Functional Significance of Prorenin Internalization in the Rat Heart

Jörg Peters, Raphaela Farrenkopf, Susanne Clausmeyer, Jutta Zimmer, Surasak Kantachuvesiri, Matthew G.F. Sharp, John J. Mullins

Abstract—Intracardiac renin is considered to be involved in the pathogenesis of cardiac hypertrophy, fibrosis, and myocardial infarction. Cardiac renin is predominantly derived from the circulation, because preprorenin is not expressed locally and uptake of renin has been demonstrated. One mechanism of internalization recently described involves the mannose-6-phosphate receptor and requires glycosylation of renin. Based on previous observations, we considered the existence of another pathway of uptake, not requiring glycosylation and predominantly involving prorenin. This hypothesis and its functional consequences were investigated in vitro and in vivo. We demonstrate that isolated adult cardiomyocytes internalize unglycosylated prorenin, which is followed by the generation of angiotensins. We further show that transgenic rats, expressing the ren-2d renin gene in an inducible manner, exhibit markedly enhanced levels of unglycosylated renin within intracellular compartments in the heart as a consequence of the induction of hepatic transgene expression and the rise of circulating unglycosylated prorenin levels. Because in this model severe cardiac damage occurs as a consequence of the rise of circulating prorenin levels, internalization of prorenin into cardiac cells is likely to play a key role in this process. (Circ Res. 2002;90:1135-1141.)

Key Words: transgenic rat ■ heart ■ prorenin ■ cardiomyocyte

Angiotensin II (Ang II) increases cardiac contractility, regulates coronary blood flow, and is considered to be a growth factor stimulating cardiac collagen synthesis, hyperplasia, and cardiac hypertrophy.1–3 There is no doubt that angiotensins are generated locally and released by the heart.3–5 Thus, it has been hypothesized that an intracardiac renin-angiotensin system (RAS) exists, which modulates cardiac function. We have, however, shown that renin transcripts encoding secretory preprorenin are neither expressed in rat cardiac tissue under basal conditions nor after myocardial infarction.6–7 Instead, there is evidence that uptake of renin from the circulation is a major source of cardiac renin.8 Some studies support the hypothesis that renin is internalized by mannose-6-phosphate receptor–mediated endocytosis, for which glycosylation of renin is required9; others suggest that renin is bound to specific but not yet characterized binding proteins.10,11 In a ren-2d transgenic rat model, TGR(mREN2)27, which expresses the mouse ren-2d renin gene under control of its endogenous promoter,12 markedly enhanced levels of nonglycosylated prorenin are associated with increased activity of the cardiac RAS and cardiac hypertrophy.12–14 This raises the possibility that circulating prorenin may be taken up into the heart even if not glycosylated. Interestingly, there is evidence that prorenin exerts its own enzymatic activity, being reversibly activated without cleavage of the profragmente.15–17 The aim of the present study was therefore to test the hypothesis that unglycosylated prorenin can be taken up into the heart in vivo and in vitro, as well as to demonstrate any functional consequences of such an uptake. Because in TGR(mREN2)27 local expression of the ren-2d transgene for cardiac renin activity cannot be ruled out, a more specific model was investigated.18 This new transgenic model allows us to study selectively the role of circulating unglycosylated prorenin, because in these rats the ren-2d gene is expressed primarily in the liver under control of the inducible Cyp1a1 promoter and circulating prorenin levels can be elevated in a controlled manner by indole-3-carbinol. In this model, the induced rise of plasma prorenin levels leads to hypertension and cardiac damage in the absence of cardiac transgene expression.

Materials and Methods

Animals and Induction of the Cyp1a1 Promoter In Vivo

For in vitro studies examining the uptake of renin into isolated cardiomyocytes, male Sprague Dawley rats (Charles River, Sulzfeld, Germany) weighing 250 to 300 g were used. For in vivo studies examining the content of renin in intracellular compartments of the heart with and without induction of the hepatic ren-2d expression, adult 12-week-old male Cyp1a1-2 transgenic rats and age-matched nontransgenic F344 Fisher rats were used. All rats were

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housed under alternating 12-hour light and dark cycles at a constant temperature between 20 and 22°C and were fed the standard laboratory diet of altromin with free access to tap water. Five rats of each group were induced with indole-3-carbinol (0.3% w/wt) for a period of 2 weeks.

**Determination of Renin and Prorenin in Plasma and Intracellular Compartments**

Plasma renin and prorenin were determined from EDTA-plasma as described previously.\(^\text{19,20}\) To prepare intracellular fractions of left ventricular tissue, rats were anesthetized with Nembutal and hearts were perfused with physiological saline through the aorta for 3 minutes at 220 mm Hg. A small piece of left ventricle was removed for 4T. TNA analysis. The remaining tissue was minced gently mechanically disrupted by 5 strokes with a motor-driven grinder in the following buffer: 0.25 mol/L sucrose, 10 mmol/L Tris/HCl, pH 7.2, 2 mmol/L EDTA and 0.1 mmol/L phenylmethylsulfon fluoride. The free intact organelles were precipitated by differential centrifugation as described previously.\(^\text{19}\) Organelle fractions were resuspended in homogenization buffer, then sonicated and centrifuged at 100 000 \(g\) for 30 minutes to separate soluble organellar content from proteins bound to membranes.

Renin and prorenin concentrations within fractions were determined as described and normalized to the total protein content within each fraction. Peak renin content was found in the 3000 g fraction. Peak renin content was found in the 3000 g fraction. For sake of clarity, only this fraction was compared. The specificity of the renin reactions was always confirmed in parallel incubations with the renin inhibitor CH732. Rat and mouse renin were distinguished by means of immunoprecipitation as previously described.\(^\text{21}\)

**Isolation of RNA and RT-PCR Analysis of Renin Transcripts in the Heart**

Rats were killed by cervical dislocation under Nembutal anesthesia. After perfusion with physiological saline via the aorta for 3 minutes, hearts were removed and a small piece of the left ventricle was immediately frozen in liquid nitrogen. Total RNA was isolated by the method of Affruf and Rougeon.\(^\text{22}\) Reverse transcription was performed using Superscript II Reverse Transcriptase (Invitrogen, Germany) with 2 \(\mu g\) of each RNA and an oligo(dT) primer containing 2 degenerate nucleotide positions at its 3’-end for cDNA synthesis. Amplification of the cDNA corresponding to ren-2\(^\text{d}\) preprorenin was done by a nested PCR based on two sets of primers. Discrimination of the ren-2\(^\text{d}\) cDNA from rat renin cDNAs was achieved by using specific sets of sense and antisense primers.\(^\text{3}\) For rat preprorenin and exon1A renin, PCR was performed as described previously.\(^\text{25}\) Sense primers for rat preprorenin corresponded to positions 91 to 111 and 467 to 4707, the antisense primers hybridized to positions 10848 to 10868 and 10698 to 10719, respectively.\(^\text{24}\) For rat exon1A, renin different sense primers were used, hybridizing to positions 3865 to 3885 and 3883 to 3904. For ren-2\(^\text{d}\), primers corresponded to positions 99 to 119, 1229 to 1250, 227 to 246, and 1098 to 1118.\(^\text{24}\) Primers for GAP-DH were chosen from the rat GAP-DH gene, positions 84 to 103, 1063 to 1082, 635 to 653, and 999 to 1020.\(^\text{3}\) Reliability of discrimination was tested and any cross-reactions were excluded.

**Construction of Plasmids Encoding for Rat and ren-2\(^\text{d}\) Renin Mutants**

The full-length rat renin cDNA\(^\text{26}\), was used for the construction of the deletion mutants corresponding to preprorenin and active renin. The cloning procedure for these deletion mutants has been described previously.\(^\text{9}\) For construction of deletion variants of the ren-2\(^\text{d}\) gene, a full-length cDNA was generated from mouse submandibular gland poly A- RNA by means of RT-PCR, using the antisense primer Ren30 (5’-GGCTTACGGCCGGCCACCAGGCAA-3’; nucleotides 1229 to 1250, according to Panther et al\(^\text{36}\)) and the sense primer Ren629 (5’-CCATGCGCCAAGATGGGACAAGGGA-3’; 52 to 49), the obtained fragment of 1226 bp was cloned into pBluescript M13 \(^\text{3}\); sequenced. From this cloned cDNA, 2 deletion mutants corresponding to preprorenin and active renin were constructed using again the antisense primer Ren30 and 2 different sense primers, which were chosen to introduce an ATG codon for in vitro translation. Primer Ren17 (5’-GGCTTACGACCTCTGCTTCC-3’; 87 to 107; T92-A, G93-T, C94-G) results in a fragment of 1164 bp with a coding sequence corresponding to preprorenin. The coding sequence of active renin was generated using primer Ren18 (5’-CAAGATGGTCTTCTGCTTGA-3’; 222 to 242; G228-T), which yields a fragment of 1029 bp. Amplification was performed using Pwo DNA polymerase (Roche Mannheim, Germany), and the obtained fragments were cloned into pBluescript M13 \(^\text{3}\) digested with EcoRV. All constructs were verified by sequencing. In vitro transcription and translation was performed as described previously.\(^\text{9}\)

**Uptake of Renin in Adult Cardiomyocytes**

Rats were killed by cervical dislocation under Nembutal anesthesia. The heart was quickly removed and placed in prewarmed (37°C) modified Hanks’ solution (in mmol/L: 120 NaCl, 5 KCl, 1.2 KH\(_2\)PO\(_4\), 15 NaHCO\(_3\), 20 HEPES, pH 7.4, containing additionally 3 CaCl\(_2\) and 1.2 MgSO\(_4\)).

Isolated cardiomyocytes were obtained by enzymatic dissociation with collagenase and DNase. The heart was cut into small pieces and placed again in prewarmed modified Hanks’ solution, this time containing 3 mmol/L CaCl\(_2\), 1.2 mmol/L MgSO\(_4\), 2% BSA, 0.34 U/mL collagenase, 0.1 mg/mL DNase, 100 U/mL penicillin G, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (antibiotic-antimycotic mix, Invitrogen). Pieces of tissue were incubated for 10 minutes at 37°C in a shaker. To increase the yield, the tissue was pipetted up and down 10 times through a 10-mL pipette. Intact pieces of tissue were allowed to sink down. The supernatant was discarded, thereby removing most erythrocytes. The remaining tissue was incubated once for 30 minutes and once for 15 minutes at 37°C. The cell suspension was gently triturated every 15 minutes as described above. After each incubation step the supernatant was filtered through a cell strainer (70 \(\mu m\)), diluted with cold modified Hanks’ solution, this time containing 1.8 mmol/L CaCl\(_2\), 1.2 mmol/L MgSO\(_4\), 1.2% BSA, and antibiotic-antimycotic mix and washed 2 times at 200g. The two cell suspensions were pooled and transferred to medium Ham’s F12.

To purify further the isolated cardiomyocytes, a Percoll gradient of 30%, containing 0.1215 mol/L NaCl, was used. Intact cardiomyocytes were found in the fraction of lowest density. All fractions were checked by microscopy and the qualified ones were pooled. Residual Percoll was washed out 3 times. After determination of protein concentration, cells were transferred to Ham’s F12 medium containing 1% BSA and antibiotic-antimycotic mix.

After a preincubation period of 1 hour, the cell suspension was divided into 4 aliquots, corresponding to 500 mg protein. Each aliquot was incubated with one of the \(^\text{14}\)S-methionine–labeled renin constructs for 90 minutes in an atmosphere of carbogen in a waterbath shaker at 37°C. Each sample was then divided into 2 parts. One half was treated for 15 minutes with 100 \(\mu g/mL\) proteinase K on ice; the other half remained untreated to distinguish internalized and extracellular proteins. Proteinase activity was terminated by 1% BSA and phenylmethylsulfon fluoride (PMSF).

The intact cardiomyocytes were precipitated by centrifugation. The pellets were washed with Ham’s F12 containing 1 mmol/L PMSF and analyzed by SDS-PAGE. The supernatants were precipitated with 4 volumes of ice-cold acetone, centrifuged, and also analyzed by SDS-PAGE to check the efficiency of proteinase K digestion. Proteinase K digestion was found to be complete (Figure 2B).

**Quantification of ren-2\(^\text{d}\) Prorenin Uptake by Adult Cardiomyocytes and Intracellular Generation of Angiotensin**

Because the autoradiograms do not allow exact quantification, cardiomyocytes were additionally incubated with larger amounts of purified ren-2\(^\text{d}\) prorenin obtained from HepG2 cells transfected with the ren-2 gene under control of the \(\alpha\)1-antitrypsin promoter. This
approach allowed us to measure intracellular renin and prorenin levels using enzymatic renin assays. To separate renin actually taken up from that simply attached, cells were washed 3 times and additionally membranes were removed by centrifugation at 50,000 g after freeze-thawing the cells. Renin content within cell extracts was determined as described above. Angiotensin levels were determined by radioimmunoassay after extraction with SepPak C18 cartridges (Waters GmbH) as described previously. The experiments were also performed using purified glycosylated rat prorenin obtained from HepG2 cells transfected with the rat renin gene under control of the CMV promotor using the vector pcDNA3.1.

**Results**

**Renin Content in Plasma and Heart**

In nontransgenic rats plasma prorenin was low but readily detectable (Figure 1A). Transgenic rats not induced exhibited slightly higher prorenin levels, which was partly ren-2d derived (Figure 1E). However, the difference was small and without any physiological consequences. Levels of active renin were not different between these groups (Figure 1B). In transgenic rats induced with indole-3-carbinol for a period of 2 weeks, prorenin levels were increased prominently in plasma, as designed (Figure 1A). Plasma active renin levels after 2 weeks of induction also increased, but far less markedly (Figure 1B). Almost all prorenin but only part of the active renin in induced transgenic rats was ren-2d derived, as indicated by immunoinhibition with a mouse renin–specific antibody (Figures 1E and 1F). This demonstrates the presence of large amounts of unglycosylated prorenin in plasma after induction. Plasma renin levels were normalized to total plasma protein levels to allow comparison of levels of renin in plasma with those in the heart. Under the same regime of induction, we previously demonstrated that the transgenic rats were severe hypertensive with systolic blood pressure of about 200 mm Hg and developed microinfarctions with myocardial necrosis in the ventricles and signs of infiltration with inflammatory cells and proliferation of fibroblasts.

In the hearts of nontransgenic and transgenic rats without induction prorenin levels were low, but active renin was readily detectable in the soluble fractions of intracellular vesicular fractions of the heart (C and D). Proportion of ren-2d renin is indicated by the inhibitory effect of a specific anti-mouse renin antibody on renin activities (E through H). Prominent increase in plasma prorenin levels after induction with indole-3-carbinol for 2 weeks (A) was due to ren-2d renin (E) and associated with an increase of ren-2d renin activity and prorenin levels in the heart (G and H). TG + I indicates nontransgenic rats; TG + nl, transgenic rats, not induced; and TG + I, transgenic rats induced for 2 weeks. I, Analysis of the expression of mouse ren-2d in the left ventricles of Cyp1a1-ren-2d transgenic rats by RT-PCR. Ethidium-bromide stained agarose-gel of the amplified cDNAs. M indicates molecular weight marker; 1, nontransgenic rat; 2, Cyp1a1-ren-2d transgenic rat, not induced; 3, Cyp1a1-ren-2d transgenic rat, induced; P, positive control: TGR(mREN2)27, expressing ren-2 in the heart under control of the ren-2 promotor; and N, negative control: sample receiving no reverse transcriptase.
Intracardiac intracellular renin levels markedly increased in transgenic rats after induction (Figures 1C and 1D) and were then predominantly ren-2d derived, because the anti-mouse renin–specific antibody inhibited Ang I generation by 92% (Figures 1G and 1H). There was a high correlation of cardiac active renin with plasma prorenin (r=0.91) but not with plasma active renin (r=0.6). Expression of the ren-2d gene was excluded by RT-PCR in all groups (Figure 1I). As expected, exon1A renin was the only endogenous transcript expected, exon1A renin was the only endogenous transcript in transgenic rats after induction (Figures 1C and 1D) and further because it is not glycosylated even when expressed by intact cells. Intracellular trypsin activation and about 15 ng Ang I · mL⁻¹ · h⁻¹ without trypsin activation). Exposure of cells to prorenin led to a marked and significant increase in intracellular levels of prorenin (A) as well as of angiotensins (C and D) and a significant, but less prominent, increase of renin activity without trypsin activation (B). *P<0.05. ren-2 indicates ren-2d derived nonglycosylated prorenin; rat, rat renin derived glycosylated prorenin.

To quantify the amount of prorenin internalized, cardiomyocytes were incubated with unlabelled purified ren-2d prorenin obtained from transfected hepatocytes (n=14). The recombinant prorenin preparations applied were able to generate 3000 ng Ang I · mL⁻¹ · h⁻¹ after trypsin activation and 20 ng Ang I · mL⁻¹ · h⁻¹ (about 0.7%) without trypsin activation. This is well in agreement with the observation by Yamauchi et al. that prorenin is able to generate Ang I to a small degree (1.6%). ren-2d prorenin was used because it is proposed to be of pathophysiological importance in ren-2d transgenic rats and further because it is not glycosylated even when expressed by intact cells. Intracellular trypsin activatable prorenin levels increased from 0.45±0.09 to 23±6.2 ng Ang I · mg⁻¹ · h⁻¹ and active renin from 0.4±0.1 to 0.76±0.2 ng Ang I · mg⁻¹ · h⁻¹ (each P<0.01; n=12; Figures 3A and 3B). Thus about 1% of prorenin supplied was internalized and became more active than in medium (3.3% in cell extracts versus 0.7% in medium). These data correlate well with our observations obtained for prorenin uptake analyzed on SDS gels. The increase of intracellular active renin may indicate

**Uptake of Renin Into Cardiac Cells In Vitro**

Uptake of unglycosylated renin by isolated cardiomyocytes was then investigated in vitro, using the direct visualization of renin protein. Cardiomyocytes were incubated with 35S-methionine–labeled active renin and prorenin derived from the ren-2d and rat renin genes (Figure 2). To distinguish membrane-bound from internalized renin, cell suspensions were divided into 2 aliquots. One was exposed to a proteinase K digest and, because extracellular proteins are accessible to degradation, contained exclusively intracellular proteins. The other one was not digested and thus contained both imported renin and renin attached to cell membranes. Both rat and ren-2d prorenin were found to be protected against proteinase digest, indicating that these have been imported into cardiomyocytes, whereas rat active renin remained extracellularly or attached to cell membranes (n=6; Figure 2A). ren-2d active renin was imported weakly.

**Figure 2. Internalization of 35S-methionine–labeled prorenin and renin into cardiomyocytes in vitro.** Autoradiogram of an in vitro transport of rat and mouse prorenin and active renin, respectively, into isolated adult cardiomyocytes. Cardiomyocytes were incubated with in vitro translated 35S-labeled proteins (lane T) for 90 minutes. After incubation, samples were divided and half of the cardiomyocytes were treated with proteinase K (lane C/H11001), the other half remained untreated (lane C–) to distinguish internalized and bound proteins. S– indicates supernatant (medium) of the cells not treated with proteinase K; S+, supernatant (medium) after treatment with proteinase K.

**Figure 3. Effect of internalization of prorenin on intracellular renin and angiotensin content.** Prorenin, renin, and angiotensin content were determined from membrane-free cell extracts containing the intracellular proteins before and after 90 minutes of incubation with or without recombinant prorenin in the medium (corresponding to an activity of 3000 ng Ang I · mL⁻¹ · h⁻¹ after trypsin activation and about 15 ng Ang I · mL⁻¹ · h⁻¹ without trypsin activation). Exposure of cells to prorenin led to a marked and significant increase in intracellular levels of prorenin (A) as well as of angiotensins (C and D) and a significant, but less prominent, increase of renin activity without trypsin activation (B). *P<0.05. ren-2 indicates ren-2d derived nonglycosylated prorenin; rat, rat renin derived glycosylated prorenin.
uptake of prorenin, probably followed by a slow increase of its endogenous activity because we did not detect cleavage on the SDS gel.

It has been shown that mannose-6-phosphate inhibited uptake of glycosylated renin, likely via competition at the mannose-6-phosphate/IGF II receptor. The pathway characterized in the present study for ren-2d prorenin presumably does not involve this receptor, because ren-2d prorenin is not glycosylated. Nevertheless, we examined the possibility of any influence of 10 mmol/L mannose-6-phosphate on the uptake of ren-2d prorenin. An inhibition of ren-2d prorenin internalization was not observed, neither when analyzed on SDS-PAGE nor by enzymatic assay (data not shown).

We also examined uptake of glycosylated rat prorenin into isolated cardiomyocytes (n=8), using the same protocol and identical levels of enzymatic activities (3000 ng Ang I · mL⁻¹ · h⁻¹ after trypsin activation). Glycosylated rat prorenin was taken up to a similar degree when compared with unglycosylated ren-2d prorenin. Intracellular trypsin activatable prorenin levels increased from 0.4±0.12 to 17±4 and renin activity from 0.3±0.07 to 0.84±0.02 (Figure 3).

**Intracellular Generation of Angiotensins**

Next, we investigated the hypothesis that prorenin taken up into cardiomyocytes is able to generate angiotensins within the cells. Prorenin incubated with medium alone or with conditioned medium previously exposed to cardiomyocytes did not generate considerable amounts of angiotensins within the incubation period of 90 minutes given (maximum angiotensin generation about 30 ng/mL). Further control experiments demonstrated that angiotensins, when supplied at concentrations of 100 or 1000 ng/mL with the medium, were partially degraded, but could not be detected within cardiomyocytes (not shown). After incubation of cardiomyocytes with 3000 ng Ang I · mL⁻¹ · h⁻¹ of unglycosylated ren-2d prorenin, however, angiotensins were detected intracellularly (n=12; Figures 3C and 3D). In contrast, after incubation of cardiomyocytes with 3000 ng Ang I · mL⁻¹ · h⁻¹ glycosylated ren-2d prorenin we could not detect any increase in intracellular angiotensin levels despite similar degree of uptake (n=6; Figure 3). In conclusion, unglycosylated prorenin is taken up by cardiomyocytes and this is of functional significance, leading to generation of angiotensins.

**Discussion**

It has long been hypothesized that prorenin exerts functions in addition to serving just as the precursor for active renin. This hypothesis has recently been strengthened by studies with an experimental transgenic rat model of hypertension, termed TGR(mREN2)27. In TGR(mREN2)27, expressing the ren-2d renin gene under control of its own promoter, we found a close association between elevated circulating prorenin but not active renin levels with the development of hypertension and cardiac hypertrophy. Long-term dexamethasone treatment partially prevented the development of hypertension and also decreased plasma prorenin, but did not change plasma levels of active renin. Bilateral adrenalectomy even normalized blood pressure as well as circulating prorenin levels (the adrenal gland being a major source of circulating prorenin in this model), and regeneration of paraaortal adrenal tissue after bilateral adrenalectomy observed in this experiment was followed by reappearance of elevated plasma prorenin levels and hypertension (J. Peters, unpublished observations, 1992). It has further been concluded that the intracardiac RAS is involved in cardiac hypertrophy and remodeling in TGR(mREN2)27. Although these studies demonstrated the role of angiotensin formation for the development of cardiac hypertrophy, they could not actually present any evidence for the existence of a local system and did not differentiate between uptake, local expression, or the classical circulating RAS.

Taking advantage of a new transgenic model, the Cyp1al-ren-2 transgenic rats, in which circulating prorenin levels can be elevated in a highly controlled manner by means of selective induction of ren-2d transgene expression, we obtained further indications about the functional importance of internalization of circulating prorenin into the heart in vivo, excluding the contribution of endogenous rat preprorenin expression in this model. Induction of hepatic prorenin expression resulted in hypertension and cardiac damage, and this was accompanied by markedly elevated intracardiac ren-2d renin content, while at the same time transcripts for ren-2d renin and for endogenous preprorenin remained absent. Internalization of unglycosylated prorenin was then confirmed by means of 2 different in vitro approaches, using isolated adult rat cardiomyocytes and either radiolabeled prorenin derived from cell-free translation in a reticulocyte lysate system or recombinant prorenin from transfected hepatocytes. There is strong evidence for uptake of renin into cardiac tissue. Katz et al detected renin isoforms of different degrees of glycosylation within the rat heart and demonstrated a prominent reduction of their levels in response to bilateral nephrectomy, which eliminates circulating renin. Accordingly, van Kesteren et al have shown that both active renin and prorenin are enriched within cardiac tissue and observed internalization into cardiac cells, which was mediated via a mannose-6-phosphate–dependent mechanism with prorenin being activated after internalization. However, in many instances a functional activity of prorenin in terms of angiotensin generation or effects on blood pressure could not be demonstrated. In our hands and in accordance with these studies, uptake of glycosylated rat prorenin also did not increase intracellular angiotensin levels. Our data strongly indicate that internalization and its functional consequences are dependent on glycosylation of the protein: uptake of ren-2d prorenin into the heart of TGR(mREN2)27 or Cyp1al-ren-2 transgenic rats, as observed in our studies, required a mechanism independent from the mannose-6-phosphate receptor. Because renin derived from the ren-2d gene cannot be N-glycosylated, the encoded protein lacked N-glycosylation sites. Glycosylation of rat or mouse prorenin proved not to be required for uptake into isolated cardiomyocytes, because the cell-free translated proteins derived from either the ren-2d or rat renin genes are not glycosylated in the in vitro system used. Furthermore, uptake of both unglycosylated rat and mouse ren-2d prorenin through this pathway was not inhibited by mannose-6-phosphate; therefore, internalization involving...
the mannose-6-phosphate receptor is excluded. The uptake of unglycosylated prorenin was as efficient as the uptake of glycosylated rat prorenin in our studies. Nevertheless the consequences appeared to be different. Whereas the uptake of unglycosylated ren-2 prorenin was associated with increased intracellular levels of angiotensins, the uptake of glycosylated prorenin was not. The difference in glycosylation may well determine the use of different pathways of internalization and/or different degrees of intracellular activation of the proteins.

Our data are further different from the results of van Kesteren et al. in that the authors found uptake of both prorenin and active renin, whereas in our experiments, active renin of mouse ren-2 and rat active renin were found to be internalized rather weakly or not at all, although they bound as efficiently to the cell membrane as prorenin. These differences again could be explained by the different glycosylation states of the renin preparations used and may also be cell-type specific (ie, neonatal versus adult cardiomyocytes, fibroblasts).

At the present, we do not know how Ang I is converted to Ang II in the heart. Because both angiotensin-converting enzyme and chymase are known to be expressed in the heart, both enzymes are good candidates to explain the generation of Ang II. This needs to be further investigated.

Sadoshima et al observed in neonatal cardiomyocytes expression of renin and generation of angiotensins without the need of renin uptake from exogenous sources. We have not yet investigated neonatal cardiomyocytes; however, even in adult cardiomyocytes, we recently demonstrated expression of an alternative renin transcript, termed exon1A renin, which could additionally provide a source for the intracellular angiotensin generation. The role of this transcript needs still to be evaluated. Its presence indicates that there is more than one local RAS present in the heart. In our model, however, exon1A renin expression is not increased and cannot explain the elevated cardiac renin levels because those are clearly mouse (ren-2) derived.

How could the increase of circulating prorenin levels in Cypl1-ren-2 transgenic rats lead to hypertension and cardiac hypertrophy? Increase of plasma active renin apparently is not crucial in this model, because elevation of blood pressure was already detected when prorenin was elevated and active renin as well as angiotensins were not. Therefore, we concluded that almost certainly ren-2 prorenin was taken up and activated by extracellular pathways, such as vessels, kidney, and the heart. In our experiments, uptake of unglycosylated prorenin in vitro into isolated cardiomyocytes was followed by increased capacity of cell extracts to generate Ang I from angiotensinogen, demonstrating enzymatic activity of internalized prorenin. Prorenin must have been either cleaved to active renin or underwent a conformational change, which resulted in activation without cleavage of the profragment. The latter possibility has gained much attention recently because it has been demonstrated that recombinant prorenin is partially active (about 1.6%) at neutral pH27 and can be cryoactivated or reversibly activated in a pH-dependent manner in vitro. The fact that we detected only a relatively small proportion of prorenin to be in the active state in cell extracts after internalization of prorenin by isolated cardiomyocytes for 90 minutes in vitro, but detected most intracardiac ren-2 prorenin in the active state in Cypl1-ren-2 transgenic rats after 2 weeks of prorenin excess in vivo may indicate slow kinetics of the intracellular conformational change of prorenin. On the other hand, because the intracellular conditions that activate prorenin are unknown at present, we have not had the opportunity to mimic them in our vitro assay of cell extracts. Finally, cleavage of the profragment should still be considered.

In conclusion, our experiments provide evidence, that prorenin is internalized into cardiomyocytes independent of its glycosylation. The uptake of unglycosylated prorenin was shown to be of functional significance, leading to elevation of intracellular angiotensin levels and to an activated intracardiac RAS in association with cardiac damage in vivo, as demonstrated with use of the new Cypl1-ren-2 transgenic model. Prorenin levels are markedly enhanced during pregnancy. are 100-fold enriched in certain body fluids, such as ovarian follicle, amniotic fluid, and vitreous fluid in diabetics with proliferative retinopathy, and are positively correlated with the progress of diabetic angiopathy. Because renin and prorenin in many species exists with different degrees of glycosylation and only a fraction is mannose-6-phosphorylated, our observations are not only a phenomenon for transgenic rats, but of general importance to cardiovascular regulation.

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