 Contribution of circulating renin to local synthesis of angiotensin peptides in the heart

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Prescott, Gary, David W. Silversides, Sui Mei Linda Chiu, and Timothy L. Reudelhuber. Contribution of circulating renin to local synthesis of angiotensin peptides in the heart. Physiol Genomics 4: 67–73, 2000.—The activity of a local cardiac renin-angiotensin system (RAS) has long been suspected in the promotion of cardiac pathologies including hypertrophy, ischemia, and infarction. All of the components of the RAS cascade have been demonstrated to be synthesized within the heart with the possible exception of the first enzyme in the cascade, renin. In the current study, we provide direct evidence that circulating renin can contribute to cardiac-specific synthesis of angiotensin peptides. Furthermore, we demonstrate this effect is independent of blood pressure and that in animals of comparable blood pressure, elevated circulating renin significantly enhances cardiac fibrosis. These results may serve to explain some of the cardiac pathologies associated with the RAS.

IN MAMMALS, the cleavage of the decapeptide angiotensin I (ANG I) from the circulating hepatic glycoprotein angiotensinogen is the rate-limiting step in the renin-angiotensin system (RAS) and is carried out by the kidney-derived aspartyl protease renin (Fig. 1A). ANG I is subsequently processed by endothelial-derived angiotensin converting enzyme to the octapeptide angiotensin II (ANG II) which exerts its effects on vasoconstriction, aldosterone release, and cell growth/apoptosis through its interaction with specific receptors (AT1-R and AT2-R). The components of the RAS as well as their corresponding mRNAs have also been reported to be expressed within certain tissues, leading to the suggestion that tissue RAS (tRAS) could influence long-term hemodynamic changes through local generation of ANG II, which can in turn affect surrounding tissues or cells. ANG II is capable of stimulating the expression of nuclear protooncogenes such as c-fos, c-jun, jun-B, egr-1, and c-myc, as well as growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-β1 (TGF-β1), and late markers of cardiac hypertrophy such as skeletal α-actin and atrial natriuretic factor (ANF) (14, 16, 17, 25), raising the possibility that locally generated angiotensin peptides act as growth factors in the heart. The resulting structural changes may be linked to decreased cardiac function.

The existence of a localized RAS in the heart has been supported by several lines of clinical evidence. For example, RAS inhibitors are beneficial in the treatment of chronic heart failure, acute myocardial ischemia, and regression of cardiac hypertrophy (9, 10, 12, 23). Both prospective and retrospective clinical studies of hypertensive patients have shown a strong correlation between elevated circulating renin levels in hypertensive patients and the risk of myocardial infarction independent of blood pressure (1, 3). Although the mRNAs for all of the components of the RAS have been detected in the heart, the mRNA for renin has variously been reported as being absent or expressed at very low levels, and there is some debate as to whether there is sufficient active renin generated within the heart to catalyze an intracardiac RAS (11, 29). Some studies have suggested that renin might be taken up by vascular tissues, including the heart; for example, the enzymatic activity of intracardiac and plasma renin in the whole animal parallel one another and both virtually disappear with removal of the kidneys, the primary source of circulating active renin (6). In situ perfusion studies also support the notion that the heart and vasculature can retain circulating renin (15, 22, 27), and two groups have reported the existence of high-affinity renin receptor proteins in rat membrane preparations (4, 26). Nevertheless, the physiological relevance of the renin binding seen in these in situ and in vitro approaches is still unclear. In the current study, we have created an in vivo model to directly test whether circulating renin can contribute to local generation of angiotensin peptides in the heart. In this model (Fig. 1B), transgenic mice that release human active renin into the circulation exclusively from the liver were mated to mice expressing human angiotensinogen exclusively in the heart. The measurement of the products of the reaction, ANG I and II, in the hearts of double-transgenic mice serves as a direct measure of the ability of circulating renin to promote...
the activity of a local cardiac RAS. Our results provide the first direct evidence that circulating renin contributes by a pressure-independent mechanism to the production of angiotensin peptides in the heart of intact animals and may point to new avenues in the treatment of certain forms of heart disease.

MATERIALS AND METHODS

All animal protocols were approved by the institutional Animal Protection Committee of the Clinical Research Institute of Montreal.

Expression of human renin and angiotensinogen in transgenic mice. To express human renin in mouse liver, a 3-kb region of the transthyretin gene promoter (a generous gift from Robert H. Costa, University of Illinois at Chicago) was cloned upstream of the human prorenin cDNA. To generate active human renin, a cleavage site for the ubiquitous prosegment of the active renin molecule, resulting in prosegment removal by endogenous proteases in the secretory pathway of expressing cells (2). Expression of human angiotensinogen in the mouse heart was achieved by cloning the cDNA downstream of a 6-kb fragment of the α-myosin heavy chain gene promoter (a generous gift from Jeffrey Robbins, University of Cincinnati).

FVB/N mouse embryos were microinjected according to standard protocols (20), and all subsequent breeding was carried out in the FVB/N line. Tissue-specific expression of the human transgenes was verified by an RNase protection assay from total tissue RNA as previously described (21).

All animal tested were male at 10 wk of age unless otherwise stated.

Biochemical and physiological characterization of transgenic mice. Renin and prorenin assays (Table 1) were performed 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°C until assayed. Human plasma renin concentration was determined by the rate of ANG I generation from an excess of human angiotensinogen to take advantage of the species specificity of the reaction between renin and angiotensinogen (21). Under the assay conditions, mouse renin generated barely detectable levels of ANG I from human angiotensinogen. Briefly, 0.25 µl (transgenic) or 5 µl (non-transgenic) of plasma was incubated with 100 ng of purified human substrate (>95%; Sigma Chemical, St. Louis, MO) at 37°C for 0, 10, 20, and 30 min in a total volume of 150 µl of buffer, pH 7.5. Reactions were stopped on ice, and subsequent steps were performed at 4°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 µl of buffer, pH 8.0. Prorenin was calculated as the difference between total and active renin content.

Blood pressures of transgenic mice were measured by tail-cuff plethysmography (model BP-2000; Visitech Systems, Apex, NC) according to previously published procedures (18). Briefly, mice were trained to the apparatus for a total of 8–9 uninterrupted days, and measurements were recorded only for the last 2 days.

The degree of cardiac hypertrophy was estimated by calculating the ratio of cardiac ventricle wet weight to body weight.

ANG I and II were measured by RIA of acid-soluble extracts of either plasma or heart tissue with a modification of the method of van Kats et al. (28). Briefly, mice were anesthetized by intraperitoneal injection with 3 mg pentobarbital sodium (MTC Pharmaceuticals, Cambridge, Ontario), and 250 µl whole blood was collected by cardiac puncture in presence of inhibitor solution (1 µM remikiren, 1 µM captopril, and 10 mM EDTA final concentration) and cleared immediately by centrifugation. Plasma samples (150 µl) were adjusted to 2 ml by addition of acid extraction buffer (80% ethanol, 0.1 M HCl) and again cleared by centrifugation at 13,000 g for 30 min. Ethanol was evaporated, and 2 ml of 1% orthophosphoric acid was added to each sample. Samples were again cleared by centrifugation, and 2 ml of 1% orthophosphoric acid was again added. The samples were loaded onto Sep-Pak hydrophobic C18 cartridges (Waters, http://physiolgenomics.physiology.org/Downloaded from http://physiolgenomics.physiology.org/ on July 11, 2017 by 10.220.33.5
Milford, MA), which was subsequently washed twice with 5 ml H2O. Angiotensin peptides were eluted with 3 ml of absolute methanol (Anachemia Canada, Montreal, Quebec). Samples were then split in two equal halves 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 metabolites, whereas the ANG II antibody used (CD3) shows 100% cross-reactivity with both ANG III and IV, but none 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.

Antihypertensive treatment. Three to four animals of each group (nontransgenic, single-transgenic for human renin and human angiotensinogen, and double-transgenic expressing both transgenes) received either tap water (vehicle) or losartan (a gift from Merck; 30 mg/kg/day in drinking water). Individually housed mice were treated for eight consecutive days in which drinking volume was measured every day and dosage was adjusted daily for fluid intake. Blood pressure measurements were performed as described above.

Histochemistry. Mice were anesthetized by intraperitoneal injection with 3 mg pentobarbital sodium (MTC Pharmaceuticals). Blood was chased from major vessels by whole body perfusion of saline solution (20 ml) through the heart, followed by in situ organ fixation using 40 ml of either 1) Bouin's fixative solution (0.9% picric acid, 10% formaldehyde, and 5% glacial acetic acid) for light microscopy or 2) 0.5% glutaraldehyde and 4% paraformaldehyde, pH 7.2, for electron microscopy. Organs were then quickly removed and postfixed in respective solution for 5 and 16 h. All fixed tissues were stored in 70% ethanol at 4°C until analyzed. For light microscopy histochemistry, tissues were dehydrated, embedded in paraffin blocks, cut into 5-μm sections, and mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical). The sections were deparaffinized, rehydrated, and washed with H2O. Staining with Sirius Red was performed as follows: rehydrated slides were stained with 0.5× hematoxylin (BDH, Toronto, Ontario) for 1 min, rinsed for 5 min with H2O and counterstained for 30 min with Sirius Red solution (saturated Bouin's solution containing 0.1% Sirius Red dye). Stained slides were again rinsed in H2O, dehydrated, and mounted for observation. For immunohistochemistry, nonspecific antibody binding was blocked by incubation with 1% donkey serum in Tris-buffered saline (TBS; 50 mM Tris-Cl, 154 mM 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) 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), followed by streptavidin-horseradish peroxidase (HRP, 1:300 dilution; Amersham). Positive staining was detected using 0.025% diaminobenzidine and 0.03% H2O2 for 8 min. The sections were then incubated with a monoclonal rat anti-mouse macrophage-specific antigen MAC2 (ATCC, Manassas, VA), EPOS-anti-α-smooth muscle actin and EPOS-anti-vimentin (Dako, Mississauga, Ontario) conjugated with HRP. Antigen retrieval treatment with 0.1% trypsin/Tris (pH 7.6) was applied for vimentin immunostaining.

For electron microscopy immunohistochemistry, tissues were embedded in LR White hard resin (London Resin, London, UK) according to the manufacturer's protocol. Sections (90 nm) were mounted on copper grids and incubated by floating on Tris-BSA buffer (20 mM Tris-Cl, 500 mM NaCl, 0.1% BSA, 0.13% NaNO3, and 0.05% Tween 20, pH 8.0) containing 2% normal goat serum (NGS) for 15 min. The grids were then transferred to a drop of human prorenin antibody BRI-6 diluted 1:200 in Tris-BSA containing 2% NGS for an overnight incubation at 4°C. After rinsing in Tris-BSA, sections were incubated on a drop of goat anti-rabbit IgG immunogold conjugate (15 nm; British BioCell International, Cardiff, UK) at a dilution of 1:30 in Tris-BSA for 1 h at 25°C. After rinsing, sections were stained using uranyl acetate and lead citrate before examination with the electron microscope (JEOL model JEM 1200 EX).

RESULTS

Expression of transgenes. Mice transgenic for the human renin cDNA under the control of the transthyretin promoter revealed expression of the transgene in the liver as shown by RNase protection assays (Fig. 2A). A small amount of expression was also detectable in the brain in agreement with previous studies documenting promoter activity in the choroid plexus (30). Expression was not detected in the other organs tested, including the heart and kidneys. In situ hybridization of sections from mouse heart and liver further confirmed that the human renin transgene was not ex-
pressed in the heart of transgenic animals while being generally expressed in hepatocytes (data not shown). Expression of the human angiotensinogen transgene under control of the myosin heavy chain promoter was detectable in the heart of transgenic mice (Fig. 2B). Some expression was also detected in the kidney and the lungs of the founder line used in this study, but this expression should have no bearing on generation of angiotensin peptides in the heart.

Physiological and biochemical characterization of single- and double-transgenic mice. Expression of human active renin in the liver of transgenic mice (TTRhRen-A3) leads to release and detection of human renin in the circulation, of which ~86% is active renin (Table 1). These mice also exhibit a significant elevation of blood pressure and cardiac hypertrophy compared with nontransgenic littermates. In contrast, the human angiotensinogen-expressing mouse lines (MHChAgt-2) showed no increase in blood pressure or heart weight compared with nontransgenic animals. The blood pressure and degree of cardiac hypertrophy seen in the double transgenic mice was identical to that seen in the mice expressing only active human renin in the liver.

Function of circulating renin in the heart. To test whether circulating renin can contribute to cardiac RAS activity, transgenic mice expressing human renin in the liver (TTRhRen-A3) were mated to mice expressing human angiotensinogen exclusively in the heart (MHChAgt-2). Double-transgenic mice were tested for circulating and cardiac content of angiotensin peptides (Fig. 3). The results demonstrate that while the single-transgenic animals showed either low or undetectable angiotensin peptides in the heart, double-transgenic mice exhibited a dramatic increase in cardiac content of both ANG I and II. Notably, the circulating levels of the angiotensin peptides did not increase in double-transgenic mice compared with single-transgenic and nontransgenic controls, suggesting that enhanced production of angiotensins in the double-transgenic mice was restricted to the heart. These results were reproduced in matings between additional founder lines of transgenic mice (not shown) and are consistent with the ability of circulating renin to act on its substrate within the heart.

Effects of blood pressure on cardiac activity of circulating renin. The entry of circulating renin into the heart could be mediated by either a specific capture mechanism (e.g., receptor/acceptor protein) or by passive diffusion. In the latter case, diffusion might be enhanced by a pressure gradient from the circulation. Indeed, mice expressing active human renin in the liver are hypertensive compared with their nontransgenic littermates (see above). To test whether renin was taken up from the circulation by a pressure-dependent mechanism, double-transgenic animals were treated with an antihypertensive agent for a period of 1 wk to normalize their blood pressure, and their cardiac and circulating levels of ANG I were compared with that of vehicle-treated littermates. Results (Table 2) demonstrate that even though anti-hypertensive treatment led to a significant reduction in the blood pressure of the double-transgenic animals, there was no decrease in the ratio of cardiac to circulating ANG I in the treated animals compared with untreated littermates. These data suggest that the contribution of circulating renin to the cardiac RAS is not mediated by a pressure-dependent mechanism.

Function of locally generated angiotensin peptides. To test for function of locally derived angiotensin peptides, hearts of single- and double-transgenic mice were stained with the connective tissue-specific stain, 

![Fig. 3. Concentration of ANG I and II in plasma and hearts of nontransgenic (NT), single-transgenic, and double-transgenic animals. Numbers in parentheses represent the number of animals analyzed in each group. *P < 0.001 compared with nontransgenic controls, suggesting that enhanced production of angiotensins in the double-transgenic mice was restricted to the heart. These results were reproduced in matings between additional founder lines of transgenic mice (not shown) and are consistent with the ability of circulating renin to act on its substrate within the heart.](http://physiolgenomics.physiology.org/)

Table 2. Treatment of transgenic mice with losartan

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Human Protein Expressed (tissue)</th>
<th>Treatment</th>
<th>SBP, mmHg</th>
<th>ANG I in Heart, ng ANG I/g wt wt</th>
<th>ANG I in Plasma, ng ANG I/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic</td>
<td>None</td>
<td>Vehicle</td>
<td>125 ± 9</td>
<td>0.045 ± 0.036</td>
<td>0.318 ± 0.056</td>
</tr>
<tr>
<td>TTRhRen-A3 × MHChAgt-2</td>
<td>Active renin (liver) + angiotensinogen (heart)</td>
<td>Vehicle</td>
<td>153 ± 6*</td>
<td>1.654 ± 0.109†</td>
<td>0.381 ± 0.012</td>
</tr>
<tr>
<td>TTRhRen-A3 × MHChAgt-2</td>
<td>Active renin (liver) + angiotensinogen (heart)</td>
<td>Losartan</td>
<td>129 ± 3</td>
<td>1.970 ± 0.100†</td>
<td>0.327 ± 0.082</td>
</tr>
</tbody>
</table>

Values are means ± SD of single determinations on 3–4 individual animals. *P < 0.05 and †P < 0.001 compared with nontransgenic mice by ANOVA using Student’s t-test.

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Sirius Red. Results show a dramatic increase in perivascular and interstitial fibrosis in the hearts of double-transgenic mice (Fig. 4), suggesting that locally derived angiotensin peptides contribute to cardiac fibrosis.

Distribution of human renin captured by peripheral tissues. Immunohistochemistry was performed on tissues of transgenic animals expressing human renin in the liver (TTRhRen-A3) using an antibody with selectivity for human renin/prorenin (Fig. 5). A dark, punctate staining pattern for human renin was seen in the hearts exclusively in transgenic animals, which was restricted to cells in the periphery of small vessels (Fig. 5A). This staining did not colocalize with vimentin (fibroblasts and some pericytes) and only partially with α-smooth muscle actin (smooth muscle and some pericytes) and MAC2 (macrophages) (data not shown). Immunoelectron microscopy (Fig. 6) revealed that the renin-containing cells in the heart were elongated perivascular cells in which dense cell bodies stained for human renin. Punctate, perivascular staining for human renin was also seen in the pituitaries, testes, ovaries, and lungs of human renin transgenic animals, whereas human renin was not detected in any of these tissues in nontransgenic littermates (data not shown). Taken together with the finding that the heart does not express the human renin transgene (see above), these results suggest that renin captured from the circulation is stored in a discrete cell type of the heart and other vascular tissues.

DISCUSSION

The current study provides the first in vivo demonstration that a chronic elevation in circulating renin leads to an increase in local synthesis of angiotensin peptides within a target tissue. Although human renin and angiotensinogen should have little biochemical

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interaction with their mouse homologs (24), the ensu-
ing products of their reaction (ANG I, ANG II, and metabolites) are identical in the two species. This should, in theory, allow the study of the human transgenes without interference from the mouse RAS, and several studies have shown that overexpression of the human transgenes using their natural promoters does not lead to hypertension in transgenic mice (5, 13, 19). However, in our study, overexpression of human active renin in the liver led to hypertension that was clearly ANG II-mediated (responded to ANG II receptor antagonists, data not shown). The most likely explanation for this finding is the cleavage of the liver-derived mouse angiotensinogen by the human renin due to its high local concentration. This hypertension leads to some degree of cardiac hypertrophy that is not increased when these animals are mated to mice expressing human angiotensinogen in the heart. For this reason we were not able in the current study to test for the role of locally generated angiotensin peptides in the development of pressure-independent cardiac hypertrophy. However, the current study demonstrates that in animals with comparable levels of hypertension and cardiac hypertrophy (TTRhRen-A3 and TTRhRen-A3 × MHChAgt-2), high circulating renin leads to an increase in intracardiac angiotensin peptides and results in an increase in cardiac interstitial and perivascular fibrosis. Such a mechanism might explain in part why elevated circulating renin has been identified as a risk factor for myocardial infarction in hypertensive pa-
tients (1, 3). Like these clinical studies, our results also suggest that the contribution of circulating renin to angiotensin peptide generation in the heart is independ-
ent of blood pressure.

Evidence of function of renin in the heart is accom-
pained by its detection in very discrete perivascular cells. To enter the heart, circulating renin would have to initially bind and traverse the endothelial cell layer in the lumen of blood vessels. Our current results suggest that this renin is subsequently transcytosed to the interstitium and stored in granular structures of perivascular cells. The identity of these cells is still uncertain, although they partially colocalize with cells stained by an antibody to macrophages (MAC2, data not shown). It is uncertain whether the cells that concentrate renin in the heart are the actual site of renin catalytic activity; attempts to stain the heart of double transgenic animals with antibody against ANG I have not been successful (data not shown), suggesting that the generated ANG I either is not stored or is generated in a diffuse compartment (perhaps the interstitial space). Indeed, de Lannoy et al. (7, 8) have recently shown that angiotensin peptides generated in the isolated, perfused rat heart are derived primarily from the interstitial fluid. These investigators also noted little exchange of angiotensins between the interstitial and intravascular compartments, suggesting that the action of locally generated peptide was re-
stricted to the interstitial space. Thus the renin-stain-
ing cells seen in the hearts of our transgenic mice could either be storing renin for local release or be in the process of clearing the renin from the cardiac intersti-
tium after its action on locally derived angiotensino-
ogen.

Two groups have characterized high-affinity vascular renin-binding proteins in tissue membrane prepar-
ations from the rat (4, 26). However, the properties of these proteins varied significantly between the two studies, raising the possibility that more than one type of renin-binding protein exists in the lumen of the vasculature. The identification of such binding pro-
teins lends support to the existence of a tissue-re-
stricted RAS that would control local levels of ANG II independently of the blood pressure modulating activity of the circulating RAS. Development of specific inhibitors to renin/prorenin binding proteins could, therefore, provide an effective way to block the local RAS and provide a new avenue for the treatment of various forms of cardiovascular disease.

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