Chantal Mercure, Gary Prescott, Marie-Josée Lacombe, David W. Silversides, Timothy L. Reudelhuber

Abstract—Elevated levels of circulating prorenin, the precursor of renin, have been reported to precede the appearance of microvascular complications in diabetes mellitus. Although several studies using animal models have attempted to address the link between elevated prorenin and the tissue remodeling and damage associated with both hypertension and diabetes mellitus, the results have been contradictory, and the mechanism whereby prorenin might contribute to these pathologies remains a subject of debate. To directly test the role of prorenin in these pathologies, we generated transgenic mice with selective increases (13- to 66-fold) in circulating native or active site-mutated prorenin. Systolic blood pressure was either unchanged or increased (+25 mm Hg) in native prorenin-expressing mice, whereas the mice expressing active site-mutated prorenin showed no significant differences in systolic blood pressure compared with control animals. There was no increase in cardiac fibrosis or renal glomerular sclerosis in any of the transgenic animals tested, even at an advanced age (18 months). Captopril (an angiotensin-converting enzyme inhibitor) rapidly normalized blood pressure of hyperproreninemic mice, whereas infusion of the putative antagonist of the prorenin receptor (handle region peptide) had no effect. These results suggest that the primary consequence of chronic elevations in circulating prorenin is an increase in blood pressure and do not support a role for prorenin as the primary causative agent in cardiac fibrosis or renal glomerular injury. The lack of effect seen with active site-mutated prorenin and the efficacy of angiotensin-converting enzyme inhibition are also consistent with prorenin acting through the generation of angiotensin II to raise blood pressure. (Hypertension. 2009;53:1062-1069.)

Key Words: hypertension ■ renin-angiotensin system ■ prorenin ■ transgenic mice ■ renin ■ cardiac remodeling ■ glomerulosclerosis

Renin derived from the kidney catalyzes the rate-limiting step of the renin-angiotensin system (RAS) and thereby plays an important role in the regulation of blood pressure and fluid balance. Renin is first synthesized as a precursor, prorenin, a portion of which is converted to active renin by the proteolytic removal of an amino-terminal prosegment before its secretion by the renal juxtaglomerular cells (reviewed in Reference 1). Prorenin is also present in the blood plasma at levels 5 to 10 times those of renin and is produced not only by the kidney but also by other tissues, such as adrenal and pituitary glands, testis, ovary, placenta, and eye.1,2 Prorenin levels increase with age and in diabetic patients,3 where a 2- to 3-fold elevation in circulating prorenin has been reported to precede the appearance of microvascular disease.4,5 Furthermore, Veniant et al6 reported that transgenic rats with a 400-fold increase in circulating prorenin exhibited severe cardiac remodeling and renal lesions in the absence of hypertension. These findings have raised concerns that prorenin, per se, might contribute to the observed cardiovascular pathologies. Because prorenin exhibits <1% of renin enzymatic activity in vitro,7 it is difficult to imagine how it would mitigate cardiovascular pathologies. However, several reports have demonstrated that circulating prorenin can be taken up by tissues and contribute to local angiotensin peptide generation in vivo8 by a mechanism that does not require the proteolytic removal of the prosegment.9 Indeed, prorenin acquires enzymatic activity on binding to the recently described (pro)renin receptor ((P)RR),10 providing a possible explanation for the enzymatic activity of intact prorenin in tissues. Moreover, studies in tissue culture cells have demonstrated that both prorenin and renin trigger intracellular mitogen-activated protein kinase activity by binding to (P)RR.11 Because this signaling cascade is not inhibited by preincubating cells with classical RAS inhibitors,12,13 prorenin and renin could trigger signaling that does not depend on the canonical activity of the RAS.

The ability of prorenin to trigger intracellular signaling has renewed interest in its possible role in conditions in which it is known to be elevated. Uddin et al14 have reported that a decapeptide corresponding with a short fragment of the prorenin prosegment (handle region peptide or HRP) blocks the interaction of prorenin with (P)RR. Furthermore, this group reported that infusion of HRP prevented the formation of
glomerulosclerosis in diabetic rats and reduced cardiac fibrosis in hypertensive rats, leading the authors to conclude that prorenin contributes to these pathologies.

To directly address the role of prorenin in cardiac remodeling and renal damage and to determine its mechanism of action, we have generated a series of transgenic mice with chronic elevations in circulating prorenin either in its native state or with a mutation that prevents its ability to contribute to tissue angiotensin II (Ang II) generation. Our results fail to support a role for chronically elevated prorenin as a direct mediator of cardiac and renal damage and demonstrate that, at sufficiently high levels, prorenin can lead to hypertension that responds to classical RAS inhibitors.

**Methods**

**Animals**

The mice used in this study were housed in a 12/12-hour light/dark cycle with free access to normal mouse chow and water. All of the experiments described herein were approved by the institutional animal ethics committee and are in compliance with guidelines issued by the Canadian Council on Animal Care.

**Construction of Plasmid Expression Vectors**

The expression vector for native mouse prorenin (TTRmProren) was constructed by placing the cDNA encoding mouse prorenin (Ren-1; National Center for Biotechnology Information accession No. X16642) downstream from a 3-kb region of the transh rerin gene promoter (TTR; a gift from Dr Robert Costa, University of Illinois at Chicago) and upstream from the rabbit β-globin 3′ nontranslated region (Figure 1A). Mouse prorenin was rendered incapable of exhibiting renin activity (TTRmProrenin-mut) by converting an aspartic acid in the renin active site (position 32) to an asparagine (its nearest structural neighbor). The substitution was carried out by site-directed mutagenesis using the overlap extension PCR technique. The following oligonucleotides were used: Mut-forward primer (5′-AAAGTCATCTTTAACACGGGT-3′), Mut-reverse primer (5′-AACCCGTTGAAAAGATGACTT3′), Ext-forward primer (5′-CCCAAGCTTATGAGCGACAGAGGGAGGGATGCCTCTCTGGGCACTG-3′), and Ext-reverse primer (5′-ATGTCGGGGGAGGGTGCGATTGCTG-3′). All of the expression vectors were verified by sequencing of double-stranded DNA.

**Generation and Maintenance of Transgenic Mouse Lines**

FVB/N mouse embryos were microinjected with appropriate expression vectors according to standard protocols. Breeding of all of the mice was carried out in the FVB/N line, keeping all of the transgenic animals heterozygous for the transgene. The presence of the transgene was determined by PCR analysis of DNA obtained by tail biopsies. All of the animals studied were males at 8 to 12 weeks of age unless otherwise noted. All of the control animals used were nontransgenic littermates.

**Determination of Transgene Expression Levels**

Transgene expression was quantitated by RNase protection assay (RPA) methodology. Total tissue RNA of liver samples, as described previously, was used to determine the expression of the transgenes in the liver of founder lines expressing either native (TTRmProren-3 and -7) or mutated mouse prorenins (TTRmProren-2mut and -3mut). Histone H4 mRNA is included as a control for RNA loading. Total RNA from transfected cells (positive control) and nontransgenic animals (control mouse) were used as controls. The relative expression level (TG/histone H4) of each transgenic line (bottom) was normalized to that in TTRmProrenin-3 (arbitrarily set at 1).

**Determination of Plasma Renin and Prorenin Concentrations**

Plasma renin concentration (PRC) was determined by incubating mouse plasma (0.1 μL, diluted in water; 50 μL final volume) with 50 μL of nephrectomized rat plasma containing angiotensinogen levels equivalent to 1000 pg of angiotensin I. The angiotensin I generated was quantitated by radioimmunoassay, as described previously. Total renin concentration (TRC; prorenin plus active renin) was determined likewise after incubation of the plasma samples with trypsin. Prorenin levels were calculated as TRC – PRC.

**Blood Pressure Measurements**

Systolic blood pressure measurement was performed by tail-cuff plethysmography (BP-2000 system, Visitech Systems), as described previously. Briefly, mice were trained to the apparatus for a total of 7 uninterrupted days, and measurements were recorded for the following 3 days. In certain cases, transgenic mice were implanted with telemeters (PA-20, Data Sciences International), and recordings were acquired beginning 1 week after surgery.

**Physiological Manipulations**

Mouse HRP (NH₂-RIPLK-KMPSV-COOH) was prepared by solid-phase synthesis and purified by high-performance liquid chromatography. Osmotic minipumps (Alza Corp) were implanted subcutaneously in anesthetized (2% isoflurane) 12-week-old male transgenic TTRmProren-7 mice for the administration of mouse HRP (1 mg/kg). The converting enzyme inhibitor captopril (Sigma-Aldrich) was administered in drinking water at a dose of 10 mg/kg per day.
Organ Histomorphology

For histological examinations, 12-week-old or 18-month-old transgenic and control animals were euthanized by CO₂ inhalation, and the kidneys and hearts were excised, rinsed rapidly to remove blood in PBS, and fixed by immersion for 4 hours in Bouin’s fixative (0.9% picric acid, 10% formaldehyde, and 5% glacial acetic acid). Fixed tissue was stored in 70% ethanol at 4°C until analyzed. Paraffin-embedded tissue sections (5 μm) of hearts were stained with Sirius Red F3BA (0.5% in saturated aqueous picric acid, Sigma-Aldrich) for assessment of interstitial and perivascular collagen content, as described previously. Quantification of fibrosis was performed using an image analysis system (Northern Eclipse 5.0, EM-PIX Imaging Inc). Seven fields were analyzed on 3 independent sections per mouse to determine the collagen area/total area. A single investigator unaware of the experimental groups performed the analysis. Kidney sections were stained with periodic acid-Schiff and examined for evidence of glomerulosclerosis by a board-certified veterinary pathologist who was unaware of the experimental groups.

Modulation of Cardiac RNA Expression

Total RNA was isolated from the cardiac ventricles and kidneys of 12-week-old control and transgenic mice with TRIzol (Invitrogen). Quantitative reverse-transcription PCR was carried out on cDNA generated with the Omniscript RT kit (QIAGEN, Inc) with the Quantitect SYBR Green PCR kit (QIAGEN) in a MX3005 real-time PCR machine (Stratagene). The oligonucleotides were designed to amplify the cDNA of mouse atrial natriuretic peptide (ANP), transforming growth factor β-1 (TGF), collagens I and III, and fibronectin and 40S ribosomal protein S16 and are described in Table S1 of the online data supplement (available at http://hyper.ahajournals.org). All of the samples fell within the linear range of the assay with a correlation coefficient of >0.95. Expression of RNA was normalized to that of S16 and arbitrarily set to 1.0 for control.

Albinurina

To test for effects of transgene expression on albuminuria, 10- to 12-week-old male control and transgenic mice were housed in metabolic cages for 48 hours with free access to food and water. Overnight urine samples were collected under paraffin oil and frozen until analyzed. No difference in water intake or urine production was noted between the different groups (data not shown). Albumin content of urine was determined using the Mouse Albumin Elisa Quantiﬁcation Kit (catalog E90-134) from Bethyl Laboratories according to the manufacturer’s instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 3.0 for Windows, (GraphPad Software) using 1-way ANOVA with Bonferroni’s multiple comparison post test. Results were considered significant with P<0.05.

Results

Generation of Transgenic Mice

To test for nonenzymatic properties of circulating prorenin in our mouse model, we mutated aspartic acid 32 of mouse Ren-1 prorenin to asparagine. This mutagenesis did not affect the synthesis and release of the Ren-1 protein from expressing cells (Figure S1). The effectiveness of the mutation in eliminating renin enzymatic activity was also confirmed by performing renin assays of culture supernatant collected from transfected cells (data not shown).

The transgenes used place the mouse prorenin cDNA (Ren-1) under the control of the transthyretin (prealbumin) promoter (Figure 1A). In mice, this promoter drives transgene expression in the liver, as demonstrated previously. The relative level of transgene mRNA expressed in the liver of transgenic mice was measured by RNase protection (Figure 1B) and varied between the different transgenic lines. By comparing the level of transgene expression to that of the endogenous histone H4 RNA, we were able to derive an estimate of the relative expression levels of the transgene in the various lines (Figure 1B).

To determine the effect of transgene expression on circulating prorenin and renin, an angiotensin I generation assay was performed on mouse plasma samples collected from 12-week-old male mice (Table 1). Expression of native mouse prorenin (Ren-1) in the liver of transgenic mice leads to a ~13-fold increase in prorenin in the TTRmProren-3 mouse line and a 28-fold increase in the TTRmProren-7 mice. This difference in circulating prorenin levels corresponds almost perfectly with the difference measured in transgene expression in these 2 lines (1.8-fold; Figure 1), suggesting that transgene expression level correlates with prorenin production in this model. In contrast, plasma active renin (PRC) was significantly depressed in both the transgenic TTR-mProren-3 and TTRmProren-7 mice compared with nontransgenic littermates (Table 1). This latter observation suggests a downregulation of the endogenous renin in this transgenic mouse line.

Because the mutant mouse prorenin (TTRmProren-mut) lacks enzymatic activity, measurement of transgene-derived prorenin by the angiotensin I generation assay is not possible for the TTRmProren-2mut and -3mut mouse lines. However, endogenous plasma renin and prorenin are unchanged as compared with those in control mice (compare TRC; Table
had a systolic pressure that was elevated by TTRmProren-7, which has a 28-fold increase in native prorenin, (TTRmProren-2mut or TTRmProren-3mut). In contrast, the or in either of the lines expressing active site-mutated prorenin in the circulation. Tail-cuff plethysmography revealed no difference in the systolic pressure of the native prorenin-expressing TTRmProren-3 line (data not shown). Blood pressure measurements by telemetry in control and in transgenic mice (Table 1). The heart rate in all of the transgenic lines as compared with control nontransgenic littermates (Table 1). To confirm these effects, we implanted telemetric blood pressure probes in control, TTRmProren-7, and TTRmProren-2mut mice and recorded 24-hour systolic blood pressures. As can be seen in Figure 2, whereas both control and TTRmProren-2mut mice have systolic pressures that oscillate around a mean of 110 to 120 mm Hg, TTRmProren-7 mice are mildly hypertensive, with a mean systolic pressure of ~145 mm Hg. These data demonstrate that high levels of prorenin in the circulation can result in an increase in blood pressure but that this increase requires a functional, active site in prorenin.

To determine whether high circulating prorenin can contribute to tissue remodeling or injury, we compared the kidneys and hearts from the highest prorenin-expressing lines (TTRmProren-7 and mProren-2mut) with those of control mice. At 12 weeks of age, routine histological staining (hematoxylin/eosin) revealed no evidence of kidney remodeling or injury in these groups (data not shown), although there was some evidence of cardiac hypertrophy in the hypertensive TTRmProren-7 mice (Table 2). In addition, although there was some evidence of cardiac hypertrophy in the hypertensive TTRmProren-7 mice at 12 weeks of age, there was no evidence of increased fibrosis in the transgenic mice expressing the highest levels of either native or active site-mutated prorenin (Table 3). To rule out more subtle or long-term effects of the high circulating prorenin levels, we compared the degree of cardiac fibrosis and glomerular injury in 18-month–old control and transgenic mice. As shown in Figure 3, there was no indication of overt glomerulosclerosis in either of the transgenic lines, as determined by periodic acid-Schiff staining (Figure 3, left). Furthermore, Sirius Red staining revealed no change in interstitial or perivascular fibrosis in the hearts of either of the prorenin-expressing transgenic lines as compared with control mice (Figure 3, right). Thus, there is no evidence of increased cardiac fibrosis or frank glomerular injury in transgenic mice with chronically increased plasma prorenin (native or active site-mutated), even at advanced age.

Physiological Characterization of Transgenic Animals

To test for the physiological effects of high circulating prorenin, we measured cardiovascular parameters in our control and transgenic mice (Table 1). The heart rate in all of the transgenic lines was not different from that in control animals (data not shown). Blood pressure measurements by tail-cuff plethysmography revealed no difference in the systolic pressure of the native prorenin-expressing TTRmProren-3 line or in either of the lines expressing active site-mutated prorenin (TTRmProren-2mut or TTRmProren-3mut). In contrast, the TTRmProren-7, which has a 28-fold increase in native prorenin, had a systolic pressure that was elevated by ~25 mm Hg as compared with control nontransgenic littermates (Table 1).
expressed less TGF, collagen I, and collagen III in the heart. In contrast, the expression of fibronectin was not significantly different in the hearts of the animals in the 3 groups, although there was a trend toward an increase in both of the hypertensive animals. Moreover, this expression pattern was mirrored in the kidney, although the differences in expression of collagen III and fibronectin did not reach statistical significance. Taken together, these results suggest that hypertension induced by chronic elevation of prorenin results in a larger increase in cardiac ANP expression and a lower induction of TGF and profibrotic genes than are seen in Ang II–induced hypertension.

Mechanism of Hypertension in TTRmProren-7 Mice
To determine whether the hypertension seen in TTR-mProren-7 mice might be caused by mechanisms other than Ang II (eg, by intracellular signaling induced by the interaction of native prorenin with [P]RR),14 we tested whether the blood pressure could be reduced by the action of the putative antagonist of (P)RR, HRP. TTRmProren-7 mice were implanted with telemetric blood pressure probes and subsequently administered mouse HRP (RIPLKKMPSV) by osmotic minipump for 11 days. The minipumps were loaded with 1 mg/kg of mouse HRP, which is 10 times the dose shown in previous mouse and rat studies to reverse diabetes mellitus–associated glomerulosclerosis.15,16 As shown in Figure 5, there was no evidence of a decrease in blood pressure in the TTRmProren-7 mice in the 11 days of HRP infusion. In contrast, administration of the angiotensin-converting enzyme inhibitor captopril at a dose of 10 mg/kg per day in the drinking water resulted in a rapid normalization of blood pressure in the TTRmProren-7 mice (Figure 5, right). Blood pressure was also reduced in these mice in response to an angiotensin receptor blocker (Candesartan cilexitil; data not shown), confirming that the hypertension in these mice was mediated by the actions of Ang II.

Discussion
The major conclusion to be drawn from the current study is that the chronic and very high levels of circulating prorenin that were achieved in our transgenic mice did not result in an increase in cardiac fibrosis or renal glomerular injury. In particular, we saw no evidence of the renal damage that has been correlated previously with high circulating prorenin levels in both diabetic humans4,5 and in mouse and rat models of diabetes mellitus,15,16 suggesting that prorenin, per se, is not the primary cause of these pathologies at the levels that we achieved in this study. These results differ significantly from those reported by Veniant et al6 in which transgenic rats with chronic elevations of rat prorenin released from the liver exhibited renal vascular lesions and cardiac hypertrophy without significant hypertension. The reason for the difference in the results of these 2 studies is not entirely clear but could be attributed to the extremely high plasma prorenin levels achieved in the rat model (≥400-fold increase over normal). Because these authors did not test for the effects of classical RAS blockade in preventing the observed pathologies, it is not possible to determine whether the prorenin was acting through tissue production of Ang II. Using an inducible transgene approach, Peters et al22 recently reported results that are strikingly different from those of Veniant et al.6 Induction of mouse Ren-2 expression in transgenic rats for a period of 12 weeks resulted in suppression of plasma active renin, an increase in circulating prorenin of ≥200-fold, and resulted in dose-dependent hypertension with no evi-

Table 3. Cardiac Remodeling Induced by Prorenin Overexpression in Transgenic Mice

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Fibrosis, %</th>
<th>Body Weight, g</th>
<th>Ventricle Weight, mg</th>
<th>VW/BW, mg/g N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic control</td>
<td>1.21±0.28</td>
<td>29.06±1.56</td>
<td>106.3±6.5</td>
<td>3.67±0.24 28</td>
</tr>
<tr>
<td>TTRmProren-7</td>
<td>1.34±0.27</td>
<td>29.27±2.34</td>
<td>127.2±10.4*</td>
<td>4.37±0.45* 41</td>
</tr>
<tr>
<td>TTRmProren-2mut</td>
<td>0.90±0.09</td>
<td>29.95±2.12</td>
<td>111.9±9.0</td>
<td>3.74±0.25 17</td>
</tr>
</tbody>
</table>

Results represent the mean±SEM. VW/BW indicates the ratio of ventricular weight to body weight.
*P<0.001 vs nontransgenic control animals.

Figure 3. Tissue effects of chronic hyperproreninemia. Representative tissue sections from 18-month-old male control and transgenic mice were stained with periodic acid–Schiff (PAS) to detect renal glomerulosclerosis (left) and with Sirius Red to detect cardiac collagen (right). No differences were detected in the degree of glomerulosclerosis or cardiac fibrosis in either transgenic animal as compared with control mice.
dence of glomerulosclerosis at the end of the treatment period. The current results confirm that this lack of effect is not because of the limited treatment period or the use of prorenin from a different species, because our mice have elevated mouse prorenin throughout life and show none of the predicted effects on cardiac fibrosis or renal glomerular damage even after 18 months. It should also be noted that the levels of prorenin achieved in this study and those of Veniant et al. and Peters et al. are extremely high, even as compared to those seen in human or rodent models of diabetes mellitus, where prorenin levels range from normal to 2- to 3-fold above normal. Thus, if prorenin had a deleterious effect, our model would likely have detected it.

Interestingly, overexpression of prorenin in the circulation in our mice did not lead to a consequent increase in circulating renin activity. In fact, both the TTRmProren-7 and TTRmProren-3 mice exhibited a decrease in the plasma content of active renin (Table I). The lack of active renin production by the transgene is not unexpected, because the encoded prorenin is being made in the liver, which does not have a prorenin processing enzyme. However, this finding reinforces the conclusion that prorenin free in the plasma does not contribute to Ang II production even when present at very high levels. Somewhat surprisingly, although the TTRmProren-7 line was hypertensive, the TTRmProren-3 line, which has approximately half of the increase in circulating prorenin (13- versus 28-fold), was not. However, the decreased active renin in both lines is consistent with the prorenin leading to a compensatory suppression of active renin release from the kidney. Perhaps in the TTRmProren-7 mice this natural compensatory mechanism was inadequate to counter the hypertensive effect of the higher prorenin levels.

The existence of such a compensatory mechanism is supported by the finding that endogenous active renin was not decreased in either of the 2 lines of mice expressing comparable amounts of active site-mutated prorenin (TTRProren132mut and TTRProren132mut), which would be incapable of contributing to the generation of Ang II. Nevertheless, the increase in blood pressure in the TTRmProren-7 line is clearly attributed to RAS activation, because it responds rapidly to angiotensin-converting enzyme inhibition (Figure 5). The simplest explanation for these results is that prorenin derived from the circulation is acting locally, either in the blood vessel wall or in a tissue, to raise blood pressure by generation of Ang II. Furthermore, the increase in blood pressure in the TTRmProren-7 line is clearly attributed to RAS activation, because it responds rapidly to angiotensin-converting enzyme inhibition (Figure 5). The simplest explanation for these results is that prorenin derived from the circulation is acting locally, either in the blood vessel wall or in a tissue, to raise blood pressure by generation of Ang II and that this results in a compensatory decrease in renin production by the kidney.

Indeed, we have reported previously that prorenin derived from the circulation could be used to generate angiotensin peptides within tissues in the absence of classical activation by prosegment removal. The prorenin-activating properties of the recently described prorenin/renin receptor ([P]RR) could provide an explanation for such tissue-restricted activity of prorenin.

The absence of obvious cardiac fibrosis and glomerulosclerosis in old (18 months) hyperproreninemic, hypertensive TTR-mProren-7 mice was a surprising and unexpected result. The hypertension is clearly attributed to Ang II production, because it could be reversed with an angiotensin-converting enzyme inhibitor, it was absent from mice transgenic for prorenin with an active site mutation, and it did not respond
to infusion of high doses of HRP peptide (ie, was not consistent with a non-Ang II mechanism). In fact, the hypertension caused by prorenin resulted in a significantly different induction of genes associated with remodeling and fibrosis than that seen in mice infused with Ang II. The induction of cardiac ANP, as well as the observed cardiac hypertrophy in the prorenin-overexpressing mice, confirms that the hearts are responding to the high blood pressure. In spite of this cardiovascular challenge, the animals fail to mount the TGF and collagen responses seen in the Ang II–infused mice, and these results likely explain the absence of the expected cardiac fibrosis in the hypertensive, hyperproreninemic mice. The explanation for these differences will require further investigation but are not inconsistent with a protective, as well as a prohypertensive, role for prorenin.

Our results also fail to explain the reported role of HRP in reversing cardiovascular damage. Although we cannot rule out the possibility that treatment with HRP for longer periods of time or with higher doses might have increased its effectiveness, the ratio of HRP:prorenin that we used was comparable to that used by Ichihara et al to prevent glomerulosclerosis in diabetic mice. Ichihara et al were the first to report that chronic HRP infusion reduced cardiac fibrosis in the spontaneously hypertensive rat in the absence of an effect on blood pressure. More recently, Susic et al also reported a blood pressure–independent reduction of cardiac remodeling, as well as a reduction in serum creatinine by HRP (called PRAM-1 by the authors) infusion in salt-fed spontaneously hypertensive rats. Thus, the protective action of HRP does not appear to require a blood pressure effect, although its mechanism of action is still debated: although HRP has been reported to block prorenin binding to (P)RR in biochemical assays, several recent reports have failed to see an effect on prorenin-induced binding and signaling using intact cells. Furthermore, HRP does not appear to be effective in preventing renal damage in a rat model of renovascular hypertension. Thus, the mode of action of HRP and the conditions under which it exerts its effects will require further clarification. However, because the high circulating prorenin levels that we achieved in this study did not generate the pathologies on which HRP appears to act, it is possible that HRP is not merely reversing the actions of prorenin but rather has a mode of action that is independent of the RAS.

Perspectives
The 2- to 3-fold elevation of circulating prorenin associated with diabetic complications has prompted a concern that prorenin exerts a pathogenic effect on tissues, and some experiments in animal models of hypertension and diabetes mellitus have reinforced these concerns. The discovery and characterization of the prorenin receptor (P)RR also raise the possibility that prorenin could have pathophysiological effects independent of its contribution to Ang II synthesis that
would, therefore, not be effectively treated with current RAS inhibitors. However, we detected no deleterious effects of overexpressing active site-mutated prorenin on blood pressure, cardiac fibrosis, or glomerulosclerosis in our mice. Because the mitogen-activated protein kinase cascades activated by prorenin binding to (P)RR in vitro are similar to those activated by Ang II,29 they might be expected to lead to significant cardiovascular consequences in our model. Our results cannot entirely rule out the possibility that elevated circulating prorenin at levels higher than those achieved in this study would have resulted in pathology or that prorenin could contribute to pathologies in the context of other complications, such as sustained diabetes mellitus. However, they are not consistent with a primary causative role of prorenin in inducing cardiac fibrosis or renal glomerular damage, and they suggest that the consequences of elevated prorenin, if any, might be beneficial.

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**Disclosures**

None

**References**

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Chronic increases in circulating prorenin are not associated with renal or cardiac pathologies

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Short title: Cardiovascular effects of circulating prorenin

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METHODS

Biosynthesis of mutated prorenin

In order to test for non-enzymatic properties of circulating prorenin in our mouse model, we mutated aspartic acid 32 of mouse Ren-1 prorenin to asparagine (its nearest structural neighbor). To ensure that this mutagenesis did not affect the biosynthesis of the Ren-1 protein, we inserted the coding sequences of the native and mutated Ren-1 prorenins into the RSV-globin expression vector {226} and transfected these into cultured HeLa cells. HeLa cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a humidified incubator at 10% CO₂. Cells were plated at a density of 1 X 10⁶ cells in 35-mm wells and transfected 6 h later with 2.0 μg of the appropriate expression vector using liposomes (Effectin, Invitrogen, Burlington, Ontario, Canada). HeLa cells were replated in 25-mm wells 48 h after transfection. The next day, the cells were depleted of methionine for 1 h in methionine-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed fetal calf serum, labeled with 300 μCi of [³⁵S] methionine per well for 90 minutes and the culture supernatants were then immunoprecipitated with a rabbit anti-human prorenin (BRI-6) and protein A-Sepharose. Immunoprecipitated proteins were fractionated by SDS-PAGE, and gels were subjected to fluorography.

RESULTS

Pulse-labeling analysis confirmed that the active site mutation of the Ren-1 prorenin did not affect its synthesis and release from expressing cells (Figure S1). The effectiveness of the mutation in eliminating renin enzymatic activity was also confirmed by performing renin assays of culture supernatant collected from transfected cells (data not shown).
Figure S1: Detection of labeled mouse prorenin in culture supernatant from cells transfected with an expression vector encoding the native (TTRmProren) or active site-mutated mouse Ren-1 prorenin (TTRmProren-mut) vectors by immunoprecipitation. Arrow indicates the expected migration of mouse prorenin. A human renin expressing vector (pRhR1100) was used as a positive control.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
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</tr>
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**Table S1:** Oligonucleotides used for quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) measurement of mouse mRNA expression. F, forward. R, reverse.