Tissue Activity of Circulating Prorenin

Gary Prescott, David W. Silversides, and Timothy L. Reudelhuber

Both renin and its biosynthetic precursor, prorenin, are secreted into the circulation of mammals. Although the circulating levels of prorenin can exceed those of renin by as much as 100-fold in certain conditions, there is no evidence that prorenin contributes to the synthesis of circulating angiotensin peptide synthesis or increased blood pressure (BP). In the current study, we have generated a transgenic mouse line that overexpresses human prorenin in the liver and have mated these mice to a second mouse line expressing human angiotensinogen in the heart. Double-transgenic progeny display a selective increase in angiotensin I content in the heart (but not the plasma) as compared to the single-transgenic mice. These results are consistent with a model in which circulating prorenin is taken up by tissues where it can contribute to the local synthesis of angiotensin peptides. This finding may explain some of the pathologies associated with high circulating prorenin levels. Am J Hypertens 2002; 15: 280–285 © 2002 American Journal of Hypertension, Ltd.

Key Words: Renin-angiotensin system, transgenic mice, heart, renin, enzyme precursors.

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The rate-limiting reaction of the circulating renin-angiotensin system (RAS) is the cleavage of the decapeptide angiotensin I (Ang I) from circulating hepatic angiotensinogen by the kidney-derived aspartyl protease renin (Fig. 1a). Ang I is subsequently processed by endothelial-derived angiotensin converting enzyme to the octapeptide Ang II, which exerts its effects on vasoconstriction, aldosterone release, and cell growth/apoptosis through its interaction with specific receptors (angiotensin II type 1 and type 2 receptors [AT1-R and AT2-R]). Although the RAS is an endocrine system, several tissues contain or synthesize components of the system (reviewed in Refs. 1–3), raising the possibility that locally produced Ang II could act in an autocrine or paracrine fashion to regulate tissue function.

Renin is synthesized as a proenzyme precursor, prorenin. The active form of renin present in the circulation results from the proteolytic removal of the 43-amino-acid amino terminal prosegment of prorenin. Although circulating active renin is derived exclusively from the kidneys, the kidneys and other tissues also secrete prorenin into the circulation where it is normally present at 5 to 10 times the level of renin. Circulating prorenin has no detectable enzymatic activity as evidenced by a lack of increase in either circulating Ang II or blood pressure (BP) in animals injected with large quantities of prorenin. 4, 5 Nevertheless, several lines of circumstantial evidence link high circulating prorenin to end-organ damage. Dramatic elevations in circulating prorenin (to as much as 100 times the level of renin) are associated with renal damage in some diabetic patients. 5–10 Transgenic rats with similar increases in cir-

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From the Laboratory of Molecular Biochemistry of Hypertension, Clinical Research Institute of Montreal (GP, TLR) and CRR, Faculté de Médecine Vétérinaire de L’Université de Montréal (DWS), St. Hyacinthe, Québec, Canada.

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Address correspondence and reprint requests to Dr. Timothy L. Reudelhuber, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7, Canada; e-mail: reudel@ircm.qc.ca

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Circulating prorenin also exhibit renal damage and cardiac hypertrophy in the absence of an obvious increase in either circulating angiotensin peptides or hypertension, raising the possibility that prorenin is contributing to these pathologies at a tissue level.\(^\text{11}\) Indeed, substantial evidence has accumulated that both prorenin and renin can bind cell surface acceptors present in the vasculature and in numerous tissues.\(^\text{12,13}\) In addition, there is evidence that prorenin can be activated in tissues or cultured cells by mechanisms that may not require the proteolytic removal of the pro-segment.\(^\text{14–16}\) In the current study, we have created an in vivo model to test whether circulating prorenin can contribute to local generation of angiotensin peptides within tissues. In this model (Fig. 1b), transgenic mice, which release human prorenin into the circulation exclusively from the liver, were mated to mice expressing human angiotensinogen exclusively in the heart. The measurement of the product of the reaction, Ang I, in the hearts of double-transgenic mice serves as a direct measure of the ability of circulating prorenin to promote the activity of a local tissue RAS. Our results provide the first direct evidence that circulating prorenin contributes to the production of angiotensin peptides in the tissues of intact animals and supports a role for prorenin in some cardiovascular pathologies.

**Methods**

**Animals**

All animal protocols were approved by the Institutional Animal Protection Committee of the Clinical Research Institute of Montreal. Transgenic mice were generated in the FVB/N strain obtained from the Jackson Laboratory (Bar Harbor, ME). Maintenance breeding was carried out in the same strain.

**Transgene Construction, Generation of Transgenic Mice, and Expression of the Transgene**

To target the expression of human prorenin to the mouse liver, a 3-kb region of the transthyretin gene promoter was cloned upstream of the human prorenin cDNA. FVB/N mouse embryos were microinjected with the plasmid according to standard protocols\(^\text{17}\) and all subsequent breeding was carried out in the FVB/N line. Genomic integration of the transgene was determined by polymerase chain reaction (PCR) analysis of DNA obtained from tail biopsies and tissue-specific expression of the human transgene was verified by an RNase protection assay from total tissue RNA as previously described.\(^\text{16}\) All animals tested were male at 10 weeks of age unless otherwise stated.

**Physiologic Measurements**

Plasma renin concentration (PRC) and total renin concentration (TRC) were measured as follows: blood samples obtained by orbital puncture of mice lightly anesthetized with ether were collected into ice-cold microcentrifuge tubes containing EDTA and immediately centrifuged to isolate plasma. Plasma was stored at \(-20^\circ\text{C}\) until assayed. Human PRC was determined by the rate of Ang I generation from an excess of human angiotensinogen. Under the assay conditions, mouse renin generated insignificant levels of Ang I from human angiotensinogen. Briefly, 0.25 \(\mu\text{L}\) (transgenic) or 5 \(\mu\text{L}\) (nontransgenic) of plasma was incubated with 100 ng of purified human substrate (>95%; Sigma Chemical Co., St. Louis, MO) at \(37^\circ\text{C}\) for 0, 20, 40, and 60 min in a total volume of 150 \(\mu\text{L}\) of buffer at pH 7.5. Reactions were stopped on ice and subsequent steps performed at \(4^\circ\text{C}\). The Ang I generated was measured by radioimmunoassay (RIA). Total renin concentration was determined after incubation with trypsin (0.3 mg/mL; Boehringer Mannheim, Germany) at room temperature for 10 min in a total volume of 50 \(\mu\text{L}\) of buffer at pH 8.0 (these conditions of trypsin digestion gave maximal prorenin activation). Prorenin was calculated as the difference between total and active renin content.

Tail-cuff plethysmography (BP-2000 system, Visitech Systems, Apex, NC) was performed according to previously published procedures.\(^\text{18}\) Briefly, mice were trained to the apparatus for a total of 7 uninterrupted days and measurements were recorded and averaged for the following 3 days. The degree of cardiac hypertrophy was estimated by calculating the cardiac mass index (the ratio of cardiac ventricle wet weight to total body weight).

Angiotensin I and II were measured by RIA of acid-soluble extracts of either plasma or heart tissue as previously described. Mice were anesthetized by intraperitoneal injection with 3 mg of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 250 \(\mu\text{L}\) of whole blood was collected by cardiac puncture in presence of inhibitor solution containing 1 \(\mu\text{mol/L}\) Remikiren (a specific human renin inhibitor received as a gift from F. Hoffmann-La Roche AG, Basel, Switzerland), 1 \(\mu\text{mol/L}\) captopril, and 10 \(\mu\text{mol/L}\) EDTA (final concentration) and cleared immediately by centrifugation. Plasma samples (150 GL) were adjusted to 2 mL by addition of acid extraction buffer (80% ethanol, 0.1 mol/L HCl) and again cleared by centrifugation at 13,000 \(g\) for 30 min. Ethanol was evaporated and 2 mL of 1% ortho-phosphoric acid was added to each sample. Samples were again cleared by centrifugation, 2 mL of 1% ortho-phosphoric acid was again added. The samples were loaded onto Sep-Pak hydrophobic c18 cartridges (Waters Corp., Milford, MA), which were subsequently washed twice with 5 mL of \(\text{H}_2\text{O}\). Angiotensin peptides were eluted with 3 mL of absolute methanol (Anachemia Canada Inc., Montreal, Quebec, Canada). Samples were then split in two equal aliquots for the separate measurement of Ang I and II. Lyophilized peptides were quantitated by RIA. The Ang I antibody used is specific for Ang I peptide with no detectable cross-reactivity with Ang II or its metabolites, whereas the Ang II antibody used (CD3) shows no cross-reactivity with Ang I (data not shown). For measurement of Ang I
and II contained in heart tissue, animals were euthanized and excised hearts were pressed repeatedly onto blotting paper to remove excess blood before being flash frozen in liquid nitrogen. Frozen hearts were pulverized with a mortar and pestle, and the powder was immediately homogenized in 2 mL of the acid extraction buffer. After clearing by centrifugation, the samples were treated as described above for the determination of Ang I and II content.

**Organ Histomorphology**

For histology, the livers, kidneys, and hearts of anesthetized transgenic and control animals were fixed in situ by organ perfusion using Bouin’s fixative (0.9% picric acid, 10% formaldehyde, 5% glacial acetic acid). Organs were then quickly removed, post-fixed in Bouin’s fixative for 5 h, washed in 70% ethanol, and imbedded in paraffin.

Hematoxylin and eosin-stained tissue sections were examined for lesions and vascular hypertrophy by standard protocols. In addition, the hearts of non-, single-, and double-transgenic mice were assessed for cardiomyocyte hypertrophy, interstitial and perivascular fibrosis by histologic staining with sirius red.

For immunostaining of human prorenin sections (5 μm thick) of Bouin’s fixed and paraffin-embedded tissues were mounted on 3-aminopropyltriethoxysilane (APTES)-coated slides (Sigma), deparaffinized, rehydrated, and washed with H2O. Nonspecific antibody binding was blocked by incubation with 1% donkey serum in Tris-buffered saline (TBS; 50 mmol/L Tris-HCl, 154 mmol/L NaCl, pH 7.4) for 1 h at 25°C. Tissue sections were incubated with rabbit polyclonal antibody to human renin (BRI-6, 1:600; a generous gift from Daniel F. Catanzaro, Weill Medical College of Cornell University, NY) in TBS containing 5% Carnation milk powder. The sections were then incubated with a biotinylated donkey anti-rabbit IgG (1:200 dilution; Amersham, Oakville, Ontario, Canada), incubated with rabbit polyclonal antibody to human renin (BRI-6, 1:600; a generous gift from Daniel F. Catanzaro, Weill Medical College of Cornell University, NY) in TBS containing 5% Carnation milk powder. The sections were then incubated with a biotinylated donkey anti-rabbit IgG (1:200 dilution; Amersham, Oakville, Ontario, Canada), followed by streptavidin-horseradish peroxidase (HRP, 1:300 dilution; Amersham). Positive staining was detected using 0.025% diaminobenzidine (DAB) and 0.03% H2O2 for 8 min. The sections were dehydrated, mounted with Permount (Fisher Scientific Ltd., Nepean, Ontario, Canada), and photographed using Nomarski optics. No staining was detected when the first antibody was omitted (data not shown).

**Results**

**Expression of Transgenes**

Mice transgenic for the human prorenin cDNA under the control of the transthyretin promoter showed expression of the transgene in the liver as shown by RNase protection assays (Fig. 2). Although a small amount of expression was detectable in the stomach, expression was not detected in the other organs tested, including the heart and kidneys. Expression of the transgene in the stomach was not a general finding with this promoter (additional data not shown) and likely reflects an integration site artifact in this line of mice. Expression of the human angiotensinogen transgene was targeted to the heart using the α-myosin heavy chain promoter and the transgene expression pattern in these mice (MHChAgt-2) has been described previously.

**Physiologic and Biochemical Characterization of Transgenic Mouse Lines**

Expression of human prorenin in the liver of transgenic mice (TTRhProren-B7) leads to release and detection of human prorenin in the circulation of which more than 99% is the enzymatically inactive prorenin form (Table 1). Mice transgenic for human prorenin showed no elevation in BP as compared to nontransgenic control littersmates (Table 1). In fact, 10-week-old male mice showed a slight, but significant decrease in their BP as compared to controls; however, this difference was not apparent in female mice of the same age or in older (14 weeks) male mice (data not shown). The finding of lower BP in young male mice remains an unexplained finding but could result from variations in the time course of transgene expression specifically in males or other factors. The BP seen in the double-transgenic mice was identical to that seen in the mice expressing only the human prorenin in the liver (Table 1). The human angiotensinogen-expressing mouse line (MHChAgt-2) showed comparable BP to nontransgenic animals. No significant differences were observed for the cardiac mass index between all of the mouse groups (Table 1).

**Tissue Uptake of Human Prorenin**

Immunohistochemistry was performed on tissues of transgenic animals expressing human prorenin in the liver (TTRhProren-B7) using an antibody with selectivity for...
human (pro)renin. Punctate staining was observed in the heart of transgenic animals exclusively, in cells bordering the lumen of small blood vessels (Fig. 3a). Specific staining was also observed in the pituitary and lungs (data not shown). However, no staining was detectable in sex organs (ovary and testis).

**Enzymatic Activity of Prorenin Taken Up By Tissues**

To test whether prorenin taken up by tissues can contribute to tissue RAS activity, transgenic mice expressing human prorenin in the liver (TTRhProren-B7) were mated to mice expressing human angiotensinogen exclusively in the heart (MHChAgt-2). Double-transgenic mice were tested for circulating and cardiac content of Ang I (Fig. 4). The results demonstrate that although the single-transgenic animals showed either low or undetectable levels of Ang I in the heart, double-transgenic mice exhibited a significant increase in cardiac Ang I content. Notably, the circulating levels of the Ang I did not increase in double-transgenic mice as compared to single- and nontransgenic controls, suggesting that enhanced production of angiotensin in the double-transgenic mice was restricted to the heart. Moreover, a significant decrease in plasma Ang I was observed.

### Table 1. Physiological characterization of transgenic mice

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Human Protein Expressed (tissue)</th>
<th>PRC (ng Ang I/mL/h)</th>
<th>TRC (ng Ang I/mL/h)</th>
<th>SBP (mm Hg)</th>
<th>CMI (1000x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nontransgenic</td>
<td>none</td>
<td>1.0 ± 0.8</td>
<td>2.3 ± 1.0</td>
<td>137 ± 5</td>
<td>3.80 ± 0.18</td>
</tr>
<tr>
<td>TTRhProren-B7</td>
<td>prorenin (liver)</td>
<td>1.5 ± 0.7</td>
<td>50.8 ± 5.6</td>
<td>124 ± 9†</td>
<td>3.90 ± 0.07</td>
</tr>
<tr>
<td>MHChAgt-2</td>
<td>angiotensinogen (heart)</td>
<td>ND</td>
<td>ND</td>
<td>135 ± 6</td>
<td>4.03 ± 0.23</td>
</tr>
<tr>
<td>TTRhProren-B7 X MHChAgt-2</td>
<td>prorenin (liver) + angiotensinogen (heart)</td>
<td>ND</td>
<td>ND</td>
<td>126 ± 5‡</td>
<td>3.97 ± 0.20</td>
</tr>
</tbody>
</table>

PRC = plasma renin concentration; TRC = total renin concentration; SBP = systolic blood pressure; CMI = cardiac mass index; ND = not determined.

Values are mean ± SEM of single determinations on 5 to 12 individual animals.

The level of prorenin can be roughly calculated by TRC–PRC.

*P < .05 compared to nontransgenic mice by ANOVA using Student t test.

†Difference not significant as compared to MHChAgt-2 mice.
for the prorenin-expressing mice. These results were reproduced in matings between additional founder lines of transgenic mice (data not shown) and are representative of a total of three independent experiments.

**Histology of Transgenic Mouse Tissues**

Routine histologic staining (hematoxylin and eosin) of hearts and kidneys of non-, single-, and double-transgenic animals revealed no apparent changes in heart physiology in double-transgenic mice compared with single- and nontransgenic mice (data not shown). Sirius red staining was used to assess any changes in interstitial and perivascular connective tissue in the heart. No differences were observed between groups of transgenic animal relative to the distribution and density of fibrosis (data not shown).

**Discussion**

The present study provides the first in vivo demonstration that a chronic elevation in circulating prorenin leads to uptake of prorenin by tissues and an increase in local synthesis of angiotensin peptides within that tissue. Human renin and angiotensinogen have little biochemical interaction with their mouse homologs, although the ensuing products of their reaction (Ang I, Ang II, and metabolites) are identical in the two species. This allows the study of the human renin and angiotensinogen transgenes without interference from the mouse RAS and metabolites, thus permitting activity on mouse angiotensinogen. Because we observed no increase in plasma Ang I content in the double-transgenic animals as compared with single- and nontransgenic mice, we confirmed that the site of angiotensin peptide generation is within the heart, with no apparent leakage of those peptides in circulation. Notably, we failed to see an increase Ang II content in the heart of double-transgenic animals. Although the explanation for this result is not known, it is possible that the levels of Ang I peptides generated within the cardiac interstitium do not permit significant conversion to and detection of Ang II. Furthermore, we did not observe any of the hallmarks of high cardiac Ang II including cardiac hypertrophy or increased fibrosis in the double-transgenic mice demonstrating that a local increase in Ang I alone is not sufficient to induce cardiac pathologies. Thus, although we failed to demonstrate a physiologic consequence of prorenin uptake and activity in the heart, it is possible that prorenin uptake plays a significant role in other tissues where it could encounter endogenous angiotensinogen. Such a possibility would need to be tested using native mouse prorenin as the human protein used in these studies does not have detectable activity on mouse angiotensinogen.

The nature of the activation step for circulating prorenin taken up by tissues is not known. Previous studies have demonstrated that human prorenin can be enzymatically active without the removal of its 43-amino-acid prosegment within tissues. Cell culture studies have shown that endothelial cells, cardiomyocytes, and fibroblasts can bind and internalize not only renin, but also prorenin whereupon prorenin can be activated to renin. It has been suggested that this finding explains why in the normal heart virtually no prorenin can be detected. We had previously reported that crossing a transgenic mouse line expressing human angiotensinogen in the heart with one that has active human renin in the circulation leads to a much greater accumulation of cardiac Ang I (1.65 ng Ang I/mg wet weight) than that seen in the current study (0.27 ng Ang I/mg wet weight; a sixfold difference). In comparing these levels to the levels of human active renin in the circulation of the mice in the previous studies (3307 ng Ang I/mL/h) and the levels of human prorenin achieved in the mice in the current study (50 ng Ang I/mL/h; a 66-fold difference) it is tempting to speculate that nearly all of the prorenin taken up by the heart must be activated. However, this assumption could be skewed by the availability of substrate (might be limiting in the previous study) and conclusive demonstration of the degree of prorenin activation after tissue uptake in this model will require further experiments.

Several groups have reported on the existence of (pro)renin-binding proteins or receptors. Nguyen et al and Sealey et al, with the use of radiolabeled (pro)renin, demonstrated high-affinity renin-binding sites/receptors (Kd 1 nmol/L) in human mesangial cells and in membranes prepared from multiple rat tissues including the heart and vasculature. Because these sites bind prorenin and renin equally well, it is possible that they could account for prorenin uptake within tissues. The demonstration in this study that the prorenin being taken up can have enzymatic activity may serve to explain the cardiovascular pathologies observed in animals with high levels of circulating prorenin and may point to a novel therapeutic target for their prevention in humans.

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**References**


