Regulation of Angiotensin II Receptor Expression by Nitric Oxide in Rat Adrenal Gland

Makoto Usui, Toshihiro Ichiki, Makoto Katoh, Kensuke Egashira, Akira Takeshita

Abstract—We recently reported that administration of N\textsuperscript{-}nitro-l-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) production, activates the vascular and cardiac renin-angiotensin systems and causes vascular thickening and myocardial hypertrophy in rats with perivascular and myocardial fibrosis. It has been reported that aldosterone may contribute to the development of cardiac fibrosis, but it is not known whether inhibition of NO synthesis affects angiotensin II (Ang II) receptor gene expression and aldosterone secretion. The aim of this study was to investigate the effect of NO inhibition on the expression of Ang II receptors in the adrenal gland and on aldosterone secretion in rats. Wistar King A rats received normal water, L-NAME alone (1 mg/mL in the drinking water), or L-NAME and the α1-adrenergic receptor blocker bunazosin (0.1 mg/mL in the drinking water) for 1 week. After 1 week of treatment with L-NAME, systolic blood pressure, plasma aldosterone concentration (PAC), and mRNA level and number of Ang II type 1 receptor (AT\textsubscript{1}-R) were increased. Plasma renin activity, serum angiotensin-converting enzyme activity, and the number of AT\textsubscript{2}-R were unchanged. Although addition of bunazosin to L-NAME restored systolic blood pressure to the control level, PAC and AT\textsubscript{1}-R numbers remained significantly higher than those of control level. These results suggest that the increased AT\textsubscript{1}-R number and PAC induced by the inhibition of NO synthesis were independent of blood pressure and systemic renin-angiotensin system. Therefore, hypertension and myocardial fibrosis induced by NO blockade may be due in part to an elevation of PAC caused by increased AT\textsubscript{1}-R in the adrenal gland. (Hypertension. 1998;32:527-533.)

Key Words: nitric oxide ■ receptors, angiotensin ■ plasma aldosterone concentration ■ L-NAME ■ adrenal gland

Angiotensin II (Ang II) has a strong vasopressor effect and also regulates electrolyte balance and drinking behavior. Its actions are mediated by specific receptors located on various target organs, including the adrenal gland, kidney, uterus, brain, and arterioles.\textsuperscript{1,2} Recently, it was discovered that Ang II receptors are heterogeneous and could be classified into at least 2 subtypes: Ang II type 1 receptor (AT\textsubscript{1}-R) and type 2 receptor (AT\textsubscript{2}-R). It is generally accepted that most of the biological effects of Ang II known to date are mediated by the AT\textsubscript{1}-R. AT\textsubscript{1}-R antagonists reduce blood pressure in renal hypertensive rats and inhibit Ang II–induced aldosterone release from adrenal cortex, epinephrine secretion, and water drinking.\textsuperscript{3–7} Recent molecular cloning of the AT\textsubscript{1}-R in rats revealed the existence of 2 subtypes, ie, AT\textsubscript{1A}-R and AT\textsubscript{1B}-R.\textsuperscript{8–12} The AT\textsubscript{2}-R is abundantly expressed in adrenal medulla, but its function has not been identified.

Endothelium plays an important role in the homeostasis of vascular tone by producing endothelium-derived substances.\textsuperscript{13–15} An important endothelium-derived relaxing factor has been identified to be nitric oxide (NO) or a related compound.\textsuperscript{16,17} In addition, NO inhibits platelet aggregation and leukocyte adhesion, which suggests that NO may have a negative effect on the growth and/or proliferation of blood vessels.\textsuperscript{13,15,18–22} Several reports have shown that NO antagonizes the biological function of Ang II. NO inhibits Ang II–induced migration of vascular smooth muscle cells.\textsuperscript{23} NO also prevents Ang II–induced [\textsuperscript{3}H]thymidine incorporation and cell proliferation in rat mesenteric arteriolar smooth muscle cells.\textsuperscript{24}

We.\textsuperscript{25–27} and other investigators\textsuperscript{26–31} have recently reported that long-term blockade of NO synthesis with chronic administration of N\textsuperscript{-}nitro-l-arginine methyl ester (L-NAME) caused systemic arterial hypertension, microvascular structural changes (medial thickening and perivascular fibrosis), and myocardial hypertrophy in rats and pigs. L-NAME–induced microvascular structural changes were prevented by angiotensin-converting enzyme (ACE) inhibition and Ang II receptor antagonism,\textsuperscript{32–34} suggesting that the renin-angiotensin system (RAS) is activated by inhibition of NO. However, it has not been determined whether inhibition of NO synthesis modulates Ang II receptor expression and aldosterone secretion in the adrenal gland. In the present study, we investigated the regulation of adrenal Ang II receptor expression and plasma aldosterone concentration (PAC) in rats treated with L-NAME.

Methods

The following protocols were reviewed and approved by the Committee of the Ethics on Animal Experiment in the Faculty of...
Angiotensin II Receptor Expression and Nitric Oxide

Drugs and Materials

We used L-NAME (Sigma Chemical Co), bunazosin (Esai Pharmaceutical Co), PD123319 (Warner-Lambert Co), and [3H]a-DCP and [3H]-Sar, Ile4-Ang II (New England Nuclear).

Animal Preparation and Tissue Preparation

Eight-week-old male Wistar King A (WKA) rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine. Three groups of rats were studied; the first group (C group) received normal drinking water, the second (L group, n=10) received L-NAME in drinking water (1 g/L), and the third (L+ group, n=10) received L-NAME and the a1-adrenergic blocker bunazosin (0.1 g/L) in the drinking water. The daily intake of L-NAME was approximately 100 mg · kg⁻¹ · d⁻¹ in the L group. All rats were fed with normal chow and were housed in a viral-antigen-free facility for 1 week. All hemodynamic and biochemical studies were performed after 1 week of treatment.

After 1 week of treatment, systolic blood pressure (SBP; tail-cuff method) and body weight were measured. All rats were anesthetized with an injection of sodium pentobarbital (50 mg/kg IP) and killed by exsanguination. Blood was collected into prechilled tubes containing EDTA disodium salt for the measurements of plasma renin activity (PRA) and plasma aldosterone concentration (PAC). Blood was collected in plain tubes for the measurement of serum ACE activity, serum corticosterone concentration (SCC), and serum potassium and sodium concentrations. The plasma and serum were separated by centrifugation at 3000 rpm for 20 minutes at 4°C and stored at −80°C until measurement of PRA, PAC, serum activity, SCC, and serum potassium and sodium concentrations. PRA was measured as the rate of angiotensin I generation from angiotensinogen, determined with a radioimmunoassay (SRL Co Ltd). Serum ACE activity was measured using a fluorometric assay described by Cheung and Cushman and Hayashi et al. Serum ACE activity was calculated as nanomoles His-Leu generated per milliliter of serum per hour. SCC was also determined with a radioimmunoassay method (SRL Co Ltd). Left and right adrenal glands were removed, frozen in liquid nitrogen, and stored at −80°C. Both adrenal glands from 6 rats in each group were pooled and used for RNA extraction for Northern blot analysis, and adrenal glands from another 4 rats were used for the radioligand binding assay.

cDNA Probes for Northern Blot Analysis

The cDNA probes were prepared as described previously. To obtain a rat AT1A-R-specific probe, total RNA from rat kidney was reverse transcribed (Ready-To-Go T-Primed First-Strand kit, Pharmacia Biotech AB), and the resultant cDNA was amplified by polymerase chain reaction (PCR) with the following primers: sense primer 5'-TGGCTTACGACAAAAAGACCA-3' and antisense primer 5'-CAAAAGGAAGACTGTAGATGGT-3'. The AT1A-R-specific probe was prepared in the same way, except that total RNA from rat adrenal gland was used. A noncoding fragment (395 bp: +1246 to +1641) was used as a template for making cDNA probes. PCR was carried out by 25 cycles of denaturation at 95°C for 60 seconds, annealing at 60°C for 60 seconds, and polymerization at 72°C for 60 seconds. The 379-bp products for AT1A and 395-bp PCR products for AT1B were subcloned into the pBluescript II KS(+) vector (Stratagene). The specificity of the AT1A-R and the AT1B-R probes was confirmed by a lack of cross-hybridization between AT1A-R and AT1B-R cDNAs. The cDNA probe for AT2 was provided by Dr Tadashi Inagami, Vanderbilt University, Nashville, Tenn.

RNA Extraction and Northern Blots

Total RNA was extracted by the guanidine thiocyanate-phenol-chloroform extraction method (Isogent; Wako Pure Chemical Ltd). Total RNA (20 μg) was electrophoresed in a 1% agarose formalde-
Each binding assay was carried out in duplicate. The protein concentration was determined by the BCA protein assay (Pierce Chemical Co) using BSA as a standard. To calculate the maximal number of Ang II binding sites ($B_{\text{max}}$) and the binding constant ($K_d$), the values of specific binding of Ang II to membrane were plotted according to the method of Scatchard.39

**Statistical Analysis**

Results are expressed as mean±SEM. Statistical analysis was performed using 1-way ANOVA followed by Fisher’s test for multiple comparison. A value of $P<0.05$ was considered statistically significant.

**Results**

**Body Weight and Hemodynamic Variables**

Body weights, SBPs, and heart rates before treatment were comparable among the 3 groups (Table 1). Body weight in the C group increased, whereas it did not increase during the 1-week treatment period in the L and L+B group.

SBP in the L group increased, but drug treatment did not change SBP in the C and L+B groups. After treatment, heart rate in the L group was less than in the C group but was comparable to that in the L+B group.

**$AT_{1A}$, $AT_{1B}$, and $AT_2$ mRNA Levels in Adrenal Gland**

The expression levels of $AT_{1A}$-R, $AT_{1B}$-R, and $AT_2$-R mRNA in the rat adrenal glands were determined by Northern blot analysis (Figures 1 through 3). Densitometric analysis indicated that the ratio of $AT_{1A}$-R mRNA to GAPDH mRNA was elevated in the L (1.68±0.15-fold, $P<0.01$) and L+B (1.58±0.14-fold, $P<0.01$) groups compared with that in the C group. Similarly, the ratio of $AT_{1B}$-R mRNA to GAPDH mRNA was elevated in the L (1.79±0.28-fold, $P<0.01$) and L+B (1.63±0.20-fold, $P<0.05$) groups compared with that in the C group. The ratio of $AT_2$-R mRNA to GAPDH mRNA did not differ among the 3 groups.

**Ligand Binding**

Figure 4 shows the saturation curves and the scatchard plots of the binding of [125I]-Sar,1Ile8-Ang II to Ang II receptors in the membrane fraction of adrenal gland from the 3 experimental groups. The calculated maximal binding sites ($B_{\text{max}}$) and dissociation constants ($K_d$) are summarized in Table 2. The differences in binding constant were not statistically significant among the 3 groups (Table 2). Numbers of total receptor and $AT_1$-R in the L and L+B groups were significantly increased. The $AT_2$-R number was slightly increased in the L and L+B groups; however, this difference was not statistically significant (Table 2 and Figure 4). Therefore, the increased $AT_1$-R number accounted for the increased Ang II receptor number in the L and L+B groups.

Figure 5 shows the total, $AT_1$, and $AT_2$ receptor density in the adrenal gland of 3 groups measured using 5 nmol/L [125I]-Sar,1Ile8-Ang II. Consistent with the changes in total receptor density, the $AT_1$-R density in the L and L+B groups expressed as mean±SEM. $AT_{1A}$ mRNA was significantly increased in the L group (1.7-fold, $P<0.01$ vs control) and L+B group (1.6-fold, $P<0.01$ vs control).
was significantly \((P<0.01)\) increased compared with that in the C group.

**PAC, PRA, Serum ACE Activity, Serum Potassium Concentration, and SCC**

PAC was significantly increased in the L and L + B groups compared with that in the C group, but PRA, serum ACE activities, and serum potassium concentration were not different among the 3 groups (Table 3). SCC was slightly increased in the L and L + B groups, but there was no significant statistical difference among the 3 groups (Table 3).

**Discussion**

The present study demonstrates three major findings. First, inhibition of NO synthesis by L-NAME in rats increased both AT1A-R and AT1B-R mRNA expression and the AT1-R number in the adrenal gland, but it did not increase AT2-R mRNA expression or AT2-R number. Second, inhibition of NO synthesis increased PAC without significant increases of PRA, serum ACE activity, SCC, or serum potassium concentration. Third, normalization of blood pressure in L-NAME-treated rats did not affect the expression levels of AT1A-R and AT1B-R mRNA, the AT1-R number of the adrenal gland, or PAC. This study is the first to show that inhibition of NO synthesis upregulates the AT1 receptors in vivo.

As shown previously, 14 SBP in the L group was increased after 1 week of treatment (204±9 mm Hg) compared with that in the C group (140±5 mm Hg). The mRNA levels of AT1A-R and AT1B-R in the L group were increased 1.7-fold and 1.8-fold, respectively. Although bunazosin reduced blood pressure to a level comparable to that in the C group, it did not affect the mRNA level and the number of AT1-R. These results suggest that the upregulation of AT1-R (both mRNA level and receptor number) by L-NAME was independent of blood pressure elevation. Although bunazosin reduced blood pressure to the control level, heart rate in the L + B groups remained decreased. Although the mechanism is not clear, it may suggest that bradycardia induced by L-NAME is not due to an elevation of blood pressure but to inhibition of NO itself.

Our results are consistent with those of Cahill et al. 40 They reported that NO-generating drugs decreased Ang II receptors in cultured rat vascular smooth muscle cells. However, the precise mechanism of the downregulation of AT1-R by NO donor or the upregulation of AT1-R by L-NAME is unknown.

Differential regulation of the transcription of AT1A-R and AT1B-R gene has been reported. 37,41 The AT1A-R mRNA levels in the kidney are significantly increased, whereas renal AT1B-R mRNA levels are markedly decreased by low dietary sodium intake. 37 The AT1B-R mRNA levels in the adrenal gland are reduced by treatment with the AT1-specific antagonist TCV 116, but AT1A-R mRNA levels are unchanged. In this study, both AT1A-R and AT1B-R mRNA levels were upregulated by L-NAME treatment. Therefore, some stimuli upregulate the expression of both receptors, whereas others differentially affect AT1A-R and AT1B-R gene transcription. The gene expression of AT2-R is modulated by many cytokines, 42 growth factors, 43 and protein kinase C. 44 In the adrenal...

**Figure 3.** Northern blot analysis of AT2-R mRNA in rat adrenal gland. Left, Adrenal AT2 mRNA and GAPDH mRNA in control rats (C), rats treated with L-NAME (L), and rats treated with L-NAME plus bunazosin (L+B; L+BUNA). A representative autoradiogram is shown. Right, Densitometric data in which AT2 mRNA levels were normalized by GAPDH mRNA level in 3 groups (n=6). AT2 mRNA level was not changed among the 3 groups. mRNA level of control rats was expressed as 1.0.

**Figure 4.** Saturation curves (left) and scatchard plot analyses (right) of \([125I]-\text{Sar, Ile}_8\text{-Ang II}\) binding to Ang II receptors in membrane from rat adrenal glands from the control, L-NAME, and L-NAME plus bunazosin (L+B) groups (n=4). Membrane protein (20 μg) was incubated with \([125I]-\text{Sar, Ile}_8\text{-Ang II}\). Ang II (0.5 to 20 nmol/L) in a final volume of 300 μL assay buffer containing 0.1% BSA. Each point represents mean value of 4 rats in each group. \(K_d\) value was not changed among the 3 groups, whereas maximal binding of Ang II (Bmax) was significantly increased in the L (\(P<0.01\) vs control) and L+B (\(P<0.05\) vs control) groups. Data are shown in Table 3.
TABLE 2. Characterization of Ang II Receptors in L-NAME-Treated Rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>(K_d), nmol/L</th>
<th>Total</th>
<th>AT1</th>
<th>AT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>1.51±0.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L group</td>
<td>1.62±0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L+B group</td>
<td>1.24±0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bmax, fmol/mg protein</th>
<th>Total AT1</th>
<th>AT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>652.7±18.4</td>
<td></td>
</tr>
<tr>
<td>L group</td>
<td>976.2±72.8</td>
<td></td>
</tr>
<tr>
<td>L+B group</td>
<td>934.1±62.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=4).

*P<0.05, †P<0.01 vs C group.

TABLE 3. Effects of Treatments on Biochemical Variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>L-NAME</th>
<th>L-NAME+ Bunazosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng Ang I \cdot mL(^{-1}) \cdot h(^{-1})</td>
<td>31±5</td>
<td>32±3</td>
<td>32±3</td>
</tr>
<tr>
<td>Serum ACE activity, nmol \cdot mL(^{-1}) \cdot h(^{-1})</td>
<td>1.8±0.1</td>
<td>1.8±0.1</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>PAC, pg/mL</td>
<td>393±79</td>
<td>852±50†</td>
<td>783±134*</td>
</tr>
<tr>
<td>SCC, ng/mL</td>
<td>302±34</td>
<td>345±42</td>
<td>388±36</td>
</tr>
<tr>
<td>K, mmol/L</td>
<td>4.3±0.4</td>
<td>4.4±0.8</td>
<td>4.3±0.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n=6).

*P<0.05, †P<0.01 vs C group.

gland, blockade of NO synthesis did not affect the mRNA level and the receptor number of AT1-

Ang II is a strong secretagogue of aldosterone from adrenal cortex and also important for basal aldosterone release.\(^{20}\) Takemoto et al\(^{34}\) reported that the inhibition of NO synthesis by L-NAME for 8 weeks increased PRA and serum ACE. In this study, we showed that L-NAME administration for 1 week increased PAC but not PRA or serum ACE activity. Bunazosin did not affect PAC. These findings suggest that increased PAC induced by L-NAME was not dependent on the systemic RAS or blood pressure. Because not only Ang II but also corticotropin or serum potassium concentration affect aldosterone secretion, we examined SCC and serum potassium concentration in this study. SCC was slightly increased in the L and L+B groups, but there was no significant statistical difference among 3 groups, and there was no correlation between PAC and SCC. Therefore, it suggests that corticotropin level is not significantly different among 3 groups. In this study, PAC was increased in L and L+B groups, but serum potassium was not increased in these groups. Some compensatory mechanism may work, but the mechanism is not clear. Because PRA, serum ACE activity, and SCC did not change, it is likely that increased PAC caused by L-NAME treatment resulted from the increase in the AT1-R in the adrenal glands. However, we did not exclude the possibility that the local RAS in the adrenal gland was activated by the blockade of NO synthesis. Our results differed from those of Simmons and Freeman,\(^{45}\) which suggested that the aldosterone secretion rate was attenuated in rats treated with L-NAME or N\(^\text{\textsuperscript{\textbullet}}\)nitro-L-arginine (L-NNA) compared with that in control animals; however, Simmons and Freeman examined aldosterone secretion rate only 30 minutes after L-NAME or L-NNA injection. We administered L-NAME for 1 week and examined PAC. The difference between our results and those of Simmons and Freeman may be derived from the difference in the period of L-NAME treatment.

Recently, many studies have reported that aldosterone is involved in cardiac hypertrophy and fibrosis, which, together with myocardial cell death, may contribute to progressive myocardial remodeling.\(^{46–51}\) We have previously shown that chronic treatment with L-NAME in rats caused vascular thickening and myocardial hypertrophy with perivascular fibrosis.\(^{34}\) Therefore, the results of this study suggest the possibility that aldosterone may play a role in perivascular and myocardial fibrosis in these models. However, it is necessary to confirm whether aldosterone antagonist is effective in preventing or attenuating the myocardial remodeling in L-NAME–treated animals. Aldosterone stimulates sodium uptake in the distal tubule of the kidney, resulting in water retention. Therefore, increased PAC may play a role in maintaining high blood pressure in L-NAME–treated rats. Under normal conditions, NO may inhibit aldosterone secretion by suppressing AT1-R expression.

Our finding that NO modulates the Ang II receptor may have an important implication for the understanding of the role of these molecules in the progression of atherosclerosis. In the rodent model, ACE inhibitor prevents neointimal formation induced by balloon injury. These data suggest that Ang II plays an important role in the growth and proliferation of vascular smooth muscle cells and remodeling of the vascular wall. Our data clearly show that blockade of NO enhanced the expression of Ang II receptor and its biological function in the adrenal gland independent of systemic blood pressure or the systemic RAS. Therefore, it is possible that vascular thickening and perivascular fibrosis observed in rats with chronic treatment of L-NAME are due to upregulation of...
Ang II receptor in vascular wall. Studies examining the Ang II receptor in vascular wall are in progress and will address the role of Ang II receptor in the pathogenesis of structural changes of coronary artery in L-NNAME–treated rats.

In conclusion, these data demonstrate that the inhibition of NO synthesis by L-NNAME increased both AT1A-R and AT1B-R mRNA expression and AT1-R number in the rat adrenal gland independent of blood pressure and the systemic RAS. AT2-R mRNA expression and AT2-R number did not change with L-NNAME, and the inhibition of NO synthesis increased PAC but not PRA or serum ACE activity. Our findings suggest that inhibition of NO synthesis causes AT1-R upregulation in the adrenal gland and increases PAC. Therefore, L-NNAME–induced hypertension and myocardial fibrosis may be partially due to the elevation of PAC induced by increased AT1-R in the adrenal gland.

Acknowledgments
This study was supported in part by grants-in-aid for scientific research (06670725, 06404034, 07557346) from the Ministry of Education, Science, and Culture, Tokyo; by research grants from the Uehara Memorial Foundation, Tokyo, the Study Group of Molecular Cardiology, Tokyo, and the Kaibara Morikazu Science Promotion Foundation, Fukuoka; and a Kimura Memorial Heart Foundation research grant for 1996, Kurume, Japan. The authors would like to thank Fumiko Amano and Tomoko Takebe for their excellent assistance.

References


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Hypertension. 1998;32:527-533
doi: 10.1161/01.HYP.32.3.527

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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