Changes in Renal Angiotensin II Receptors in Spontaneously Hypertensive Rats by Early Treatment With the Angiotensin-Converting Enzyme Inhibitor Captopril

Jian-Nan Wu, David Edwards, Kathleen H. Berecek

Abstract We tested the hypothesis that in utero treatment with the angiotensin-converting enzyme inhibitor captopril could change the affinity, density, and/or subtypes of angiotensin II (Ang II) receptors in the kidneys of spontaneously hypertensive rats (SHR). Newborn, 7-day-old, and 4-month-old SHR and Wistar-Kyoto (WKY) rats were used. SHR and WKY rat breeders were treated with captopril (0.4 mg/mL, 100 mg/kg per day) in drinking water, and their pups were maintained on captopril treatment until experimentation. Control groups were untreated, age-matched SHR and WKY rats. The density, affinity, and subtypes of renal Ang II receptors were determined using radioligand binding techniques and receptor antagonists specific for Ang II receptor subtypes 1 and 2 (losartan, an AT₁-specific antagonist, and CGP 42112B, an AT₂-specific antagonist). AT₁ receptor density in kidneys was higher than AT₂ receptor density in both neonatal and adult rats. AT₁ receptor density in kidneys increased approximately twofold from birth to 7 days of age in all groups. Newborn and 7-day-old SHR showed significantly greater Ang II receptor densities in kidneys than other rat groups because of significantly greater densities of both AT₁ and AT₂ receptors. At 4 months of age, there were no significant differences in Ang II receptor densities in kidneys between captopril-treated and control SHR. Our data indicate that the expression of AT₁ and AT₂ receptors in kidneys is differentially regulated during development. Enhanced activity of the renal renin–Ang II system in newborn and probably fetal SHR may be involved in the pathogenesis of hypertension. The decrease in the density of kidney AT₁ receptors in newborn SHR after captopril treatment may play a role in the prevention of hypertension in this model. (Hypertension. 1994;23[part 2]:819-822.)

Key Words • angiotensin II • angiotensin-converting enzyme inhibition • captopril • renin-angiotensin system

Angiotensin II (Ang II) plays an important role in the homeostasis of blood pressure and fluid-electrolyte balance. Previous studies from our laboratory and others have shown that early treatment of young spontaneously hypertensive rats (SHR) with angiotensin-converting enzyme (ACE) inhibitors prevented the full expression of hypertension even after treatment had been stopped for 7 months. The permanent antihypertensive effect of ACE inhibitors administered to fetal and neonatal animals has been attributed to a reduction in brain renin-angiotensin system (RAS) activity, a remodeling of cardiovascular structures, and/or a change in sodium metabolism. Renin, angiotensinogen, and ACE mRNA levels have been demonstrated in rat kidney. Ang II receptors, predominantly type 1 (AT₁) in the adult, are widely distributed in kidney. Enhanced activity of the RAS in the kidney has been proposed to play a role in the pathogenesis and maintenance of hypertension. During the early phase of development, the activity of the renal RAS is markedly increased. Circulating renin and Ang II are also increased in newborn and young animals. Furthermore, renin and angiotensinogen gene expression in newborn SHR are significantly higher than in adult rats. Functional Ang II receptors have been demonstrated in kidney during fetal and neonatal life. Renal kidneys respond to changes in blood volume, participate in the regulation of arterial blood pressure, and contribute to fluid and electrolyte homeostasis. The RAS plays an important role in regulating blood pressure during fetal life, but its influence on renal hemodynamics and function appears later during development. These findings suggest that alterations in the activity of the renal RAS, including the density of Ang II receptors, in maturing and adult kidneys may contribute to the pathogenesis of hypertension in SHR. Furthermore, ACE inhibitors may produce their antihypertensive effects by a decrease in the sensitivity or number of Ang II receptors in the kidney.

The present study tested the hypothesis that early administration of captopril alters the affinity and/or density of Ang II receptors in neonatal and adult kidneys. The affinity and density of Ang II receptors in kidney were studied in newborn, 7-day-old, and 4-month-old SHR and Wistar-Kyoto (WKY) rats with the use of radioligand binding techniques. Ang II receptor subtypes in kidneys were also characterized in all groups with the use of nonpeptide antagonists specific for the AT₁ or AT₂ angiotensin receptor subtype.
Methods

Animal Preparation

Newborn, 7 day-old, and 4-month-old SHR and WKY rats, offspring of breeders obtained from Harlan Sprague Dawley Inc, were used. At 3 months of age, 10 mating pairs of SHR and WKY rats were given captopril (Bristol-Myers Squibb Pharmaceutical Research Institute) in their drinking water at a dose of 0.4 mg/mL (100 mg/kg per day). Pups were maintained on captopril treatment until experimentation (SHRCAP and WKYCAP). Control SHR and WKY rats (SHRCON and WKYCON) were given tap water. All rats were housed at constant temperature (24°C) and humidity (60±5%), with a 12-hour light/dark cycle. Standard laboratory rat chow was provided ad libitum.

Experimental Protocols

Pups were killed with an intraperitoneal injection of an overdose of sodium pentobarbital (Nembutal, Abbott Laboratories), and adult rats were killed by decapitation without prior anesthesia. Anesthesia markedly affects the release of hormones and peptide receptor binding properties. Rats were preconditioned to guillotine before death. Ang II receptors were characterized by standard radioligand binding techniques and Scatchard analysis of binding data. The kidneys were removed, wrapped with aluminum foil, and then immediately frozen in liquid nitrogen (~80°C). After weighing, the tissue was placed into a 20 mL/g volume of hypotonic phosphate buffer ([mmol/L] Na2HPO4 8.1, KH2PO4 1.46, NaCl 136, KCl 2.68, EDTA 5, pH 7.4) containing 1,10-phenanthroline (0.1 mg/mL) and aprotinin (0.3 trypsin inhibitory units/mL, pH 7.4), making the final concentration of the tissue 0.1 g/mL.

For the experiments, 100 μL of resuspended membranes was incubated with 400 μL reaction buffer in duplicate in 75×12 mm polypropylene tubes at 37°C for 60 minutes. Time-course studies had shown that the maximal binding plateau was achieved at 60 minutes. The final reaction mixture contained 0.1 to 10 nmol/L [3H-][Sar1,Ile8]Ang II (Pep tide Idonization Center, Washington State University, Pullman) and 0.1% heat-inactivated bovine serum albumin (BSA) in 1x phosphate-buffered saline. For estimation of the densities of AT, and AT2 receptors, losartan (DuP 753, a selective AT, receptor blocker) and CGP 42112B (a selective AT2 receptor blocker, trifluoroacetate salt of CGP 42112) were added to the final reaction solution in other sets of tubes. The final concentrations of losartan and CGP 42112B were 10⁻⁴ and 10⁻⁷ mol/L, respectively. Specific binding was determined by subtracting nonspecific binding from total binding. For determination of nonspecific binding, membrane samples were incubated in the same reaction buffer containing a 1000-fold excess of unlabeled Ang II (10⁻³ mol/L). For determination of the proportion of Ang II receptor subtypes, membrane samples were incubated in a reaction buffer containing 0.01 nmol/L to 10 μmol/L [3H][Sar1,Ile8]Ang II, losartan, or CGP 42112B. Separation of bound from free [3H]-labeled peptides was accomplished by vacuum filtration with the use of a Brandel Harvester with glass-fiber filters (FP-100 Whatman GF/B) preincubated with 0.1% heat-inactivated phosphate buffer. After filtration the filters were placed in test tubes and counted in a gamma counter. The final concentration of protein was determined by the Lowry method using BSA as a standard.

The proteins labeled [3H][Sar1,Ile8]Ang II during sample incubation was estimated using a gel filtration chromatographic assay. [3H][Sar1,Ile8]Ang II (0.08 nmol/mL) was incubated with kidney tissue under the same conditions used for the tissue receptor binding assay. After incubation at 37°C for 60 minutes, the reaction mixture was treated with the same volume of 0.5 mol/L acetic acid for 5 minutes. 125I-[Sar1,Ile8]Ang II (100 μL) that had been incubated with membranes or diluted in incubation buffer (control) was applied to a Sephadex A-25-100 column (0.5×15 cm) that had been previously equilibrated with isotonic phosphate buffer (pH 7.4). The column was eluted with elution buffer (5 mmol/L EDTA, 150 mmol/L NaCl, 0.1% BSA, pH 7.4) at a flow rate of 0.5 to 2.0 mL/min. Fractions of the column effluent were collected and counted in a gamma counter. The amount of degraded 125I-[Sar1,Ile8]Ang II was calculated by subtracting the percentage of the total radioactivity present in the Ang II peak fractions of the sample from the percentage present in the control fractions. The degradation of radioactive Ang II by the membranes was within reasonable limits. Less than 5% degradation of the radioisotope was found after incubation with kidney membranes.

Data Analysis

Data are expressed as mean±SEM. Dissociation constants (Kd) and maximal binding sites (Bmax) were estimated by LIGAND software (Elsevier-BIOSOFT) using Scatchard plots. The percentages of AT1 and AT2 receptors were estimated by the competitive binding curve-fitting program of INPLOT software (GraphPad Software Inc). The lower plateaus of the competition curves of losartan and CGP 42112B represented the percentage of AT1 receptors and the percentage of AT2 receptors, respectively. ANOVA with Newman-Keuls posttest procedure was used to evaluate whether there were significant differences in Ang II receptor affinity, density, and percentages of AT1 and AT2 receptors among the experimental groups. Statistical significance was assumed at a value of P<0.05.

Results

There were no significant differences in body weights, kidney weights, or kidney protein concentrations among the four groups of newborn and 7-day-old pups. Kidney weights (in grams) of newborn pups were SHRCON, 0.24±0.007; SHRCAP, 0.24±0.012; WKYCON, 0.21±0.016; and WKYCAP, 0.18±0.012. Kidney protein concentrations (in micrograms per milliliter) were SHRCON, 140±19; SHRCAP, 120±10; WKYCON, 139±11.6; and WKYCAP, 133±12. Kidney weights (in grams) of 1-week-old pups were SHRCON, 5.39±0.74; SHRCAP, 5.24±0.65; WKYCON, 5.62±0.29; WKYCAP, 5.50±0.33; and protein concentrations (in micrograms per milliliter) were SHRCON, 287±34; SHRCAP, 260±21; WKYCON, 275±25; and WKYCAP, 281±17. The average nose-to-rump lengths of neonatal WKYCON were significantly greater than those of SHRCAP, SHRCAP, and WKYCAP. No significant differences were found in body or kidney weights and protein concentrations among 4-month-old SHRCON, SHRCAP, and WKYCON rats. Kidney weights (in grams) of 4-month-old rats were SHRCON, 9.66±0.05; SHRCAP, 9.99±0.08; and WKYCON, 1.06±0.09. Protein concentrations (in micrograms per milliliter) were SHRCON, 701±39; SHRCAP, 671±45; and WKYCON, 760±48.

Nonspecific binding was less than 10% of the specific Ang II binding. The affinities (Kd) and densities (Bmax) of total, AT1, and AT2 receptors in each group were calculated from saturation binding curves using Scatchard analysis. The results of saturation binding experiments in kidney membranes are summarized in Table 1 (newborn and 7-day-old rats).

The estimated percentages of AT1 and AT2 receptors in kidneys are summarized in Table 2 for newborn and 7-day-old rats. Only one class of high-affinity binding sites
TABLE 1. $^{125}$I-[Sar$^1$,Ile$^8$]Angiotensin II Binding to Kidney Membranes of Control and Captopril-Treated WKY Rats and SHR at 1 and 7 Days of Age

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_d$, nmol/L</th>
<th>$B_{max}$, fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKYCON (n=6)</td>
<td>0.44±0.08</td>
<td>365.6±36.8$^*$</td>
</tr>
<tr>
<td>WKYCAP (n=4)</td>
<td>0.53±0.10</td>
<td>376.5±36.3$^*$</td>
</tr>
<tr>
<td>SHRCON (n=4)</td>
<td>0.43±0.05</td>
<td>569.1±43.2</td>
</tr>
<tr>
<td>SHRCAP (n=6)</td>
<td>0.38±0.07</td>
<td>407.3±44.5$^*$</td>
</tr>
<tr>
<td>7 Days of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKYCON (n=6)</td>
<td>0.76±0.12</td>
<td>528.9±66.8</td>
</tr>
<tr>
<td>WKYCAP (n=5)</td>
<td>0.82±0.22</td>
<td>459.7±78.8</td>
</tr>
<tr>
<td>SHRCON (n=6)</td>
<td>0.98±0.16</td>
<td>642.8±91.2</td>
</tr>
<tr>
<td>SHRCAP (n=4)</td>
<td>0.86±0.07</td>
<td>446.0±66.4</td>
</tr>
</tbody>
</table>

WKY indicates Wistar-Kyoto; SHR, spontaneously hypertensive rats; WKYCON, control WKY rats; WKYCAP, captopril-treated WKY rats; SHRCON, control SHR; and SHRCAP, captopril-treated SHR. Values are mean±SEM; n is number of experiments.

$^*$P<.05 compared with SHRCON by ANOVA with Newman-Keuls posttest procedure.

for $^{125}$I-[Sar$^1$,Ile$^8$]Ang II was found in the kidney for all rat groups. No significant differences in $K_d$ were found among the groups. SHRCON had a significantly greater density of total Ang II receptors in kidneys than the other groups because of a significantly greater density of AT$^1$ receptors (Table 1). The $K_d$ values of AT$^1$ and AT$^2$ receptors (not shown in the table) were not significantly different among groups. In general, AT$^1$ receptor density was higher than AT$^2$ receptor density in newborn SHRCON. This was not true in newborn WKYCON, in which the densities of AT$^1$ and AT$^2$ receptors were roughly equal. Newborn WKYCAP showed a lower percentage of AT$^1$ receptors in kidneys compared with SHRCON. Table 2 shows the densities of AT$^1$ and AT$^2$ receptors calculated by multiplying the percentages of each receptor subtype by the corresponding $B_{max}$. SHRCON showed significantly higher densities of both AT$^1$ and AT$^2$ receptors than all other groups.

In 7-day-old rat pups, SHRCON showed a larger number of total Ang II receptors than the other groups although this difference did not reach statistical significance (Table 2). Percentages of AT$^1$ receptors were higher than AT$^2$ receptors in all groups at 7 days of age (Table 2). The density of AT$^1$ receptors in kidneys of SHRCON was significantly greater than in all other groups.

Table 3 summarizes the affinities and densities of Ang II receptors in the kidneys of 4-month-old SHRCON. CGP 42112B did not displace Ang II binding, whereas losartan completely blocked the Ang II binding to kidney membranes derived from all groups, suggesting that Ang II receptors in the adult kidney are of the AT$^1$ subtype (data not shown). No significant differences in the affinities and densities of Ang II receptors in kidneys were found among all rat groups. However, SHRCAP showed fewer renal Ang II receptors than SHRCON.

**Discussion**

At least two Ang II receptor subtypes, AT$^1$ and AT$^2$, have been identified and characterized pharmacologically by binding affinity studies. In adult rats two subtypes of renal Ang II receptors other than AT$^1$ and

TABLE 2. Percentage of Angiotensin II Receptor Subtypes In Kidneys of Control and Captopril-Treated WKY Rats and SHR at 1 and 7 Days of Age

<table>
<thead>
<tr>
<th>Group</th>
<th>% of AT$^1$</th>
<th>% of AT$^2$</th>
<th>$B_{max}$, fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day of age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKYCON (n=6)</td>
<td>67.9±6.0</td>
<td>47.4±3.7</td>
<td>175.1±21.9$^*$</td>
</tr>
<tr>
<td>WKYCAP (n=4)</td>
<td>41.1±3.3$^*$</td>
<td>48.7±4.6</td>
<td>154.7±12.4$^*$</td>
</tr>
<tr>
<td>SHRCON (n=4)</td>
<td>60.3±2.3</td>
<td>44.2±4.4</td>
<td>343.2±13.0</td>
</tr>
<tr>
<td>SHRCAP (n=6)</td>
<td>59.2±6.3</td>
<td>39.7±2.8</td>
<td>241.1±25.7$^*$</td>
</tr>
<tr>
<td>7 Days of age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKYCON (n=6)</td>
<td>70.4±2.3</td>
<td>34.2±4.6</td>
<td>372.3±12.2$^*$</td>
</tr>
<tr>
<td>WKYCAP (n=5)</td>
<td>74.4±1.3</td>
<td>35.2±3.1</td>
<td>342.0±6.0$^*$</td>
</tr>
<tr>
<td>SHRCON (n=6)</td>
<td>72.9±1.6</td>
<td>30.0±4.3</td>
<td>468.6±10.3</td>
</tr>
<tr>
<td>SHRCAP (n=4)</td>
<td>80.4±1.2</td>
<td>25.8±5.1</td>
<td>358.6±5.4$^*$</td>
</tr>
</tbody>
</table>

WKY indicates Wistar-Kyoto; SHR, spontaneously hypertensive rats; AT$^1$ and AT$^2$, angiotensin receptor subtypes 1 and 2; WKYCON, control WKY rats; WKYCAP, captopril-treated WKY rats; SHRCON, control SHR; and SHRCAP, captopril-treated SHR. Values are mean±SEM; n is number of experiments.

$^*$P<.05, $^t$P<.01 compared with SHRCON by ANOVA with Newman-Keuls posttest procedure.

TABLE 3. $^{125}$I-[Sar$^1$,Ile$^8$]Angiotensin II Binding to Kidney Membranes of Control and Captopril-treated SHR and WKY Rats at 4 Months of Age

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_d$, nmol/L</th>
<th>$B_{max}$, fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKYCON (n=7)</td>
<td>1.4±0.08</td>
<td>223±42</td>
</tr>
<tr>
<td>SHRCON (n=11)</td>
<td>0.83±0.14</td>
<td>202±32</td>
</tr>
<tr>
<td>SHRCAP (n=7)</td>
<td>0.66±0.02</td>
<td>108±27</td>
</tr>
</tbody>
</table>

SHR indicates spontaneously hypertensive rats; WKY, Wistar-Kyoto; WKYCON, control WKY rats; SHRCON, control SHR; and SHRCAP, captopril-treated SHR. Values are mean±SEM; n is number of experiments. Data were analyzed by ANOVA with Newman-Keuls posttest procedure.

Discussion

At least two Ang II receptor subtypes, AT$^1$ and AT$^2$, have been identified and characterized pharmacologically by binding affinity studies. In adult rats two subtypes of renal Ang II receptors other than AT$^1$ and...
AT₂ were suggested because of distinct responses to different stimuli. One is the glomerular Ang II receptor, which is downregulated during sodium depletion and with high doses of Ang II infusion.²⁴,²⁵ The other is the Ang II receptor in tubular epithelia, which is upregulated during sodium depletion.²⁶ We found a single, high-affinity binding site for Ang II in the adult kidney with the use of Scatchard analysis. All radiolabeled Ang II binding was blocked by losartan but not unacted by CGP 42112B. Our data, along with the results of other studies, suggest that all of the Ang II receptors in adult kidney are of the AT₁ subtype.²⁷,²⁸

In newborn WKYCON and WKYCAP pups, approximately equal densities of AT₁ and AT₂ receptors were found in the kidneys, unlike other tissues. The densities of AT₁ receptors in kidney approximately doubled from birth to 7 days of age. These results are consistent with the previous observation that at 5 days of age the AT₁ receptor mRNA level is higher than the level at 1 day of age.₁⁴ AT₁ receptor densities in kidneys of newborn SHRCON were significantly higher than in other groups, suggesting that newborn SHR may have enhanced activity of the renal RAS. Our finding that administration of captopril to pregnant SHR lowered the density of renal AT₁ receptors in newborn SHR offspring toward the level found in WKYCON pups suggests that the decrease in the density of renal AT₁ receptors may contribute to the long-term antihypertensive effects of captopril treatment in SHR.

The physiological functions of AT₂ receptors remain unclear. The possible function of AT₂ receptors has been suggested by studies showing that the expression of AT₂ receptors is increased in association with tissue repair, a phenomenon that occurs in wound healing in the skin and neointima formation after vascular injury.²⁸,²⁹ In our study, only the developing kidney had AT₂ receptors, indicating that the function of AT₂ receptors may be involved in the development of the kidneys.

In summary, AT₁ and AT₂ receptors were observed in kidneys of neonatal SHR and WKY rats, and both appear to be regulated differentially during growth. A higher density of renal AT₁ receptors in SHR suggests that an enhanced activity of renal RAS may be involved in the pathogenesis of hypertension in SHR. Captopril treatment alters the expression of renal Ang II receptors in newborn SHR, suggesting that a decrease in the activity of renal RAS may be involved in the long-term antihypertensive effects of early treatment with ACE inhibitors in SHR.

Acknowledgments

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References

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