Mouse and rat plasma renin concentration and gene expression in (mRen2)27 transgenic rats

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Bohlender, Jürgen, Joël Ménard, Olivier Edling, Detlev Ganten, and Friedrich C. Luft. Mouse and rat plasma renin concentration and gene expression in (mRen2)27 transgenic rats. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1450–H1456, 1998.—The (mRen2)27 transgenic rat [TGR(mRen2)27] is said to have low plasma levels of active renin. We used a direct radioimmunoassay (RIA) for mouse submaxillary renin, as well as an indirect enzyme-kinetic assay based on the generation of angiotensin I with modification of the pH optimum, to measure rat and mouse plasma renin activity (PRA), plasma renin concentration (PRC), and plasma prorenin in TGR before and after lisinopril. The relationship between rat PRC and %rat kidney extract was steepest at pH 6.0 and flat at pH 8.5, whereas the relationship between mouse PRC and purified mouse renin was steepest at pH 8.5 and flat at pH 6.0. Mouse PRC was highly correlated with direct RIA measurements (r = 0.93). PRA before lisinopril was little influenced by pH, whereas the increase with lisinopril was greatest at pH 6.5. PRC before lisinopril was fourfold higher at pH 8.5 compared with that at pH 6.0. Lisinopril increased both PRC values but reversed the pH dependency. Prorenin was fourfold higher at pH 8.5 compared with that at pH 6.0 and decreased slightly with lisinopril. Renal renin concentration was higher at pH 6.0 than at pH 8.5. With lisinopril, renal renin concentration increased at both pH values. Mouse PRC was not changed by lisinopril. Ribonuclease protection assay showed both rat and mouse renin gene expression in the kidney, which increased with lisinopril. Thus TGR have circulating active rat and mouse renin and prorenin. The notion that TGR are a "low renin" model should be revised.

METHODS

Animals.

Male, heterozygous TGR(mRen2)27 and male nontransgenic Sprague-Dawley rats (age: 10–12 wk) were used for experiments unless otherwise noted. Rats were kept under standard breeding conditions at our animal facility. A standard chow (Altromin 2000) containing 0.2% NaCl was given, and free access to tap water was allowed. All experiments were done according to guidelines of The American Physiological Society after approval by local institutional boards.

Experimental Protocols

Eight TGR were treated for 14 days with the ACE inhibitor lisinopril (10 mg/l drinking water), and eight control TGR were not treated. Blood pressure was measured by tail-cuff plethysmography (short ether anesthesia) before and at the end of experiments. Blood samples (0.6 ml) were obtained 2 days apart by jugular venous puncture after surgical exposure (ketamine/xylazine anesthesia, 15 and 5 mg/100 g body wt, ip, respectively). Blood was placed into prechilled tubes (4°C) and anticoagulated with Na2-EDTA at a final concentration of 6.25 × 10−6 mol/l. Blood samples were centrifuged for 10 min at 4°C to separate plasma. Aliquots of plasma were shock frozen using liquid nitrogen and stored at −70°C. Rats were killed with an overdose of intraperitoneal ketamine. Kidneys and adrenal glands were removed rapidly, frozen in liquid nitrogen, and stored at −70°C for analysis of renin content and renin gene expression. Plasma samples were thawed only once before determination of plasma renin-angiotensin system components.

Validation of Renin Measurement Techniques

Direct radioimmunoassay for mouse renin. A polyclonal rabbit anti-mouse submaxillary gland renin antibody (RSMS/PA) was supplied by J. Ménard. The antibody detects total Ren-2 mouse renin protein, but, because of its polyclonal
nature, the antibody cannot differentiate between active renin and prorenin. Purified submaxillary mouse renin was prepared as described (19). Purity of the preparation was >80%. Protein concentrations were determined according to Lowry et al. (13). Iodinated submaxillary mouse renin was custom made by BioTeZ, (Berlin, Germany) according to published techniques (15). Specific activity of the tracer was 6 MBq/mg renin.

A liquid-phase competition radioimmunoassay (RIA) was established (6). Polystyrene RIA tubes (75 × 10 mm; Greiner, Frickenhausen, Germany) and tris(hydroxymethyl)aminomethane (Tris) buffer [Tris-HCl buffer (pH 7.4) containing 1.0 mg/ml bovine serum albumin (BSA)] were used. Diluted antibody (200 µl) was incubated overnight at room temperature, together with 200 µl of radioactive tracer (6 × 10² Bq in Tris buffer) and 100 µl of assay buffer containing various amounts of unlabeled mouse renin. Separation of bound from free tracer was performed with 100 µl of 10 mg/ml bovine serum γ-globulins (no. G-5009, Sigma, St. Louis, MO) added to the tubes for 30 min. Polyethylene glycol 20% (wt/wt) in water; 1 ml) was then added to precipitate antibody-bound tracer. The tubes were then centrifuged at 5,000 revolutions/min (CS-6R, Beckman Instruments) at 4°C for 20 min, and supernatant was removed. The radioactivity of the precipitate (bound radioactivity) was measured with a gamma counter.

The binding characteristics of the antibody were tested at final antibody concentrations from 1:12,500 to 1:125,000 and specific binding of the tracer as measured in the absence of antibody could not be detected at 1:12,500. At 1:125,000, the antibody cannot differentiate between active renin and prorenin. Purified submaxillary mouse renin was prepared as described (19). Purity of the preparation was >80%. Protein concentrations were determined according to Lowry et al. (13). Iodinated submaxillary mouse renin was custom made by BioTeZ, (Berlin, Germany) according to published techniques (15). Specific activity of the tracer was 6 MBq/mg renin.

We determined TGR PRC before and after treatment and in control animals at pH 6.0 and 8.5 as described earlier (4). The angiotensinogen concentration was expressed as micrograms of ANG I per milliliter.

Plasma and Kidney Renin in TGR

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Ribonuclease Protection Assay

Total RNA was purified from snap-frozen kidneys and adrenal glands using a standard lithium chloride urea precipitation technique (1). Messenger RNA specific for rat and mouse renin and β-actin as the internal control were detected by ribonuclease (RNase) protection assay using an Ambion RPA III kit (Biotechnology, Heidelberg, Germany) (12). The exposure time using a FujiX BAS 2000 imaging system was 36 h. Ratios of measured radioactivity with β-actin were calculated.

Interstrain Comparisons

We measured PRA (at pH 7.4) and angiotensinogen in TGR, Brown Norway rats, Sprague-Dawley rats, and spontaneously hypertensive rats (SHR) as an additional control. All rats were male and between 8 and 10 wk of age. Blood was obtained from jugular vein puncture after anesthesia (see Experimental Protocols).

Statistics

Calculated values are means ± SD. Differences were tested by paired and unpaired Student's t-test as appropriate. StatView statistics software was used on a Macintosh personal computer. The interstrain comparisons were conducted by analysis of variance and post hoc tests using the same program.
RESULTS

The characteristics of our direct RIA for submaxillary gland renin are shown in Fig. 1. Radioactive tracer was effectively bound by the antibody (Fig. 1A). As antibody concentrations decreased, the amount of bound tracer decreased in parallel. Addition of unlabeled renin significantly displaced tracer from antibody, and the effect was significantly different from that with a nonspecific background. Variations in the amount of tracer did not change these characteristics (data not shown). Sensitivity and specificity of the assay standard curve are shown in Fig. 1B. Twenty percent of antibody-bound tracer was displaced by 0.25 ng unlabeled renin/tube, and 50% was displaced by 1.3 ng renin/tube. The presence of rat plasma or rat renin in the assay did not influence the standard curve, documenting the reliability and the specificity of the assay for mouse renin.

The results from our enzyme-kinetic determinations of mouse and rat renin concentration are shown in Fig. 1, C and D. Linear relationships with expected values (r = 0.97, P < 0.005) were found in all instances. The slopes were significantly influenced by pH level. For rat renin, the slope was highest at pH 6.0 and almost negligible at pH 8.5 (<5% of the value at pH 6.0). Mouse renin produced an inverse relationship, with the highest values at pH 8.5. At pH 6.0, the slope was only ~35% of this value. Mouse renin concentrations as determined by enzyme-kinetic assay correlated linearly with direct RIA determinations (r = 0.93, P < 0.005). The conversion factors for our assay were 42.0 ngANG I·ml⁻¹·h⁻¹ per nanogram of mouse renin at pH 8.5 and 23.1 and 15.3 ngANG I·ml⁻¹·h⁻¹ at pH 7.4 and 6.0, respectively. We conclude that determination of renin concentration at pH 8.5 (RC₈.₅) is approximately representative for mouse renin concentration, whereas rat renin concentration equals RC₆.₀ = (0.35 × RC₈.₅), where RC₆.₀ is the renin concentration determined at pH 6.0.

We next studied PRA and PRC as well as prorenin concentrations at different pH levels in TGR plasma and kidneys before and after treatment with lisinopril or vehicle (control) (Fig. 2). Before the experiment, TGR in the control and treatment groups were not significantly different in terms of PRC and blood pressure values. Lisinopril treatment significantly lowered blood pressure in TGR from 205 ± 14 to 128 ± 9 mmHg. Control TGR did not show significant changes in their PRC and blood pressure values during the experiment (data not shown).

Figure 2A shows PRA at pH 6.0, 7.4, and 8.5 in untreated TGR. PRA continuously increased with pH. With lisinopril, PRA significantly increased only at pH 6.0; there was no significant change at pH 8.5. Mouse renin concentrations as determined by enzyme-kinetic assay correlated linearly with direct RIA determinations (r = 0.93, P < 0.005). The conversion factors for our assay were 42.0 ngANG I·ml⁻¹·h⁻¹ per nanogram of mouse renin at pH 8.5 and 23.1 and 15.3 ngANG I·ml⁻¹·h⁻¹ at pH 7.4 and 6.0, respectively. We conclude that determination of renin concentration at pH 8.5 (RC₈.₅) is approximately representative for mouse renin concentration, whereas rat renin concentration equals RC₆.₀ = (0.35 × RC₈.₅), where RC₆.₀ is the renin concentration determined at pH 6.0.

Fig. 1. A: relationship between percentage of bound-to-total (bound/total) radioactivity in assay and antibody dilution for vehicle and unlabeled renin (3 ng/tube mouse renin). As antibody concentrations became more dilute, amount of bound/total radioactivity decreased. Unlabeled renin displaced curve to lower left. NSB, nonspecific binding. B: assay standard curve shows relationship between percent displacement and mouse renin concentration. Four media were tested: buffer, nephrectomy plasma (Nephr. Pl.), and two concentrations of rat kidney extract (kidney 1:10 and kidney 1:2). C: pH dependence of rat renin in relationship between ANG I generation and %kidney extract. Optimum lies at pH 6.0. D: relationship between ANG I generation and expected mouse renin concentrations at 3 different pH values.
lisinopril. In untreated rats PRC was greater at pH 8.5 than at pH 6.0. With treatment, PRC determinations at both values increased but were quantitatively different. There was a massive rise in PRC at pH 6.0. In terms of specific renin concentrations, mouse PRC increased by a factor of 1.3, whereas rat PRC increased by a factor of ~10. Both increases were significant as determined by paired t-test (P < 0.05). Figure 2C shows prorenin determinations at pH 6.0 and 8.5 before and after lisinopril. Plasma prorenin was almost exclusively mouse prorenin. Rat prorenin was below the detection level of the assay. Lisinopril treatment did not significantly influence prorenin concentrations. Figure 2D shows kidney tissue renin concentration under these conditions. Control renin determinations were slightly different at the two pH values; at pH 6.0 the values were higher. With lisinopril treatment, kidney rat renin concentrations increased markedly by a factor of 5. Mouse renin concentration increased by only a factor of 2. Figure 2E shows determinations of transgenic mouse renin by direct RIA. Transgenic PRC were ~20–30 ng/ml. There was no influence by lisinopril treatment. Rat angiotensinogen decreased from 0.89 ± 0.17 to 0.67 ± 0.2, whereas values remained unchanged in control TGR.

Results from our RNase protection assay in kidneys and adrenal glands are shown in Fig. 3. Mouse and rat renin mRNA were both detectable in kidneys from untreated control TGR (Fig. 3A). There was an increase in mouse and rat renin gene expression by a factor of
2–3 and 10, respectively, with lisinopril treatment. In the adrenal gland only mouse renin mRNA expression was detectable (Fig. 3B). No significant change occurred with lisinopril treatment.

As an additional control, we measured PRA and angiotensinogen (n = 8 for all groups) in TGR(mRen2)27, Brown Norway rats, Sprague-Dawley rats, and SHR. These data are shown in Table 1. We found that PRA in TGR was significantly higher than that in the other strains. Furthermore, angiotensinogen levels were significantly lower in TGR compared with those in Brown Norway rats and SHR.

**DISCUSSION**

We established a direct RIA for mouse Ren-2 renin for determinations of plasma and tissue renin concentrations in TGR(mRen2)27. Furthermore, we developed indirect enzyme-kinetic assay techniques based on the generation of ANG I to specifically determine mouse and rat PRC. Differences in the pH optimum were used. To allow comparison, we standardized our mouse renin enzyme-kinetic assay for absolute renin concentrations as measured by direct RIA. Our data clearly demonstrate that most, if not all, active renin in TGR plasma is of transgenic origin. Treatment with an ACE inhibitor increased the concentrations of both rat and mouse renins in plasma and kidneys. In contrast, transgene expression in the adrenal glands did not change significantly. These results suggest that short-term changes in active renin concentrations are under the physiological control of the kidney and, at least in part, are dependent on ANG II-related feedback mechanisms. The kidney and plasma, both endogenous and transgenic active renin behave similarly but with quantitatively different effects. Our results stress the importance of the pH value during enzyme-kinetic determinations of renin, which may lead to misinterpretations of the activity and dynamics of the plasma renin-angiotensin system in TGR.

Previous authors have suggested that the renin in TGR plasma is predominantly mouse renin. The first evidence was reported by Yamaguchi et al. (30), who found that renin activity in TGR plasma and tissue could not be inhibited by a monoclonal antibody directed against rat renin. These preliminary observations were confirmed with an inhibitory antibody directed against mouse renin (23). Véniant et al. (29) used a semiquantitative enzyme-kinetic assay based on the different pH optima of mouse and rat renin to study the dynamosics of the TGR plasma renin-angiotensin system during postnatal development. They demonstrated that mouse plasma renin increases, whereas rat renin decreases during TGR postnatal development, and they showed a correlation between plasma mouse renin concentrations and blood pressure. Tokita et al. (27) suggested that enhanced kinetics of mouse renin with rat angiotensinogen may play a role in causing TGR hypertension. Publications (3, 7, 11, 21, 30) concerning the renin-angiotensin system in TGR have traditionally relied on enzyme-kinetic assays to measure plasma renin. The renin concentrations as measured in these assays were expressed as the amount of ANG I generated in the presence of excess substrate, assuming a linear relationship between the amount of ANG I generated and the actual renin concentrations in the sample. Various incubation protocols were used, and the results from TGR and Sprague-Dawley rats were compared. However, comparing such indirect measurements is dependent on the assumption that the kinetics of mouse renin and rat renin acting on rat angiotensinogen are the same. This assumption is not the case, because mouse renin has a ~60-fold higher catalytic efficiency for cleaving rat angiotensinogen than for cleaving rat renin (24).

To address these problems, we developed a new, direct, and competitive RIA for mouse renin. Ours is not the first such assay (14, 15, 18, 26). We relied on a new immunoassay with a polyclonal anti-mouse submaxillary gland renin (anti-mouse Ren-2) antibody. We demonstrated specific and concentration-dependent binding of radioactively (125I) labeled renin tracer to the anti-mouse renin antibody and its specific displacement in the presence of unlabeled renin in our assay. Antibody dilutions were sufficiently high for an efficient RIA. Nonspecific binding of radioactivity was not negligible and was probably a consequence of our relatively simple methods for separating bound from unbound tracer. However, specific binding was significantly greater, allowing us to obtain a reliable and reproducible standard curve. The sensitivity of this mouse renin assay was ~300 pg renin/tube with a sufficient upper limit spanning about one order of magnitude.

We then established enzyme-kinetic assays to differentiate mouse from rat renin concentrations based on their respective pH optima. The pH characteristics for rat and mouse renin in our enzyme-kinetic assays were similar to those described earlier (16, 17). The assays produced linear relationships with values predicted from renin dilutions or with mouse renin concentrations as measured by direct RIA. Rat renin was almost inactive at pH 8.5, which allowed us to measure the concentrations and dynamics of mouse and rat renin in TGR. The concentrations of total transgenic protein in TGR plasma were similar when measured by direct RIA and enzyme-kinetic assay. Active mouse renin concentrations were in the range of 0.5–1.0 ng/ml, equivalent to ~14–28 fmol/ml. This value was higher

**Table 1.** PRA and angiotensinogen values for four different rat strains: TGR, Brown Norway rats, Sprague-Dawley rats, and SHR

<table>
<thead>
<tr>
<th>Strain</th>
<th>PRA, ngANG I·mL⁻¹·h⁻¹</th>
<th>Angiotensinogen, µgANG I·ml⁻¹</th>
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<tbody>
<tr>
<td>TGR</td>
<td>8.6 ± 1.7*</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>Brown Norway</td>
<td>1.7 ± 1.3</td>
<td>0.77 ± 0.10</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>2.8 ± 1.7</td>
<td>0.73 ± 0.10</td>
</tr>
<tr>
<td>SHR</td>
<td>2.5 ± 1.8</td>
<td>0.88 ± 0.14</td>
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</tbody>
</table>

Values are means ± SD for 4 rat strains (8 rats/group). Plasma renin activity (PRA) was measured at physiological pH. TGR, transgenic rats; SHR, spontaneously hypertensive rats. *Different from all other strains (P < 0.001); † different from Brown Norway and SHR (P < 0.05).
than that predicted by others from theoretical considerations, namely, 1–2 fmol/ml (28). We cannot exclude the possibility that some transgenic prorenin was activated during freezing and thawing of the plasma samples. However, determinations of mouse PRC were in the expected range.

In a similar experiment, Tokita et al. (27) used an anti-Ren-2 mouse renin antibody, albeit at different pH characteristics of TGR plasma, to study TGR plasma renin before and after treatment with an ACE inhibitor. They suggested that mouse renin is the main circulating species, whereas, after ACE-inhibitor treatment, rat renin appeared to be the main circulating renin species. Renal renin was found to be mainly rat renin. With our assays, we were able to comment more precisely and specifically on plasma and renal renin and their respective dynamics. In our experiment, lisinopril treatment massively increased rat PRC, whereas mouse PRC increased only slightly. In the kidney both renin species were detected, whereas both tissue renin concentrations and mRNA expression levels paralleled rat and mouse PRC before and after lisinopril treatment. The mouse renin in the kidneys appeared relatively insensitive to the negative feedback control exerted by ANG II compared with rat renin. The adrenal gland has previously been shown (25) to be a major source of transgenic plasma prorenin circulating at relatively high concentrations. Our results confirm these observations. We noted a slight decrease in transgenic prorenin with lisinopril treatment. However, this phenomenon could have resulted from a biochemically incomplete activation of prorenin by trypsin before the renin assay. Furthermore, the transgene expression in the adrenals did not change with lisinopril treatment. Such changes have been seen only at very high ACE inhibitor doses (11). Because we were interested in mRNA studies, we were not able to measure tissue renin concentrations in adrenal glands. We conclude from our data that, in TGR, the short-term regulation of active plasma renin by the kidney is still intact while both renin species participate in this regulation. However, this feature can only be observed during pharmacological blockade of an overactive negative feedback mechanism exerted by locally generated or plasma-derived ANG II. The source of this excess ANG II could be either the presence of transgenic mouse renin in the kidney or the secretion of mouse renin or generation of ANG II by extrarenal tissues. In this context, absolute concentrations of renin in plasma do not reflect the complexity of the situation.

As an additional control, we measured PRA and angiotensinogen values in TGR and three other strains, namely, Brown Norway rats, Sprague-Dawley rats, and SHR. We found that PRA values in TGR were significantly higher than in the other three strains, whereas angiotensinogen values were significantly lower than in Brown Norway rats and SHR. The PRA results concur with those reported by Campbell et al. (7), although they did not take the precaution of considering the confounding variables of pH and differences in cleavage efficiency rates of the two renins harbored by TGR. We can explain the lower angiotensinogen levels in TGR on the basis of angiotensinogen consumption. We did not measure ANG II levels in our rats because such measurements have already been reported by Campbell et al. (7). The investigators found that plasma ANG II and ANG I levels in TGR were fourfold higher than in controls. Their findings are in accord with the high renin values we report.

Our assays were designed to monitor the dynamics of both mouse and rat PRC values in TGR. Lowering the pH to 5.0 or 4.5 could possibly further increase the specificity of our rat renin assay. However, at extreme pH levels renin and angiotensinogen could become unstable. The decrease in cleavable angiotensinogen with lisinopril treatment in TGR is related to a known phenomenon occurring at very high PRC. Because PRA at physiological pH indicates the overall activity of the plasma renin-angiotensin system, namely, the actual capacity of plasma to generate ANG I, PRA measurements are valid to monitor the TGR renin-angiotensin system during pharmacological interventions. Conclusions about PRC values necessitate a differential approach. Otherwise, any comparison between Sprague-Dawley rats and TGR will not be valid. Because both renin species respond differentially to pharmacological stimulation, dose-response curves involving renin-dependent effects will become complex. The direct assay we describe will help in further research on these issues. In any event, earlier views on active PRC in TGR, as well as the notion of the TGR as a “low” active renin model with little if any renal renin transgene expression, should be revised.

This study was supported by a grant-in-aid from Hoffmann-LaRoche (Basel, Switzerland).

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Received 3 September 1997; accepted in final form 9 January 1998.

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