Regulation of type 1 ANG II receptor in vascular tissue: role of $\alpha_1$-adrenoreceptor

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Du, Yong, Jinggaxin Qiu, Sharon H. Nelson, and Donna H. Wang. Regulation of type 1 ANG II receptor in vascular tissue: role of $\alpha_1$-adrenoreceptor. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1224–R1229, 1997.—Angiotensin II (ANG II) and norepinephrine (NE) are important regulators of vascular function and structure. Recent studies showed that there are multiple interactions between these two potent vasoconstrictor agents. The present experiment was designed to investigate the effect of NE on the expression of the type 1 ANG II receptor (AT1) in the aorta and cultured vascular smooth muscle cells (VSMC) of rats. Rats were subcutaneously infused with either NE (0.5 $\mu$g·kg$^{-1}$·min$^{-1}$, $n = 6$) or the $\alpha_2$-adrenoreceptor antagonist prazosin (3.5 $\mu$g·kg$^{-1}$·min$^{-1}$, $n = 6$) for 2 wk. Body weight and tail cuff systolic blood pressure were not modified compared with the vehicle control ($P > 0.05$). Northern blot analysis showed that AT1 mRNA levels in aorta were decreased by 38% in NE-treated rats and increased 117% in prazosin-treated rats ($P < 0.05$) compared with control. To determine whether NE directly regulates expression of vascular AT1 mRNA and AT1 receptor density, Northern blot analysis and radioligand binding experiments were performed in cultured VSMC. Incubation of VSMC with NE (10$^{-7}$ M) led to 44% decrease in AT1 mRNA levels ($P < 0.05$) and 39% decrease in AT1 receptor density ($P < 0.05$). Prazosin, but not the $\alpha_2$-adrenoreceptor antagonist yohimbine, prevented NE-induced decrease in AT1 mRNA and AT1 receptor density in these cells. Taken together, our results indicate that vascular AT1 gene expression and receptor protein are regulated by ambient NE levels, and NE-induced downregulation of AT1 mRNA and receptor protein is mediated, at least in part, by activating $\alpha_1$-adrenoreceptors.

THE RENIN-ANGIOTENSIN SYSTEM and the sympathetic nervous system have each been implicated in the primary causes of certain forms of clinical hypertension and congestive heart failure. Of the effects on cardiovascular tissues, these two systems influence vascular tone and growth of smooth muscle cells (24, 28, 29). The effects of the pressor substances of these two systems, angiotensin II (ANG II) and norepinephrine (NE), are triggered by their interaction with specific receptors on the vascular wall. It has been demonstrated that the $\alpha_1$-adrenergic receptor mediates sympathetic vasoconstriction of the blood vessel (12), whereas most vascular ANG II receptors in all species studied to date are mainly of the type 1 ANG II receptor (AT1) (4, 7, 30) that mediates contractile and growth effects of ANG II in vascular smooth muscle (5, 31, 32).

It is well known that there are multiple interactions between the renin-angiotensin system and the sympathetic nervous system. For example, ANG II facilitates sympathetic neurotransmission at several sites, including the central nervous system (22), adrenal medulla (21), sympathetic ganglia (17), and presynaptic noradrenergic nerve terminals (1, 14, 18). On the other hand, stimulation of the sympathetic nervous system leads to renin secretion and ANG II generation (11). It is conceivable that these two systems interact at or beyond the receptor levels. Indeed, it has been shown that NE negatively regulates ANG II receptors in cultured brain neurons through its interaction with $\alpha_1$-adrenergic receptors (25). However, it is unknown whether NE, the primary pressor substance of sympathetic innervation of blood vessels, participates in ANG II receptor regulation in the vascular tissues. An understanding of vascular ANG II receptor regulation by the sympathetic nervous system may provide insights into the overall mechanisms by which these two pressor systems interact. Therefore, the present study was designed 1) to investigate the effect of NE on AT1 gene expression and AT1 receptor density in the aorta and cultured vascular smooth muscle cells (VSMC) of rats and 2) to identify the specific pathway responsible for NE-induced regulation of the vascular AT1 receptor.

MATERIALS AND METHODS

Animal groups. Seven-week-old male Wistar rats weighing 179 ± 4 g (Charles River Laboratories, Wilmington, MA) were randomly divided into four groups. Group 1 received 0.9% NaCl (C1, $n = 6$), group 2 received NE dissolved in 0.9% NaCl (NE, $n = 8$), group 3 received 50% dimethyl sulfoxide (DMSO) (C2, $n = 6$), and group 4 received prazosin + 50% DMSO (Pr, $n = 6$). All the rats were anesthetized with a single intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (1 mg/kg), and osmotic minipumps were implanted subcutaneously between the scapulae. Physiological saline or NE (0.5 µg·kg$^{-1}$·min$^{-1}$) was infused continuously for 2 wk by osmotic minipumps (Alzet model 2ML2, Alza). At the end of the 2-wk treatment period, rats infused with NE and saline were decapitated. Blood samples (3 ml) were collected in heparinized tubes containing 5 ml of sodium metabisulfite at 4°C and were centrifuged at 3,000 revolutions/min for 15 min. Plasma was then frozen at −20°C for measurement of plasma NE levels with the use of high-performance liquid chromatography (8). Rats infused with prazosin and DMSO were anesthetized with the dose of ketamine and xylazine described above, and the left carotid artery was catheterized for the measurement of mean arterial pressure (MAP). MAP responses to bolus injections of 3 µg/kg $\alpha_1$-adrenoreceptor agonist phenylephrine were assessed to evaluate the effectiveness of blockade of $\alpha_1$-adrenoreceptors with prazosin. All animal procedures were in accordance with...
the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Blood pressure measurement. Indirect tail cuff systolic blood pressures were routinely obtained in all rats by use of a Narco Bio-Systems Electro-Sphygmomanometer (Houston, TX). The pressures were measured in conscious rats every 3 days for 14 days, beginning 1 day before surgery. The blood pressure value for each rat was calculated as the average of three separate measurements at each session.

Cell culture. Seven to fifteen passages of VSMC from the thoracic aorta of male Sprague-Dawley rats (kindly provided by Dr. Marschall Runge, University of Texas Medical Branch) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 0.68 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator with a 95% air-5% CO2 atmosphere at 37°C. After 70% confluence was reached, the medium was replaced by serum-free DMEM, and the cells were cultured for 48 h to become quiescent (10). The cells were divided and treated with phosphatase-buffered saline (PBS) (control), NE (10-7 M), prazosin (Pr, 10-7 M), NE + Pr, yohimbine (Yo, 10-7 M) and NE + Yo (20). The cells were then collected and used for RNA extraction (after 12-h treatment) or radioligand binding assay (after 24-h treatment).

cDNA probes. To make AT1cDNA probes, a clone pUC19 containing a 2.3-kb fragment of the rat AT1 receptor cDNA was digested with Kpn I and EcoR I to obtain a 790-bp fragment (–180 to +610) (kindly provided by Dr. Tadashi Inagami, Vanderbilt University, Nashville, TN) (13). Because this fragment contains the AT1 coding region where AT1a and AT1b cDNAs exhibit high nucleotide sequence identity, it was used as a template for making AT1 cDNA probes that hybridize to both AT1a and AT1b mRNA. Probes were labeled with 32P-labeled dCTP using a MultiPrime DNA labeling system (Amersham, Arlington, IL) to a specific activity of 3 × 106 counts·min-1·µg-1. The labeled probes were separated from unincorporated nucleotide using MiniSpin G-50 DNA purification spin columns (Worthington Bio, Freehold, NJ).

RNA extraction and Northern blots. Total RNA of aorta and VSMC was extracted with use of the guanidine thiocyanate-phenol-chloroform extraction protocol (6). Ectrophoresis of 30 µg of denatured RNA from each preparation was carried out in 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to a positively charged nylon membrane. The membrane was baked at 80°C for 2 h in a vacuum oven. After prehybridization for 5 h at 42°C in 50% deionized formamide, 5× Denhart’s solution, 5× saline sodium citrate (SSC), 0.5% sodium dodecyl sulfate (SDS), and 200 µg/ml of denatured salmon sperm DNA, the membrane was hybridized with the 32P-labeled probes for 18–20 h. The membrane was then washed successively in 2×, 1×, and 0.5× SSC (2 times, 10 min each) containing 0.1% SDS. The washing temperature was 65°C for each probe. Blots were exposed to XAR-5 X-ray film (Eastman Kodak, Rochester, NY). To correct the differences in RNA loading, Northern blots were incubated at 90°C for 10 min in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0) to strip off the AT1 cDNA probes and rehybridized with 32P-labeled probe for 18S rRNA. Autoradiographic signals were scanned with a laser densitometer (Ultrascan XL Laser Densitometer, Bromma, Sweden). Relative gene expression was calculated as the ratio of AT1 mRNA to 18S rRNA and expressed as a percentage of the respective control group.

Ligand binding assay. Cultured VSMC cells were divided into four groups and treated with the same doses of NE, prazosin, NE + prazosin, and PBS as described above. After 24 h, cells were collected and homogenized in hypotonic buffer (20 mM sodium phosphate, pH 7.1–7.2). Homogenates were then centrifuged at 48,000 g for 20 min at 40°C. Cell membrane was resuspended in assay buffer [50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, and 0.1 mM bacitracin, pH 7.2] and recentrifuged. After resuspension in assay buffer, an aliquot of the cell membrane suspension was used for protein assay with the use of modified Bradford method (Bio-Rad, Hercules, CA) (3). To measure total ANG II receptors, 10 µg protein were incubated with 125I-labeled [Sar,Ile]ANG II (kindly provided by Dr. Robert C. Speth, Washington State University, Pullman, WA) in a final volume of 200 µl assay buffer containing 0.1% bovine serum albumin. To measure the AT1 receptor density, 0.5 nM 125I-[Sar,Ile]ANG II was used in the presence or absence of the specific AT1 antagonist losartan (1 µM). Nonspecific binding was measured in the presence of 1 µM unlabeled ANG II. Binding assay was performed for 120 min at room temperature and was followed by immediate filtration through glass fiber filters (Whatman GF/C, Hillboro, OR). The filter-bound radioactivity was counted in a gamma spectrometer (Beckman, LS 3801, Irvine, CA). Receptor affinity and concentration were calculated by Scatchard analysis using the GraphPad InStat software.

Statistical analysis. Results are means ± SE. The data were analyzed either by unpaired Student’s t-test (between 2 groups) or by one-way analysis of variance followed by Tukey-Kramer multiple comparison tests (for multiple groups). Differences were considered statistically significant at P < 0.05.

RESULTS

There were no significant differences in the body weights of the rats in the different groups at the end of the experiments [(in g) C1, 270 ± 6; NE, 263 ± 6; C2, 283 ± 7; Pr, 300 ± 12]. Systolic blood pressures were not modified by the infusion of NE or prazosin compared with respective controls [before implantation of osmotic pumps (in mmHg): C1, 117 ± 5; NE, 118 ± 4; C2, 117 ± 4; Pr, 110 ± 5 and at the end of the experiments: C1, 109 ± 5; NE, 114 ± 7; C2, 115 ± 5; Pr, 118 ± 4]. We did not measure MAP in rats infused with NE and saline because these rats were decapitated for the measurement of plasma NE levels at the end of the experiments. The MAP at the end of the experiments for prazosin- and DMSO-infused rats was 95 ± 3 mmHg and 97 ± 5 mmHg (P > 0.05), respectively.

Plasma NE levels were measured in rats infused with saline or NE to evaluate the effectiveness of NE administration. Plasma NE levels were about three times higher in NE-infused rats (107 ± 43 pmol/ml) than in saline-infused rats (30 ± 5 pmol/ml), but this difference did not reach statistical significance (P > 0.05). Plasma NE levels in NE-infused rats were 1.07 × 10-7 M, which were almost equal to the concentration used in the in vitro experiments (10-7 M). To evaluate the efficacy of α1-adrenergic receptor blockade by prazosin, intra-arterial blood pressure responses to bolus injection of α1-adrenergic receptor agonist phenylephrine were measured in rats infused with DMSO or prazosin. The pressor response to 3 µg/kg phenylephrine was 44 ± 7 mmHg in DMSO-infused rats but was undetectable in prazosin-treated rats, indicating that α1-
adrenergic receptors were effectively blocked by prazosin.

AT1 mRNA content in the rat aorta was determined by Northern blot analysis in all four experimental groups. Blots were then stripped and rehybridized to 18S rRNA probes. To determine if DMSO treatment has an effect on aortic AT1 mRNA levels, RNA samples from the aorta of DMSO- or saline-infused rats were analyzed first. We found that there was no significant difference in aortic AT1 mRNA levels between DMSO-infused (AT1 mRNA/18S rRNA, 0.8 ± 0.1) and saline-infused (1.1 ± 0.1, P > 0.05) rats. Aortic AT1 mRNA levels were significantly decreased in NE-infused rats compared with saline-infused rats (Fig. 1). Conversely, aortic AT1 mRNA levels were significantly increased in prazosin-infused rats compared with DMSO-infused rats (Fig. 2).

To determine whether NE has a direct effect on AT1 gene expression in VSMC, AT1 mRNA levels were determined by Northern blot analysis in cultured VSMC treated with NE and/or α-adrenergic receptor antagonists (Fig. 3A). Northern blot analysis (Fig. 3B) indicated that AT1 mRNA levels were decreased by 44% in NE-treated cells compared with PBS-treated cells. Prazosin and yohimbine did not modify the expression of AT1 mRNA compared with that of PBS-treated cells, which may reflect that there was no basal NE level in the culture medium. However, NE-induced downregulation of AT1 mRNA expression was fully prevented by the addition of prazosin, indicating that an α1-adrenergic receptor-mediated mechanism is operating. In contrast, NE-induced downregulation of AT1 mRNA was not modified by the addition of yohimbine, indicating that the α2-adrenergic receptor may not participate in NE-induced regulation of AT1 mRNA expression.

The radioligand binding data are summarized in Table 1. No significant difference in the binding constants was found among VSMC preparations from control, NE, prazosin, and NE + prazosin-treated groups. The total receptor number was lower in the NE-treated group than in the other three groups, but this difference did not reach statistical significance. Figure 4 shows AT1-specific binding sites that were calculated by measuring the difference in 0.5 nM NE-treated group than in the other three groups, but this difference did not reach statistical significance. Figure 4 shows AT1-specific binding sites that were calculated by measuring the difference in 0.5 nM NE.
lates AT1 receptor expression in the vascular tissue first direct evidence showing that NE negatively regulates the AT1 receptor by NE downregulates the AT1 receptor in cultured VSMC.

**DISCUSSION**

We examined the regulation of the AT1 receptor in the aorta and cultured VSMC of rats treated with NE and \( \alpha \)-adrenergic receptor antagonists. The present study contains several distinct observations. First, the data from the in vivo study demonstrate that continuous infusion of NE without alteration of the blood pressure decreases AT1 mRNA levels in the aorta of the rats. Conversely, infusion of a nondepressor dose of prazosin increases AT1 mRNA content. Furthermore, the data from the in vitro study show that NE has a negative effect on both AT1 mRNA expression and AT1 receptor density in cultured VSMC. This inhibitory effect of NE can be prevented by the \( \alpha_1 \)-adrenoceptor antagonist prazosin, but not by the \( \beta_1 \)-adrenoceptor antagonist yohimbine. To our knowledge, this is the first direct evidence showing that NE negatively regulates AT1 receptor expression in the vascular tissue through an \( \alpha_2 \)-adrenergic receptor-mediated mechanism.

The interactions between the renin-angiotensin system and the sympathetic nervous system have been found in various tissues at different levels (1, 14, 19, 21). ANG II works within the central nervous system to stimulate sympathetic outflow and promotes the release of catecholamines from the adrenal medulla (2). ANG II also potentiates the pressor effects of the peripheral sympathetic nervous system. This potentiation is achieved through facilitation of peripheral adrenergic neurotransmission, inhibition of NE reuptake, and enhancement of postsynaptic response of VSMC to \( \alpha_1 \)-adrenoceptor stimulation (9). On the other hand, low-frequency renal sympathetic nerve stimulation promotes renin release through activation of \( \beta_1 \)-adrenoceptors (19). All of this evidence indicates that the renin-angiotensin and sympathetic nervous systems interact positively with each other.

In opposition to these positive interactions, our present results indicate that the sympathetic nervous system negatively regulates the vascular AT1 receptor in vivo and in vitro. Our results are consistent with observations reported in other tissues. For example, it has been shown in brain neuronal cultures of Wistar-Kyoto rats that NE decreases AT1 receptor density and its gene expression (33). Also, renal denervation or sympathetic blockade with guanethidine increases glomerular ANG II receptor density in normotensive and hypertensive rats (23), suggesting that the sympathetic nervous system exerts an inhibitory effect on AT1 receptor expression in glomeruli. These studies provide clear evidence for heterologous downregulation of the AT1 receptor by NE. As a cautionary note, because it is well known that NE causes renin release and an increase in circulating ANG II, the contribution of activation of the renin-angiotensin system to NE-induced downregulation of AT1 mRNA in the present in vivo experiments cannot be ruled out.

The question arises as to the possible mechanisms by which NE induces downregulation of the AT1 receptor in the vascular tissue. Among the many possibilities is that NE activates \( \alpha \)-adrenergic receptors and, through modulation of one or more signaling pathways, downregulates AT1 receptor gene expression. Support of this hypothesis comes from our observations that 1) blockade of the \( \alpha_1 \)-adrenoceptor with prazosin increases AT1 mRNA levels in the aorta of the rat; this increase does not appear to be an artifact of the effect of DMSO because our preliminary experiments showed that there is no significant difference in aortic AT1 mRNA levels between DMSO and saline-infused rats, and 2) the inhibitory effect of NE on both AT1 mRNA and receptor protein in cultured VSMC was prevented by cotreatment with prazosin but not with the \( \alpha_2 \)-adrenergic receptor antagonist yohimbine. It is possible that yohimbine at 0.1 \( \mu \)M may not be sufficient to completely block \( \alpha_2 \)-adrenoceptors. However, the fact that yohimbine had no effect whatsoever does serve to rule out involvement of this receptor type. We have shown that a

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Table 1. Changes in ANG II receptors in cultured VSMC in response to NE and/or prazosin treatment

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<th>Bmax, fmol/mg protein</th>
<th>( K_d ), pM</th>
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<tbody>
<tr>
<td>Control</td>
<td>228 ± 23</td>
<td>482 ± 81</td>
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<tr>
<td>NE</td>
<td>153 ± 25</td>
<td>548 ± 161</td>
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<tr>
<td>Prazosin</td>
<td>201 ± 14</td>
<td>434 ± 49</td>
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<tr>
<td>NE + prazosin</td>
<td>221 ± 31</td>
<td>637 ± 173</td>
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Values are means ± SE; n = 3 for each condition. Bmax, maximal binding capacity; \( K_d \), dissociation constant; VSMC, vascular smooth muscle cells; NE, norepinephrine.

\( ^{125} \text{I}-\text{[Sar, Ile]} \text{ANG II} \) binding in the presence or absence of 1 \( \mu \)M losartan. AT1 receptor density was decreased by 39% in NE-treated compared with PBS-treated VSMC (\( P < 0.05 \)). This decrease was abolished by the addition of prazosin, indicating that activation of the \( \alpha_1 \)-adrenoceptor by NE downregulates the AT1 receptor in cultured VSMC.

![Fig. 4. Bar graph showing AT1 receptor density in cultured VSMC treated with PBS (control), NE (10\(^{-7}\) M), prazosin (Pr, 10\(^{-7}\) M), or NE + Pr for 24 h. Values were obtained by measuring difference of 0.05 nM \( ^{125} \text{I}-\text{[Sar, Ile]} \text{ANG II} \) binding in presence or absence of 1 \( \mu \)M losartan. Results are expressed as means ± SE of 3 experiments. \(* P < 0.05 \) vs. control; \( ^{+} P < 0.05 \) vs. NE.](http://apregu.physiology.org/ by 10.223.33.6 on September 21, 2017)
nonpressor dose of ANG II infusion (25 ng·kg⁻¹·min⁻¹) for 2 wk downregulates AT₁ mRNA levels in both the aorta and mesenteric resistance arteries (27). This homologous downregulation of the AT₁ receptor in the vascular tissue seems to be mediated by both transcriptional and posttranscriptional mechanisms (16). The results from the present study showed that the changes in AT₁ receptor density parallel the changes in AT₁ mRNA levels in cultured VSMC, indicating that NE-mediated downregulation of AT₁ receptors occurs, at least partially, via a diminished AT₁ receptor mRNA level. Although it is unknown whether NE-induced heterologous downregulation of AT₁ receptors occurs at transcriptional or posttranscriptional levels, the data represent a clear example of cross talk between the two plasma membrane receptors.

The physiological and pathophysiological significance of NE on AT₁ receptor expression in the circulatory system of the rats is that the vascular AT₁ receptor is a central component of the renin-angiotensin system, and regulation of its expression is likely to be important in cardiovascular responsiveness. On the basis of our data and those of others, we propose the following events: a positive interaction between ANG II and NE through an action on the vascular AT₁ and α₁-adrenergic receptor to produce a greater vasoconstriction than that produced by either alone. At the same time, persistent increased ANG II and NE will downregulate the AT₁ receptor, providing a “negative feedback” mechanism to attenuate the action of ANG II. Thus cyclic upregulation of ANG II and NE, a “feed-forward” mechanism, followed by downregulation of AT₁ receptor by a negative feedback mechanism may play an important homeostatic role between the renin-angiotensin and sympathetic nervous systems in blood pressure regulation and the normotensive rat. Conversely, in hypertensive rats, disruption of the negative feedback mechanism will prevent downregulation of AT₁ receptor. This ultimately leads to hyperactive renin-angiotensin and sympathetic nervous systems that may contribute to blood pressure increase in hypertensive rats. Support of this hypothesis comes from the study showing that the inhibitory effect of NE on the AT₁ receptor expression in brain neurons of Wistar-Kyoto rats is absent in neurons of spontaneously hypertensive rats (33). Obviously, whether such an alteration occurs in the vascular tissue and leads to overactivity of the effect of ANG II in hypertensive animal models remains to be explored.

In conclusion, we have provided the first direct evidence that NE regulates the vascular AT₁ receptor through a negative feedback mechanism both in vivo and in vitro. The mechanisms underlying this regulation are through the α₁-adrenergic receptor. These studies suggest that, in vasculature, AT₁ expression is regulated by ambient NE levels. Reciprocal regulation between the renin-angiotensin system and sympathetic nervous systems may play an important role in the control of blood pressure hemostasis. By extension, it seems reasonable to assume that lack of negative feedback on AT₁ receptor by ANG II and/or NE may exist in hypertensive rats.

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