Differential Regulation of Angiotensin II Receptor Subtypes in the Adrenal Gland
Role of Aldosterone

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Abstract—It has been shown that aldosterone potentiates the action of angiotensin II (Ang II) in cultured rat vascular smooth muscle cells solely by increasing the number of Ang II receptors. The mechanisms responsible for aldosterone–Ang II interactions in the adrenal gland are unknown. The present study was designed to investigate the effect of aldosterone on expression of Ang II receptor subtypes (AT₁ and AT₂) in the adrenal gland. Seven-week-old male Wistar rats were treated for 2 weeks with either aldosterone (0.05 µg/h, n=14) or vehicle (n=14) by use of implanted osmotic minipumps. Systolic blood pressure was not altered by aldosterone treatment. Plasma aldosterone levels were higher in aldosterone-treated rats (181±53 pg/mL) compared with vehicle-treated rats (33±21 pg/mL, P<0.05). Northern blot analysis and radioligand binding assay showed that adrenal AT₁ mRNA levels and AT₁ receptor density in aldosterone-treated rats were not statistically different from those of vehicle-treated rats. However, immunohistochemical studies showed that the highest adrenal AT₁ receptor expression was shifted from the zona glomerulosa to the zona fasciculata after aldosterone treatment. In contrast, adrenal AT₂ mRNA and AT₂ receptor density in aldosterone-treated rats were decreased by approximately 50% and 40%, respectively, compared with vehicle-treated rats (P<0.05). Aldosterone-induced decrease in adrenal AT₂ receptor expression occurred mainly in the medulla. Thus, aldosterone differentially modulates the expression of AT₁ and AT₂ receptors in the adrenal gland. Although the function of the AT₂ receptor in the adrenal gland is largely unknown, our data indicate that aldosterone may modulate the effect of Ang II by altering the location of AT₁ receptors and by reducing the number of AT₂ receptors in the adrenal gland. (Hypertension. 1998;32:65-70.)

Key Words: aldosterone ■ receptors, angiotensin II ■ adrenal gland ■ Northern blot ■ radioligand assay ■ immunohistochemistry

The renin-angiotensin-aldosterone system is one of the most potent systems that regulate blood pressure, electrolyte balance, and extracellular fluid volume. The effects of the principal substances of this system, angiotensin II (Ang II) and aldosterone, are triggered by their interaction with specific receptors in a variety of tissues. In the adrenal gland, Ang II receptors are classified into two major subtypes: AT₁ and AT₂. The AT₁ receptor subtype accounts for 80% of the Ang II receptors in the rat and bovine adrenal gland, with the other 20% being the AT₂ receptor subtype.¹² It has been shown that Ang II–induced aldosterone production is mediated by activation of the AT₁ receptor in the adrenal zona glomerulosa cells.³⁴ Although the physiological role of the AT₂ receptor in the adrenal gland is largely unknown, it has been reported that Ang II stimulates secretion of endogenous ouabain from bovine adrenocortical cells via activation of the AT₂ receptor.⁷

Several lines of evidence have shown that modulation of the expression of the Ang II receptor is important in the regulation of Ang II action.⁶⁷ We have recently shown that sodium deficiency increases the expression of genes encoding AT₁ receptor subtypes and elevates the AT₁ receptor density in the adrenal gland.⁸ The increase in AT₁ receptor density in the adrenal gland during sodium restriction may contribute to increased aldosterone production induced by Ang II. In addition, blockade of the binding of Ang II to the AT₁ receptor by losartan prevents the increase in mRNA expression of AT₁ receptor subtypes and AT₁ receptor density induced by sodium depletion, suggesting that these changes in the adrenal gland are mediated by activation of the renin-angiotensin system accompanying low sodium intake.⁸ Although it is known that sodium restriction also increases aldosterone production, and that aldosterone increases Ang II receptor density in cultured rat vascular smooth muscle cells,⁹¹⁰ the effects of aldosterone on expression of Ang II receptor subtypes in the adrenal gland have not been defined. Therefore, in the present study, we used a combination of Northern blot, radioligand binding, and immunohistochemistry to test the hypothesis that infusion of aldosterone differentially regulates the expression of AT₁ and AT₂ receptors in the adrenal gland.
Methods

Animals and Treatment Groups
Seven-week-old male Wistar rats (Charles River Laboratories, Wilmington, Mass) weighing between 150 and 175 g were randomly divided into two groups: group 1 (Ald) was subcutaneously infused with aldosterone at 0.05 μg/h in saline plus 15% ethanol alcohol (n = 14), and group 2 (Con) was subcutaneously infused with vehicle (saline plus 15% ethanol alcohol, n = 14). It has been shown that aldosterone infused at 0.05 μg/h subcutaneously does not change systolic blood pressure in rats. All the rats were anesthetized with a single intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (12 mg/kg). Alzet miniosmotic pumps (model 2002, Alza Corp) with a capacity of 226±6 μL and infusion rate of 0.5±0.02 μL/h were filled with either 0.9% saline plus 15% ethanol alcohol or aldosterone in 0.9% saline plus 15% ethanol alcohol at a concentration that was adjusted depending on the weight of the rat. The pumps were implanted subcutaneously between the scapulae. Sterile technique was used, and the rats were given penicillin G (10 000 U IM) after the surgery. The rats were housed in pairs, fed regular food and tap water ad libitum, and maintained on a 12-hour light/dark cycle for a total of 14 days.

Systolic Blood Pressure
Indirect tail-cuff blood pressure measurements were routinely obtained in all rats to assess the blood pressure using a Narco Bio-Systems Electro-Sphygmomanometer. The pressures were measured in conscious rats every 3 days for 14 days, beginning 1 day before the placement of the minipumps. The blood pressure value for each rat was calculated as the average of 5 separated measurements at each session.

Plasma Aldosterone Levels
The rats were decapitated and bled into chilled EDTA tubes. Blood samples were centrifuged at low temperatures (4°C) to obtain plasma for aldosterone assay. Plasma aldosterone concentration was then determined by using a commercially available radioimmunoassay kit for aldosterone (Diagnostic Products Corp).

Tissue Preparation
To obtain tissue for Northern blot and radioligand binding assay, a midline abdominal incision was made, and adrenal glands were promptly removed, frozen in liquid nitrogen, and stored at -80°C. Adrenal glands from 7 rats in each group were used for RNA extraction for Northern blot analysis, and adrenal tissues from the other 7 rats were used for the radioligand binding assay.

AT1 and AT2 cDNA and Probe Preparation
cDNA probes were prepared as described previously. Briefly, a 0.8-kb fragment (178 to +562) from the coding region of rat AT1\textsubscript{a} cDNA\textsuperscript{14} (a generous gift from Dr Tadaaki Inagami, Vanderbilt University, Nashville, Tenn) was used as a template to make AT\textsubscript{1} probes. Because this fragment contains the AT\textsubscript{1}α coding region where AT\textsubscript{1}α and AT\textsubscript{1β} cDNAs exhibit high nucleotide sequence identity, these probes detect both AT\textsubscript{1}α and AT\textsubscript{1β}. A 1.23-kb fragment (+16 to +1249) from the coding region of rat AT\textsubscript{2} cDNA\textsuperscript{15} was used as a template to make AT\textsubscript{2} probes. The probes were labeled with \textsuperscript{32}P-dCTP using a multiprimer DNA labeling system (Amersham Co) to a specific activity of 3×10\textsuperscript{6} cpm/μg. The labeled probes were separated from unincorporated nucleotides by Mini-Spin G-50 DNA purification spin columns (Worthington Biochemical).

RNA Extraction and Northern Blots
Total RNA of adrenal gland was extracted using the guanidine thiocyanate-phenol-chloroform extraction protocol.\textsuperscript{16} Electrophoresis of 20 μg denatured RNA from each preparation was carried out in a 1% agarose gel containing 2.2 mol/L formaldehyde. RNA was transferred to a positively charged nylon membrane (Fisher Co). The membrane was baked at 80°C for 2 hours in a vacuum oven (Fisher Co). After prehybridization for 5 hours at 42°C in 50% deionized formamide, 5× Denhardt’s solution, 5× SSC, 0.5% SDS, and 200 μg/mL denatured salmon sperm DNA, the membrane was hybridized with the \textsuperscript{32}P-labeled probes for 18 to 20 hours at 42°C. The blot was then washed successively in 2×, 1×, and 0.5× SSC (two times, 10 minutes each) containing 0.1% SDS at 65°C. To control for differences in RNA loading, Northern blots were incubated at 90°C for 10 minutes in 20 mmol/L Tris-HCl (pH 8.0) to strip off the cDNA probes and rehybridized with \textsuperscript{32}P-labeled probe for 18S rRNA. Blots were exposed to XAR-5 x-ray film (Eastman Kodak Co) with two intensifying screens. Autoradiographic signals were scanned with a laser densitometer (Ultrascan XL, Pharmacia). Results of relative gene expression are expressed as the ratios of AT\textsubscript{1}mRNA and AT\textsubscript{2} mRNA to 18S rRNA.

Ligand Binding Assay
Whole adrenal tissues were homogenized in hypotonic buffer (20 mmol/L sodium phosphate, pH 7.1 to 7.2). Homogenates were then centrifuged at 48 000g for 20 minutes at 4°C. Cell membranes were resuspended in assay buffer (50 mmol/L sodium phosphate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L bacitracin, pH 7.2) and recentrifuged. After resuspension in assay buffer, an aliquot of the cell membrane suspension was used for protein assay using a modified Bradford method (Bio-Rad).\textsuperscript{17} To measure the AT\textsubscript{1} receptor density, 20 μg protein was incubated with 125 pmol/L to 2 mmol/L \textsuperscript{125}I-[Sar\textsuperscript{10},Ile\textsuperscript{11}]Ang II (kindly provided by Dr Robert C. Speth, Washington State University) in a final volume of 200 μL assay buffer containing 0.1% bovine serum albumin in the presence or absence of the specific AT\textsubscript{1} receptor antagonist PD123319 (10 μmol/L).\textsuperscript{2} To measure the AT\textsubscript{2} receptor density, \textsuperscript{125}I-[Sar\textsuperscript{10},Ile\textsuperscript{11}]Ang II was used in the presence or absence of the specific AT\textsubscript{2} receptor antagonist losartan (10 μmol/L). Nonspecific binding was measured in the presence of 1 μmol/L unlabeled Ang II. Binding assays were performed for 120 minutes at room temperature and followed by immediate filtration through glass-fiber filters (Whatman GF/C). The filter-bound radioactivity was counted in a gamma spectrometer (Beckman, LS 3801). Receptor affinity and concentration were calculated by Scatchard analysis using GraphPAD InStat software.

Immunohistochemistry
Immunohistochemistry was performed as described previously. Briefly, the adrenal gland was fixed in 4% paraformaldehyde−0.1 mol/L phosphate buffer, pH 7.4, at 4°C overnight and was embedded in paraffin. Tissue sections (5 μm) were cut and mounted on slides. The endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol, and nonspecific binding sites of secondary goat antibody were blocked with 3% normal goat serum and 1% nonfat dry milk in PBS. The sections were sequentially incubated at room temperature with primary antibody diluted in 1.5% normal goat serum and 0.5% nonfat dry milk in PBS [1:100 for AT\textsubscript{1} receptor antibody (Santa Cruz Biotechnologies Inc) and 1:100 for AT\textsubscript{2} receptor antibody (a generous gift of Dr Robert M. Carey, University of Virginia Health Sciences Center, Charlottesville)], biotinylated antibody, and avidin-peroxidase (ABC kit, Vector Laboratories). Staining was visualized with diaminobenzidine (Fast DAB tablets, Sigma). Slides were counterstained with hematoxylin.

Statistical Analysis
Results are expressed as mean±SE. The data were analyzed by either unpaired Student’s t test (between 2 groups) or 2-way ANOVA followed by Tukey-Kramer multiple comparison test (for multiple groups). Differences were considered statistically significant at \( P<0.05 \).

Results
There was no significant difference in body weight between the two groups of rats at the end of the experiment (Con, 243±3 g; Ald, 240±4 g). Systolic blood pressures were not modified by the infusion of aldosterone compared with vehicle (Con, 112±6 mm Hg; Ald, 107±4 mm Hg at day 1;
Con, 120±3 mm Hg; Ald, 125±2 mm Hg at the end of the experiment).

Plasma aldosterone levels were measured in each of the two groups to evaluate the effectiveness of aldosterone administration. Plasma aldosterone levels were approximately 5 times higher in aldosterone-infused rats (181±53 pg/mL) than in vehicle-treated rats (33±21 pg/mL) (P<0.05).

AT$_1$ and AT$_2$ receptor mRNA content in the adrenal gland of each of the two groups of rats was determined by Northern blot analysis. Blots were then stripped and rehybridized to 18S rRNA probes. Densitometric analysis (Figure 1) indicated that the ratio of AT$_1$ mRNA to 18S rRNA was not statistically different between aldosterone-infused (1.24±0.28) and vehicle-treated (0.92±0.14) rats. In contrast, densitometric analysis (Figure 2) indicated that the ratio of AT$_2$ mRNA to 18S rRNA in aldosterone-infused rats (0.70±0.14) was significantly decreased compared with vehicle-treated rats (1.36±0.15).

The maximal binding (B$_{max}$) and dissociation constants (K$_d$) of the AT$_1$ and AT$_2$ receptors in the adrenal gland of each group were determined with the use of radioligand binding assay. The calculated AT$_1$ B$_{max}$ (Figure 3) was not altered by aldosterone treatment (944±129 fmol/mg protein) compared with control (1016±134 fmol/mg protein). In contrast, the calculated AT$_2$ B$_{max}$ (Figure 4) was decreased approximately 40% by aldosterone treatment (429±61 fmol/mg protein) compared with vehicle treatment (698±91 fmol/mg protein, P<0.05). There was no significant difference in the binding constants of both AT$_1$ and AT$_2$ receptors among adrenal preparations from two groups (Table).

Immunohistochemical localization of the AT$_1$ and AT$_2$ receptors in the adrenal gland was determined in both groups (Figure 5). The AT$_1$ receptor immunohistochemical signal was detected strongly in the zona glomerulosa in vehicle-treated rats (Figure 5A and 5C). AT$_1$ receptor signal also was detected in the zona fasciculata, zona reticularis, and medulla of vehicle-treated rats (Figure 5A). In contrast, heavy immunohistochemical staining for the AT$_1$ receptor was observed in the zona fasciculata in aldosterone-infused rats (Figure 5B and 5D). Adrenal staining for the AT$_1$ receptor was reduced but remained detectable in the zona glomerulosa, zona reticularis, and medulla of aldosterone-infused rats (Figure 5B).
The AT$_2$ receptor immunohistochemical signal was detected strongly in the adrenal medulla in vehicle-treated rats (Figure 5E and 5G). AT$_2$ receptor signal was also detected in the adrenal zona glomerulosa and zona fasciculata but was absent from the zona reticularis of vehicle-treated rats (Figure 5E). Aldosterone treatment reduced immunohistochemical staining for the AT$_2$ receptor in the medulla (Figure 5F and 5H). Method controls in which AT$_1$ (Figure 5I) and AT$_2$ (Figure 5J) receptor antibody was replaced by normal rabbit IgG were negative.

**Discussion**

Although it is well known that Ang II is a principal regulator of aldosterone production in the adrenal gland, knowledge of the feedback regulation of aldosterone on the action of Ang II in the adrenal gland remains incomplete. The present experiment was designed to test the hypothesis that infusion of aldosterone differentially regulates the expression of AT$_1$ and AT$_2$ receptors in the adrenal gland. We found that aldosterone shifts adrenal AT$_1$ receptor expression without altering total AT$_1$ receptor density and significantly decreases adrenal AT$_2$ receptor mRNA content and AT$_2$ receptor density. This appears to be the first indication of a role for aldosterone as a regulator of the action of Ang II through modulation of AT$_1$ and AT$_2$ receptor expression in the adrenal gland.

The dose of aldosterone used was carefully chosen from analysis of the literature so that blood pressure responses in our experiment would be comparable to those of other reports. Accordingly, we chose a 0.05-μg/h dose of aldosterone because, in agreement with other reports, this dose did not cause detectable changes in systolic blood pressure in aldosterone-infused rats compared with rats infused with vehicle only. In addition, this dose allowed discrimination of effects of aldosterone on AT$_1$ and AT$_2$ receptor expression in the adrenal gland because plasma aldosterone levels were approximately 5 times higher in aldosterone-infused compared with vehicle-infused rats.

Northern blot analysis and radioligand binding study show that adrenal AT$_1$ receptor mRNA levels and receptor density are not altered by aldosterone infused at the specific dose used in this experiment. We have previously shown that sodium deficiency increases both AT$_1$ receptor mRNA content and AT$_1$ receptor density in the adrenal gland. These increases appear to be mediated by activation of the AT$_1$ receptor by Ang II, since blockade of the AT$_1$ receptor with losartan prevents these increases induced by low sodium intake. Likewise, Lehoux et al have shown that low sodium or high potassium intake increases adrenal AT$_1$ receptor expression, and they suggested that increased aldosterone secretion induced by low sodium or high potassium intake involves increases in AT$_1$ receptor mRNA levels in the adrenal gland. Furthermore, Ullian et al have shown that in cultured rat vascular smooth muscle cells, aldosterone potentiates Ang II-stimulated, phospholipase C-dependent intracellular signals solely by coupling to an increased number of Ang II receptors. Although adrenal AT$_1$ receptor density is not altered by aldosterone infusion in the present experiment, it appears that there is a shift in adrenal AT$_1$ receptor expression in aldosterone-infused rats, indicated by immunohistochemical studies; ie, the highest AT$_1$ receptor expression is relocated from the zona glomerulosa to the zona fasciculata after aldosterone treatment. Because AT$_1$ receptors in the zona glomerulosa mediate Ang II–induced secretion of aldosterone, it is tempting to speculate that attenuated AT$_1$ receptor expression in the zona glomerulosa induced by aldosterone infusion represents a means of negative feedback regulation in aldosterone secretion.

The suppressive effects of aldosterone on AT$_2$ receptor mRNA content and receptor density in the adrenal gland have not been previously reported and deserve comment. It is well known that aldosterone alters plasma electrolyte concentrations and levels of neurohormonal factors. It is possible that aldosterone-induced changes in these parameters have direct or indirect effects on adrenal AT$_2$ receptor expression.

It has been shown in cultured bovine adrenal cells and PC12W cells that Ang II downregulates both AT$_1$ and AT$_2$ receptors through different mechanisms; AT$_1$ receptor is regulated through internalization/degradation of the occupied receptor and inhibition of transcription, whereas AT$_2$ receptor is regulated mainly by decrease in the stability of its mRNA. Moreover, results from the same group showed that the phorbol ester phorbol 12-myristate 13-acetate decreases both AT$_1$ mRNA and receptor binding on PC12W cells, suggesting that the hormonal regulation of AT$_2$ receptors is mediated through protein kinase C activation. Because it is difficult to separate the effects of aldosterone-induced changes in plasma electrolyte concentrations and levels of neurohormonal factors on AT$_2$ receptor expression in vivo, future studies using in vitro models may provide insight into the mechanisms by which aldosterone inhibits adrenal AT$_2$ receptor expression.

Although the function of the AT$_1$ receptor in the adrenal gland is relatively clear, the function of the adrenal AT$_2$ receptor is largely unknown. It has been shown that Ang II stimulates secretion of endogenous ouabain from cultured bovine adrenocortical cells via activation of the AT$_2$ receptor. Furthermore, in the rat zona glomerulosa and PC12W cells, stimulation of AT$_2$ receptors appears to reduce guanylate cyclase activity. Because modulation of the expression of the Ang II receptor is associated with changes in the Ang II action, it is conceivable that aldosterone may modulate Ang II action by suppressing the expression of the AT$_2$ receptor in the adrenal gland. This possibility awaits further in vivo and in vitro investigation.

In conclusion, we have demonstrated that infusion of aldosterone shifts adrenal AT$_1$ receptor expression without decreasing AT$_1$ receptor density. Moreover, aldosterone infusion decreases adrenal AT$_2$ receptor mRNA and AT$_2$ receptor density. Aldosterone-induced modulation of AT$_1$ and AT$_2$ receptor expression in the adrenal gland may be important in the adaptation to low salt diets and other conditions in which aldosterone is increased.
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References


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