 740 kBeq/µg) and served as the secondary antibody. The results of this assay were expressed as µU/g using the human kidney renin standard MRC 68/356 as a reference.

Binding of renin to cardiac membranes

Cardiac membranes were prepared from freshly obtained porcine left ventricular tissue (20 g) as previously described.[4] The membrane fraction contained both plasma- and sarcoplasmic reticulum-membranes. Semipurified kidney renin was prepared from porcine kidney.[4] Twenty-five µL of the cardiac membrane fraction, containing ≈125 µg protein, was incubated in a shaking waterbath at 37°C and pH 7.4 for 2 hours with 250 µL semi-purified porcine kidney renin at six different concentrations (20 to 400 pmol Ang I/min per mL). Non-specific binding was measured by incubating renin with membranes that had been heated for 10 minutes at 95°C. The incubation was terminated by the addition of 3 mL of ice-cold 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, followed by rapid vacuum filtration through a Whatman GF/B filter. Filters were washed four times with 3 mL phosphate buffer and incubated with sheep angiotensinogen to measure renin according to the routine enzyme kinetic assay. The dissociation constant (K_d) and the maximum number of binding sites (B_{max}) were calculated from plots according to Scatchard.[26]

Measurements of angiotensinogen, ACE and serum albumin

Angiotensinogen was measured as the maximum quantity of Ang I that was generated during incubation at pH 7.4 and 37°C with a high concentration of human kidney renin (10 mU/mL) in the presence of inhibitors of ACE and angiotensinases[27]. The normal range in plasma is 745 to 2340 pmol/mL.[27,28]

ACE was measured with a commercial kit (ACE Color, Fujirebio; normal range, 7 to 20 mU/mL).[29]

Serum albumin was measured with single radial immunodiffusion (LC and NOR-Partigen plates, Behringwerke) according to the method of Mancini et al.[30] The normal range is 22 to 43 mg/mL.[28]

Statistical analysis

Differences between cardiac transplant recipients and donors were evaluated for statistical significance by using Student's *t*-test or Mann-Whitney's *U*-test for unpaired observations. Statistical significance was accepted at $P<0.05$.

Results

Levels of renin, prorenin, angiotensinogen and ACE in cardiac tissue

The specific renin inhibitor remikiren, at concentrations of 10^{-8} mol/L, caused virtually complete inhibition of the Ang I-generating activity in the acid-and-plasmin treated cardiac tissue homogenates (Figure 1). The inhibition curve (relating the degree of inhibition to the inhibitor concentration) for cardiac tissue homogenate treated with acid followed by plasmin was identical to the curves for untreated plasma and the human kidney renin standard, which supports the assumption that the measured Ang I-generating activity of cardiac tissue homogenate is a valid estimate of true renin. The IC_{50} value was $\approx 3 \times 10^{-10}$ mol/L, which is in accordance with reports in the literature.[22] The inhibition curve for untreated cardiac tissue homogenate differed somewhat from the inhibition curves for plasma renin and kidney renin (Figure 1); the difference can be accounted for by assuming that 30% of the Ang I-generating activity in untreated cardiac homogenate was caused by pseudorenin.

We therefore measured true renin in untreated cardiac tissue homogenates as the difference between the Ang I-generating activity in the absence of remikiren and the Ang I-generating activity in the presence of 10^{-5} mol/L remikiren.

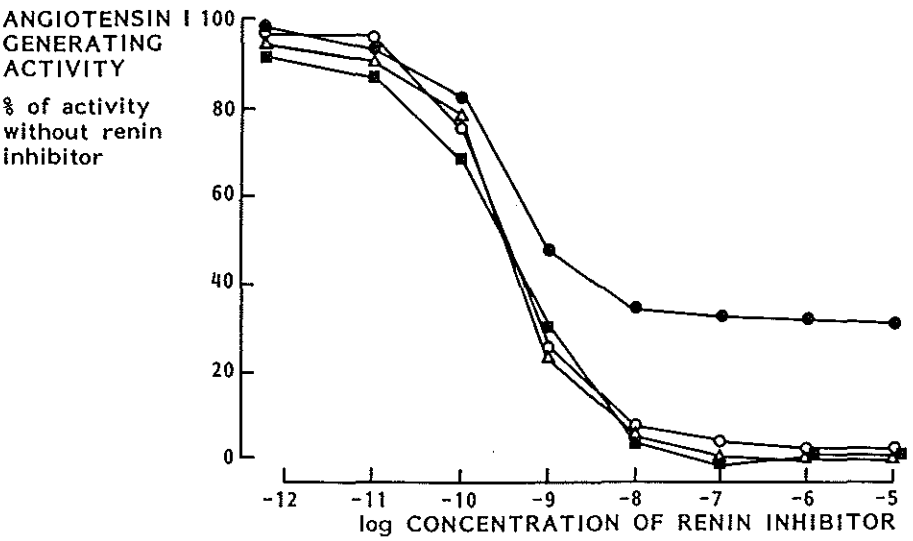

Figure 1. Inhibition of angiotensin I-generating activity by increasing concentrations of the renin inhibitor remikiren. Closed circles indicate untreated cardiac tissue homogenate; open circles, cardiac tissue homogenate pretreated with acid and plasmin; open triangles, human kidney renin standard MRC 68/356; and closed squares, plasma.

Comparison between the results of the enzyme kinetic and immunoradiometric assays of renin in cardiac tissue homogenate and plasma from 5 subjects with DCM showed good agreement (Figure 2), confirming that the enzyme kinetic assay is indeed a valid measurement of true renin. According to the enzyme kinetic assay, the plasma concentration of naturally occurring renin in subjects with DCM was 80±23% (mean±SD) of the renin concentration after prorenin activation compared with 65±33% according to the immunoradiometric assay (difference not significant). The cardiac tissue concentration of naturally occurring renin in these subjects was 59±19% of the renin concentration after prorenin activation in the enzyme kinetic assay and 77±11% in the immunoradiometric assay (difference not significant).

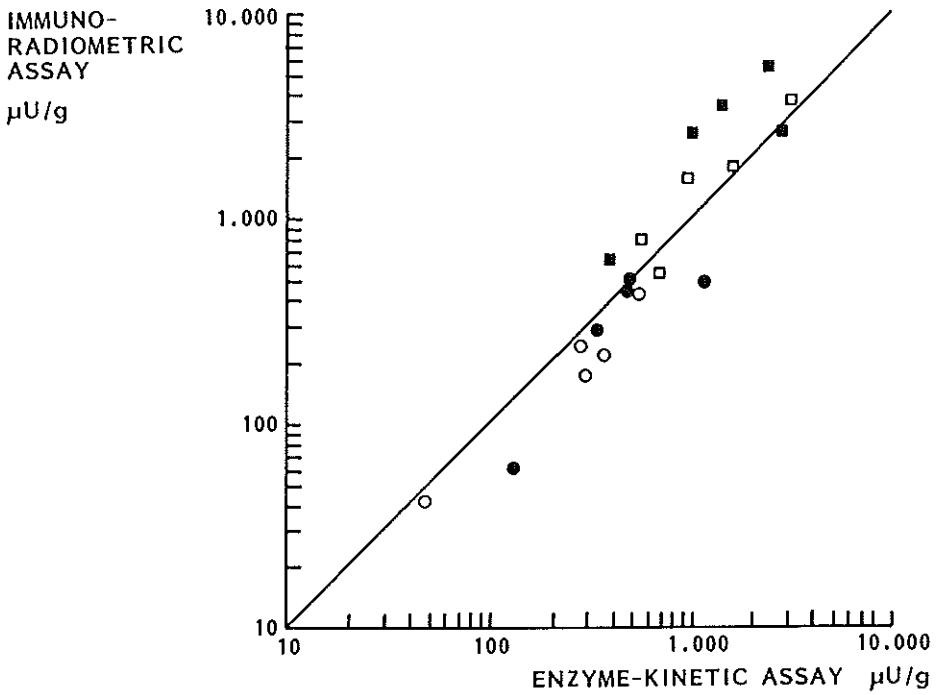

Figure 2. Renin measured by enzyme kinetic assay vs. renin measured by immunoradiometric assay in cardiac tissue (circles) and plasma (squares), before (open symbols) and after (closed symbols) prorenin activation ($r=.92$, $P<.01$).

Figure 3 is a comparison of the cardiac tissue levels of renin, prorenin, angiotensinogen, ACE and serum albumin between the DCM patients and the donors. The levels of renin and prorenin were more than fivefold higher in the patients, whereas angiotensinogen was lower. Cardiac ACE was not different between the two groups. The cardiac tissue level of serum albumin was somewhat higher in the DCM patients than in the donors.

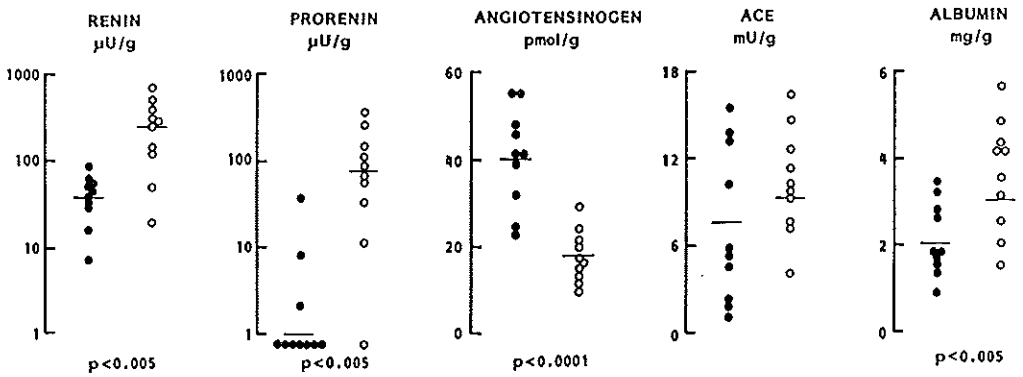

Figure 3. Renin, prorenin, angiotensinogen, ACE and albumin in cardiac tissue of heart donors (closed circles) and patients with dilated cardiomyopathy (open circles). Prorenin was below the detection limit in 7 donors and one patient. Medians (renin and prorenin) and means (angiotensinogen, ACE and albumin) are shown. Differences between donors and patients were tested by Mann-Whitney's *U*-test (renin and prorenin) or Student's *t*-test (angiotensinogen, ACE and albumin) for unpaired observations. Plasma levels at the time of heart transplantation was available for 6 of the 10 DCM cases.

Plasma renin in these subjects ranged from 472 to 3028 μU/mL, which is more than sevenfold the normal level. Plasma prorenin ranged from 180 to 1214 μU/mL, and in 3 subjects it was above normal. Plasma angiotensinogen ranged from 77 to 484 pmol/mL, which is below the normal range. Plasma ACE ranged from 5.8 to 28.9 mU/mL, and in 3 subjects it was above normal. Albumin in plasma was normal; it ranged from 22 to 34 mg/mL.

The cardiac tissue-to-plasma concentration ratio for serum albumin was ≈12% (Figure 4), which is in agreement with the fact that the localization of this protein is restricted to the extracellular fluid. Albumin (molecular mass, 70 kD) is known to be present in the interstitial fluid albeit in lower concentrations than in plasma.[31]

The cardiac tissue-to-plasma concentration ratio for angiotensinogen (molecular mass, 65 kD) was 6%, which is in keeping with the contention that cardiac angiotensinogen is also localized in the extracellular fluid. The lower ratio for angiotensinogen than for albumin may suggest that the angiotensinogen consumption rate, and therefore the Ang I production rate, is higher in cardiac tissue than in circulating plasma.

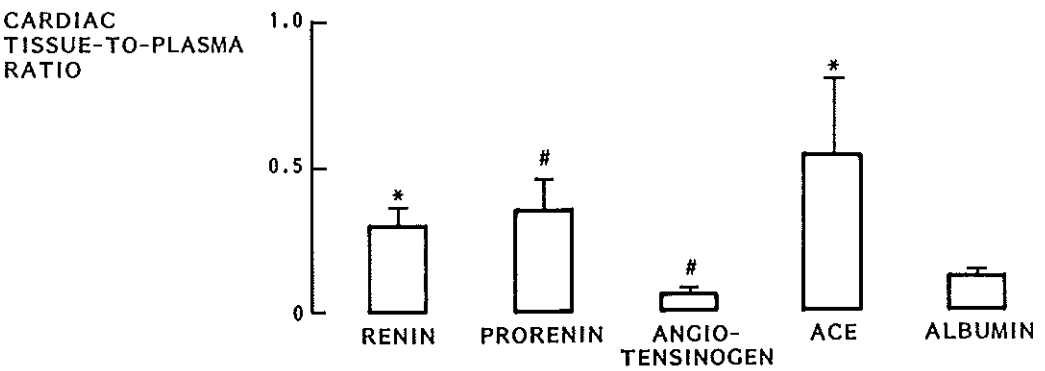

Figure 4. Cardiac tissue-to-plasma concentration ratios for renin, prorenin, angiotensinogen, ACE and albumin in patients with DCM. Data are means±SD *P<.005 albumin; #P<.05 albumin (Student's t-test for paired observations).

The cardiac tissue levels of renin and angiotensinogen were negatively correlated (Figure 5), most likely because increased renin leads to increased substrate consumption. The cardiac tissue-to-plasma ratio for ACE was 54%, which was much higher than the ratio for serum albumin. Most of the ACE in cardiac tissue is probably cell membrane-bound[4]. The cardiac tissue-to-plasma concentration ratios for renin and prorenin (molecular mass, 48 and 54 kD respectively) were 30% and 36%, which was also higher than the ratio for serum albumin. This suggests that the localization of these proteins is not restricted to the extracellular fluid compartment.

The cardiac tissue levels of renin in the subjects with DCM were directly correlated with the plasma levels of renin (log [cardiac renin] = 0.50 x log [plasma renin]+0.93; r=.84, $P< 0.05$). There was no significant correlation between the prorenin levels in cardiac tissue and plasma.

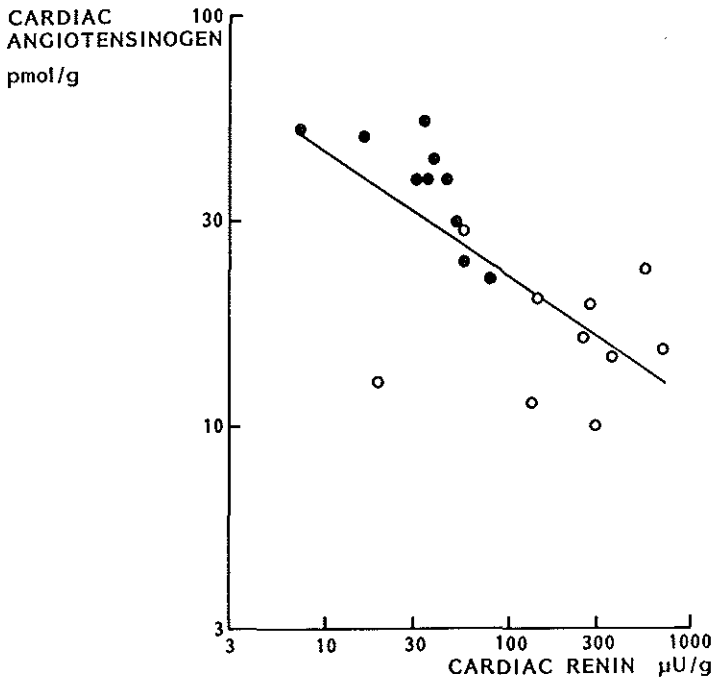

Figure 5. Relationship between cardiac renin and cardiac angiotensinogen in heart donors (closed circles) and patients with dilated cardiomyopathy (open circles) (r=.72, $P<.05$).

Binding of renin to cardiac membranes

Cardiac membranes prepared from porcine left ventricular tissue (n=8) bound porcine renin in a dose-dependent way (Figure 6). Binding was maximal within 15 minutes. According to Scatchard analysis, K_d was 0.21±0.11 nmol Ang I/min per mL and B_{max} was 0.5±0.3 pmol Ang I/min per mg protein. One nanomole of pure porcine renin generates during incubation at 37°C with saturating concentrations of porcine angiotensinogen ≈200 nmol Ang I/min.[32,33]

With our semipurified preparation of porcine kidney renin we found the maximum reaction velocity (V_{max}) when incubated with sheep angiotensinogen to be similar to the V_{max} when incubated with angiotensinogen prepared from nephrectomized pigs.[34] Thus K_d was in the order of 10^{-9} mol/L, and B_{max} was ≈2 fmol/mg protein.

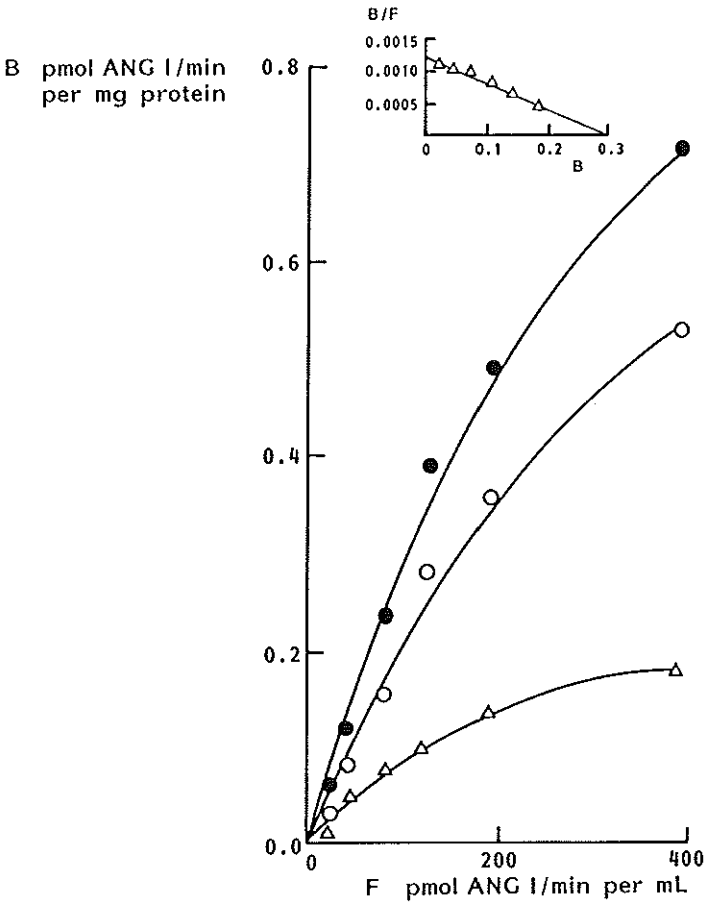

Figure 6. Binding of semipurified porcine kidney renin to cardiac membranes prepared from porcine left ventricular tissue. Specific binding (open triangles) was taken as the difference between total binding (closed circles) during incubation of renin with intact cardiac membranes, and non-specific binding (open circles) during incubation of renin with cardiac membranes after their denaturation by heating. K_d and B_{max} were calculated from a plot according to Scatchard[26] (inset).

Discussion

The results of the present study demonstrate the presence of increased concentrations of renin and prorenin in left ventricular tissue from patients with DCM. The cardiac levels of renin and prorenin were more than fivefold the cardiac levels in the donors. In addition, the cardiac tissue-to-plasma concentration ratios for renin and prorenin (molecular mass, 48 kD and 54 kD, respectively) were about 3 times the ratio for serum albumin (molecular mass, 70 kD), indicating that the levels of renin and prorenin in cardiac tissue were too high to be explained by admixture with blood or by diffusion from the blood into the interstitial fluid. In normal porcine left ventricular tissue, the renin level was also higher than can be explained by its localization in extracellular fluid.[4]

Purified membrane fractions prepared from porcine left ventricular tissue contained renin,[4] and specific binding of renin and prorenin to rat renal and other tissue membranes has been reported.[35,36] In the present study, we observed high-affinity binding of porcine renin to porcine cardiac membranes. Since the membrane fraction contained both plasma membranes and sarcoplasmic reticulum, it is unclear whether renin binding was specific for plasma membranes. Chemical cross-linking studies also suggest the presence of renin-binding proteins in rat vascular tissue membranes.[37] Our findings in the human heart, which indicate that renin and prorenin in cardiac tissue are not restricted to the extracellular fluid, may therefore be explained by binding to cell membranes.

ACE, which is known to be a membrane-bound enzyme, was also found in cardiac tissue in concentrations that were higher than could be explained by its presence in the extracellular fluid. Cardiac ACE levels did not differ between the DCM patients and the donors. It should be noted, however, that 8 of the 10 patients with DCM were receiving ACE inhibitor treatment at the time of cardiac transplantation. This may have led to some underestimation of ACE activity in these patients. In normal hearts, ACE appears to be limited to the vascular endothelium and the endocardium.[38,39]

After myocardial infarction in humans, ACE can also be detected in the remaining viable cardiomyocytes near the infarct scar of the aneurysmal left ventricle,[38] as well as in fibroblasts, vascular smooth muscle cells and macrophages in the scar area itself.[38] After coronary occlusion in rats, ACE was demonstrated in fibroblasts in the healthy hypertrophying part of the heart.[40]

The patients with DCM had markedly increased levels of renin and reduced levels of angiotensinogen in circulating plasma at the time of transplantation. These are characteristic findings in patients with end-stage heart failure who are treated with diuretics and ACE inhibitors.[41,42] The cardiac level of angiotensinogen in these patients was approximately one third of the level in the donors and was negatively correlated with the renin concentration. This is probably related to the fact that substrate consumption is increased when renin is increased. An interesting finding was that the cardiac tissue-to-plasma concentration ratio for angiotensinogen (molecular mass, 65 kD) was half the ratio for albumin (molecular mass, 70 kD). This is evidence in favor of consumption of angiotensinogen that is present in the cardiac extracellular fluid through the reaction with renin, thereby implying local Ang I production occurs in the heart.

In the DCM patients in whom measurements of renin were available in cardiac tissue as well as in plasma, the tissue and plasma levels showed a positive correlation. Extrapolation of the regression line to normal plasma concentrations yielded a tissue concentration of renin close to the concentration measured in the donor hearts. Thus, there was no evidence to suggest that the regulation of cardiac renin is independent of the regulation of circulating renin. A previous study by our group of the effect of nephrectomy on the cardiac levels of renin in healthy pigs showed that most, if not all, renin in cardiac tissue originates from the kidney.[4] Studies, in which intravenous bolus injections of radiolabeled renin were given to rats and monkeys, have demonstrated that the enzyme accumulated in the heart.[43,44]

Prorenin in cardiac tissue was not significantly correlated with prorenin in plasma. It should be noted, however, that the measurement of prorenin is not as accurate as the measurement of renin. Renin was measured as the Ang I-generating activity without prior in-vitro activation of prorenin, whereas prorenin was measured as the difference between the Ang I-generating activity after prorenin activation and

the Ang I-generating activity before activation. The results of this subtraction method are sufficiently accurate when renin is low compared with prorenin, as is the case in normal plasma. Results are less accurate when the renin-to-prorenin ratio is higher, as was the case in both plasma and cardiac tissue in the patients with DCM. Another explanation for the apparent lack of a correlation between the measured levels of prorenin in cardiac tissue and plasma might be that part of the plasma-derived prorenin is activated in cardiac tissue. Such in vivo activation of plasma-derived prorenin in extrarenal tissues, however, has never been demonstrated.

In addition to the evidence presented here, there is growing evidence from the literature to support the existence of local angiotensin formation in cardiac tissue. Perfusion of the isolated rat heart with renin leads to Ang I and II release into the coronary effluent[3] and to Ang I release into the interstitial fluid.[45] Without the addition of renin to the perfusion fluid, the cardiac release of Ang I and II was practically zero. The study reported here provides no data on Ang I and II levels in cardiac tissue and in plasma. Special precautions are required when samples for measuring these peptides are collected, and these precautions could not always be taken in the setting of this study. It appears logical to assume that the elevated renin concentrations in the hearts affected by DCM will promote cardiac Ang I and II production. Experiments in intact pigs showing reduced cardiac contractility in response to intracoronary administration of the renin inhibitor remikiren, support that cardiac Ang II production has functional significance[46]; the time course of this response was not correlated with the effects of the inhibitor on the levels of Ang I and II in the circulation. Intracardiac Ang I and II production may also participate in the long-term processes of hypertrophy and remodeling after myocardial infarction.[47]

In summary, our results add to the growing evidence in support of local Ang I and II production by the heart. So far, attempts to show that the production of these peptides is independent of renin from the circulation have failed, at least in normal hearts. The present study also fails to provide such evidence in DCM patients with severe heart failure; rather, our results are in keeping with the contention that renin from the circulation is taken up by the heart and that tissue binding of renin is part of the uptake process.

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CHAPTER 7

Summary and Concluding Remarks

General Introduction

Since the discovery of the kidney-derived pressor substance renin by Tigerstedt one hundred years ago, investigations on the renin-angiotensin system have taken an enormous flight. Not only are various compounds of this system now recognized as important effector molecules, a diversity of receptors that can bind these compounds has also been discovered. In view of the success of the renin-angiotensin system blockers in the treatment of cardiovascular diseases, much attention has been paid in the past ten years to the concept of 'local' angiotensin production in heart and blood vessels. At first such production was thought to depend on locally synthesized renin. More recent evidence suggests that it is circulating (i.e., renal) renin which contributes mostly to this local production of angiotensin II. In the present thesis we have studied whether cultured cardiac cells synthesize renin or its inactive precursor, prorenin. Furthermore, we have investigated how circulating renin (and prorenin) may reach tissue sites, and whether prorenin is activated to renin at these sites. Finally, the hypertrophic/hyperplastic effects of angiotensin II were studied in cardiac cells, taking into account the various receptors that are stimulated by this peptide.

Chapters 2 and 3

Demonstration of renin-angiotensin system (RAS) components in cardiac tissue cannot be taken as definite evidence for synthesis of these components in the heart, due to interference with plasma-derived RAS components. To avoid the problems arising from ex-vivo measurements, renin, prorenin, angiotensinogen, ACE, angiotensin I and angiotensin II were measured in the medium and cell lysate of neonatal rat cardiac myocytes and fibroblasts, cultured under serum-free conditions. Measurements were also made in the fetal calf serum- and horse serum-containing medium which had been used to obtain cell adherence and confluency prior to the serum-free period, and in medium of serum-deprived cardiomyocytes exposed to cyclic stretch.

All RAS components were detectable in unconditioned serum-supplemented medium. Prorenin, but none of the other RAS components, could be detected in medium of serum-deprived cells. However, its levels were low and the angiotensin I-generating activity corresponding with these low prorenin levels could not be inhibited by the specific rat renin inhibitor CH-732.

This suggests that the measured prorenin was most likely 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 angiotensin I under serum-free conditions, both cardiomyocytes and fibroblasts generated angiotensin II in a captopril-inhibitable manner. Lysates of serum-deprived cells did not contain renin, prorenin, angiotensinogen, angiotensin I or angiotensin II in detectable quantities. Stretch increased protein synthesis by 20% and was not accompanied by angiotensin release into the medium.

Taken together, these results suggest that 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 angiotensin II concentrations of physiological relevance. These cells do synthesize ACE, thereby allowing the synthesis of angiotensin II at cardiac tissue sites when renin and angiotensinogen are provided via the circulation.

Although angiotensin II is a well-known stimulator of cardiovascular growth and remodelling via AT_1 receptors, it did not appear to be a prerequisite to observe a hypertrophic response of cardiac cells following stretch. Additional experiments were therefore performed to study the effects of exogenous angiotensin II on protein and DNA synthesis in neonatal rat cardiac myocytes and fibroblasts. In view of the possibility that angiotensin II may inhibit growth via AT_2 receptors, the effects of exogenous angiotensin II were also investigated in cardiac cells in which the protein and DNA synthesis had been stimulated with endothelin-1. Total protein and total DNA, as well as [^{3}H]leucine and [^{3}H]thymidine incorporation were measured following incubation with either vehicle, angiotensin II, endothelin-1 or angiotensin II + endothelin-1, both in the presence or absence of the AT_1 receptor blocker losartan or the AT_2 receptor blocker PD123319.

In myocytes, endothelin-1 increased total protein as well as [^{3}H]leucine and [^{3}H]thymidine incorporation. Angiotensin II did not affect any of these parameters, nor did it influence the endothelin-1-induced responses. However, in the presence of PD123319, angiotensin II stimulated [^{3}H]leucine and [^{3}H]thymidine incorporation.

In fibroblasts, endothelin-1 and angiotensin II did not significantly affect total DNA and [^{3}H]thymidine incorporation. Angiotensin II tended to increase total protein in these cells, an effect which was significant only in the presence of PD123319.

Angiotensin II stimulated [^{3}H]leucine incorporation in fibroblasts. This effect was absent with losartan and enhanced in the presence of PD123319. These data demonstrate that AT_1 receptor-mediated proliferative effects of angiotensin II in neonatal cardiac cells may become apparent only when its AT_2 receptor-mediated antigrowth effects are blocked (Figure 1). The net growth effect of angiotensin II therefore depends on the cellular AT_1/AT_2 receptor ratio. Angiotensin II did not interfere with endothelin-1-induced effects.

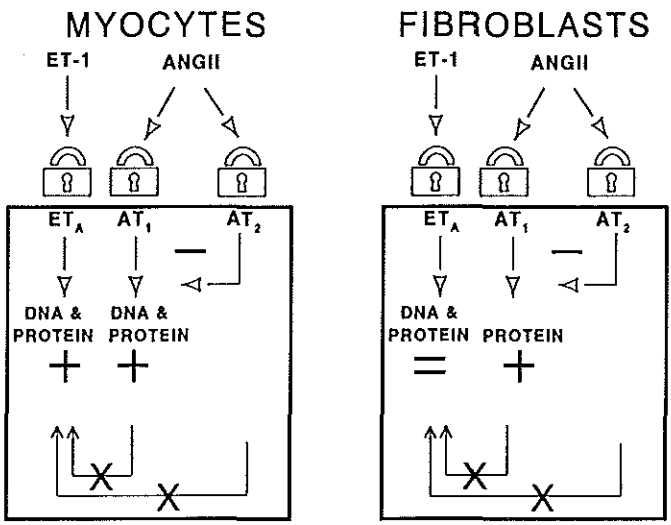

Figure 1. Growth-stimulatory and growth-inhibitory effects of angiotensin II (ANGII) and endothelin-1 (ET-1) in neonatal rat cardiac myocytes and fibroblasts.

Chapters 4 and 5

Since (pro)renin does not appear to be synthesized in the heart and vasculature under normal circumstances, local generation of angiotensin II at these sites may depend on (pro)renin that is taken up from the circulation. Here, the binding and internalization of recombinant human renin and prorenin, as well as the activation of prorenin to renin, were studied in 1) neonatal rat cardiac myocytes and fibroblasts and 2) human umbilical vein endothelial cells (HUVECs).

The cells were incubated with recombinant human prorenin or renin in the presence or absence of putative inhibitors of renin internalization. Surface-bound and internalized enzyme were distinguished by the acid-wash method.

Prorenin and renin were bound and internalized at 37°C in a dose-dependent manner by all cells. At 4°C, (pro)renin remained surface-bound and was not internalized. Prorenin that had been treated with endoglycosidase H to remove N-linked oligosaccharides was also not internalized. Prorenin was activated by the cells, and cell-activated prorenin was only found in the internalized fraction, whereas the surface-bound prorenin remained inactive. Thus, it appears that the activation of prorenin took place at the time of its internalization or thereafter.

Addition of mannose 6-phosphate to the medium caused a dose-dependent inhibition of renin and prorenin internalization. Mannose 1-phosphate, glucose 6-phosphate and α-methylmannoside had no effect. Ammonium chloride and monensin, which interfere with the normal trafficking and recycling of internalized receptors and ligands, inhibited the activation of prorenin. In HUVECs the activation of prorenin was monitored by a direct immunoradiometric assay (IRMA) with the use of a monoclonal antibody directed against the -p24-Arg to -1p-Arg C-terminal propeptide sequence of prorenin. The results indicated that activation was associated with proteolytic cleavage of the propeptide.

These findings provide an explanation for recent experimental data indicating that angiotensin I and II are produced at cardiac and other extrarenal tissue sites by the action of renal renin. This local angiotensin generation may occur either on the cell surface or intracellularly, and may involve both renin and locally activated prorenin.

Chapter 6

A local renin-angiotensin system in the heart is often invoked to explain the beneficial effects of ACE inhibitors in heart failure. The heart however produces little or no renin under normal conditions. To investigate whether cardiac renin production occurs under pathological conditions, the cardiac tissue levels of renin and other components of the renin-angiotensin system were compared in 10 potential heart donors who died of non-cardiac disorders and 10 subjects with dilated cardiomyopathy (DCM) who underwent cardiac transplantation.

Cardiac levels of renin and prorenin in DCM patients were higher than in the donors. The cardiac and plasma levels of renin in DCM were positively correlated and extrapolation of the regression line to normal plasma levels yielded a tissue level close to that measured in the donor hearts. The cardiac tissue-to-plasma concentration (T/P) ratios for renin and prorenin were threefold the ratio for albumin, which indicates that the tissue levels were too high to be accounted for by admixture with blood and diffusion into the interstitial fluid. Cell membranes from porcine cardiac tissue bound porcine renin with high affinity.

The T/P ratio for ACE, which is membrane-bound, was fivefold the ratio for albumin. Cardiac angiotensinogen was lower in DCM patients than in the donors and its T/P ratio was half that for albumin, which is compatible with substrate consumption by cardiac renin. These data, obtained in patients with heart failure, suggest that also under pathological conditions local angiotensin production in the heart depends on renin that is taken up from the circulation. Membrane-binding may be part of the uptake process.

Concluding Remarks

The experiments described in this thesis focus on the cardiac synthesis of components of the renin-angiotensin system, the role of angiotensin II in the development of cardiac hypertrophy, and the uptake of (pro)renin by cultured cardiac and vascular cells.

Despite many reports in the literature on the synthesis and release of angiotensin II by cardiac cells,[1-4] we were unable to show that these cells are capable of synthesizing renin and angiotensinogen. Thus, either renin is not involved in the generation of angiotensin II by these cells, or the renin (and angiotensinogen) required for angiotensin II synthesis are derived from the serum 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 low or undetectable levels of angiotensin I and II in the heart following bilateral nephrectomy.[5,6] Therefore, the second option (uptake from serum) seems the most likely explanation. This would be in accordance with our earlier in-vivo findings, which suggested that cardiac renin, under normal circumstances, is derived from the circulation and therefore of renal origin.[5]

Binding to the cation-independent mannose 6-phosphate receptor (CI-MPR) might be (one of) the mechanism(s) by which cardiac cells, as well as endothelial cells, sequester circulating renin.

This receptor does not distinguish between renin and its inactive precursor, prorenin, as both enzymes contain the mannose 6-phosphate signal. Moreover, prorenin, once bound and internalized by the CI-MPR, is rapidly activated to renin. At present, it is not known whether locally activated prorenin contributes to angiotensin generation in the heart and vascular wall. Such local angiotensin generation may occur intracellularly, or outside the cell, after the release of cell-activated prorenin into the fluid surrounding the cells (Figure 2). The concept of prorenin contributing to local angiotensin generation is attractive, in view of the fact that the levels of prorenin in the circulation are tenfold higher than those of renin. However, since the CI-MPR is also involved in the clearance of enzymes (e.g., insulin-like growth factor II),[7] it is also conceivable that this receptor contributes to the clearance of (pro)renin. The latter would still imply a regulatory role with regard to local angiotensin production.

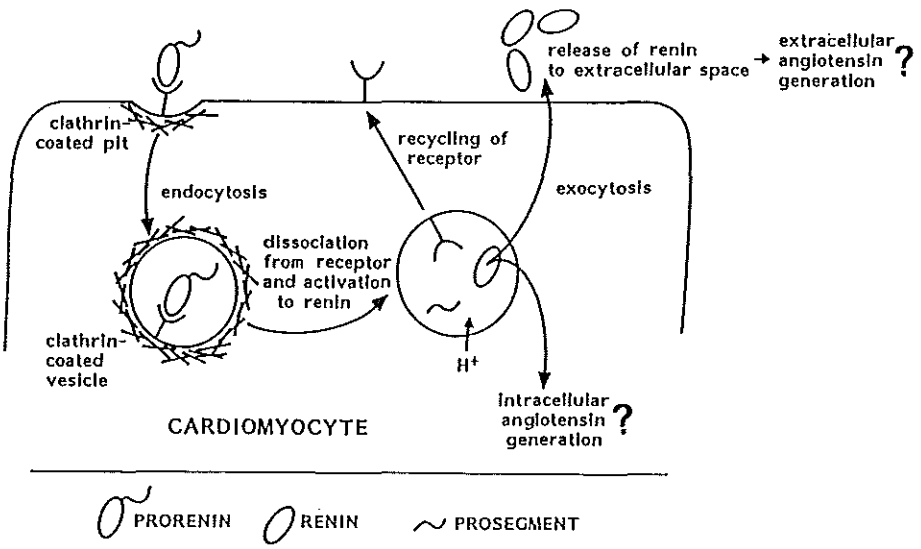

Figure 2. Binding and activation of prorenin by cardiomyocytes. After binding to the cell surface, the receptor-prorenin complex is internalized in a clathrin-coated pit, that pinches off to become a coated vesicle. The clathrin coat then depolymerizes, thereby leading to the formation of an endosome. 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.

It has been suggested, on the basis of mRNA measurements, that cardiac renin synthesis is switched on under pathological conditions.[8,9] Our findings in cardiac tissue of subjects with heart failure do not support this possibility. Cardiac renin in these subjects rose in parallel with plasma renin. Thus, most likely also under pathological conditions the majority of cardiac renin is derived from the circulation. The density of renin binding sites (CI-MPRs?) may however change under these circumstances.

ACE appears to be the only RAS component involved in cardiac angiotensin II generation that does not have to be sequestered from the circulation. After the addition of (pro)renin and angiotensinogen, cultured cardiac cells should therefore be capable of synthesizing angiotensin II. It is not yet known where this angiotensin II is located. It might be present in the cells, either because it has been synthesized intracellularly or because, after its extracellular generation, it has been internalized via AT_1 receptor-mediated endocytosis.[10]

Several studies suggest that neonatal rat cardiomyocytes release angiotensin II from intracellular storage sites after the induction of stretch, and that this angiotensin II contributes to the hypertrophic response observed after stretch.[2-4] 'Stretch' is assumed to lead to a myocyte phenotype that mimics that of load-induced cardiac hypertrophy. In the present study, stretch did lead to an increase in protein synthesis rate, but was not accompanied by autocrine angiotensin II release. Thus, the hypertrophic response we observed was not due to angiotensin II. This does not rule out the possibility that angiotensin II under certain circumstances may contribute to myocyte hypertrophy. In fact, experiments in which angiotensin II was added to the culture medium of cardiac cells showed that angiotensin II was capable of inducing a (modest) hypertrophic response, at least at concentrations > 10^{-9} M (i.e., more than 1000 times higher than the detection limit of our angiotensin assay) and in the presence of an AT_2 receptor antagonist. The latter is necessary since angiotensin II mediates antigrowth effects via AT_2 receptors, which mask its AT_1 receptor-mediated growth-stimulatory effects. Interestingly, this inhibitory effect was observed in relationship with the AT_1 receptor only, and not with other growth-stimulating receptors such as the ET_A receptor (Figure 1).

Future Studies

This study is the first to show binding and activation of prorenin by extrarenal cells. In addition, the counterregulatory role of the AT_2 receptor with regard to the AT_1 receptor-mediated growth-stimulatory effects was demonstrated in neonatal rat cardiac cells. Future studies should now address the following questions:

How is prorenin activated to renin in extrarenal cells?
We have observed that the intracellular activation of prorenin by cardiac cells and HUVECs is accompanied by proteolytic cleavage of the prosegment. It remains to be determined in which cellular compartment(s) the activation takes place, and what enzyme(s) is/are involved in this process. Such studies could be performed in cultured cells, using subsequent centrifugation steps to separate the various cellular compartments.

Is the CI-MPR the only receptor involved in (pro)renin binding?
All our studies on (pro)renin binding have been performed in isolated cells obtained from neonatal rats and human umbilical veins. The number of CI-MPRs in these cells is most likely much higher than in non-fetal/neonatal cells.[11,12] Therefore, our studies should be extended to cells of adult animals. Moreover, the effect of carbohydrate removal (or more specifically, mannose 6-phosphate removal) should be studied in detail, to address the question whether mannose 6-phosphate-independent binding occurs. Alternatively, binding studies (involving a range of (pro)renin concentrations) might be performed, preferably with ^{125}I-labelled (pro)renin, in the presence and absence of substances that prevent the binding of (pro)renin to the CI-MPR. Using this approach, high affinity (pro)renin binding sites have been demonstrated in rat tissue membranes that were not blocked by mannose 6-phosphate.[13]

Is (pro)renin binding affected by pathological conditions?
After the determination of the number of (pro)renin binding sites in cultured cells, binding studies should also be performed in tissue samples obtained from different patient groups (e.g., with and without heart failure). We did not obtain evidence for cardiac renin synthesis under pathological conditions.

However, the number of binding sites and/or the binding affinity might change under these conditions, thus allowing the heart (and other organs) to sequester more or less (pro)renin from the circulation, depending on the circumstances. For instance, Katz et al. observed that the rat heart preferentially retains high-mannose renin glycoforms after bilateral nephrectomy.[14]

Does (pro)renin binding and/or internalization result in an intracellular response?
(Pro)renin binding may lead to angiotensin II formation, and thereby induce an AT receptor-mediated response. Alternatively, (pro)renin binding to a receptor may be sufficient by itself to stimulate an intracellular second messenger cascade. The latter has been shown in human mesangial cells, where renin binding leads to an increase in DNA synthesis in the absence of angiotensin generation.[15] Thus, second messenger formation by cultured cardiac cells should be evaluated in response to (pro)renin, with or without the addition of angiotensinogen.

Does angiotensin release after stretch depend on the presence of (pro)renin in the culture medium prior to stretch?
In cardiomyocytes, we were unable to demonstrate angiotensin II release in response to cyclic stretch. The levels of (pro)renin in the serum used to culture the cells prior to stretch may have been too low to lead to significant cellular angiotensin generation and/or storage. Therefore, these experiments might be repeated after loading the cells with (pro)renin, which, in combination with the angiotensinogen present in the serum, should lead to significant angiotensin II formation.

How do pathological conditions affect the response to angiotensin II?
The AT_1 receptor-mediated growth-stimulatory effects of angiotensin II are counteracted by the AT_2 receptor. The number of AT_2 receptors is high in fetal tissue, and declines to low levels in adult animals. However, the AT_1/AT_2 receptor ratio may change considerably under pathological conditions (e.g., after myocardial infarction or in subjects with heart failure)[16-18] either due to a downregulation of AT_1 receptors, or to an upregulation of AT_2 receptors. Thus, the angiotensin II-induced hypertrophic responses should also be evaluated in cells obtained from infarcted and failing hearts.

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Nederlandse Samenvatting

Hoofdstuk 1

Honderd jaar geleden werd door Tigerstedt een stof ontdekt in nierextracten die de bloeddruk verhoogde, het renine. Sindsdien heeft het onderzoek naar het renine-angiotensine systeem (RAS) een enorme vlucht genomen. Het is nu bekend dat verschillende componenten van dit enzym-systeem (de belangrijkste is angiotensine II) effecten kunnen bewerkstelligen en dat hierbij diverse receptoren zijn betrokken. Het startpunt van het RAS is het (inactieve) prorenine dat kan worden omgezet in (actief) renine. Renine gaat een reactie aan met angiotensinogeen waardoor angiotensine I ontstaat dat vervolgens door het angiotensine-converterend enzym (ACE) wordt omgezet in angiotensine II.

Mede dankzij het succes van de blokkers van het renine-angiotensine systeem bij de behandeling van hart- en vaatziekten, is de afgelopen tien jaar veel aandacht besteed aan de hypothese dat angiotensine II lokaal (in het hart en de bloedvaten) wordt geproduceerd. Eerst dacht men dat deze produktie afhing van lokaal gesynthetiseerd renine. Recente bevindingen suggereren echter dat renine uit de bloedsomloop, dat door de nieren aan het bloed wordt afgegeven, het meeste bijdraagt aan de lokale produktie van angiotensine II.

In dit proefschrift is onderzocht of gekweekte hartcellen renine en diens inactieve voorloper, prorenine, kunnen produceren. Tevens is bestudeerd hoe renine en prorenine uit de bloedsomloop in hart en bloedvaten terecht kunnen komen en of op deze plaatsen het prorenine omgezet kan worden in het renine. Tenslotte is onderzocht of angiotensine II in hartcellen een groei-stimulerende werking heeft en welke angiotensine receptoren hierbij betrokken zijn.

Hoofdstukken 2 en 3

Het aantonen van RAS componenten in hartweefsel is geen bewijs dat deze componenten in het hart gemaakt worden, aangezien hartweefsel ook RAS componenten bevat die afkomstig zijn uit bloed. Om deze problemen van ex-vivo metingen te vermijden, werden de concentraties van renine, prorenine, angiotensinogeen, ACE, angiotensine I en angiotensine II gemeten in het kweekmedium en celextract van myocyten (spiercellen) en fibroblasten (tussencellen) uit neonatale ratteharten. De metingen werden uitgevoerd in celcultures zonder serum.

Ter vergelijking werden ook bepalingen uitgevoerd in het foetaal kalfsserum- en paardeserum-bevattende medium dat gebruikt werd om de cellen zo optimaal mogelijk te kweken (dus voor ze werden blootgesteld aan serum-vrije omstandigheden), en in medium van cellen die, onder serum-vrije condities, cyclisch uitgerekt en gerelaxeerd werden ("stretch").

Alle RAS componenten waren meetbaar in serum-bevattend medium dat niet met de cellen in aanraking was geweest. Prorenine, maar geen van de andere RAS componenten, kon ook worden gedetecteerd in medium van cellen die onder serum-vrije omstandigheden verkeerden. De prorenine concentraties onder deze condities waren echter zeer laag, en de angiotensine I-vormende activiteit die overeen kwam met deze lage prorenine concentraties kon niet worden geremd met de specifieke rat renine remmer CH-732. Dit duidt erop dat het hier niet om rat prorenine gaat, maar waarschijnlijk om prorenine dat afkomstig is uit het kalfs- en/of paardeserum waaraan de cellen eerder bloot waren gesteld. Onder serum-vrije condities bleken zowel cardiomyocyten als fibroblasten angiotensine I in angiotensine II om te kunnen zetten, en deze omzetting was volledig blokkeerbaar met de ACE remmer captopril. Extracten van serum-vrij gekweekte cellen bevatten geen renine, prorenine, angiotensinogeen, angiotensine I of angiotensine II in meetbare concentraties. Stretch van myocyten verhoogde de eiwit synthese met 20%, maar resulteerde niet in angiotensine afgifte aan het medium.

Samenvattend wijzen deze resultaten erop dat cardiomyocyten en fibroblasten geen renine, prorenine of angiotensinogeen produceren in concentraties die hoog genoeg zijn om te resulteren in angiotensine II concentraties van fysiologische betekenis. Deze cellen produceren wel ACE, hetgeen de synthese van angiotensine II in hartweefsel mogelijk maakt als renine en angiotensinogeen via de circulatie worden aangevoerd. Hoewel van angiotensine II bekend is dat de cardiovasculaire groei en remodelling via AT_1 receptoren stimuleert, bleek het niet een vereiste te zijn voor de hypertrofe respons van cardiale cellen na stretch.

Daarom werden vervolgens experimenten uitgevoerd om het effect van exogeen angiotensine II op de eiwit - en DNA synthese te bestuderen in cardiomyocyten en fibroblasten van neonatale ratten. Ook werd de mogelijkheid van een groei-remmend effect van angiotensine II via AT_2 receptoren onderzocht onder condities waarbij de eiwit- en DNA synthese gestimuleerd waren met endotheline-1.

De eiwit - en DNA concentraties, alsmede de inbouw van [^{3}H]leucine (een maat voor eiwit synthese) en van [^{3}H]thymidine (een maat voor DNA synthese) werden bepaald na incubatie met placebo, angiotensine II, endotheline-1, of angiotensine II + endotheline-1, zowel in de aan- of afwezigheid van de AT_1 receptor blokker losartan als de AT_2 receptor blokker PD123319.

In myocyten verhoogde endotheline-1 de hoeveelheid eiwit en de inbouw van [^{3}H]leucine en [^{3}H]thymidine. Angiotensine II had geen effect op deze parameters, noch beïnvloedde het de effecten van endotheline-1. Echter, in de aanwezigheid van PD123319 stimuleerde angiotensine II de inbouw van [^{3}H]leucine en [^{3}H]thymidine. In fibroblasten hadden zowel endotheline-1 als angiotensine II geen significant effect op de DNA concentraties en de [^{3}H]thymidine inbouw. Angiotensine II leek de eiwit concentratie in deze cellen iets te verhogen, maar dit effect was alleen significant in de aanwezigheid van PD123319. Angiotensine II stimuleerde tevens de [^{3}H]leucine inbouw in fibroblasten. Dit effect was afwezig na voorbehandeling met losartan en versterkt in aanwezigheid van PD123319.

Deze bevindingen tonen aan dat de AT_1 receptor-gemedieerde proliferatieve effecten van angiotensine II in neonatale hart cellen alleen zichtbaar worden als de AT_2 receptor gemedieerde anti-groei effecten geblokkeerd worden. Het netto groei effect van angiotensine II is zodoende afhankelijk van de cellulaire AT_1/AT_2 receptor ratio. Angiotensine II had geen invloed op de effecten van endotheline-1.

Hoofdstukken 4 en 5

Omdat (pro)renine niet in het hart en de bloedvaten geproduceerd lijkt te worden onder normale omstandigheden, zal de lokale produktie van angiotensine II op deze plaatsen afhankelijk zijn van (pro)renine dat uit de circulatie is opgenomen. Om dit nader te onderzoeken, is de binding en opname van recombinant humaan renine en prorenine bestudeerd in myocyten en fibroblasten uit neonatale ratteharten, en in humane navelstreng endotheel cellen (HUVECs). Tevens is gekeken of deze cellen prorenine kunnen omzetten in renine.

De cellen werden geïncubeerd met recombinant humaan renine en prorenine in de aan- en afwezigheid van mogelijke remmers van renine internalisatie.

Met de zogenaamde 'zure-was' methode werd onderscheid gemaakt tussen (pro)renine dat zich aan het celoppervlak bevindt en (pro)renine dat door de cellen is opgenomen (en dus in de cellen aanwezig is).

Wanneer de experimenten werden uitgevoerd bij 37°C, bleken de cellen zowel prorenine als renine dosisafhankelijk te binden en te internaliseren. Tijdens incubatie bij 4°C trad alleen binding aan het oppervlak (en geen internalisatie) op. Prorenine dat voorbehandeld was met endoglycosidase H, een enzym dat stikstof-gebonden oligosaccharides verwijdert, werd niet gebonden. Prorenine werd door de cellen geactiveerd. Het geactiveerde prorenine was alleen aanwezig in de geïnternaliseerde fractie; het celoppervlak-gebonden prorenine bleef inactief. Het lijkt er dus op dat de activering van prorenine plaatsvindt tijdens de internalisatie of daarna.

De toevoeging van mannose 6-fosfaat aan het medium veroorzaakte een dosis-afhankelijke remming van de (pro)renine internalisatie. Mannose 1-fosfaat, glucose 6-fosfaat en α-methylmannoside hadden geen remmend effect. Ammoniumchloride en monensine, stoffen die ingrijpen op de cyclus die receptor-ligand complexen in de cel doorlopen, bleken de activatie van prorenine te remmen.

In HUVECs werd het activatieproces van prorenine nader bestudeerd met behulp van een directe immunoradiometrische assay, welke gebruik maakt van een monoklonaal antilichaam tegen het -p24-Arg tot -1p-Arg C-terminale propeptidegedeelte van prorenine. De resultaten duidden erop dat de activatie van prorenine daadwerkelijk gepaard gaat met de proteolytische afsplitsing van het prosegment.

Deze bevindingen plaatsen recente experimentele data betreffende renaal renine-afhankelijke angiotensine produktie in hart- en ander extrarenaal weefsel in perspectief. Deze lokale angiotensine generatie zou aan het celoppervlak en/of intracellulair plaats kunnen vinden, en zowel renine als lokaal geactiveerd prorenine kunnen hierbij betrokken zijn. De (pro)reninebinding door extrarenaal weefsel verloopt mogelijk via mannose 6-fosfaat receptoren.

Hoofdstuk 6

Een lokaal renine-angiotensine systeem in het hart wordt vaak genoemd ter verklaring van de gunstige effecten van ACE remmers bij hartfalen. Het hart produceert echter weinig of geen renine onder normale omstandigheden.

Om te onderzoeken of renine produktie in het hart wel onder pathologische condities plaatsvindt, zijn de concentraties van renine en andere componenten van het renine-angiotensine systeem bepaald in de harten van 10 hartklepdonoren (overleden door niet-cardiovasculaire aandoeningen) en van 10 patiënten met ernstig hartfalen (gedilateerde cardiomyopathie, DCM) die een harttransplantatie ondergingen.

De concentraties van renine en prorenine waren hoger in de harten van DCM patiënten dan in de harten van donoren. In de DCM patiënten bleek een positieve correlatie te bestaan tussen de renine concentraties in hartweefsel en bloed plasma. Extrapolatie van de regressielijn naar normale plasma renine concentraties gaf een weefsel concentratie die overeenkwam met de gemeten waarden bij de donoren. De hartweefsel/plasma (W/P) ratio's van renine en prorenine waren drie keer zo hoog als de W/P ratio van albumine. Dit geeft aan dat de weefsel concentraties van renine en prorenine te hoog zijn om veroorzaakt te worden door vermenging van hartweefsel met bloed en/of door diffusie vanuit het bloed naar de cardiale interstitiële vloeistof. Celmembranen bereid uit hartweefsel van varkens bonden varkens renine met hoge affiniteit. De W/P ratio van ACE, een membraan-gebonden enzym, was vijf keer zo hoog als de ratio van albumine. Cardiaal angiotensinogeen was lager in DCM patiënten dan in donoren, en de W/P ratio van angiotensinogeen bedroeg slechts de helft van die voor albumine, hetgeen suggereert dat angiotensinogeen in hartweefsel door renine wordt omgezet in angiotensine I.

Deze resultaten, verkregen in patiënten met hartfalen, duiden erop dat ook onder pathologische omstandigheden de lokale angiotensine produktie in het hart afhankelijk is van renine uit de circulatie. Membraanbinding kan deel uitmaken van het opname-proces.

Hoofdstuk 7

In dit hoofdstuk worden enkele concluderende opmerkingen gemaakt naar aanleiding van de bevindingen uit de voorgaande hoofdstukken. Ondanks de vele studies waarin wordt beschreven dat hartcellen angiotensine II maken, konden wij niet aantonen dat deze cellen zelf het hiervoor benodigde renine synthetiseren. Het lijkt er dus op dat dit renine uit de bloedbaan moet worden opgenomen, bijvoorbeeld door binding aan een receptor.

Een receptor die hierbij betrokken zou kunnen zijn is de mannose 6-fosfaat receptor (MPR). Deze receptor is aanwezig op hartcellen (myocyten en fibroblasten) en endotheelcellen, en bindt niet alleen renine maar ook prorenine. Bovendien bleek prorenine, na binding en internalisatie door de cellen, snel in renine omgezet te worden. Zodoende bestaat de mogelijkheid dat zowel renine als lokaal geactiveerd prorenine bijdragen aan de lokale produktie van angiotensine II.

Waar deze produktie precies plaatsvindt kan nog niet worden gezegd: op het celoppervlak, in de cellen, of in de vloeistof tussen de cellen na de afgifte van geactiveerd prorenine door de cellen? Aangezien de MPR ook betrokken is bij klaringsprocessen (bijv. van insulin-like growth factor II) kan op dit moment niet worden uitgesloten dat de receptor een rol speelt bij de klaring van renine en/of prorenine. Dit laatste zou overigens nog altijd betekenen dat de receptor een regulerende rol speelt bij de lokale produktie van angiotensine II.

Op grond van veranderingen in mRNA concentraties werd eerder door anderen geconcludeerd dat de renine synthese in het hart met name op gang komt onder pathologische omstandigheden. Onze bevindingen in de harten van patiënten met hartfalen bevestigen deze conclusie niet. Het lijkt er dus op dat zelfs onder pathologische omstandigheden het meeste cardiale renine afkomstig is uit de circulatie. Wel zouden onder dergelijke omstandigheden de (pro)renine receptoren (MPR?) in aantal kunnen veranderen.

ACE is waarschijnlijk de enige RAS component benodigd voor de synthese van angiotensine II die in het hart zelf wordt gemaakt. Als aan hartcellen dus renine en angiotensinogeen wordt aangeboden moeten zij in staat zijn om zelf angiotensine II te maken. Of dit angiotensine II wordt opgeslagen in de cellen is vooralsnog onbekend. Opgeslagen angiotensine II zou de bron kunnen zijn van het angiotensine II dat in hoge concentraties in het medium van hartcellen wordt aangetroffen na celstretch. Volgens sommigen speelt dit angiotensine II een cruciale rol bij het op gang brengen van de hypertrofe respons na de introductie van stretch. Hoewel onze bevindingen in cardiomyocyten bevestigen dat na stretch een hypertrofe respons optreedt, kon geen afgifte van angiotensine II worden aangetoond. Dus ook zonder angiotensine II kan hypertrofie optreden.

Wel kan angiotensine II onder bepaalde omstandigheden toch een groei-stimulerende rol spelen, zo bleek uit experimenten waarbij angiotensine II van buiten af aan de cellen werd toegediend. Ook dan trad hypertrofie op, althans wanneer de AT_2 receptoren geblokkeerd waren. Deze receptoren remmen namelijk de AT_1 receptor-gemedieerde groeirespons. De angiotensine II concentraties waarbij de groei-stimulerende effecten optraden lagen ver boven de ondergrens van onze angiotensine II assay.

Verder onderzoek is nodig om de vragen die bovenstaande experimenten hebben opgeworpen te beantwoorden: hoe vindt de activatie van prorenine precies plaats, is de MPR de enige receptor die betrokken is bij (pro)reninebinding, veranderen de (pro)reninebindingsplaatsen in aantal onder pathologische omstandigheden, leidt (pro)reninebinding tot een intracellulaire respons, waar hangt de angiotensine release na stretch van af, en hoe zit het met de rol van de AT_2 receptoren?

Abbreviations

ACE	angiotensin-converting enzyme
Ang	angiotensin
AT_1	angiotensin II type 1 receptor
AT_2	angiotensin II type 2 receptor
CD-MPR	cation-dependent mannose 6-phosphate receptor
CHO	chinese hamster ovary
CI-MPR	cation-independent mannose 6-phosphate receptor
DAG	diacylglycerol
DCM	dilated cardiomyopathy
DMEM	Dulbecco's modified Eagle's medium
HUVEC	human umbilical vein endothelial cell
ET-1	endothelin-1
FAK	focal adhesion tyrosine kinase
HPLC	high-performance liquid chromatography
IGFII	insulin-like growth factor II
IP_3	inositol 1,4,5-triphosphate
$InsP_n$	inositolphosphates
MAP	mitogen-activated protein
M-6-P	mannose 6-phosphate
MPR	mannose 6-phosphate receptor
PA	phosphatidic acid
PBS	phosphate-buffered saline
PIP_2	phosphatidylinositol 4,5-biphosphate
PLC-β	phospholipase C-β
PKC	protein kinase C
PLD	phospholipase D
RAS	renin-angiotensin system
RnBP	renin-binding protein
STAT	signal transducers and activators of transcription
TCA	trichloric acid
TGF-β	transforming growth factor-β

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Lieve Maarten en Femke, jullie zijn het mooiste dat me ooit is overkomen.

Curriculum Vitae

Toos van Kesteren werd op 24 december 1966 geboren te Schiedam. Na het behalen van het VWO diploma aan de scholengemeenschap Spieringshoek te Schiedam in 1985, werkte zij twee jaar als leerling röntgenlaborante in het Eudokia Ziekenhuis te Rotterdam. In 1987 begon zij met de studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden. Zij liep stages bij Algemene Heelkunde (AZL, Leiden), waar onder leiding van dr H.J. Smeets onderzoek werd gedaan naar de preventie van orgaanschade na aortachirurgie in een varkensmodel en bij Endocrinologie (AZL, Leiden), waar onder leiding van dr C.W.G.M. Löwik het effect van IL-1 op osteoclast-vorming en -functie bestudeerd werd. Als hoofdstage deed zij onderzoek naar de rol van plasminogeen activatie bij wondheling in gekweekte huidcellen en gereconstrueerde epidermis, onder leiding van dr P.H.A. Quax en dr M. Ponec op het Gaubius laboratorium (IVVO-TNO, Leiden) en op de afdeling Dermatologie (AZL, Leiden). De studie werd in september 1993 afgerond na een stage bij het Instituto di Cardiologia, Policlinico Gemelli, Rome, Italië, waar zij onderzoek deed naar deleties van mitochondriaal DNA in anoxisch hart-weefsel onder supervisie van prof. dr A. Maseri en dr G. Sperti.

Vanaf januari 1994 is zij werkzaam geweest als assistent in opleiding bij de vakgroepen Farmacologie en Inwendige Geneeskunde I, welke deel uitmaken van de Cardiovasculaire Onderzoekschool Erasmus Universiteit Rotterdam (COEUR). Het in dit proefschrift beschreven onderzoek aan het cardiale renine-angiotensine systeem is uitgevoerd onder leiding van de promotoren prof. dr M.A.D.H. Schalekamp, prof. dr P.R. Saxena en co-promotor dr A.H.J. Danser.