Prorenin Contributes to Angiotensin Peptide Formation in Transgenic Rats With Rat Prorenin Expression Targeted to the Liver

Duncan J. Campbell, Habib Karam, Joël Ménard, Patrick Bruneval, John J. Mullins

Abstract—We reported previously that targeted expression of rat prorenin to the liver under the control of the human α1-antitrypsin promoter increased plasma prorenin levels by several-hundred-fold in male transgenic rats and caused cardiac hypertrophy, severe renal lesions, and myocardial fibrosis by 20 weeks of age, despite normal blood pressure. We examined the evolution of the phenotype of male transgenic rats over 12 months and the effects of binephrectomy on the renin-angiotensin (Ang) system. Plasma prorenin levels were >1000-fold higher than in wild-type littersmates, whereas plasma and renal Ang II levels were no different from wild-type (WT) levels, and kidney renin levels were suppressed in transgenic rats. In contrast to our earlier report, transgenic rats had increased systolic blood pressure at 3 to 12 months of age, and only modest renal lesions and myocardial fibrosis were evident after 6 months of age. Binephrectomy reduced plasma renin activity and concentration and prorenin levels by 50% to 80% and Ang II levels by 90% in WT rats. By contrast, binephrectomy increased plasma renin activity and concentration and prorenin levels by 52.0-, 13.0-, and 5.8-fold, respectively, without change in Ang II levels in transgenic rats. We conclude that, in the animals studied in this report, elevated prorenin levels did not cause renal lesions or myocardial fibrosis during the first 6 months of age. Ang peptide formation consequent to the increased prorenin levels prevented reduction of Ang II levels after binephrectomy and was likely to have contributed to hypertension, cardiac hypertrophy, and suppression of kidney renin levels in these transgenic rats. (Hypertension. 2009;54:1248-1253.)

Key Words: prorenin ■ transgene ■ hypertension ■ angiotensin II ■ nephrectomy ■ nephrosclerosis ■ cardiac fibrosis

Prorenin, the biosynthetic precursor of renin, contains a prosegment that masks the active site, thereby preventing access by the renin substrate, angiotensinogen.1,2 Renal juxtaglomerular cells are the only known site of production of renin, and the kidney produces both renin and prorenin, whereas a number of extrarenal tissues produce prorenin.1,2 Plasma prorenin concentrations are 10- to 20-fold higher than renin concentrations in humans.3,4 Whether plasma prorenin has biological activity in vivo is a matter of controversy.1,2 Partial conversion to renin and a low degree of intrinsic activity because of transitory unfolding of the prosegment may contribute to angiotensin (Ang) formation by prorenin. In addition, prorenin binding to the (pro)renin receptor may initiate signal transduction by mechanisms independent of Ang peptide formation, and the (pro)renin receptor may activate prorenin by promoting unfolding of the prosegment.1,2,5

We reported previously the development of a transgenic rat model with high plasma prorenin levels that suggested a direct pathogenic effect of prorenin.6 These transgenic rats, designated TGR(hAT-rpR), had rat prorenin expression targeted to the liver by a human α1-antitrypsin promoter and exhibited sexual dimorphism, with plasma prorenin levels increased ∼600-fold in males but only 2- to 3-fold in females.6 Despite the absence of elevation of plasma renin activity (PRA) or systolic blood pressure (SBP) by 20 weeks of age, male transgenic (hAT-rpR) rats exhibited cardiac hypertrophy, and histological analysis revealed severe renal lesions, hypertrophic cardiomyocytes, interstitial and perivascular fibrosis in the heart, and increased aortic wall thickness.6 To examine further the mechanism of the phenotype of these (hAT-rpR) rats we studied these animals ≤12 months of age, measured Ang II levels in plasma and kidney, and examined the effects of removal of renal renin by binephrectomy on the renin-Ang system. Because expression of the rat prorenin transgene in (hAT-rpR) rats showed sexual dimorphism, and because only male rats showed renal, cardiac, and aortic abnormalities,6 our study was confined to male rats. In
contrast to our initial report, we found that male (hAT-rpR) rats were hypertensive by 3 months of age, developed only modest renal lesions and cardiac fibrosis after 6 months of age, and had normal aortic wall thickness. Plasma and kidney Ang II levels in (hAT-rpR) rats were higher than in WT littermates, and binephrectomy experiments confirmed this difference in Ang II levels in (hAT-rpR) rats.

Materials and Methods

The generation of (hAT-rpR) rats by the Centre for Genome Research, Edinburgh, has been described.6 This transgenic rat line was created by inserting a rat prorenin cDNA fused to a human α1-antitrypsin promoter into the genome of the Fischer F344 rat. In December 1996, 9 male and 12 female heterozygous (hAT-rpR) rats, strain 85-26, and 8 WT male Fischer F344 rats were received at the Institut National de la Sante et de la Recherche Médicale U367. The colony was maintained by mating heterozygous male and female (hAT-rpR) rats. Heterozygous male (hAT-rpR) rats and their WT male littermates were used for all of the experiments, carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The rat experiments were completed in 2000. For detailed Materials and Methods, please see the online Data Supplement, available at http://hyper.ahajournals.org.

Results

SBP, Body Weight, and Left Ventricular and Kidney Weights

Male (hAT-rpR) rats had higher SBP than male WT rats from 3 to 12 months of age (Table 1). This difference in SBP between male WT and (hAT-rpR) rats was also seen in the original breeding stock received from Edinburgh: 142±2 mm Hg (mean±SEM; n=8 to 9; P=0.0003) at 6 months of age and 147±2 versus 218±7 mm Hg (n=6; P=0.0001) at 12 months of age. By contrast, female (hAT-rpR) rats received from Edinburgh did not show increased SBP at either 4 months (129±3 mm Hg; n=12) or 10 months of age (138±5 mm Hg; n=6).

Body weights were similar for male WT and (hAT-rpR) rats (Table 1). Left ventricular weight was higher in (hAT-rpR) than in WT rats at 6 and 12 months of age, and the left

Table 1. Change With Age in SBP, Body Weight, Left Ventricular Weight, and Kidney Weight in Male WT and Male (hAT-rpR) Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 mo</th>
<th>6 mo</th>
<th>9 mo</th>
<th>12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mm Hg</td>
<td>143±2</td>
<td>160±3*</td>
<td>149±2</td>
<td>177±6*</td>
</tr>
<tr>
<td>BW, g</td>
<td>281±6</td>
<td>280±6</td>
<td>366±6</td>
<td>356±6</td>
</tr>
<tr>
<td>LVW, g</td>
<td>0.53±0.01</td>
<td>0.60±0.03</td>
<td>0.65±0.02</td>
<td>0.81±0.03*</td>
</tr>
<tr>
<td>PRA, ng of Ang I/mL per hour</td>
<td>1.88±0.02</td>
<td>2.17±0.09†</td>
<td>1.67±0.03</td>
<td>2.30±0.03*</td>
</tr>
<tr>
<td>KW, g</td>
<td>0.97±0.04</td>
<td>0.95±0.03</td>
<td>1.11±0.04</td>
<td>1.08±0.04</td>
</tr>
<tr>
<td>KW:BW ratio, mg/g</td>
<td>3.44±0.08</td>
<td>3.48±0.07</td>
<td>2.84±0.07</td>
<td>3.07±0.04</td>
</tr>
</tbody>
</table>

Data shown are mean±SEM. BW indicates body weight; LVW, left ventricular weight; KW, kidney weight; NS, not significant. For SBP, n=17 for WT rats and n=14 for (hAT-rpR) rats. For BW, n=13 for WT rats and n=14 for (hAT-rpR) rats. For LVW, LV:BW ratio, and KW:BW ratio, n=6 for WT and (hAT-rpR) rats at 3, 6, and 9 months of age and n=13 to 14 at 12 months of age. SBP and BW were analyzed by repeated measures ANOVA; LVW, LV:BW ratio, KW, and KW:BW ratio were analyzed by 2-way ANOVA.

Table 2. Change With Age in PRA, Plasma Renin, Prorenin, Ang II, and Renal Renin Concentrations in Male WT and Male (hAT-rpR) Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 mo</th>
<th>6 mo</th>
<th>9 mo</th>
<th>12 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng of Ang I/mL per hour</td>
<td>0.78±0.12</td>
<td>1.95±0.30*</td>
<td>0.71±0.07</td>
<td>1.81±0.12*</td>
</tr>
<tr>
<td>PRC, ng of Ang I/mL per hour</td>
<td>9.6±1.5</td>
<td>17.4±2.5†</td>
<td>9.4±0.9</td>
<td>23.6±1.5*</td>
</tr>
<tr>
<td>Prorenin, ng of Ang I/mL per hour</td>
<td>22.2</td>
<td>23 583±3 2669*</td>
<td>19±1</td>
<td>23 563±4 263*</td>
</tr>
<tr>
<td>Angiotensinogen, ng of Ang I/mL</td>
<td>893±24</td>
<td>818±33</td>
<td>999±88</td>
<td>832±58</td>
</tr>
<tr>
<td>Plasma Ang II, fmol/mL</td>
<td>15±3</td>
<td>13±2</td>
<td>43±3</td>
<td>29±5</td>
</tr>
<tr>
<td>Renal renin, µg of Ang Ing of protein per hour</td>
<td>1.7±0.2</td>
<td>0.6±0.4†</td>
<td>6.3±0.9</td>
<td>0.8±0.1*</td>
</tr>
</tbody>
</table>

Data shown as mean±SEM. NS indicates not significant. For PRA, PRC, and prorenin, n=11 to 14 for WT rats and n=13 to 14 for (hAT-rpR) rats. For angiotensinogen, n=11 for WT and (hAT-rpR) rats at 3 months of age and n=7 to 8 for WT and (hAT-rpR) rats at 12 months of age. For plasma Ang II and renal renin, n=5 to 6 for WT and (hAT-rpR) rats at 3, 6, and 9 months of age and n=13 to 14 for WT and (hAT-rpR) rats at 12 months of age. Data were analyzed by 2-way ANOVA.

*P<0.01 and †P<0.05 for comparison of WT with (hAT-rpR) rats after adjustment for multiple comparisons.
ventricular weight:body weight ratio was higher in (hAT-rpR) than in WT rats at 3, 6, and 12 months of age (Table 1). Although there was a slight difference in kidney weight, there was no difference in the kidney weight:body weight ratio between WT and (hAT-rpR) rats (Table 1).

Plasma Renin, Prorenin, Angiotensinogen, and Ang II and Kidney Renin and Ang II

PRA and plasma renin concentration (PRC) values of (hAT-rpR) rats were 1.6- to 3.0-fold higher, and plasma prorenin levels were >1000-fold higher than in WT rats (Table 2). Plasma angiotensinogen levels of (hAT-rpR) rats were 8% to 17% lower than in WT rats (Table 2). By contrast, there were no differences between WT and (hAT-rpR) rats in plasma Ang II levels. Kidney renin levels in (hAT-rpR) rats were suppressed to 13% to 35% of the levels in WT rats (Table 2); these kidney renin levels were similar to those of WT rats maintained from 3 to 12 months of age on a diet with 2% sodium chloride (0.6±0.2 μg of Ang I/mg of protein per hour; n=7) and were not further suppressed when (hAT-rpR) rats were maintained on a diet with 2% sodium chloride for the same period (0.4±0.1 μg of Ang I/mg of protein per hour; n=8). Renal Ang II levels of (hAT-rpR) rats (113±16 fmol/g of wet weight, mean±SEM; n=6) were not different from the levels in WT rats (164±17 fmol/g of wet weight).

Effects of Binephrectomy

Measurements were made in both sham-nephrectomized and binephrectomized WT and (hAT-rpR) rats (Figure 1). PRA, PRC, plasma prorenin, and angiotensinogen were measured on both the day of surgery and 24 hours after surgery, whereas plasma Ang II was measured only 24 hours after surgery. Sham nephrectomy caused similar changes in PRA, PRC, and prorenin levels in WT and (hAT-rpR) rats. Sham nephrectomy increased PRA by ~2-fold and did not change PRC or prorenin levels in WT rats, whereas sham nephrectomy increased PRA by ~6-fold and PRC by ~1.7-fold and did not change prorenin levels in (hAT-rpR) rats. There were, however, marked differences between WT and (hAT-rpR) rats in the effects of binephrectomy. In comparison with the values before surgery, binephrectomy reduced PRA by ~80%, PRC by ~60%, and prorenin by ~50% in WT rats. By contrast, binephrectomy increased PRA by ~52.0-fold, PRC by ~13.0-fold, and prorenin by ~5.8-fold in (hAT-rpR) rats, reaching an average prorenin level of 201.638 ng of Ang I/mL per hour. The changes in plasma angiotensinogen levels were similar for WT and (hAT-rpR) rats. Sham surgery increased angiotensinogen levels by ~3-fold, and nephrectomy increased angiotensinogen levels by ~7-fold in both WT and (hAT-rpR) rats. In comparison with sham nephrectomy, binephrectomy reduced plasma Ang II levels by ~90% in WT rats; by contrast, there was a nonsignificant increase in Ang II levels of (hAT-rpR) rats to ~124 fmol/mL after binephrectomy (Figure 1).

Renal and Cardiac Histology

There were no differences between WT and (hAT-rpR) rats in renal histology at 3 and 6 months of age, although the glomerular, tubulointerstitial, and vascular injury scores of (hAT-rpR) rats were higher than in WT rats at 9 and 12 months of age (Figures 2 and S1, available in the online Data Supplement). There were no differences between WT and (hAT-rpR) rats in myocardial collagen density at 3, 6, and 9 months of age, although collagen density (both interstitial and perivascular) was increased above the level in WT rats at 12 months of age (Figures 3 and S2). There were no differences between WT and (hAT-rpR) rats in aortic medial area or thickness at any age (Figure 3).

Discussion

This study confirmed our original report of elevated prorenin levels, cardiac hypertrophy, and suppressed kidney renin levels in (hAT-rpR) rats. In contrast to our original report, however, we found that (hAT-rpR) rats in the present study
were hypertensive with mildly elevated PRA and PRC, and the markedly elevated prorenin levels were not associated with histological lesions during the first 6 months of age; only modest renal and cardiac lesions were evident after 6 months of age, and (hAT-rpR) rats had no aortic medial hypertrophy. Plasma and renal Ang II levels in (hAT-rpR) rats were similar to WT levels, but, contrary to WT rats, plasma Ang II levels did not fall after binephrectomy in (hAT-rpR) rats. These data demonstrate in vivo Ang peptide formation consequent to the increased prorenin levels, which likely contributed to the hypertension and suppression of kidney renin levels in (hAT-rpR) rats. Increased blood pressure may have contributed to the cardiac hypertrophy and renal and cardiac lesions in (hAT-rpR) rats. We were unable to test the role of Ang II by treating (hAT-rpR) rats with an Ang receptor blocker, because this strain of rats is no longer extant; new transgenic strains would need to be established to perform additional experiments, and, although a close approximation could be generated, it is not possible to remake the identical strain.

We studied the same transgenic rat line 85-26 described in our original report, and we are unable to explain why the phenotype of (hAT-rpR) rats in the present study differed from that described in our original report.6 We previously showed a critical influence of genetic background on the phenotype of mouse prorenin transgenic (mRen-2)27 rats,7 but we have no information regarding whether the genetic background of (hAT-rpR) rats in this study was different from that of (hAT-rpR) rats in our original report, because they are no longer extant in either center. Differences in diet and animal housing may have also contributed to the differences in phenotype. The altered phenotype of (hAT-rpR) rats was evident in the breeding stock received from Edinburgh in that their blood pressure was elevated when first measured after arrival in Paris and remained elevated, and their renal and cardiac lesions were modest when examined at 12 months of age. There was a trend for a higher SBP in (hAT-rpR) than in WT rats at 15 and 20 weeks of age in our original study, which may have failed to achieve statistical significance because the number of rats provided insufficient statistical power. Moreover, the measurement of SBP by tail-cuff plethysmography in rats briefly anesthetized with 2% halothane in our original study may have made hypertension more difficult to detect than SBP measurement in conscious rats, as performed in the present study.

Other transgenic models have failed to provide evidence for a direct pathogenic role of prorenin. Peters et al8 concluded that ≈180-fold elevation of prorenin levels, per se, did not cause glomerulosclerosis in rats transgenic for the Ren gene under the transcriptional control of the cytochrome P450 Cyp1a1 promoter. Moreover, mice with human prorenin expression targeted to the liver and human angiotensinogen expression targeted to the heart had no abnormality of heart size or fibrosis, despite plasma prorenin levels 20-30 fold higher than in control mice.9 Furthermore, mice with mouse prorenin expression targeted to the liver showed no increase in cardiac fibrosis or renal glomerulosclerosis despite hypertension and 13- to 28-fold elevation of plasma prorenin levels, and captopril treatment normalized blood pressure in these mice.10

Although PRA and plasma angiotensinogen levels were similar for (hAT-rpR) and WT rats in our initial study,6 PRA and PRC were 1.6- to 3.0-fold higher, with an 8% to 17% reduction in angiotensinogen levels in (hAT-rpR) rats than in control mice.9 Furthermore, mice with mouse prorenin expression targeted to the liver showed no increase in cardiac fibrosis or renal glomerulosclerosis despite hypertension and 13- to 28-fold elevation of plasma prorenin levels, and captopril treatment normalized blood pressure in these mice.10

Figure 2. Change with age in renal glomerular, tubular interstitial, and vascular injury scores in male WT (open columns) and (hAT-rpR) rats (closed columns). Data shown as mean ± SEM; n = 6 for WT and (hAT-rpR) rats aged 3, 6, and 9 months; n = 19 to 20 for WT and (hAT-rpR) rats aged 12 months. **P < 0.01 in comparison with WT rats.

Figure 3. Change with age in myocardial collagen density, abdominal aortic medial area and thickness in male WT (open columns) and (hAT-rpR) rats (closed columns). Data shown as mean ± SEM; n = 6 for WT and (hAT-rpR) rats aged 3, 6, and 9 months; n = 19 to 20 for WT and (hAT-rpR) rats aged 12 months. **P < 0.01 in comparison with WT rats.
in (hAT-rpR) rats, and a critical issue in the interpretation of the PRA and PRC values for these rats was the possible inadvertent activation of prorenin during handling of plasma and performance of the assays, despite precautions. The similar Ang II levels in WT and (hAT-rpR) rats suggest that the higher PRA and PRC levels in (hAT-rpR) rats were consequent to inadvertent prorenin activation, although the modest decrease in plasma angiotensinogen levels was consistent with higher PRA and PRC in (hAT-rpR) rats.

The marked suppression of kidney renin levels in (hAT-rpR) rats was confirmation of our earlier study in which we also found >90% suppression of renin mRNA detected by hybridization in situ of the juxtaglomerular apparatus of the kidney. This evidence for markedly reduced renin secretion suggests that the (hAT-rpR) kidney made little contribution to the elevated PRA and PRC in these rats. The failure of a high-sodium diet to reduce kidney renin levels in (hAT-rpR) rats was consistent with their kidney renin levels being predominantly attributed to plasma prorenin trapped in the tissue that was subsequently activated during tissue processing before renin assay. These data, therefore, suggest that the major part of renin activity and Ang II detected in the plasma of intact (hAT-rpR) rats was a consequence of the elevated prorenin levels of hepatic origin. This interpretation is strongly supported by the failure of plasma Ang II levels to explain why Ang II levels were not suppressed by the high-sodium diet to reduce kidney renin levels in (hAT-rpR) rats and was not subject to the same regulatory influences as kidney renin production in WT rats. For example, the increase in prorenin levels after binephrectomy was probably related to the acute-phase response of the α1-antitrypsin promoter of the prorenin transgene. The different regulation of hepatic prorenin production may, therefore, explain why Ang II levels were not suppressed by the increased blood pressure of (hAT-rpR) rats, as would be expected when renin is secreted from juxtaglomerular cells in similarly hypertensive WT rats. The failure of Ang II levels to increase in parallel with the increases in PRA and PRC after binephrectomy suggests that much of the increases in PRA and PRC were attributable to in vitro activation of the increased prorenin levels during PRA and PRC measurement.

The present data enable estimation of the contribution of prorenin to Ang formation in (hAT-rpR) rats in vivo by comparing the plasma Ang II and prorenin levels of anephric and intact rats and adjusting for differences in the angiotensinogen level, given that essentially all of the plasma Ang II is derived from prorenin in vivo. Anephric (hAT-rpR) rats had plasma Ang II levels of ≈124 fmol/mL; adjusting for the 6-fold elevation in angiotensinogen levels, which would approximately double Ang I formation by a constant renin concentration, this corresponded with an Ang II level of ≈60 fmol/mL for an animal with normal angiotensinogen levels, which corresponded with a PRC of ≈60 mg of Ang I/mL per hour in a WT rat. Given the prorenin level of anephric (hAT-rpR) rats (201 638 ng of Ang I/mL per hour), a PRC level of 60 ng of Ang I/mL per hour corresponded with ≈0.03% activity of prorenin in vivo. It also suggests that prorenin levels of intact (hAT-rpR) rats aged 9 to 12 months (≈50 000 ng of Ang I/mL per hour) were sufficient to produce all of the Ang II measured in plasma. We acknowledge that these calculations have limitations, but the estimated contribution of prorenin to Ang II levels in the (hAT-rpR) rat is consistent with their hypertension and suppressed renal renin levels.

Our study does not provide information about the mechanism by which prorenin contributed to Ang formation, whether by a low degree of intrinsic activity of prorenin because of unfolding of the prosegment or by cleavage of the prosegment to produce renin. The (pro)renin receptor was proposed to bind and activate prorenin, thereby facilitating Ang peptide formation in tissues. One tissue where this might occur is the kidney, because the (pro)renin receptor is expressed in glomeruli, tubules, and vessels of the rat kidney. Moreover, a soluble form of the (pro)renin receptor is reported to be present in plasma. However, our finding that renal Ang II levels of (hAT-rpR) rats were no different from the levels in WT rats provides no support for this or any other mechanism of local prorenin activation in kidney, and our estimate of ≈0.03% activity of rat prorenin, on the basis of plasma Ang II levels, suggests very little systemic activation of prorenin in vivo.

In summary, we found that the phenotype of (hAT-rpR) rats studied in this report was similar to that described in our initial report with respect to the elevated prorenin levels, cardiac hypertrophy, and suppressed kidney renin levels but differed from our original report in that (hAT-rpR) rats in the present study were hypertensive with mildly elevated PRA and PRC, and the markedly elevated prorenin levels were not associated with histological lesions during the first 6 months of age. Genetic or environmental factors may be responsible for the different pathological and blood pressure responses to the elevated plasma prorenin levels seen in (hAT-rpR) rats described in this study and in our initial report. The failure of Ang II levels to fall after binephrectomy demonstrated that prorenin contributed to Ang II levels in (hAT-rpR) rats. We propose that Ang peptide formation consequent to the increased prorenin levels contributed to the hypertension, cardiac hypertrophy, and suppression of kidney renin levels in (hAT-rpR) rats. Furthermore, the similar renal Ang II levels of WT and (hAT-rpR) rats did not support a role for the (pro)renin receptor in the activation of prorenin in the kidney.

**Perspectives**

The 10- to 20-fold higher concentration of prorenin than renin in human plasma raises many questions about the role that prorenin may play in health and disease states. Our studies of the (hAT-rpR) rat indicate that, although prorenin may contribute to Ang peptide formation in vivo, the contribution of physiological levels of prorenin to Ang formation is much less than that of renin. Additional studies are required to define the contribution of prorenin to Ang peptide formation in specific tissues, such as those that produce prorenin, and in conditions associated with elevated prorenin levels, such as diabetes mellitus and pregnancy.

**Acknowledgments**

We thank Marie-Francoise Gonzalez and Thanh-Tam Guyene for assistance with these experiments and Didier Heudes for assistance with histological analysis of cardiac and aortic tissues.
Sources of Funding
This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, the Association Claude Bernard (Paris, France), a Kidney Research United Kingdom (formerly National Kidney Research Fund) award (to J.J.M.), a Wellcome Trust Programme Grant WTO53646, the Wellcome Trust Cardiovascular Research and Functional Genomics initiatives, and the EURATools consortium. D.J.C. is recipient of a senior research fellowship from the National Health and Medical Research Council of Australia (grant 395508). J.J.M. is recipient of the Wellcome Trust Principal Fellowship.

Disclosures
D.J.C. has had research contracts with Solvay Pharmaceutical Company and Novartis and has been a member of an advisory board for Novartis. H.K. is an employee of Novartis. J.M. is a consultant for Actelion and for Novartis.

References
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Hypertension. 2009;54:1248-1253; originally published online October 19, 2009;
doi: 10.1161/HYPERTENSIONAHA.109.138495

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Prorenin contributes to angiotensin peptide formation in transgenic rats with rat prorenin expression targeted to the liver

Duncan J. Campbell, Habib Karam, Joël Ménard, Patrick Bruneval, and John J. Mullins

From the St. Vincent’s Institute of Medical Research and the Department of Medicine (D.J.C.), University of Melbourne, St. Vincent’s Hospital, Fitzroy, Victoria, Australia; Institut National de la Santé et de la Recherche Médicale U367 (H.K., J.M.), Paris, France; Department of Pathology, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, and Université Paris Descartes (P.B.), Paris, France; Department of Molecular Physiology (JJM), Centre for Cardiovascular Science, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, UK

Correspondence: Dr. D. J. Campbell
St. Vincent's Institute of Medical Research
41 Victoria Parade, Fitzroy, Victoria 3065, Australia
Ph: 61 3 9288 2480
Fax: 61 3 9416 2676
Email: dcampbell@svi.edu.au
Materials and Methods

Experimental procedures
Rats were housed in groups of 4-5 animals, lights on from 08.00-19.00, and were fed a normal rat diet containing 0.3% sodium and 0.9% potassium (UAR, Epinay-sur-Orge, France), and tap water ad libitum. Systolic blood pressure (SBP) and body weight were measured every 1-3 months; SBP was measured in conscious rats using tail-cuff plethysmography.

Genotyping
All rats were genotyped by PCR of DNA obtained from tail biopsies. The 3 primers used were: forward primer A: 5'-CCA GCC CAG ACC TTC AAA GTC -3', corresponding to nucleotides 303-319 in exon 3 (numbering from Genbank BCO78878); forward primer B: 5'-CCT GGC AGA TCA CAA TGA AGG -3', corresponding to nucleotides 804-824 in exon 6; and reverse primer C: 5'-GCA TTA GCA ACT ACA GGG AGC -3', corresponding to nucleotides 915-935 in exon 7. Thus, primer pairs AC and BC produced products of 637 and 132 nucleotides, respectively, from the transgene, and 3452 and 509 nucleotides, respectively, from the wildtype (WT) gene (gene sequence from Genbank NC_005112). We previously reported segregation of the transgene was consistent with a single insertion site in the genome and transgene expression was confined to the liver.1 We confirmed expression of the prorenin transgene in liver of male (hAT-rpR) rats by Northern blot analysis of liver RNA in the present study.

Collection of blood and tissues
Blood (1 mL) for measurement of plasma renin activity (PRA), plasma renin concentration (PRC), prorenin and angiotensinogen was taken from the jugular vein under light anesthesia with ketamine-xylazine (15 and 5 mg/kg body weight, respectively, by intra-peritoneal injection)2 into a syringe with ~0.05 vol 2.5 mmol/L Ω-phenanthroline, 0.1 mol/L EDTA to inhibit angiotensin converting enzyme and angiotensinase activity, and centrifuged at 4,000 g for 10 min. Plasma was stored at -80°C for subsequent assay. Blood (≤10 mL) for measurement of angiotensin peptides was collected from the aorta into a syringe containing 0.5 mL 0.1 mmol/L rat renin inhibitor kindly provided by Dr Hiwada,3 0.01 mmol/L MK 422 and 1 mmol/L EDTA. The organs were sampled immediately after blood collection. The left and right ventricles were dissected, blotted and weighed. The kidneys were cut longitudinally into 2 equal portions and one portion was frozen in liquid nitrogen for renin measurement. The other portion of kidney, heart and abdominal aorta were placed in alcoholic Bouin's fixative for histological and morphometric analysis.

Binephrectomy experiments
The effect of binephrectomy was studied in male rats aged 3 months. Before surgery, under anesthesia with ketamine-xylazine, blood was collected from the jugular vein for measurement of PRA, PRC, prorenin, and angiotensinogen. Rats were then subjected to either sham operation or bilateral nephrectomy. During sham operation, the kidneys were exposed and manipulated, but not removed. The kidneys removed during nephrectomy were immediately placed in liquid nitrogen for measurement of kidney angiotensin peptides. Twenty-four hours after surgery, blood was again collected from the jugular vein of anesthetized rats for measurement of PRA, PRC, prorenin, and angiotensinogen, and then blood was collected from the aorta for angiotensin measurement.
Measurement of PRA, PRC, plasma prorenin and angiotensinogen, and renal renin
Because of the very high prorenin levels of (hAT-rpR) rats, particular care was taken during measurement of PRA and PRC to minimize the possible inadvertent activation of prorenin. Plasma was thawed only once for these assays. PRA was measured by the *in vitro* production of angiotensin (Ang) I at pH 7.4 in the absence of exogenous angiotensinogen. PRC was measured by the *in vitro* production of Ang I at pH 7.4 in the presence of an excess of angiotensinogen provided by binephrectomized rat plasma. Total renin was measured after trypsin activation of plasma, and prorenin was calculated from the difference between total renin and PRC. Angiotensinogen was measured by incubating plasma with an excess of pure mouse submaxillary gland renin, sufficient to release all Ang I from angiotensinogen. For measurement of renal renin, the frozen kidney was thawed and refrozen 3 times, then homogenized, centrifuged, and renin measured in appropriate dilutions of the supernatant as described for PRC. Renal renin was expressed in µg Ang I liberated per mg supernatant protein per h, and protein was measured by the method of Bradford.

Measurement of Ang II
Blood for Ang II measurement was immediately centrifuged at 4°C for 10 min, and plasma immediately extracted on polymeric Oasis hydrophilic–lipophilic balanced (HLB) cartridges (Waters Corporation, Mass). The eluates were dried and reconstituted with 0.5 ml 0.1 mol/L Tris HCl buffer, pH 7.5, containing 5 mmol/L EDTA and 0.1% BSA, and Ang II measured by radioimmunoassay as described previously. For measurement of Ang II in kidney, ~0.1 g frozen kidney was homogenized in 2 mol/L acetic acid at room temperature, centrifuged at 4°C, and 1 mL supernatant was extracted on an HLB cartridge, before radioimmunoassay for Ang II as described above.

Analysis of renal histology
Kidneys from male (hAT-rpR) and WT rats, fixed in alcoholic Bouin's solution, were embedded in paraffin, and 2 sagittal sections (4 µm) were stained with Masson’s trichrome and examined under light microscopy. Two investigators blinded as to the experimental groups graded the severity of the morphological changes semiquantitatively as described previously.

For assessing glomerulosclerosis, 50 superficial and 50 juxtamedullary glomeruli in each kidney were observed at X400 magnification and graded (0 to 4) depending on the degree of injury. Grade 0 denoted a normal glomerulus. Slight glomerular damage (grade 1) included abnormalities such as a mild increase in mesangial matrix and/or hyalinosis with focal adhesions involving <25% of the glomerulus. Grade 2 represented sclerosis of 25-50%, grade 3 represented sclerosis of 50-75%, and grade 4 represented sclerosis of 75-100% of the glomerulus. A glomerular damage index was calculated for the 100 glomeruli and corresponded to the sum of grades assigned to all glomeruli.

For tubulo-interstitial lesions (interstitial inflammation and fibrosis, tubular atrophy and casts) every third field of each kidney (total of 20 fields/kidney) was assessed at X100 magnification and assigned an injury grade (0 to 3). Grade 0 represented normality, grade 1 denoted lesions involving <10%, grade 2 denoted lesions involving 10-25%, and grade 3 denoted lesions...
involving >25% of the field. The score corresponding to tubulo-interstitial lesions was obtained for each kidney by summing the grades given to each field, as above.

Vascular lesions in each kidney were attributed grades of severity from 0 to 4 in 20 fields at X200 magnification. This grade was based on both the severity of vascular wall thickening and the extent of fibrinoid necrosis in afferent arterioles, intralobular arterioles and small arteries. Grade 0 denoted normal vessels, grade 1 denoted mild vascular wall thickening, grade 2 denoted moderate thickening, grade 3 denoted severe thickening (onion skin pattern), and grade 4 denoted fibrinoid necrosis. The vascular lesion score was obtained using the same procedure described above.

**Analysis of cardiac fibrosis and aortic medial hypertrophy**

Heart and abdominal aorta from male (hAT-rpR) and WT rats, fixed in alcoholic Bouin's solution, were embedded in paraffin, and 2 coronal ventricular sections (4 µm) taken at the equator of the heart. For the aorta, 3 sequential sections (4 µm) from the mid-portion were mounted on a glass slide. The sections were stained for collagen with picrosirius red. Cardiac and aortic measurements were determined by quantitative morphometry using a video camera (Sony, Tokyo, Japan) connected to an image analysis processor (Nachet 1500, Nachet, Evry, France) and a microcomputer (Macintosh II, Apple, Cupertino, CA). Left ventricular myocardial collagen density (both interstitial and perivascular) was calculated as the area of collagen staining expressed as a percentage of the total myocardial area. For the aortic sections, the total medial cross-sectional area was measured, and the medial thickness was calculated as the ratio of this medial area over the average mid-circumference of the aortic wall. The investigator responsible for the morphometric analysis was blinded as to each experimental group.

**Statistical analysis**

Data are expressed as means ± SEM. Calculations were done with Statview 5.0.1 statistical software (SAS Institute Inc.), and a value of P < 0.05 was considered significant. SBP and body weight data were analyzed by repeated measures analysis of variance (ANOVA). Other data were analyzed by 2-way ANOVA, except renal histology was analyzed by Mann-Whitney U test. Data were logarithmically transformed when necessary to normalize variances. The Bonferroni correction was used for multiple comparisons.

**References**


Figure S1.

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Figure S1. Representative kidney sections of wild type (WT) and transgenic (hAT-rpR) rats aged 3, 6, 9, and 12 months. Glomerular, tubulo-interstitial, and vascular histology of (hAT-rpR) rats was similar to that of WT rats at 3 and 6 months of age. At 9 and 12 months of age, (hAT-rpR) rats showed glomerulosclerosis, tubulo-interstitial inflammation and fibrosis, tubular atrophy and casts, and vascular wall thickening. When glomerular, tubulo-interstitial and vascular lesions were present, they had a patchy distribution and the same field might contain both normal and abnormal structures. Masson’s trichrome stain; bar = 50 µm.
Figure S2. Representative heart sections of WT and transgenic (hAT-rpR) rats aged 12 months. In hAT-rpR rats, the myocardium was involved by interstitial fibrosis between the myocytes, by scar fibrosis replacing myocytes, and by perivascular fibrosis around vessels (arrow). Sirius red staining collagen; bar = 50µm.