Adrenal and Circulating Renin-Angiotensin System in Stroke-Prone Hypertensive Rats

Shokei Kim, Masatoshi Tokuyama, Masayuki Hosoi, and Kenjiro Yamamoto

The plasma and adrenal renin-angiotensin system in stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats were examined in animals at 5, 11, 18, and 25 weeks of age. Plasma active renin was significantly increased in 18- and 25-week-old SHRSP with impaired renal function, whereas there was no difference in the plasma prorenin level or renal renin content between the two strains at all ages examined. Thus, the rate of activation of prorenin seems to be enhanced in the kidney of SHRSP with malignant hypertension. Adrenal renin contents were severalfold higher in SHRSP than WKY rats at all ages. However, adrenal angiotensin peptides were not increased in SHRSP aged 5 and 11 weeks. In 18-week-old SHRSP, adrenal angiotensin II (Ang II) and III (Ang III) levels were fourfold and 1.8-fold higher, respectively, than in WKY rats, accompanied by 1.5-fold higher plasma aldosterone. Increased adrenal angiotensin and plasma aldosterone were also found in 25-week-old SHRSP. Zonal distribution studies indicated that the elevated Ang II and III in SHRSP were derived mainly from the capsular tissue (the zona glomerulosa). To examine the contribution of circulating angiotensin to the adrenal angiotensin content, effects of bilateral nephrectomy on adrenal angiotensin and renin were examined in 18-week-old rats. At 24 hours after nephrectomy, plasma angiotensin, prorenin, and active renin were decreased to almost negligible concentrations. Conversely, in both adrenal capsular and decapsular tissues of SHRSP and WKY rats, neither angiotensin nor renin was significantly decreased after nephrectomy. These results suggest that the increase in adrenal capsular Ang II contents in SHRSP may be partly due to an enhanced local production or Ang II.

**Key Words** • renin • renin-angiotensin system • malignant hypertension • angiotensin II • rats, inbred SHR

Multiple lines of evidence indicate that various extrarenal organs, including adrenal gland, possess an intrinsic renin-angiotensin system. Dissociation of hypotensive effects of angiotensin converting enzyme inhibitor with plasma angiotensin II (Ang II) levels have been noted in hypertensive patients and in rats. Either angiotensin converting enzyme inhibitor or Ang II antagonist can significantly alleviate the hypertension in spontaneously hypertensive rats (SHR) without high plasma renin levels. Thus, the extrarenal renin-angiotensin system may play an important role in the pathophysiology of hypertension.

Stroke-prone SHR (SHRSP), a substrain of SHR developed by Okamoto et al, are a useful model of human malignant hypertension. The renin-angiotensin system also participates in the pathophysiology of malignant hypertension in SHRSP, as shown by previous reports. However, previous research done on the renin-angiotensin system of SHRSP has been limited to the circulating system.

In preliminary experiments, we found that Ang II contents in the whole adrenal gland of 25-week-old SHRSP are higher than those of Wistar-Kyoto (WKY) rats. In the present study, we did detailed studies on adrenal and plasma renin-angiotensin systems in SHRSP of various ages. We examined true plasma prorenin in SHRSP using our recently developed methods.

**Methods**

**Chemicals**

Bovine serum albumin (BSA, fraction V) was obtained from Seikagaku Kogyo, Tokyo. Trypsin from bovine pancreas (type III, 10,200 Nε-benzoyl-L-arginine ethyl ester [BAEE] units/mg protein), soybean trypsin inhibitor (type I-S), bovine γ-globulin, and α-methylmannoside were from Sigma Chemical Co., St. Louis, Mo. Polyethylene glycol 6,000 was from Fluka, Buchs, Switzerland. Iodine-125-labeled angiotensin I (Ang I) and Ang II and carrier-free sodium iodide were from New England Nuclear, Boston, Mass. Protein A-Sepharose and concanavalin A (con A)-Sepharose 4B were from Pharmacia, Uppsala, Sweden. Synthetic angiotensin peptides were purchased from Peptide Institute, Inc., Osaka, Japan.

**Animals**

SHRSP and Wistar-Kyoto rats were kindly donated by Dr. K. Okamoto (Kinki University School of Medicine, Osaka, Japan) and were maintained by selective breeding.
mating at Santen Pharmaceutical Co., Ltd., Osaka, Japan. Animals were fed a standard laboratory chow (CE-1; Clea Japan, Tokyo) and were given tap water ad libitum. Five-, 11-, 18-, and 25-week-old male animals of both strains were decapitated, and trunk blood was collected into prechilled tubes containing ethylenediaminetetraacetic acid (EDTA) (2 mg/ml) and Trasylol (500 kallikrein inhibitor units/ml). The plasma was separated by centrifugation at 3,000 rpm for 15 minutes at 4°C. Bilateral kidneys and adrenal glands were rapidly removed, weighed, frozen in liquid nitrogen, and stored together with plasma at −80°C until use.

Zonal Distribution of Adrenal Angiotensin and Renin and Effects of Nephrectomy on Adrenal Angiotensin and Renin Contents

In 18-week-old SHRSP and WKY rats, zonal distribution of adrenal angiotensin and renin and effects of nephrectomy on the zonal distribution were examined. Rats were bilaterally nephrectomized by flank incision 24 hours before decapitation. Nonnephrectomized and 24-hour-nephrectomized rats were decapitated as described above. After collection of trunk blood, both adrenal glands were removed, separated into the capsular tissue (containing the zona glomerulosa) and the decapsular tissue (the zona fasciculata/zona reticularis and the medulla), weighed, frozen in liquid nitrogen, and stored at −80°C until use.

Homogenization of Adrenal Gland and Kidney

For measurement of adrenal angiotensin peptides, whole adrenal glands, capsular tissues, and decapsular tissues were heated at 100°C in boiled distilled water (0.5 ml) for 5 minutes to inactivate proteases, then homogenized in 0.05N HCl solution (total volume, 1 ml). The supernatant was obtained by centrifugation at 13,000 rpm at 4°C for 1 hour and was assayed for immunoreactive peptide contents as described below.

For measurement of active renin, inactive renin, immunoreactive renin, or molecular mass, whole kidney, adrenal gland, capsular tissue, and decapsular tissue were homogenized in 50 mM sodium phosphate (pH 7.0) containing 0.1 M NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 13,000 rpm at 4°C for 1 hour to obtain the supernatant. For kidney, 10% homogenate was prepared for all experiments. In the case of adrenal tissue, 20% homogenate was prepared for measurement of active renin and inactive renin, and 5% homogenate was prepared for the determination of immunoreactive renin and molecular mass and for con A chromatography.

Measurement of Plasma and Adrenal Angiotensins

Plasma and adrenal angiotensin peptides were measured according to our previously described methods. In brief, the sample of plasma (1 ml) and adrenal extracts (1 ml) was applied to a Sep-Pak C8 cartridge column (Waters Associates, Milford, Mass.). The angiotensins retained by the cartridge were eluted with a mixture of methanol/water/trifluoroacetic acid (80/19.9/0.1 vol/vol/vol) (2 mg/ml) and Trasylol (500 kallikrein inhibitor units/ml). The plasma was separated by centrifugation at 3,000 rpm for 15 minutes at 4°C. Bilateral kidneys and adrenal glands were rapidly removed, weighed, frozen in liquid nitrogen, and stored together with plasma at −80°C until use.

Measurement of Active Renin

Active renin was measured as the rate of Ang I generation, as described. In brief, the samples of plasma and of renal and adrenal extracts were incubated with nephrectomized rat plasma containing an excess of renin substrate (2 μM angiotensinogen), and the Ang I generated was measured by RIA. Adrenal active renin was regarded as the renin activity inhabitable by specific anti-mature rat renin serum.

Inhibition of the Angiotensin I-Generating Activity of Adrenal Extract and Plasma by Anti-Renin Serum

To compare the inhibitory manner of the renin activity by anti-renin serum between adrenal extract and plasma, the samples (100 μl) of adrenal extracts or plasma were incubated with serially diluted anti-renin serum or nonimmunized rabbit serum as control at 4°C for 24 hours, and the residual renin activity was measured as described above.

Measurement of Plasma Prorenin

Plasma prorenin was measured by high-performance liquid chromatography (HPLC) coupled with trypsin activation according to our previously described methods. In brief, the sample of plasma (200–300 μl) from nonnephrectomized and 24-hour-nephrectomized SHRSP and WKY rats was applied to a G3,000SW column (0.75×60 cm; Tosoh, Yamaguchi, Japan) equilibrated with 50 mM sodium phosphate (pH 7.4) containing 0.2 M NaCl and 5 mM EDTA. The elution was performed at a flow rate of 0.5 ml/min, and fractions were collected every 30 or 60 seconds. Inactive renin in each fraction was activated with 0.31 mg/ml trypsin at 23°C for 30 minutes, the optimum condition noted in preliminary experiments. Renin activity after trypsin activation (total renin) was measured as described above. Inactive renin in each fraction was calculated as the difference between total and active renin. Preliminary experiments showed that the recovery of pure rat renal active renin24 and purified recombinant rat prorenin from a G3,000SW column was 91±3% (n=6) and 89±3% (n=6), respectively.

To determine whether the inactive renin fraction recovered from the HPLC column was true prorenin, the inactive renin fraction was immunoprecipitated with two kinds of anti-prorenin prosegment immunoglobulin.
G (IgG). The anti-Pro N IgG and anti-Pro C IgG used recognize the amino-terminal and the carboxyl-terminal portions of the prosegment of rat prorenin, respectively, as described. The immunoprecipitation using protein A-Sepharose was carried out as described. Percent inhibition of the enzymatic activity of inactive renin fraction after trypsin activation by anti-mature rat renin IgG was determined as described.

**Trypsin Treatment of Adrenal and Renal Extracts**

To detect prorenin or inactive renin in the adrenal gland and kidney, adrenal and renal extracts (50 μl) were incubated with various concentrations (0, 0.01, 0.1, 0.3, or 1 mg/ml) of trypsin at 23°C for 15 or 30 minutes (total volume, 100 μl), followed by the termination of the reaction with the addition of soybean trypsin inhibitor (2 mg/mg trypsin). The renin activity after trypsin treatment (total renin) was measured as described above and subtracted from active renin.

**Direct Radioimmunoassay of Immunoreactive Renin**

To measure immunoreactive renin in plasma and in renal and adrenal extracts, direct RIA of renin was developed. To obtain a renin tracer, pure rat renal renin was labeled with [125I] by the chloramine T method and purified by gel permeation HPLC on G3,000SW as described. The titer of anti-renin serum used was ×1,000, and this antiserum was specific for rat renin, as shown by no cross-reactivity to mouse submaxillary renin, human renin, and rat cathepsin D and E. The RIA buffer was 50 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl, 3 mM EDTA, 1 mM PMSF, and 0.5% BSA; this was used to dilute all reagents. The assay incubation mixture consisted of 100 μl standard pure rat renal renin (390 pg to 100 ng) or serially diluted sample (plasma, renal and adrenal extracts, and chromatographic fractions of plasma), 100 μl anti-renin serum (dilated to 1:1,000), and 100 μl iodine-125-labeled renin and 700 μl RIA buffer (total volume, 1 ml). After incubation at 4°C for 48 hours, the immunocomplex was separated from the unbound fraction by addition of 0.1% bovine y-globulin and 12.5% polyethylene glycol 6,000, followed by centrifugation at 3,000 rpm at 4°C for 20 minutes. The radioactivity in the precipitate was counted with a gamma-counter (Packard model 500C, Rockville, Md.).

**Molecular Mass of Adrenal Renin**

To estimate the molecular mass of adrenal renin, adrenal extracts (300–500 μl) were subjected to HPLC on G3,000SW as described.

**Concanavalin A Chromatography of Adrenal Renin**

To examine whether adrenal renin is glycosylated, adrenal extracts pooled from 25-week-old SHRSP or WKY rats in 50 mM Tris-HCl (pH 7.2) containing 0.2 M NaCl, 2 mM PMSF, and 0.2 M NaCl and 1 mM PMSF as previously described. After washing with 8 ml of the equilibration buffer, the column was eluted with the equilibration buffer containing 0.2 M α-methylmannoside. The flow rate was 12 ml/hr. Fractions of 1 ml were collected into plastic tubes containing 50 μl equilibration buffer/10% BSA.

**Measurement of Plasma Angiotensinogen**

Angiotensinogen was indirectly measured by incubating plasma samples with an excess of pure rat renal renin (50 ng/ml) as described. The generated Ang I was measured by RIA.

**Determination of Aldosterone, Blood Urea Nitrogen, and Plasma Creatinine**

Plasma aldosterone concentration was determined by RIA using kits (Sorin Biomedica S.P.A., Italy). Blood urea nitrogen (BUN) and plasma creatinine were measured with kits (Wako Chemicals, Osaka, Japan).

**Figure 1.** Line graphs show gel permeation high-performance liquid chromatography (HPLC) profile of plasma from nonnephrectomized and 24-hour nephrectomized 18-week-old stroke-prone spontaneously hypertensive rats (SHRSP). Panel A: HPLC separation of nonnephrectomized SHRSP plasma resulted in a single peak of inactive renin and active renin with molecular mass of 48 and 40 kDa, respectively. Panel B: Molecular mass of inactive renin from 24-hour-nephrectomized rats (65 kDa) was significantly different from that of inactive renin from nonnephrectomized rats (48 kDa) and of angiotensinogen (59 kDa). Active renin was not detected throughout HPLC. Retention time of protein standards including bovine serum albumin (68 kDa), ovalbumin (45 kDa), α-chymotrypsinogen (25 kDa), and cytochrome C (12.5 kDa) is indicated by arrows. *Vo* void volume; *Ang* angiotensin.
Table 1. Molecular Mass and Immunoreactivity of Plasma Inactive Renin Recovered From HPLC on G3,000SW

<table>
<thead>
<tr>
<th>Molecular mass and immunoreactivity</th>
<th>Intact</th>
<th>Nephrectomized</th>
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</thead>
<tbody>
<tr>
<td>Molecular mass (kd) (n=6)</td>
<td>47.6±0.4</td>
<td>47.8±0.5</td>
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<tr>
<td>%Immunoreactivity</td>
<td></td>
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<tr>
<td>Anti-Pro N IgG (n=4)</td>
<td>65±3</td>
<td>68±2</td>
</tr>
<tr>
<td>Anti-Pro C IgG (n=4)</td>
<td>90±3</td>
<td>92±3</td>
</tr>
<tr>
<td>Anti-Pro N IgG combined with Anti-Pro C IgG (n=3)</td>
<td>91±2</td>
<td>88±2</td>
</tr>
<tr>
<td>%Inhibition of the activity by anti-renin IgG (n=4)</td>
<td>92±3</td>
<td>91±4</td>
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</table>

Anti-Pro N IgG and anti-Pro C IgG recognize the amino-terminal and the carboxyl-terminal portions of the prosegment of prorenin, respectively. Anti-renin IgG recognizes the portion of mature renin. HPLC, high-performance liquid chromatography; WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; IgG, immunoglobulin G.

Table 2. Body Weight, Blood Pressure, Hematocrit, and Plasma Urea Nitrogen and Creatinine in WKY Rats and SHRSP

<table>
<thead>
<tr>
<th>Animals</th>
<th>Body weight (g)</th>
<th>BP (mm Hg)</th>
<th>Hct (%)</th>
<th>BUN (mg/dl)</th>
<th>Plasma creatinine (mg/dl)</th>
</tr>
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<tr>
<td>5 weeks old</td>
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<tr>
<td>WKY (n=8)</td>
<td>108±2</td>
<td>107±4</td>
<td>45.2±0.5</td>
<td>12.0±0.6</td>
<td>0.47±0.01</td>
</tr>
<tr>
<td>SHRSP (n=8)</td>
<td>93±3*</td>
<td>113±2</td>
<td>44.5±0.5</td>
<td>10.3±0.7</td>
<td>0.46±0.01</td>
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<td>11 weeks old</td>
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</tr>
<tr>
<td>WKY (n=5)</td>
<td>282±5</td>
<td>121±3</td>
<td>44.6±0.5</td>
<td>11.4±0.7</td>
<td>0.47±0.01</td>
</tr>
<tr>
<td>SHRSP (n=5)</td>
<td>234±7*</td>
<td>201±8*</td>
<td>44.0±0.8</td>
<td>11.6±0.6</td>
<td>0.46±0.01</td>
</tr>
<tr>
<td>18 weeks old</td>
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<tr>
<td>WKY (n=6)</td>
<td>377±6</td>
<td>135±5</td>
<td>46.3±0.8</td>
<td>14.6±1.4</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>SHRSP (n=6)</td>
<td>329±7*</td>
<td>249±9*</td>
<td>46.6±0.4</td>
<td>19.4±0.7*</td>
<td>0.68±0.03†</td>
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<td>25 weeks old</td>
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<tr>
<td>WKY (n=7)</td>
<td>391±5</td>
<td>130±2</td>
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<td>11.8±0.5</td>
<td>0.61±0.02</td>
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<tr>
<td>SHRSP (n=7)</td>
<td>320±4*</td>
<td>254±6*</td>
<td>43.7±0.4</td>
<td>32.5±3.2*</td>
<td>1.01±0.06*</td>
</tr>
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</table>

WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; BP, systolic blood pressure; Hct, hematocrit; BUN, blood urea nitrogen.

*p<0.01, †p<0.05 compared with age-matched WKY rats.

Measurement of Blood Pressure

Systolic blood pressure was measured by the tail-cuff method with a sphygmomanometer (model PS-100, Riken Kaihatsu, Tokyo). Each value is the average of three consistent readings.

Statistical Method

Results are expressed as mean±SEM. Statistical analysis was performed by use of unpaired Student’s t test. Values of p<0.05 were considered to be statistically significant. Correlation coefficients were calculated by the method of least squares.

Results

Method for Measurement of True Plasma Prorenin

As shown in Figure 1A, the HPLC profile of plasma from 18-week-old SHRSP showed a single peak of inactive renin (trypsin-activatable renin) with a molecular mass of 48 kd, a value higher than that of active renin (40 kd). As shown in Table 1, this inactive renin fraction recovered from HPLC was immunoprecipitated with anti-Pro C IgG recognizing the carboxyl-terminal portion of the prosegment by 92±3% (n=4), and the enzymatic activity of this inactive renin after trypsin treatment was inhibited by specific IgG against mature rat renin (anti-renin IgG) by 91±4% (n=4). Thus, the inactive renin fraction recovered from HPLC was true prorenin, and HPLC separation of plasma resulted in the disappearance of inactive renin not caused by either prorenin or renin (the trypsin-induced artifact). There was no difference in molecular mass and the immunoreactivity to specific antibody against the prorenin prosegment or mature renin between the HPLC fraction of inactive renin from SHRSP and WKY rats at the age of 5, 11, 18, and 25 weeks. These observations on SHRSP and WKY rats are consistent with our previous data on Wistar rats that HPLC separation of plasma coupled with trypsin treatment allows for the measurement of true prorenin.

On the other hand, inactive renin from 24-hour-nephrectomized SHRSP and WKY rats had a molecular mass of about 65 kd and was immunoreactive to specific antibody against the prorenin prosegment or mature renin to a negligible degree (Figure 1B and Table 1). Thus, this inactive renin is neither prorenin nor renin. These observations are in good agreement with our findings on nephrectomized Wistar rats.

Table 2. Body Weight, Blood Pressure, Hematocrit, and Plasma Urea Nitrogen and Creatinine in WKY Rats and SHRSP

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WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; BP, systolic blood pressure; Hct, hematocrit; BUN, blood urea nitrogen.

*p<0.01, †p<0.05 compared with age-matched WKY rats.
TABLE 3. Plasma Concentrations of Angiotensinogen, Active Renin, Prorenin, Angiotensin, and Aldosterone in WKY Rats and SHRSP

<table>
<thead>
<tr>
<th>Animals</th>
<th>Angiotensinogen (ng Ang I - ml⁻¹)</th>
<th>Active renin (ng Ang I - hr⁻¹ - ml⁻¹)</th>
<th>Prorenin (ng Ang I - hr⁻¹ - ml⁻¹)</th>
<th>Ang I (pg/ml)</th>
<th>Ang II (pg/ml)</th>
<th>Ang III (pg/ml)</th>
<th>Aldosterone (pg/ml)</th>
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</table>

WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; Ang, angiotensin.

*P<0.05, t/P<0.01 compared with age-matched WKY rats.

Plasma Renin-Angiotensin-Aldosterone System in SHRSP and WKY Rats

As shown in Table 2, SHRSP had a normal blood pressure at age 5 weeks, an elevated blood pressure at age 11 weeks, and severe established hypertension at 18 and 25 weeks of age. Both BUN and plasma creatinine of 18-week-old SHRSP were significantly higher than those of age-matched WKY rats, and these parameters were further increased in 25-week-old SHRSP (Table 2).

As shown in Table 3, at 5 weeks of age, there was no difference in plasma active renin, Ang I, II, and III, and aldosterone concentrations between SHRSP and WKY rats. However, plasma prorenin and angiotensinogen in SHRSP were lower than those in WKY rats, and these parameters were further increased in 25-week-old SHRSP (Table 2).

As shown in Table 3, at 5 weeks of age, there was no difference in plasma active renin, Ang I, II, and III, and aldosterone concentrations between SHRSP and WKY rats. However, plasma prorenin and angiotensinogen in SHRSP were lower than those in WKY rats. At 11 weeks of age, no difference in these parameters in plasma was found between the two strains of rats. In 18-week-old SHRSP, the plasma active renin concentration was 1.8-fold higher than that in the age-matched WKY rats, and was associated with 1.7-, 1.9-, 1.6-, and 1.5-fold higher plasma concentrations of Ang I, II, and III and aldosterone, respectively, in SHRSP. These components in the plasma were further increased in 25-week-old SHRSP. In contrast to the increase in plasma active renin, plasma prorenin levels were not elevated in 18- and 25-week-old SHRSP. Thus, the percentage of active renin to total renin (active renin plus prorenin) in plasma was significantly higher in SHRSP than WKY rats at the ages of 18 weeks (57.5±5.1% versus 44.3±1.7%; p<0.05) and 25 weeks (70.9±4.0% versus 31.1±2.0%; p<0.01) (Figure 2).

In SHRSP, there was a close correlation of plasma Ang II with creatinine (r=0.859; n=26; p<0.01) and BUN (r=0.769; n=26; p<0.01). Direct Radioimmunoassay of Renin in Plasma and in Renal and Adrenal Extracts

As shown in Figure 3, the dilution curves for renal extracts from SHRSP and WKY rats were parallel to

FIGURE 2. Bar graph shows percent active renin in the plasma of 5-, 11-, 18-, and 25-week-old Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP). % Active renin represents the percentage of active renin to total renin (active renin and prorenin) in plasma. N.S., not significant.

FIGURE 3. Line graph shows standard curve for direct radioimmunoassay of renin and dilution curves for plasma and renal extracts from 25-week-old stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats. Ordinate (B/B₀ x 100) is the percentage of bound radioactivity in the presence and absence of unlabeled renin. Aboissa shows the amount of pure renin (○) and the sample volume of renal extracts from 25-week-old SHRSP (●) and WKY rats (○) (1.5, 3, and 6 µl) and plasma from 25-week-old SHRSP (●) and WKY rats (○) (12.5, 25, 50, and 100 µl), which are plotted on a logarithmic scale.
the standard curve for the RIA, thereby indicating the immunological identity between renin in renal extracts and pure renal renin. Thus, the amount of renin in renal extracts could be directly measured (Table 4).

On the other hand, the dilution curves for plasma from SHRSP and WKY rats were not parallel to the standard curve, findings consistent with our previous data obtained for Wistar rat plasma. Although the reason for this is unknown, it is possible that multiple renin forms with different antigenicity or substance (or substances) interfering with binding of antibody to renin may exist in rat plasma. Thus, this RIA system did not allow us to accurately quantify the amount of immunoreactive renin in plasma. However, all the plasma samples from 25-week-old SHRSP (n=7) caused a greater displacement of iodine-125-labeled renin from the antiserum than those from WKY rats (n=7), thereby supporting the notion that plasma from 25-week-old SHRSP contains a larger amount of renin than that from WKY rats. When chromatographic fractions of active renin or prorenin obtained by HPLC of plasma were subjected to direct RIA, immunoreactive renin was not detected.

Our RIA system was also applied to the direct measurement of renin in adrenal extracts. In the case of adrenal extracts (19–63 ng Ang I·hr⁻¹·ml⁻¹) from 5- and 25-week-old WKY rats and 5-week-old SHRSP, no immunoreactive renin was detected (n=4 for each group). Only in the case of adrenal extracts from 25-week-old SHRSP (n=2), whose renin activity was 381 and 409 ng Ang I·hr⁻¹·ml⁻¹, respectively, could immunoreactive renin be detected. Their immunoreactive renin concentrations were 5.21 and 5.33 ng/ml, respectively. The specific activity (the renin activity divided by immunoreactive renin) was 73.3 and 76.7 μg Ang I·hr⁻¹·μg renin⁻¹, respectively, similar to that of renal renin (Table 4).

Table 4. Renal Active Renin and Immunoreactive Renin Contents and Specific Activity

<table>
<thead>
<tr>
<th>Animals</th>
<th>Active renin (μg Ang I·hr⁻¹·g⁻¹)</th>
<th>Immunoreactive renin (μg renin/g)</th>
<th>Specific activity* (μg Ang I·hr⁻¹·μg renin⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=8)</td>
<td>1,264±50</td>
<td>14.3±1.4</td>
<td>89.1±3.4</td>
</tr>
<tr>
<td>SHRSP (n=8)</td>
<td>1,192±99</td>
<td>13.3±1.2</td>
<td>90.5±3.6</td>
</tr>
<tr>
<td>11 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=5)</td>
<td>1,066±51</td>
<td>11.6±0.6</td>
<td>92.5±6.6</td>
</tr>
<tr>
<td>SHRSP (n=5)</td>
<td>1,033±68</td>
<td>12.2±1.0</td>
<td>85.5±5.2</td>
</tr>
<tr>
<td>18 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=6)</td>
<td>825±46</td>
<td>9.8±0.7</td>
<td>85.6±5.1</td>
</tr>
<tr>
<td>SHRSP (n=6)</td>
<td>804±87</td>
<td>10.0±0.9</td>
<td>81.6±6.3</td>
</tr>
<tr>
<td>25 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=7)</td>
<td>781±71</td>
<td>9.6±1.1</td>
<td>83.1±4.2</td>
</tr>
<tr>
<td>SHRSP (n=7)</td>
<td>805±84</td>
<td>9.7±1.1</td>
<td>84.6±6.1</td>
</tr>
</tbody>
</table>

Ang, angiotensin; WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats.

*Specific activity, renin activity divided by immunoreactive renin content.

As shown in Table 4, there was no significant difference in renal active renin and immunoreactive renin contents between SHRSP and WKY rats at all ages examined. The specific activity (the renin activity divided by immunoreactive renin) was similar between the two strains and among all ages examined. These results indicate that the decrease in renal renin activity with age can be explained by the decreased amount of renal renin rather than the appearance of renin isoenzyme with lower enzymatic activity.

Trypsin treatment of renal extracts from 5-, 11-, 18-, and 25-week-old SHRSP and WKY rats (n=4 for each group) did not cause the increase in the renin activity, thereby not allowing for the detection of prorenin or inactive renin in renal tissues.

Renin and Angiotensin Contents in Whole Adrenal Tissues of SHRSP and WKY Rats

As shown in Figure 4, in SHRSP at all ages examined, adrenal active renin contents were severalfold higher than those in WKY rats. Conversely, at age 5 and 11 weeks, there was no significant difference in adrenal Ang I, II, and III contents between SHRSP and WKY rats, findings indicating a dissociation between adrenal renin and angiotensin peptide contents. Ang II content in 18-week-old SHRSP was fourfold higher than in WKY rats (9,494±2,568 versus 2,365±107 pg/g tissue; p<0.05), in association with a 1.8-fold higher concentration of adrenal Ang III (806±105 versus 443±23 pg/g tissue; p<0.01), whereas the Ang I content was not increased in 18-week-old SHRSP (446±78 versus 404±35 pg/g tissue; p>0.05). At 25 weeks of age, adrenal Ang I, II, and III in SHRSP were 2.3, 4.3, and 1.8-fold higher, respectively, than in WKY rats.

The renin activity of adrenal extracts from 5-, 11-, 18-, and 25-week-old SHRSP and WKY rats (each, n=2) was not increased by trypsin treatment. Thus, as in the case of the kidney, neither prorenin nor inactive renin could be detected in the adrenal gland.

Inhibition of the Angiotensin I-Generating Activity of Adrenal Extract by Anti-renin Serum

As shown in Figure 5, the pattern of inhibition of the adrenal renin activity by anti-renin serum was similar between 25-week-old WKY rats and SHRSP. The maximum percent inhibition of the activity by anti-renin serum was 98% (the mean of two experiments) for the plasma of both 25-week-old WKY rats and SHRSP and
89% and 90% (each, the mean of two experiments) for the adrenal extract of 25-week-old WKY rats and SHRSP, respectively.

**Molecular Mass and Affinity for Concanavalin A of Adrenal Active Renin**

The molecular mass of adrenal active renin was 40±1 kd (n=3), 39±2 kd (n=3), 41 kd (the mean of two experiments), and 40 kd (the mean of two experiments) for 25-week-old WKY rats, 25-week-old SHRSP, 5-week-old WKY rats, and 5-week-old SHRSP, respectively (Figure 6A). The total recovery of adrenal active renin from the column was more than 85% for all experiments.

Con A chromatography showed that 84% and 86% of adrenal active renin from 25-week-old SHRSP and WKY rats, respectively, were retained by a con A column and eluted with 0.2 M α-methylmannoside (Figure 6B). The total recovery of adrenal active renin from the column was 73% and 70% for SHRSP and WKY rats, respectively.

**Effects of Nephrectomy on Plasma Renin-Angiotensin-Aldosterone System**

To examine whether the elevated adrenal angiotensin content in SHRSP is a result of the increased uptake of circulating angiotensin, the adrenal capsular and decapsular angiotensin and renin contents were compared between 18-week-old SHRSP and WKY rats at 24 hours after nephrectomy. As shown in Figure 7, in both SHRSP and WKY rats, bilateral nephrectomy remarkably decreased plasma active renin, prorenin, and Ang I, II, and III, yet increased plasma aldosterone. There was no difference in plasma active renin (1.22±0.32 versus 0.52±0.03 ng Ang I·hr⁻¹·ml⁻¹), prorenin (not detected in both groups), Ang I (21.4±3.4 versus 14.8±1.7 pg/ml), Ang II (5.4±1.1 versus 3.2±0.6 pg/ml), and Ang III (0.42±0.12 versus 0.33±0.14 pg/ml) between 24-hour-nephrectomized 18-week-old SHRSP and WKY rats. On the other hand, plasma aldosterone concentrations remained higher in SHRSP compared with WKY rats after nephrectomy (1,027±102 versus 719±30 pg/ml; p<0.05).

**Effects of Nephrectomy on Adrenal Capsular and Decapsular Angiotensin and Renin Contents**

In contrast to a dramatic decrease in plasma Ang I, II, and III and renin 24 hours after nephrectomy, in both SHRSP and WKY rats, these parameters in the adrenal capsular or decapsular tissues were not significantly decreased 24 hours after nephrectomy (Figures 8 and 9). In the capsular tissue, active renin and Ang II and III contents (7,180±439 ng Ang I·hr⁻¹·g⁻¹, 7,069±729 pg/g, and 958±95 pg/g, respectively) in nephrectomized SHRSP were 4.9-, 2.3-, and 1.9-fold higher, respectively, than in nephrectomized WKY rats (Figure 8), whereas no difference in Ang I content was found between the two strains of nephrectomized rats (503±56 versus 482±81 pg/g). In the decapsular tissue, renin content (341±29 ng Ang I·hr⁻¹·g⁻¹) in nephrectomized SHRSP was 2.3-fold higher than that in ne-
Relation Between Plasma and Adrenal Angiotensin II Concentrations in SHRSP

Combined data from all SHRSP examined show that correlation between plasma Ang II and adrenal Ang II concentrations was weak \( r = 0.26; n = 26; p < 0.01 \).

Relation Between Renin and Angiotensin II Concentrations in the Plasma and in the Adrenal Gland of SHRSP

Figure 10 shows the relation between renin and Ang II in plasma and in adrenal gland from all SHRSP examined. There was a close correlation between renin and Ang II in the plasma \( r = 0.944; n = 26; p < 0.01 \). On the other hand, the correlation between renin and Ang II in the adrenal gland was weak \( r = 0.369; n = 26; p < 0.01 \).

Discussion

The concentration and molecular weight of plasma active renin in SHRSP have been sufficiently examined by various groups of investigators. Plasma active renin concentrations in SHRSP are increased with the development of malignant hypertension. Shibuta et al noted that the increased plasma active renin in SHRSP is associated with an increase in urinary protein and renal vascular lesions. Volpe et al speculated that the rise in plasma active renin of SHRSP may in part contribute to end-organ damage and stroke. However, the plasma Ang II concentrations remained to be determined. In the present study, we found that plasma Ang II levels as well as active renin were increased in SHRSP with renal dysfunction. Furthermore, a close correlation between plasma Ang II and creatinine or BUN was seen in SHRSP. These observations support the previous assumption that the circulating renin-angiotensin system may contribute to the development of renal dysfunction and end-organ damage in SHRSP.

**Figure 6.** Line graphs show gel permeation high-performance liquid chromatography on G3,000SW (panel A) and concanavalin A chromatography (panel B) of adrenal active renin from 25-week-old stroke-prone spontaneously hypertensive rats. Retention time of protein standards is indicated by arrows. Ang, angiotensin.

**Figure 7.** Line graphs show effects of nephrectomy on plasma active renin, prorenin, angiotensin, and aldosterone levels of 18-week-old Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP). Ang, angiotensin; C, nonnephrectomized rats; NX, 24-hour-bilaterally nephrectomized rats. *p < 0.05, **p < 0.01 compared with WKY rats; N.S., not significant.
Figure 8. Line graphs show effects of nephrectomy on adrenal capsular renin and angiotensin contents in 18-week-old Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP). C, nonnephrectomized rats; NX, 24-hour-bilaterally nephrectomized rats; Ang, angiotensin. **p < 0.01 compared with WKY rats. N.S., not significant.

Figure 9. Line graphs show effects of nephrectomy on adrenal decapsular renin and angiotensin contents in 18-week-old Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP). C, nonnephrectomized rats; NX, 24-hour-bilaterally nephrectomized rats; Ang, angiotensin. *p < 0.05 compared with WKY rats. N.S., not significant.

Exacerbation of malignant hypertension, although the rise in plasma active renin seems to be the consequence rather than the cause of renal injury.

In various animals as well as humans, trypsin treatment of plasma causes a significant increase in the renin activity (the Ang I-generating activity). This trypsin-induced renin activity is generally called inactive renin. Using immunological techniques and HPLC, we obtained evidence that trypsin treatment of unfractionated rat plasma leads not only to the activation of prorenin, the biosynthetic precursor of renin, but also to the occurrence of a significant amount of Ang I-generating activity not related to prorenin or renin (the trypsin-induced artifact). Thus, trypsin treatment of crude rat plasma does not allow for measurement of true prorenin, although the nature of the trypsin-induced artifact is not fully understood. We developed the new method for measurement of true rat plasma prorenin, using HPLC separation of rat plasma on G3,000SW before trypsin activation.

In the present study, we first examined true plasma prorenin in genetically hypertensive rats. At all ages, including the malignant phase, plasma prorenin from SHRSP was similar to that seen in WKY rats with respect to the molecular mass and immunoreactivity, thereby differing from the finding obtained for active renin that plasma active renin from SHRSP in the malignant phase has a significantly larger molecular mass than that from WKY rats. It should be noted that despite the progressive increase in plasma active renin in SHRSP, there was no elevation of plasma prorenin concentration in SHRSP.
very small, if present at all, since prorenin is known to be readily converted to mature renin within both the adrenal gland and kidney. Second, prorenin in these tissues might be converted to active renin during the homogenization procedure, although the homogenization buffer contained protease inhibitors. Third, the method for activation used in the present study might not be optimum, although various concentrations of trypsin were used and two different incubation times (15 and 30 minutes) were used. The accurate measurement of tissue prorenin must await the development of specific and sensitive direct RIA of prorenin.

Previous investigations revealed a significant hypotensive action and prevention of organ damage of angiotensin converting enzyme inhibitor or Ang II antagonist in SHRSP in the phase of normal plasma renin level; hence, the extrarenal renin-angiotensin system may be involved in the pathogenesis of malignant hypertension in SHRSP. Adrenalec-tomy markedly decreases blood pressure in SHRSP as well as SHR but does not in normotensive WKY rats. Adrenal mineralocorticoids are suggested to be responsible for the abnormal vascular reactivity observed in SHRSP. Investigations on comparisons of plasma catecholamine levels between SHRSP and WKY rats showed that plasma catecholamines are higher in SHRSP than in WKY rats, and the adrenal-medullary activity is enhanced in SHRSP. These findings, taken together with the finding that Ang II acts on adrenal zona glomerulosa cells and medullary cells to stimulate the biosynthesis and release of aldosterone and catecholamines, respectively, suggested that we determine adrenal capsular and decapsular angiotensin and renin contents of SHRSP. The availability of specific anti-mature rat renin antibody allowed for the measurement of true adrenal renin. Adrenal Ang I, II, and III could be differentially measured by HPLC coupled with specific RIA. Adrenal renin and Ang II contents measured in the present study were about 10-fold and 100-fold higher, respectively, than those of plasma, findings in good agreement with reported data.

SHRSP showed severalfold higher adrenal renin contents than WKY rats at all ages examined, a finding similar to the observation obtained for SHR. Of note are the observations that despite the rise in adrenal renin, adrenal Ang I, II, and III were not increased in 5- and 11-week-old SHRSP. Thus, there is a dissociation between adrenal renin and angiotensin contents in SHRSP in the phase of no severe hypertension. On the other hand, in 18- and 25-week-old SHRSP with malignant hypertension, both thyroid Ang II and III contents were higher than those of WKY rats. Zonal distribution studies (Figures 8 and 9) showed that the increased adrenal Ang II and III contents are caused mainly by the capsular portion (the zona glomerulosa).

In the present study, the physicochemical characteristics of adrenal active renin from SHRSP and WKY rats were examined and compared with each other. No difference was found between the two strains of rats with respect to the molecular mass, immunoreactivity, and affinity for con A. Furthermore, all these properties of adrenal active renin are similar to those observed for active renin from the kidney and plasma. Thus, the increase in adrenal renin activity in SHRSP may be caused by the increased amount of active renin rather than the appearance of renin isoenzyme with higher enzymatic activity. This speculation is supported by the observation that the specific activity of adrenal renin

FIGURE 10. Scatterplots show relations between renin and angiotensin II levels in plasma (left panel) and adrenal gland (right panel) of 5-, 11-, 18-, and 25-week-old stroke-prone spontaneously hypertensive rats. Ang, angiotensin.
from 25-week-old SHRSP was similar to that of renal renin.

The regulating mechanism of adrenal angiotensin content remains to be elucidated. Husain et al.\(^8\) examined the effects of dehydration on rat adrenal capsular and decapsular angiotensin content and Ang II receptor binding activity and found that adrenal capsular angiotensin content of dehydrated rats is determined primarily by receptor-mediated uptake of circulating angiotensin rather than the local Ang II production. Autoradiography after injection of iodine-125–labeled Ang II\(^47,48\) showed that the labeled peptide is preferentially taken up by adrenal glomerulosa cells and medullary cells by endocytosis followed by transport into the lysosome, thereby supporting the notion that adrenal angiotensin is at least in part derived from the circulation. On the other hand, in vitro studies using adrenal capsular tissue\(^13,16\) or cultured dispersed glomerulosa cells\(^14,16\) provided evidence that glomerulosa cells can synthesize and secrete Ang II as well as renin. Kifor et al.\(^9\) have found that superfused rat adrenal capsules can release Ang II to a much higher level than the originally contained Ang II. Thus, adrenal angiotensin content is possibly affected by the local production as well as uptake of circulating angiotensin.

The present study revealed a weak correlation between plasma Ang II and adrenal Ang II of SHRSP, thereby indicating that the increase in adrenal angiotensin content of SHRSP cannot be explained only by an increased uptake of circulating Ang II. To further examine the contribution of circulating angiotensin to the adrenal angiotensin, we studied effects of bilateral nephrectomy on the adrenal capsular and decapsular angiotensin contents. Nephrectomy dramatically decreased plasma angiotensin and active renin, in agreement with reported data.\(^4\) Plasma prorenin was not detectable in either group of rats 24 hours after nephrectomy, as in the case of nephrectomized Wistar rats.\(^31,32\) Interestingly, in contrast to plasma angiotensin and renin, in both adrenal capsular and decapsular tissues of SHRSP and WKY rats, angiotensin and renin were not significantly decreased after nephrectomy. The capsular Ang II and III remained higher in SHRSP than in WKY rats after nephrectomy, although there was no difference in plasma angiotensin levels between nephrectomized SHRSP and WKY rats. Thus, the increased adrenal capsular Ang II in SHRSP may result in part from the enhanced local production of Ang II, which might be responsible for a higher plasma aldosterone in nephrectomized SHRSP compared with nephrectomized WKY rats. However, further study is needed to elucidate the regulating mechanism of adrenal angiotensin contents, because the rate of receptor-mediated internalization and degradation of Ang II may also affect adrenal Ang II levels.

It is established that renin is the limiting factor in the circulating renin-angiotensin system.\(^40\) In the present study, the close correlation between active renin and Ang II in the plasma of SHRSP (Figure 10) confirms that in SHRSP, renin is also the most important determinant of the circulating Ang II level. Conversely, the correlation between renin and Ang II in the adrenal gland of SHRSP was weak. The dissociation between adrenal angiotensin and renin was evident in 5- and 11-week-old SHRSP and 24-hour-nephrectomized SHRSP. In addition, investigations on subcellular fractions of the rat adrenal capsules\(^9\) and on localization of adrenal angiotensin converting enzyme\(^7\) and angiotensinogen\(^11\) showed that the adrenal contents of both angiotensin converting enzyme and angiotensinogen are much smaller than adrenal renin and are not detectable in zona glomerulosa cells. Therefore, renin may not be the limiting factor of the adrenal renin-angiotensin system.

In conclusion, the present study shows that the adrenal capsular Ang II content is increased in SHRSP with malignant hypertension. This may result in part from the enhanced local production of Ang II in the SHRSP. In addition, the rate of activation of prorenin and the ensuing release of active renin in the kidney of SHRSP are accelerated with the development of renal impairment.

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