Abnormal Regulation of Adrenal Angiotensin II Receptors in Spontaneously Hypertensive Rats

BARTON BRADSHAW AND THOMAS J. MOORE

SUMMARY The aldosterone response to angiotensin II is blunted in spontaneously hypertensive rats (SHR). To determine whether this blunting is due to a defect in angiotensin II receptors, we assessed angiotensin II binding to intact adrenal glomerulosa cells in SHR and normotensive Wistar-Kyoto rats (WKY) that had been fed high or low sodium diets before sacrifice. In rats on high salt intake, we observed no difference between the two strains in either receptor affinity ($K_d = 1.0-1.2 \text{ nM}$) or binding capacity (36,000-38,000 receptors/cell). When sodium-restricted, WKY increased receptor content more than fourfold to 167,000 sites/cell. SHR increased receptor number to only 103,000 sites/cell, which was significantly ($p<0.01$) less than the WKY increase. The cause of the abnormal receptor regulation remains unclear. Two known receptor regulators, the plasma angiotensin II level and the state of potassium balance, were similar in the two strains. Our results suggest that the blunted aldosterone response to angiotensin previously reported in SHR is due to abnormal angiotensin receptor up-regulation in the adrenal gland in response to sodium restriction. (Hypertension 11: 49-54, 1988)

KEY WORDS adrenal gland • angiotensin II receptors • spontaneously hypertensive rats • hypertension

ANGIOTENSIN II (Ang II) is a major stimulator of aldosterone production, and the level of sodium intake regulates the degree of aldosterone responsiveness to Ang II: Sodium restriction increases and sodium loading decreases the aldosterone response. In the Aoki-Okamoto strain of spontaneously hypertensive rats (SHR), this sodium regulation of adrenal sensitivity is defective, preventing the expected increased aldosterone response during sodium restriction. In the normal rat, sodium-regulated shifts in adrenal sensitivity are due, at least in part, to parallel shifts in the number of Ang II receptors on adrenal glomerulosa cells. Since SHR are unable to increase adrenal sensitivity, we postulated that these animals are unable to regulate their adrenal Ang II receptors appropriately. In the present studies, we evaluated the ability of the SHR to change glomerulosa cell Ang II receptors during changes in sodium intake, as well as the factors known to regulate adrenal receptors.

Materials and Methods

Male Aoki-Okamoto SHR and normotensive Wistar-Kyoto rats (WKY) were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA). Diets (Ralston Purina, St. Louis, MO, USA) contained either 1.6% sodium (high salt) or 0.05% sodium (low salt). Both diets contained 1.1% potassium. [125I]monoiodo-Ang II (activity, 2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Unlabeled Ang II and DNase were obtained from Sigma Chemical (St. Louis, MO, USA). Essential and nonessential amino acid mixtures were obtained from Gibco (Grand Island, NY, USA). Crude collagenase (Type 1; specific activity, 149 u/mg) was purchased from Cooper Biomedical (Malvern, PA, USA). Dibutyl phthalate and phenylmethylsulfonyl fluoride (PMSF) were obtained from Aldrich Chemical (Milwaukee, WI, USA), while dinonyl phthalate was purchased from Eastman Kodak (Rochester, NY, USA).
USA). Reagents for Krebs-Ringer buffer solution were obtained from Fisher Scientific (Medford, MA, USA). Bovine serum albumin was obtained from Miles Laboratory (Naperville, IL, USA).

Experimental Design

Renin and Aldosterone Levels in SHR and WKY

Although the blunted aldosterone response to Ang II has been documented in several previous studies, we reassessed plasma renin activity (PRA) and plasma aldosterone levels as well as the aldosterone response to Ang II in vitro to document that there had been no change in the defect in SHR since those earlier studies.

Ten to 12 rats of each strain (11 weeks of age) were maintained in individual metabolic cages on either a high or low sodium diet and were given distilled water with 5% dextrose ad libitum. After 5 days on the diets, animals were killed by decapitation and blood from the trunk was collected in cold test tubes containing 15 mg of EDTA. Samples were stored on ice for no longer than 15 minutes before centrifugation at 4°C for 15 minutes at 1200 g. Plasma for PRA and aldosterone assay was quick frozen in acetone/dry ice and stored at −20°C until assayed.

To assess in vitro aldosterone responsiveness to angiotensin, the adrenal glands of these animals were removed and pooled and glomerulosa cell suspensions were prepared as described in a subsequent section. Glomerulosa cells for this single experiment were then incubated in a modified Krebs-Ringer bicarbonate solution containing 1.25 mM calcium, 0.1% bovine serum albumin, 2% glucose, 0.19% l-glutamine, 13.1 ml/L each of an essential and a nonessential amino acid mixture. Cells (150,000–200,000/aliquot) were incubated in either the presence or absence of 10^{-8} M Ang II for 1 hour at 37°C in an atmosphere of 95% O₂, 5% CO₂ in a final medium volume of 0.5 ml. Aldosterone in these incubates was assayed in duplicate and reported as nanograms per 10^6 cells.

Adrenal Ang II Receptor Regulation

To assess adrenal Ang II receptor regulation, SHR and WKY (10–11 weeks of age) were again maintained for 5 days in individual metabolic cages on either a high or low sodium diet. Insofar as possible, adrenal receptors were assayed simultaneously in animals of both strains that had been maintained, killed, and studied under identical experimental conditions. Metabolic balance was assessed the day before death by measuring 24-hour urinary sodium, potassium, and creatinine excretion. The animals were killed by decapitation, the adrenals removed, and the capsules collected. For receptor studies, the adrenal capsules of five to seven animals of each strain were pooled, glomerulosa cells prepared (see the next section), binding assayed in duplicate, and the results considered as one experiment. Blood from the trunks of these animals was collected for plasma Ang II levels in cold test tubes containing 7.2 mg of EDTA, and plasma was quick frozen in tubes containing 15 μl of 15% PMSF and stored at −20°C until assayed. Values for urinary electrolyte excretion and plasma Ang II levels were obtained from individual animals and results presented as the mean for each strain on each diet.

Preparation of Glomerulosa Cell Suspension

Techniques for preparation of dispersed rat adrenal glomerulosa cell suspensions have been described previously.4 Adrenal capsules were placed in iced, modified Krebs-Ringer bicarbonate solution containing 4% bovine serum albumin, 2% glucose, 0.19% l-glutamine, and 13.1 ml/L each of an essential and a nonessential amino acid mixture (KRBA). The capsules were then digested with collagenase (3.75 mg/ml) and DNase (0.05 mg/ml) for 50 minutes at 37°C in a shaking incubator. After the collagenase incubation, the cellular material was strained through nylon mesh and centrifuged at 120 g for 10 minutes, and the cell pellet was washed twice with fresh KRBA. The pellet was then resuspended in KRBA, and the cell number was determined by light microscopy using a Riechert hemacytometer. Cell viability (by trypan blue exclusion) using these preparative methods is approximately 90%.

Ang II Receptor Assay

Receptor binding was assayed by incubating 400-μl aliquots of the glomerulosa cell suspension (adjusted to contain 100,000 cells) with tracer amounts of [^{125}I]Ang II (final concentration, 20 pM) and with various concentrations (1 × 10^{-10} M to 1 × 10^{-6} M) of unlabelled Ang II (final volume, 500 μl). Cells were incubated at 22°C for 90 minutes in a shaking water bath. To separate bound from free hormone, 200-μl duplicate aliquots of the incubates were microcentrifuged (Microfuge B, Beckman Instruments, Irvine, CA, USA) for 90 seconds through mixture of a 1-dimethyl phthalate 5-dibutyl phthalate oils in 400-μl microfuge tubes. The cell pellet was cut off and counted as bound, the supernatant was counted as free in a Packard gamma counter (Rockville, MD, USA). Binding data are reported as specific binding of Ang II after subtraction of nonspecific binding (i.e., binding observed in the presence of excess [10^{-4} M] unlabeled Ang II). Under these conditions, specific binding of 20 pm [^{125}I] Ang II ranged from 2.5 to 5.6% of total tracer on the low salt diet and from 0.8 to 2.2% on the high salt diet; nonspecific binding was always less than 0.2% of total tracer added.

Other Assays

Urinary electrolyte concentrations were measured by direct potentiometry with an ion-selective electrode (Nova Analyzer I, Nova Biomedical, Waltham, MA, USA), and creatinine was measured on a Beckman creatinine analyzer (Model II, Somerset, NJ, USA). Plasma Ang II and PRA were measured by radioimmunoassay as previously reported.41 Our Ang II antiserum shows 0.03% cross-reactivity with angiotensin I but recognizes Ang II and des-Asp Ang II (angiotensin III; Ang III) equally. Aldosterone from plasma samples and from acutely stimulated isolated glomerulosa
cells was assayed by the Coat-a-count assay kit (Diagnostic Products, Los Angeles, CA, USA). Aldosterone levels using this assay correspond well with levels using our previous assay. Its intra-assay and interassay coefficients of variation are less than 8% and less than 10%, respectively, and the lower limit of detectability is 1.6 ng/dl.

**Data Analysis**

Data are presented as means ± SEM. For receptor analysis, affinity and number were estimated from binding data analyzed using the curve-fitting program LIGAND. Differences in binding between diets and between strains were assessed using the Fisher exact test. Urinary sodium and potassium excretion were normalized per milligram of creatinine excreted to allow for any differences in completeness of urine collection and variation in body mass. Urinary electrolytes, creatinine, PRA, plasma Ang II, and plasma aldosterone levels were determined on individual animals and presented as the mean of each strain on each diet. Differences in these parameters were assessed using Student's t test for unpaired data.

**Results**

**Renin and Aldosterone Levels in SHR and WKY**

PRA, plasma aldosterone levels, and in vitro aldosterone production from both strains on each diet are shown in Table 1. In sodium-restricted animals of both strains, PRA and plasma aldosterone levels were higher than those in sodium-loaded animals. PRA levels were similar in SHR and WKY on both diets. In animals on the high salt diet, plasma aldosterone and in vitro aldosterone production did not differ significantly between strains. However, in sodium-restricted animals, SHR tended to have lower plasma aldosterone levels than WKY (122 ± 33 vs 162 ± 27 ng/dl; p < 0.1, > 0.05). Furthermore, in vitro aldosterone production in cells from sodium-restricted animals was lower in SHR than in WKY. These data confirm the presence in SHR of a defect in aldosterone production that was reported earlier.

**Table 1. Plasma Renin, Aldosterone, and In Vitro Aldosterone Stimulation in WKY and SHR**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low salt</th>
<th>High salt</th>
<th>Low salt</th>
<th>High salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA (ng Ang I/ml hr)*</td>
<td>8.6 ± 0.6</td>
<td>9.5 ± 0.1</td>
<td>5.5 ± 0.6</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>Plasma aldosterone* (ng/dl)</td>
<td>162 ± 27</td>
<td>122 ± 33</td>
<td>17 ± 3</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Aldosterone with 10−9 M Ang II (ng/106 cells/hr)</td>
<td>69.1</td>
<td>7.5</td>
<td>12.4</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Ang I = angiotensin I.  
*Data represent the means ± SEM of values from 10 to 12 rats of each strain on each diet.  
†Results of a single experiment using adrenal capsules of 10 to 12 rats of each strain on each diet.

**Adrenal Ang II Receptor Regulation**

The urinary electrolyte excretion and plasma Ang II levels for both rat strains on both diets are displayed in Table 2. On the day before death, SHR and WKY showed no differences in sodium or potassium excretion. In addition, urine was collected daily in six animals of each strain during the 5 days of low sodium intake. This cumulative potassium excretion was similar in SHR and WKY, confirming equivalent potassium balance in the sodium-restricted state. Normalized per milligram of creatinine excreted, the total potassium excretion was 2280 ± 170 μEq/rat/5 days in SHR versus 2130 ± 190 μEq/rat/5 days in WKY. The plasma Ang II level was slightly higher in SHR than WKY on both diets, but the differences were not significant.

The affinity of the receptor was very similar in both SHR and WKY and was unaffected by sodium intake: WKY high salt Kd = 0.9 ± 0.2 nM (n = 5 experiments), WKY low salt Kd = 1.2 ± 0.1 nM (n = 7); SHR high salt Kd = 0.8 ± 0.2 nM (n = 4), SHR low salt Kd = 1.2 ± 0.1 nM (n = 7). In all cases, Scatchard plots were linear and computerized analysis (LIGAND) indicated a receptor with a single affinity state (Figure 1).

On the high salt intake, the numbers of receptors on SHR and WKY adrenal cells were nearly identical (38,000 ± 13,000 vs 36,000 ± 9,000 cells). Similar to what has been reported for Sprague-Dawley rats, both SHR and WKY increased the number of adrenal Ang II receptors during sodium restriction (see Figure 1; Figure 2). The degree of up-regulation was quite different in the two strains, however. WKY displayed a fourfold increase in receptor number (167,000 ± 16,000 sites/cell), whereas SHR showed only a 2.5-fold increase (103,000 ± 9,000 sites/cell). Thus, although both strains significantly increased receptor number with sodium restriction (p < 0.01 for both), SHR displayed significantly fewer receptors than WKY on the low salt diet (p < 0.01).

**Discussion**

Studies in several species have shown that sodium restriction increases the aldosterone response to Ang II stimulation. More recent work has suggested a possible mechanism for this effect: Sodium restriction increases the number of angiotensin receptors on adrenal glomerulosa cells in the rat. SHR are unable to increase aldosterone responsiveness during sodium restriction, and we postulated that this defect may be due to an inability to up-regulate adrenal angiotensin receptors.

In our studies of plasma renin and aldosterone and the in vitro aldosterone response to Ang II, we confirmed the blunted aldosterone response exhibited by sodium-restricted SHR. Thus, this defect appears to be stable in this strain over the 5 years since it was originally demonstrated. The actual levels of plasma renin and aldosterone as well as the aldosterone response in vitro in our low salt studies are somewhat lower than those in some previous reports. We believe this is
TABLE 2. Metabolic Balance Data and Plasma Ang II Levels in WKY and SHR

<table>
<thead>
<tr>
<th>Variable</th>
<th>WKY Low salt</th>
<th>SHR Low salt</th>
<th>WKY High salt</th>
<th>SHR High salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (ml/day)</td>
<td>30.7 ± 4.0</td>
<td>25.4 ± 4.1</td>
<td>45.2 ± 4.1</td>
<td>41.7 ± 5.3</td>
</tr>
<tr>
<td>Creatinine (mg/day)</td>
<td>4.8 ± 0.1</td>
<td>5.5 ± 0.2</td>
<td>5.5 ± 0.1</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>( U_{Na^+} ) (\mu Eq/mg creatinine)</td>
<td>4.1 ± 0.4</td>
<td>4.7 ± 0.4</td>
<td>1225 ± 37</td>
<td>1292 ± 50</td>
</tr>
<tr>
<td>( U_{K^+} ) (\mu Eq/mg creatinine)</td>
<td>45 ± 12</td>
<td>443 ± 15</td>
<td>479 ± 13</td>
<td>504 ± 21</td>
</tr>
<tr>
<td>Plasma Ang II (pg/ml)</td>
<td>92 ± 12</td>
<td>101 ± 17</td>
<td>35 ± 11</td>
<td>58 ± 14</td>
</tr>
<tr>
<td>Ang II receptor affinity ( K_d ) (nM)</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

Urinary values and plasma Ang II levels are means ± SEM of data from individual animals \((n = 32–38\) for low salt values and \(n = 21–26\) for high salt values). \( U_{Na^+} \) = urinary sodium excretion; \( U_{K^+} \) = urinary potassium excretion.

largely due to our use of 5 days of diet preparation as compared with 14 days in other studies.

Our receptor results indicate that the blunted aldosterone response could be due to blunted up-regulation of the SHR Ang II receptor. On liberal sodium intake, where aldosterone responsiveness is similar in SHR and WKY, both strains displayed the same number and affinity of adrenal receptors. When sodium-restricted, however, SHR failed to up-regulate receptors as effectively and displayed only 60% as many receptors as did WKY, paralleling their defect in aldosterone response.

In one previous report, Shimada and Fanburg\(^14\) also noted reduced angiotensin binding capacity in SHR adrenal glands. However, their studies were performed in animals on only normal sodium intake (0.4% sodium), and they assessed binding to crude homogenates of the entire adrenal gland. Since angiotensin receptors have been found in the adrenal medulla as well as on fasciculata-reticularis cells,\(^13\)\(^19\) binding studies using whole adrenal glands do not give a specific estimate of receptors on the aldosterone-producing glomerulosa cells. Our current results extend the observations of Shimada and Fanburg\(^14\) by demonstrating both that Ang II receptors are reduced specifically on glomerulosa cells and that the receptor defect in the SHR is one of abnormal regulation: receptor number is normal on high sodium intake but fails to up-regulate during sodium restriction.

**Figure 1.** Scatchard plots of Ang II binding to adrenal glomerulosa cells in a representative experiment. For each binding experiment, adrenal cells were pooled from five to seven animals of each strain (SHR or WKY) on each diet. Receptor affinity was similar in both strains, but SHR fed the low salt diet displayed fewer receptors.

**Figure 2.** Comparison of Ang II binding capacities (presented as the number of receptors per cell) in SHR and WKY fed high and low salt diets. Each point represents the result of a single experiment (an example of one such experiment is shown in Figure 1). The average of each group is shown, with the SEM as the index of dispersion. The two strains were similar on the high salt diet, but SHR fed the low salt diet displayed fewer receptors \((p<0.01)\).
The cause of the defect in receptor regulation remains unclear. The level of Ang II in plasma appears to regulate adrenal receptor number. Increasing the angiotensin level, either by sodium restriction or by continuous low-dose infusion by means of a minipump,\textsuperscript{2, 10, 20-23} increases the number of adrenal receptors, while reducing the plasma level (sodium-loading or inhibiting angiotensin converting enzyme)\textsuperscript{3-10, 24, 25} reduces angiotensin receptors. In our experiments, however, there were no significant differences between the PRA or plasma Ang II level of SHR and WKY on either high or low salt intake. In fact, Ang II levels tended to be slightly higher in SHR on both diets. Since higher levels should tend to increase receptor number, this difference does not explain the observed reduction in SHR adrenal binding. Since our Ang II assay recognizes Ang II and Ang III equally, we were unable to detect any differences between strains in the ratio of Ang II to Ang III. It is therefore theoretically possible that the altered up-regulation of SHR receptors may be due to some difference in their Ang II/Ang III ratio during sodium restriction. Little is known about the importance of Ang III as a receptor agonist. The detailed mechanism is not clear. We are not aware of any studies on Ang III in SHR and WKY. Consequently, this theoretical explanation of our data seems unlikely to us. Differences in potassium balance can also affect Ang II receptors. Potassium loading increases and potassium depletion reduces adrenal Ang II receptor number.\textsuperscript{2} Potassium loading also reduces receptor affinity for angiotensin.\textsuperscript{26} In our studies, urinary potassium excretion was similar in both strains on both diets; suggesting comparable states of potassium balance. This finding plus the fact that we observed no differences in receptor affinity indicate that differences in potassium balance do not account for the reduced receptor number in SHR.

One other potential regulator of angiotensin receptors deserves mention. Beyond their presence in plasma, all elements of the renin–angiotensin system are present in the adrenal gland.\textsuperscript{3} In the rat, the adrenal gland’s renin content (per gram of tissue) is inversely related to sodium intake.\textsuperscript{29} Although the angiotensin level, either by sodium restriction or by continuous low-dose infusion by means of a minipump,\textsuperscript{2, 10, 20-23} increases the number of adrenal receptors, while reducing the plasma level (sodium-loading or inhibiting angiotensin converting enzyme)\textsuperscript{3-10, 24, 25} reduces angiotensin receptors. In our experiments, however, there were no significant differences between the PRA or plasma Ang II level of SHR and WKY on either high or low salt intake. In fact, Ang II levels tended to be slightly higher in SHR on both diets. Since higher levels should tend to increase receptor number, this difference does not explain the observed reduction in SHR adrenal binding. Since our Ang II assay recognizes Ang II and Ang III equally, we were unable to detect any differences between strains in the ratio of Ang II to Ang III. It is therefore theoretically possible that the altered up-regulation of SHR receptors may be due to some difference in their Ang II/Ang III ratio during sodium restriction. Little is known about the importance of Ang III as a receptor agonist. The detailed mechanism is not clear. We are not aware of any studies on Ang III in SHR and WKY. Consequently, this theoretical explanation of our data seems unlikely to us. Differences in potassium balance can also affect Ang II receptors. Potassium loading increases and potassium depletion reduces adrenal Ang II receptor number.\textsuperscript{2} Potassium loading also reduces receptor affinity for angiotensin.\textsuperscript{26} In our studies, urinary potassium excretion was similar in both strains on both diets; suggesting comparable states of potassium balance. This finding plus the fact that we observed no differences in receptor affinity indicate that differences in potassium balance do not account for the reduced receptor number in SHR.

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