Regulation of Growth of the Adrenal Gland in DOC-Salt Hypertension
Role of Angiotensin II Receptor Subtypes

Fernando Eljovich, Hua-Wei Zhao, Cheryl L. Laffer, Yong Du, Donald J. DePette, Tadashl Inagami, Donna H. Wang

Abstract To investigate the role of the renin-angiotensin system in the regulation of adrenal growth in deoxycorticosterone (DOC)-salt hypertensive rats, and the adrenal gene expression of angiotensin AT1 and AT2 receptors, three groups of uninephrectomized rats + DOC pellet + 0.9% NaCl were given water (DOC), losartan (DOC-L), or ramipril (DOC-R) by gavage. Controls had sham surgery and water gavage. Tail-cuff systolic and mean intra-arterial blood pressures were significantly higher in the three DOC groups than in controls and not different among the groups. Adrenal weight of DOC was slightly but not significantly greater than that of controls, while those of DOC-L and DOC-R were greater than that of controls (P < 0.1). Northern blots showed that AT1 and AT2 gene expression was significantly reduced in DOC (by 33% and 60%), while that of AT1 (but not AT2) was significantly reduced further (versus control and DOC) in DOC-L and DOC-R. There were negative correlations between adrenal weight and AT1 (r = -0.80, P < 0.001) or AT2 (r = -0.60, P < 0.05).

We conclude that DOC-salt hypertension downregulates adrenal AT1 and AT2 gene expression by different mechanisms. Removal of the effects of angiotensin by losartan or ramipril downregulates AT1 further and promotes adrenal growth, indicating the presence of an AT1-mediated growth-inhibitory action of angiotensin II on the adrenal gland. These observations constitute an additional example of a growth-inhibitory role for the AT1 receptor, opposite to its more common growth-promoting actions in other organs and tissues. (Hypertension. 1997;29[part 2]:408-413.)

Key Words • receptors, angiotensin II • gene expression regulation • blunting, Northern • losartan • ramipril • deoxycorticosterone • rats

The circulating renin-angiotensin system has long been known to regulate vasoconstriction and aldosterone secretion, thus participating in blood pressure control and fluid homeostasis. Over the last few years, it has become apparent that local tissue renin-angiotensin systems play a major role in the regulation of growth processes. These effects have been described in tissues that are involved either in the pathogenesis or in the target organ damage of hypertension, e.g., vascular smooth muscle, cardiomyocytes, mesangium, endothelium, and neointima of injured vessels.

The actions of angiotensin II are exerted via activation of cell surface receptors. The development of specific nonpeptide antagonists for angiotensin II and their use in pharmacological and radioligand binding experiments have permitted characterization of distinct receptor subtypes for this pressor peptide. More recently, molecular probes for the study of receptor gene expression and experiments with gene transfection have been used to study the role of these receptor subtypes in mediating the actions of angiotensin II. The hemodynamic and aldosterone secretion effects of angiotensin II are mediated by the AT1 receptor. Regarding the effects on cell growth, the prevailing view is that the AT1 receptor mediates the growth-promoting actions, while the AT2 receptor mediates the opposing, antiproliferative actions of angiotensin II.

However, not all available evidence supports this view. For example, AT1 blockade by losartan does not prevent aortic hypertrophy and fibrosis during angiotensin II-induced hypertension in the rat. In contrast, AT2 blockade by specific antagonists inhibits aortic hypertrophy and fibrosis in this model. Also, AT2 blockade prevents neointimal growth after carotid artery injury in the rat. These observations indicate the presence of growth-promoting actions for the AT2 receptor.

The adrenal gland expresses the genes encoding both AT1 and AT2 receptors throughout fetal and adult life, with the former predominating in the cortex and the latter in the medulla. In bovine adrenocortical cells in culture, angiotensin II has potent mitogenic effects and induces expression of proto-oncogenes, both effects mediated by the AT1 receptor. In vivo, it has been reported that the adrenal glands of rats with experimental DOC-salt hypertension sustain weight reduction, a change that is prevented by administration of angiotensin I. The role of angiotensin II receptor subtypes in these effects of the renin-angiotensin system on adrenal growth has not been explored in this model.

The aims of this study were threefold. (1) To examine the role of the renin-angiotensin system in the regulation of the changes in growth of the adrenal gland induced by DOC-salt hypertension, (2) to define the specific pathways (AT1 versus AT2 receptor-mediated) responsible for these growth processes, and (3) to assess the effects of DOC-salt hypertension, with or without blockade of the renin-angiotensin system, on the gene expression of AT1 and AT2 receptors in the adrenal gland. We used the converting enzyme inhibitor ramipril and the AT1 receptor blocker losartan for pharmacological blockade of the
remnant-angiotensin system and measured expression of angiotensin II receptor subtypes, AT₁ and AT₂, in the adrenal glands of DOC-salt rats. Plasma renin is low in DOC-salt rats 3 weeks after induction of hypertension and pharmacological blockade of the renin-angiotensin system does not modify blood pressure. Under these conditions, the effects of ramipril and losartan on organ growth can be predominantly attributed to changes in local tissue angiotensin II, and more importantly, they are devoid of the confounding influence of changes in blood pressure by these agents.

**Methods**

**Treatment Groups**

Five-week-old male Sprague Dawley rats (Harlan Inc.), weighing between 140 and 170 g, were randomly divided into four groups (n = 7 each). Animals were anesthetized with a single intraperitoneal injection of 80 mg/kg ketamine and 0.2 mg/kg xylazine. Three groups were subjected to left nephrectomy and implantation of a DOC pellet (150 mg, Innovative Research) in the back of the neck, while the control group had sham nephrectomy. After recovery from surgery, the rats in the three DOC groups were given 0.9% NaCl and 0.2% KCl to drink ad libitum, while the controls were given tap water. In addition, animals were given water (0.5 to 1 mL) via oral gavage, once a day for 3 weeks, containing either losartan (10 mg/kg per day, DOC-L group), ramipril (10 mg/kg per day, DOC-R group), or no added drugs in the DOC-treated (DOC group) and in control (C group) animals. At the end of the 21-day treatment period, all rats were anesthetized as above, and the left carotid artery was catheterized for these measurements. The Laboratory Animals of the experiment Indirect tail-cuff systolic blood pressures were measured in conscious rats every 3 to 4 days for 21 days beginning 1 day before surgery. A Narco Bio-Systems Electro-Sphygmomanometer was used for these measurements. The blood pressure value for each rat in each session was the average of three consecutive measurements.

**Body Weight and Systolic Blood Pressure**

Body weight was recorded at the beginning and at the end of the experiment. Indirect tail-cuff systolic blood pressures were routinely measured in conscious rats every 3 to 4 days for 21 days beginning 1 day before surgery. A Narco Bio-Systems Electro-Sphygmomanometer was used for these measurements. The blood pressure value for each rat in each session was the average of three consecutive measurements.

**Adrenal Weights and Tissue Preparation**

After recording of intra-arterial MAP and measurement of the pressor effects of angiotensin I and II, the animals were killed by further injection of the anesthetic agents. A midline abdominal incision was made for removal of the adrenal glands. The perirenal fat was carefully dissected, and the glands were weighed in an analytical scale (Sartorius, Brinkmann Instruments Co, precision, 0.1 mg) and immediately frozen in liquid nitrogen for storage at −80°C. The adrenal glands from 5 rats in each group were used to extract RNA for Northern blot analysis.

**cDNA Probes**

A 0.8-kb fragment (−178 to +562) from the coding region of rat AT₁α cDNA was used as a template to make AT₁ probes. A 23-kb fragment (−16 to +1249) from the coding region of rat AT₂ cDNA was used as a template to make AT₂ probes. All probes were labeled with [32P]deoxycytidine triphosphate (Amersham) by use of a random primer DNA labeling system (Amersham). (Unincorporated nucleotides were separated using a G-50 spin column (Worthington).

**Northern Blots**

Total adrenal RNA was extracted using the guanidine thioctate-phenol-chloroform extraction protocol. Electrophoresis of 20 µg denatured RNA 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), and the blot was prehybridized for 5 hours at 42°C in hybridization buffer (50% deionized formamide, 5× Denhardt’s solution, 5× SSC, 0.5% SDS, and 200 µg/mL denatured salmon sperm DNA), followed by hybridization with the 32P-labeled 18S rRNA probe and later rehybridized with a 32P-labeled 18S rRNA probe Autoradiograms were made using XAR-5 x-ray film (Eastman Kodak Co) and an intensifying screen. Autoradiographic signals were scanned with a laser densitometer (Ultrascan XL Laser Densitometer) Result of relative gene expression are expressed as the ratios of AT₁ mRNA and AT₂ mRNA to 18S rRNA.

**Statistical Analysis**

Results are given as mean ± SEM. The significance of changes in a variable from the beginning to the end of the study was assessed with paired Student’s t tests. The significance of differences between groups was assessed by ANOVA followed by the Tukey-Kramer multiple comparison test. Relationships between variables were assessed by single linear regression analysis. All analyses were run with the JMP (version 3.0.2) statistical package of the SAS Institute Inc. A value of P < 0.05 was used to reject the null hypothesis (ie, no difference between means or no relationship between variables).

**Results**

Initial body weight was not significantly different among the four groups of rats (Table). All animals gained weight over the 3-week course of the experiment. However, the weight gain of the three groups treated with DOC was significantly less than that in controls. There was no difference in body weight gain between DOC, DOC-L, and DOC-R, indicating that blockade of the renmin-angiotensin system did not have an effect on this parameter. At the end of the study, the three groups of DOC rats had body weights that did not differ among them but were all significantly less than that in controls (Table).

**Body Weight of Rats at Beginning and End of Treatment**

<table>
<thead>
<tr>
<th>C, g</th>
<th>DOC, g</th>
<th>DOC-L, g</th>
<th>DOC-R, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beginning</td>
<td>147±2</td>
<td>156±9</td>
<td>185±9</td>
</tr>
<tr>
<td>End</td>
<td>265±5</td>
<td>196±16*</td>
<td>201±14*</td>
</tr>
<tr>
<td>Weight gain</td>
<td>117±4</td>
<td>39±6*</td>
<td>36±10*</td>
</tr>
</tbody>
</table>

C indicates sham-nephrectomized controls given tap water, DOC, un-nephrectomized + DOC pellet + 0.9% NaCl, DOC-L, DOC + losartan (10 mg/kg per day), and DOC-R, DOC + ramipril (10 mg/kg per day). Values are mean ± SEM, n = 7 rats per group.

*Significant difference (Tukey test) between the labeled group and the controls and also lack of significant difference among the three DOC groups.

†Significant weight gain from baseline (paired t test).
Tail-cuff systolic blood pressures of the DOC, DOC-L, and DOC-R groups were significantly higher than those of controls beginning on days 3, 3, and 7, respectively. Over the remainder of the experiment, systolic blood pressure of the three DOC groups rose similarly (Fig 1). At the end of the experiment, the values for the four groups were controls, 139±4; DOC, 215±8; DOC-L, 226±7; and DOC-R, 223±7 mm Hg (F=42.4, *P* < .0001, Tukey: all DOC groups higher than controls and not different between them). On day 21, MAP under anesthesia exhibited the same pattern (controls, 98±1; DOC, 131±3; DOC-L, 126±7; and DOC-R, 126±4 mm Hg; *F* = 9.7, *P* < .001, Tukey: same as that for tail cuff). Therefore, neither losartan nor ramipril prevented the increase in blood pressure produced by the combined treatment with DOC and salt in uninephrectomized rats, confirming that angiotensin II is not necessary for the development of hypertension in this model.

MAP responses to bolus injections of angiotensin I (50 ng/kg) were significantly smaller in DOC (26±4 mm Hg) than in controls (41±2), perhaps due to decreased lung angiotensin-converting enzyme levels in DOC.* Responses to angiotensin II in DOC-L (3±2) and DOC-R (3±2) were markedly diminished and significantly smaller than those in controls and DOC. Responses to angiotensin II (50 ng/kg) were not different among controls (49±2), DOC (44±7), and DOC-R (41±4), while those of DOC-L were significantly decreased (10±4). These data confirm effective AT1 receptor blockade by losartan and inhibition of the angiotensin-converting enzyme by ramipril.

Fig 2 shows that the weight of the adrenal gland of DOC-rats (177±14 μg/g body wt) was slightly but not significantly greater than that of controls (131±13). In contrast, the DOC-L (209±18) and DOC-R (236±15) groups exhibited significantly higher adrenal weights than controls, but they were not different between DOC-L and DOC-R. Thus, we found that losartan and ramipril promoted enlargement of the adrenal gland, suggesting the presence of a growth-inhibitory action of angiotensin II on this organ.

AT1 and AT2 mRNA levels in the adrenal glands were determined by Northern blot analysis in the four experimental groups (Fig 3A). Blots were then stripped and rehybridized to 18S mRNA probes. Densitometric analysis indicated that the AT1 mRNA/18S rRNA ratios differed in the four groups (F=19.9, *P* < .0001, Fig 3B). AT1 mRNA/18S rRNA ratio of DOC (1.07±0.06) was significantly lower than that in controls (1.61±0.16), a decrease of 33%. In DOC-L (0.67±0.05) and DOC-R (0.74±0.07), AT1 mRNA/18S rRNA ratios were further diminished, significantly differing from those of DOC and controls. AT2 mRNA/18S rRNA ratios were also different among the four groups (F=12.0, *P* < .0002, Fig 3C). AT2 mRNA/18S rRNA ratio of DOC (0.82±0.08) was significantly lower than that in controls (2.02±0.28), a decrease of 60%. AT2 mRNA/18S rRNA ratios of DOC-L (0.65±0.08) and DOC-R (0.73±0.22) were also significantly lower than that in controls but not significantly decreased from that of DOC.

There were no correlations between adrenal AT1 mRNA/18S rRNA or AT2 mRNA/18S rRNA ratios and blood pressures (tail cuff or intra-arterial) in control or DOC-treated animals. In contrast, significant negative correlations were detected, for all animals analyzed together, between the weight of the adrenals (normalized per gram of body weight) and the AT1 mRNA/18S rRNA (r = −.80, *P* < .0001) or AT2 mRNA/18S rRNA (r = −.60, *P* < .005) ratios, Fig 4.

**Discussion**

Most reports suggest that the growth-promoting and antiproliferative actions of angiotensin II are exerted via its AT1 and AT2 receptors, respectively.8,11 However, there are exceptions for both these subtypes in vascular tissues.2,13 In adrenocortical cells in culture, AT1 stimulates proto-oncogene expression18 and mediates proliferative effects.17 No action has been described, either on growth-promoting or inhibiting processes, for AT2 in these in vitro preparations.

The DOC-salt rat is an ideal model for the in vivo study of regulation of adrenal growth by the renin-angiotensin system because plasma renin is profoundly suppressed during the early stages of the hypertension,21 while the components of the tissue renin-angiotensin system are still detectable in several organs,22-25 including expression of renin mRNA in the adrenal gland.19 Therefore, we speculated that this model would permit investigation of the effects of re-
mipril and losartan on adrenal growth via actions on the local adrenal renin-angiotensin system and without the confounding effects of changes in blood pressure.

We confirmed that neither ramipril nor losartan modified the development of hypertension in our DOC-salt rats, as reported previously with other converting enzyme inhibitors and AT1 receptor blockers. In this regard, the DOC-salt model is unique among low-renin models of experimental hypertension. Rats subjected to partial renal ablation and Dahl-S rats given salt also develop hypertension with decreased plasma renin, but they exhibit blood pressure reduction in response to converting enzyme inhibitors and AT1 receptor antagonists. It has been reported that the adrenal gland of DOC-salt rats sustains a decrease in weight during the development of hypertension, a change that is prevented by coadministration of angiotensin I. We could not confirm these observations in our DOC-salt rats. They actually sustained a mild increase (albeit not statistically significant) in adrenal weight compared with controls. In one of the previous publications, adrenal weights were not normalized to body weights, and in the other, there were methodological differences with our experiments (Wistar rats instead of Sprague Dawley, and repeated subcutaneous injections of DOC instead of pellet implantation). We cannot speculate whether these differences account for the conflicting results.

The major findings of our experiments can be summarized as follows: (1) DOC-rats without pharmacological blockade of the renin-angiotensin system exhibited significantly reduced expression of AT1 and AT2 genes in the adrenal gland compared with controls; (2) losartan and ramipril decreased AT1 (but not AT2) gene expression further, beyond the decrease observed in untreated DOC-rats; (3) in all DOC-rats and controls, analyzed together, there were inverse relationships between the weight of the adrenal glands and AT1 or AT2 gene expression; and (4) both
losartan and ramipril produced a significant enlargement of the adrenal glands. Taken together, these observations suggest that angiotensin II exerts a tonic growth-inhibitory effect on the adrenal gland, which is mediated by the AT₁ receptor (increased adrenal weight by losartan) and perhaps also by AT₂ (inverse relationship between AT₂ and adrenal weight), although confirmation of the latter would require the use of specific AT₂ antagonists. Decreases in the expression of these receptors, of the magnitude observed in DOC-salt rats, were not enough to produce a statistically significant enlargement of the adrenal gland in the small group of animals studied. In contrast, with further decrease in expression of the AT₁ gene by losartan and ramipril, there was significant enlargement of the adrenal gland, which was possibly enhanced by diminished action of angiotensin II on AT₁ (losartan) or by decreased tissue generation of this peptide (ramipril).

It is unlikely that downregulation of AT₁ or AT₂ was due to DOC because (1) only the glucocorticoid receptor regulates angiotensin receptor gene expression, 24 (2) a GRE is only present in the gene for the AT₁ receptor subtype, and (3) binding of the glucocorticoid receptor to this GRE stimulates gene transcription, 34 which is not consistent with diminished receptor expression observed in our DOC-salt rats.

Opposing effects of nephrectomy on adrenal angiotensin receptor binding have been described. Upregulation was attributed to high serum potassium, 35 which is consistent with upregulation of adrenal AT₁ mRNA and protein by high potassium diet in normal rats. 30 Others have attributed observed downregulation of receptor binding 37 and receptor mRNA 38 to decreased angiotensin II. Although those results were obtained in anephric, not uninephrectomized, rats, they are applicable to interpretation of our findings. It is conceivable that low serum potassium and low circulating angiotensin contributed to downregulation of adrenal angiotensin II receptors in DOC-salt rats.

The most likely factor responsible for adrenal downregulation of both AT₁ and AT₂ in DOC-salt rats is high sodium intake, perhaps enhanced by the salt-retaining properties of DOC. In normal rats, adrenal angiotensin II immunoreactivity and angiotensin receptor binding correlate closely and are diminished by a high salt diet. 39 An effect of high salt diet on angiotensin gene expression has not been reported, but low-sodium diet upregulates adrenal AT₁a and AT₁b receptor mRNA, 40 AT₂ ligand binding, 10 and AT₁ receptor protein. 36 Captopril 36 and losartan 41 prevent the effects of low-salt diet, indicating that adrenal angiotensin receptor upregulation is due to increased angiotensin II by sodium deprivation. This has been confirmed by direct demonstration of adrenal angiotensin receptor upregulation by infusion of angiotensin II. 38 These findings make it likely that suppression of circulating and/or tissue angiotensin II by high salt diet and DOC was the major factor determining downregulation of AT₁ and AT₂ in our DOC-salt rats. They are also consistent with further reduction of AT₁ expression by administration of losartan or ramipril to these animals. Although ours is the first report in DOC-salt rats, others have shown downregulation of adrenal AT₁ but not AT₂ by losartan, 42 of both AT₁ and AT₂ by lisinopril, 42 and of the AT₁a and AT₁b subtypes by delapril. 43 In the present experiment, losartan and ramipril downregulated the AT₁ receptor beyond the decrease produced by DOC-salt. In contrast, downregulation of AT₂ by DOC-salt was not augmented by these compounds. This difference could be due to the more profound downregulation of AT₂ by DOC-salt, compared with AT₁, which may have made it more difficult to detect further changes in AT₂ mRNA after blockade of the renin-angiotensin system. It is also possible that downregulation of AT₁ by high salt-diet is dependent on withdrawal of angiotensin II or of its action, while that of AT₂ reflects an angiotensin-independent action of sodium. We do not have data to support either possibility.

An additional factor, norepinephrine stimulation of alpha-1 adrenoceptors, may contribute to downregulation of adrenal AT₁ in DOC-salt rats. These animals exhibit exaggerated norepinephrine release into the synaptic cleft, with spillover to the circulation. 44 In normal rats, prazosin enhances expression of adrenal AT₁a and AT₁b, demonstrating an alpha-1 inhibitory action of norepinephrine on gene expression of AT₁ receptor subtypes. 45

Regardless of its mechanisms, downregulation of adrenal expression of angiotensin II receptors has been now described in three models of experimental hypertension. Adrenal AT₁b mRNA (but not AT₁a) is decreased by 50% in Goldblatt two-kidney, one clip hypertension of Wistar rats, 40 adrenal AT₁ by 66% in hypertensive rats due to reduced renal mass and high sodium intake, 32 and adrenal AT₂ by 33% and AT₁ by 60% in DOC-salt rats in the present experiment. These models encompass the full spectrum of plasma renin activity or renin dependence of blood pressure and differ in the mechanisms of their hypertension. This suggests that downregulation of adrenal angiotensin receptor genes may play an as yet unknown compensatory role in experimental hypertension. In contrast, in spontaneously hypertensive rats, adrenal AT₁ receptors are more abundant than in Wistar-Kyoto controls, 14 suggesting that alterations in angiotensin receptor gene expression may play a pathogenic rather than a compensatory role in genetic hypertension.

In conclusion, we have shown that DOC-salt hypertension in the rat exhibits downregulation of adrenal expression of AT₁ and AT₂. In the case of AT₁, this downregulation seems to follow the same pattern as in normal rats, i.e., it is most likely dependent on removal of the action of angiotensin II by salt and DOC. A role for angiotensin II in downregulation of adrenal AT₂ mRNA was not demonstrated by these experiments. We also show that downregulation of these receptors by DOC-salt hypertension seems to withdraw a growth-inhibitory influence that angiotensin II exerts on the adrenal gland. This becomes more apparent after more profound downregulation of the AT₁ (not the AT₂) receptor by blockade of the renin-angiotensin system. The effect of losartan on adrenal weight makes it unequivocal that the AT₁ receptor mediates the growth-inhibitory action of angiotensin II on the adrenal gland, providing another example of an exception to the usual growth-promoting effects of this receptor.

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