Angiotensin in the Nucleus Tractus Solitarii Contributes to Neurogenic Hypertension Caused by Chronic Nitric Oxide Synthase Inhibition

Kenichi Eshima, Yoshitaka Hirooka, Hideaki Shigematsu, Isamu Matsuo, George Koike, Koji Sakai, Akira Takeshita

Abstract—Activation of the sympathetic nervous system and renin-angiotensin system has been suggested to contribute to the hypertension caused by chronic nitric oxide synthase inhibition. The aim of the present study was to determine whether angiotensin within the nucleus tractus solitarii (NTS) plays a role in activation of the sympathetic nervous system in this model. Rats were treated with N^ω-nitro-L-arginine methyl ester (L-NAME, 100 mg · kg⁻¹ · d⁻¹ in drinking water) for 2 weeks. Experiments were performed on anesthetized rats with denervated arterial and cardiopulmonary baroreceptors. Arterial pressure, heart rate, and renal sympathetic nerve activity (RSNA) were measured. Microinjection of an angiotensin II type 1 (AT₁) receptor antagonist (CV11974) or an angiotensin II type 2 (AT₂) receptor antagonist (PD123319) into the depressor region within the NTS (identified by prior injection of L-glutamate) was performed. Microinjection of CV11974, but not of PD123319, produced greater decreases in arterial pressure, heart rate, and RSNA in L-NAME–treated rats than in control rats. The administration of hexamethonium resulted in a larger fall in arterial pressure in L-NAME–treated rats than in control rats. The ACE mRNA level in the brain stem was greater in L-NAME–treated rats than in control rats. These results suggest that increased sympathetic nerve activity plays a role in hypertension caused by chronic nitric oxide synthase inhibition and that activation of the renin-angiotensin system in the NTS is involved at least in part in this increased sympathetic nerve activity via AT₁ receptors. (Hypertension. 2000;36:259-263.)

Key Words: nitric oxide ▪ sympathetic nervous system ▪ brain ▪ angiotensin ▪ blood pressure ▪ heart rate

It is well established that pharmacological inhibition of nitric oxide (NO) synthesis produces acute and chronic hypertension in many animal species, but the underlying mechanisms that mediate this hypertension are not fully understood. Although this hypertension was initially attributed solely to the inhibition of endothelial NO synthase (eNOS), numerous studies have demonstrated that the inhibition of neuronal NO may also play a role. The administration of an NOS inhibitor, such as N^ω-monomethyl-L-arginine (L-NMMA) or N^ω-nitro-L-arginine methyl ester (L-NAME), into the central nervous system evokes acute increases in both arterial pressure and sympathetic nerve activity. These results have been interpreted to suggest that neuronal NO plays roles in the signal transduction pathways involved in the tonic inhibition of sympathetic vasoconstrictor outflow from the brain stem. The removal of this inhibition by NOS inhibitors is postulated to activate the sympathetic nervous system and thereby result in hypertension.

Several investigators have demonstrated a great effect of sympathectomy or ganglionic blockade on L-NAME–induced hypertension, suggesting that the sympathetic nervous system is involved primarily in the maintenance, rather than the initiation, of L-NAME–induced hypertension. However, the specific sites at which NOS inhibition produces progressive neurogenic hypertension are unknown. Neuronal NOS (nNOS) is expressed in the brain stem, including the nucleus tractus solitarii (NTS) and the ventrolateral medulla (VLM). The injection of L-NMMA into the NTS results in increases in arterial pressure and renal sympathetic nerve activity (RSNA). It was demonstrated that L-NAME treatment is accompanied by a progressive attenuation of baroreceptor reflex, which might contribute to neurogenic vasocostriction and hypertension. It has also been suggested that hypertension induced by chronic NOS inhibition is mediated in part by activation of the sympathetic nervous system and the renin-angiotensin system. Plasma norepinephrine level, epinephrine level, and renin activity were increased in rats treated orally with L-NAME. The intravenous injection of both losartan and phentolamine reduced arterial pressure considerably in L-NAME–treated rats. Whole brain NOS activity was reduced by 84% in such rats.
Baseline Levels of Mean Arterial Pressure and Heart Rate Before and After Sinoaortic Denervation and Vagotomy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=15)</th>
<th>L-NAME (n=15)</th>
<th>Control (n=15)</th>
<th>L-NAME (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>99±3</td>
<td>154±1*</td>
<td>97±2</td>
<td>160±3†</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>360±10</td>
<td>330±10*</td>
<td>355±16</td>
<td>371±15‡</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; HR, heart rate; and SAD, sinoaortic denervation. The results were obtained from Protocol 1 (see Methods). Values are mean±SEM.

*P<0.05 vs control rats.
†P<0.01 vs control rats.
‡P<0.05 vs before SAD and vagotomy.

We recently reported that the chronic inhibition of NOS causes microvascular remodeling and cardiac hypertrophy associated with activation of the tissue renin-angiotensin system.16–19 An independent renin-angiotensin system exists in the brain,20–22 and the microinjection of angiotensin II into the NTS has been shown to increase arterial pressure and heart rate.23 The aim of this study was thus to determine whether brain angiotensin in the NTS plays a role in the hypertension caused by chronic NOS inhibition, which in turn activates the sympathetic nervous system. For this purpose, we examined the effects of an angiotensin II type 1 (AT1) receptor antagonist (CV11974) microinjected into the NTS on arterial pressure, heart rate, and RSNA in anesthetized rats treated with L-NAME.

Methods

General Procedures

These experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Faculty of Medicine, and were conducted according to the guidelines for animal experiments of the Kyushu University Faculty of Medicine.

Experiments were performed on male Wistar-Kyoto rats weighing 300 to 350 g. Anesthesia was induced with pentobarbital sodium (50 mg/kg IP), and an intravenous infusion of propofol (300 to 350 mg) for 2 weeks as previously described.16–19 At the concentration administered, the daily intake of L-NAME was ~30 to 40 mg.

All rats were fed a normal diet and housed singly in a special pyrogen-free facility.

Experimental Protocols

Protocol 1

Microinjections of 2 doses of CV11974 (0.1 and 1 nmol) into the NTS were made in L-NAME–treated rats as well as in control rats (15 rats of each group) to examine the effect of AT1 receptor blockade on arterial pressure, heart rate, and RSNA.

Protocol 2

A microinjection of PD123319 (0.1 nmol) into the NTS was made in L-NAME–treated rats as well as in control rats (15 rats of each group) to examine the effect of AT2 receptor blockade on arterial pressure, heart rate, and RSNA.

Protocol 3

A microinjection of vehicle (the solution dissolved CV11974) into the NTS was made in both groups of rats (15 rats of each group) to rule out nonspecific effects of microinjection on arterial pressure, heart rate, and RSNA.

Protocol 4

A microinjection of CV11974 (1 nmol) into the area postrema was made to examine the effect of AT1 receptor blockade on arterial pressure, heart rate, and RSNA.

Protocol 5

To determine whether basal sympathetic nerve activity is activated in L-NAME–treated rats, hexamethonium chloride was administered intravenously (40 mg/kg) at the end of the microinjection experiments.

Protocol 6

To determine whether the brain stem renin-angiotensin system is activated in L-NAME–treated rats, we assayed brain stem levels of ACE and AT1 receptor mRNAs in L-NAME–treated and control rats.

Histological Examination

At the end of experiments, the micropipette containing Evans’ blue was positioned at the site of the drug injection, and Evans’ blue (100 nL) was injected to determine the site of injection at postmortem examination. After completion of the experiments, the rat was deeply anesthetized with an overdose of pentobarbital, and the brain was perfused with 0.9% saline followed by 4% paraformaldehyde solution through the heart. The brain stem was removed, and frozen sections (50 μm) were cut serially. The location of the injection site was identified under a microscope.

Statistical Analysis

All values are expressed as mean±SEM. The unpaired t test was used to compare the baseline values between the groups. The paired t test was used to examine the effects of each intervention within a group. ANOVA followed by Bonferroni’s multiple comparison test was used to compare the values of changes in differences between groups. Differences were considered significant for values of P<0.05.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared from the brain stem with ISOGEN (Nippon Gene), followed by poly(A) RNA purification with an oligo(dT)/cellulose column (Takara Shuzo). Poly(A)+ RNA (5 μg/lane) was separated with electrophoresis and transferred onto a nylon membrane (Hybond N+; Amersham). Hybridization was carried out with a 32P-labeled EcoRV/EcoRI fragment of rat ACE cDNA,23 rat AT1 receptor cDNA,20 or mouse GAPDH cDNA, followed by autoradiography. Autoradiograms were scanned with a Fuji phosphorimagery system to measure amounts of ACE and AT1 receptor mRNAs normalized against those of GAPDH mRNA.

Animal Model of Chronic Inhibition of NO Synthesis

The rats were randomly separated into 2 groups. The first group (control) received untreated drinking water for 2 weeks. The second group received L-NAME (Sigma Chemical Co) in drinking water (1 mg/mL) for 2 weeks as previously described.16–19 At the concentration administered, the daily intake of L-NAME was ~30 to 40 mg.
Results

Baseline Levels of Arterial Pressure and Heart Rate
The Table shows the baseline levels of mean arterial pressure and heart rate in the L-NAME–treated and control rats before and after sinoaortic denervation and vagotomy. Mean arterial pressure in L-NAME–treated rats was higher than that in control rats (P<0.01). Heart rate did not differ between the 2 groups after sinoaortic denervation and vagotomy.

Effects on Arterial Pressure, Heart Rate, and RSNA of Microinjection of AT$_1$ Receptor Antagonist, AT$_2$ Receptor Antagonist, or Vehicle Injected Into the NTS
Prior microinjection of L-glutamate into the NTS (0.5 nmol) decreased mean arterial pressure (−28±8 versus −34±12 mm Hg) and heart rate (−18±2 versus −18±2 bpm) in both control and L-NAME–treated rats. The magnitudes of the decreases in these variables did not differ between the 2 groups.

Microinjections of CV11974 (0.1 and 1 nmol) into the depressor regions in the NTS (identified prior injection of L-glutamate) decreased arterial pressure, heart rate, and RSNA (Figure 1). The magnitudes of decreases in arterial pressure, heart rate, and RSNA evoked by CV11974 were greater in L-NAME–treated rats than in control rats (Figure 1). On the other hand, the microinjection of PD123319 (0.1 nmol) into the NTS produced no changes in these variables in either group. The microinjection of vehicle into the NTS also did not produce changes in these variables.

Effects of on Arterial Pressure and Heart Rate AT$_1$ Receptor Antagonist Injected Into the Area Postrema
The microinjection of CV11974 (1 nmol) into the area postrema produced no changes in arterial pressure or heart rate in either group.

Effects of Systemic Administration of Hexamethonium
Figure 2 shows the effects on mean arterial pressure of the intravenous injection of hexamethonium. The mean arterial pressure of L-NAME–treated rats was significantly higher than that of control rats before the injections of hexamethonium. After the intravenous injection of hexamethonium, mean arterial pressure decreased to the same levels, and the change in mean arterial pressure of L-NAME–treated rats was significantly greater than that of control rats (−100.0±7.3 versus −43.7±4.1 mm Hg, P<0.01).

ACE and AT$_1$ Receptor Expression in the Brain Stem
As shown in Figure 3, the ACE mRNA level in the brain stem of L-NAME–treated rats was higher than that in brain stem of control rats. On the other hand, the AT$_1$ receptor mRNA level did not differ between the 2 groups.

Discussion
The major finding of the present study was that AT$_1$ receptor blockade, but not AT$_2$ receptor blockade, in the NTS reduced arterial pressure, heart rate, and RSNA in rats chronically treated with L-NAME, suggesting that activation of the renin-angiotensin system in the NTS via AT$_1$ receptors plays a role in the neurogenic hypertension in this model. The intravenous injection of hexamethonium produced a greater fall in arterial pressure in rats treated with L-NAME. After hexamethonium injection, the arterial pressure of rats treated

Figure 1. Histogram of grouped results showing the changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) evoked by microinjections of 2 doses of CV11974 (0.1 and 1 nmol) into the NTS. **P<0.01 vs control.

Figure 2. Individual and summary data (mean±SEM) of effects of intravenous hexamethonium on mean arterial pressure in rats treated with L-NAME or vehicle. **Significant responses to hexamethonium (P<0.01). ††Significant differences in responses to hexamethonium between rats treated with L-NAME and those treated with vehicle (P<0.01).
with L-NAME was similar to that of control rats. These results indicate that activation of the sympathetic nervous system contributes to the hypertension in this model. Furthermore, we found an increase in ACE mRNA level in the brain stem of the L-NAME–treated rats, which supports the hypothesis of activation of the renin-angiotensin system in the brain stem in this model.

We first consider the possibility that the greater response to microinjection of CV11974 on arterial pressure in the L-NAME–treated rats was nonspecific due to the presence of a higher baseline arterial pressure. However, this is unlikely because the prior injection of L-glutamate produced similar depressor responses in the 2 groups. In addition, we also found a greater decrease in RSNA in the L-NAME–treated rats than in the control rats. Furthermore, we ruled out the possibility of a nonspecific effect of vehicle solution–dissolved CV11974.

Sander et al. found that once L-NAME–induced hypertension developed, it can be completely reversed by acute ganglionic blockade, suggesting that the sympathetic nervous system plays a role in L-NAME–induced hypertension in the chronic phase. They demonstrated that L-NAME treatment induced smaller (by \( \approx 50\% \)) increases in arterial pressure in rats with than in those without sympathectomy. Furthermore, in rats without sympathectomy that received chlorisondamine, ganglionic blockade produced larger decreases in arterial pressure in L-NAME–treated than in vehicle-treated rats. Thus, although arterial pressure was higher in L-NAME–treated than in vehicle-treated rats before ganglionic blockade, the values of arterial pressure in the 2 groups were comparable after ganglionic blockade. It has also been shown that ganglionic blockade induced by trimethaphan caused a greater decrease in arterial pressure in L-NAME–treated rats than in control rats. Our results are consistent with the results of these studies, indicating that an increase in central sympathetic drive contributes to the hypertension induced by the chronic inhibition of NOS.

There is evidence that L-NAME crosses the blood-brain barrier when administered orally. It has been demonstrated that NOS activity in the cerebral tissue was reduced by 84% with L-NAME. Previous studies have indicated that 120 minutes is required for intravenous L-NAME to cross the blood-brain barrier and reach a concentration in the central nervous system sufficient to nearly completely inhibit NOS activity, as measured in brain homogenates in many species.

The relationship between the renin-angiotensin system and the sympathetic nervous system is complex. It has been demonstrated that intravenous injection of the AT\(_1\) receptor antagonist losartan alone causes a small decrease in arterial pressure in L-NAME–treated rats but that the combination of \( \alpha_1 \)-adrenoceptor and AT\(_1\) receptor blockade was potently antihypertensive, resulting in normalization of arterial pressure in chronically NO blockade hypertensive rats. On the other hand, the intravenous injection of losartan caused a greater decrease in arterial pressure in rats treated with L-NAME than in normotensive rats. The reason for the discrepancy in results between these studies is not clear. Because an AT\(_1\) receptor antagonist was administered intravenously in those studies, activation of the renin-angiotensin system in the brain stem may be more important for increased sympathetic nerve activity, which in turn produces hypertension.

The role of angiotensin II in the NTS is not fully understood. The microinjection of angiotensin II into the NTS has been reported to have both pressor and depressor effects. The microinjection of angiotensin antagonist into the NTS has been shown to facilitate arterial baroreceptor reflex control of heart rate, suggesting that endogenous angiotensin within the NTS reduces arterial baroreflex control. The intracisternal infusion of angiotensin II caused pressor effects and activated the neurons in the NTS, VLM, and area postrema, as determined with c-fos immunohistochemistry. However, we found no effect on arterial pressure of the microinjection of AT\(_1\) receptor antagonist into the area postrema. The microinjection of L-NMMA into the area postrema has been shown to have no effect on arterial pressure and RSNA. The role played by the VLM in the increase in sympathetic nervous system caused by chronic NOS inhibition cannot be determined on the basis of the results of our study. It was recently suggested that rostral VLM neurons are activated in chronic NOS inhibition. Further studies will be needed to determine whether the renin-angiotensin system in the rostral VLM is also activated by chronic inhibition of NOS or whether the increase in neuronal activity of the rostral VLM is related to activation of the renin-angiotensin system of NTS neurons caused by chronic inhibition of NOS.
The mechanisms by which the renin-angiotensin system is activated by chronic NOS inhibition cannot be determined on the basis of the results of our study. A decrease in NO synthesis induces the synthesis of growth-promoting factors from the endothelium. It has been hypothesized that vascular and myocardial structural changes in rats treated with L-NAME are mediated by the local elaboration of growth-promoting factors, which in turn are induced by activation of the renin-angiotensin system, including ACE. Further studies are needed to clarify the mechanism of an activation of renin-angiotensin system caused by chronic NO inhibition.

In summary, our results suggest that the chronic inhibition of NOS activates the sympathetic nervous system and that activation of the renin-angiotensin system via AT1 receptors in the NTS contributes at least in part to this neurogenic hypertension.

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References

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