Rho/Rho-Kinase Pathway in the Brainstem Contributes to Hypertension Caused by Chronic Nitric Oxide Synthase Inhibition

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Abstract—Central nervous system mechanisms are involved in hypertension caused by chronic inhibition of nitric oxide (NO) synthesis. Chronic inhibition of NO synthesis might also activate the Rho/Rho-kinase pathway in the vasculature. We recently demonstrated that activation of the Rho/Rho-kinase pathway in the nucleus tractus solitarii (NTS) contributes to hypertensive mechanisms in spontaneously hypertensive rats. The aim of the present study was to determine whether activation of this pathway also contributes to neurogenic hypertensive mechanisms caused by chronic NO synthesis inhibition. The NO synthase inhibitor N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME) was administered to Wistar-Kyoto rats in their drinking water (1 mg/mL) for 2 weeks. Bilateral microinjection of Y-27632, a specific Rho-kinase inhibitor, into the NTS elicited decreases in arterial pressure, heart rate, and renal sympathetic nerve activity in control rats and L-NAME–treated rats. The magnitude of the decrease, however, was significantly greater in L-NAME–treated than in control rats. In another group of rats, the specific Rho-kinase inhibitor, Y-27632, was administered intracisternally for 2 weeks with a mini-osmotic pump from the beginning of treatment with L-NAME. Y-27632 co-treatment significantly attenuated the increase in arterial pressure. Furthermore, the expression level of membranous RhoA and phosphorylation of the target proteins of Rho-kinase, the ERM (ezrin, radixin, moesin) family members, was significantly greater in L-NAME–treated rats than in control rats. These results indicate that activation of the Rho/Rho-kinase pathway in the NTS contributes to neurogenic hypertension caused by chronic NO synthase inhibition. (Hypertension. 2004;43:156-162.)

Key Words: nitric oxide □ blood pressure □ sympathetic nervous system □ hypertension □ brain

Inhibition of nitric oxide (NO) synthesis produces hypertension in many species.\textsuperscript{1,2} Although this hypertension was initially attributed to the inhibition of endothelial NO synthesis, numerous studies suggest that inhibition of neuronal NO also plays an important role in blood pressure regulation.\textsuperscript{3-7} A previous study demonstrated that increased sympathetic nerve activity plays a role in hypertension caused by chronic NO synthesis inhibition, and activation of the renin–angiotensin system in the nucleus tractus solitarii (NTS) of the brainstem via angiotensin II type 1 receptors is involved.\textsuperscript{8} Chronic inhibition of NO synthesis might activate the Rho/Rho-kinase pathway in the cardiac tissues or vasculature.\textsuperscript{9,10} The Rho/Rho-kinase pathway regulates the phosphorylation state of myosin light chains and contributes to smooth muscle contraction.\textsuperscript{11} Y-27632, a specific Rho-kinase inhibitor, dramatically reduces blood pressure in rat models of hypertension.\textsuperscript{12} In addition, Rho-kinase activity is augmented in hypertensive blood vessels\textsuperscript{13} and inhibition of Rho-kinase induces preferential forearm vasodilatation in hypertensive patients, but not in normal subjects.\textsuperscript{14} Thus, the Rho/Rho-kinase pathway plays an important role in peripheral mechanisms of hypertension.\textsuperscript{11-14}

RhoA and Rho-kinase are also distributed in the central nervous system.\textsuperscript{15,16} Activation of the Rho/Rho-kinase pathway in the NTS contributes to maintain basal blood pressure via the sympathetic nervous system, and this pathway might contribute to hypertensive mechanisms in spontaneously hypertensive rats.\textsuperscript{17} It is unknown, however, whether activation of the Rho/Rho-kinase pathway also contributes to neurogenic hypertension caused by chronic NO synthesis inhibition. The aim of this study was to elucidate the role of the Rho/Rho-kinase pathway in the brainstem in neurogenic hypertension caused by chronic NO synthesis inhibition.

For this purpose, we microinjected Y-27632 into the NTS of Wistar-Kyoto (WKY) rats treated with the NO synthase inhibitor N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME) and monitored systolic blood pressure (SBP), heart rate (HR), and
renal sympathetic nerve activity (RSNA) under anesthesia. Furthermore, Y-27632 or vehicle was continuously infused intracisternally for 2 weeks with a mini-osmotic pump in WKY rats from the beginning of treatment with l-NAME, and SBP was measured. Finally, we compared RhoA and Rho-kinase activity in the NTS between control rats and l-NAME-treated rats.

Methods

This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences and was conducted according to the guidelines for animal experiments of Kyushu University.

Microinjection Experiments With Rho-Kinase Inhibitors

Male WKY rats (280 to 340 g, 16 to 20 weeks old) obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine were used. The rats were randomly divided into two groups. The first group (control rats) received nontreated drinking water for 2 weeks. The second group received l-NAME (Sigma Chemical Co.), in the drinking water (1 mg/mL) for 2 weeks (l-NAME-treated rats). The animals were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and a cannula (PE 50) was inserted into the right femoral artery for measurement of SBP and HR and into the femoral vein for infusion of pentobarbital (10 to 15 mg/kg per hour). The anesthetized animals were artificially ventilated and placed in a stereotaxic frame. The dorsal surface of the medulla was exposed, the microinjection sites were defined according to an atlas of the rat brain, and the depressor response was evoked by l-glutamate (40 pmol) microinjection. We then microinjected Y-27632 (0.4, 4, 40 pmol/site X 2 sites/side, bilaterally; injection volume: 80 nL/site). In addition, we measured RSNA in another experiment. The left renal nerve was divided into two groups. The first group (control rats) received nontreated drinking water for 2 weeks. The second group (l-NAME-treated rats) received l-NAME in drinking water (1 mg/mL) for 2 weeks. We then performed Western blot analysis to compare the RhoA or Rho-kinase activity in the NTS of control rats with that in l-NAME-treated rats. The animals were killed with an overdose of sodium pentobarbital, and the NTS tissue was obtained by punching out the NTS from coronal slices (2-mm thickness) of the brainstem with an 18-gauge needle (inside diameter: 950μm). The tissues were homogenized in a lysing buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, and 1 mmol/L Na3VO4 and 1 mmol/L PMSF. The tissue lysate was centrifuged and the supernatant collected. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical). An aliquot of 15 μg of protein from each sample was separated on 10% SDS-polyacrylamide gel. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore). Membranes were incubated with rabbit anti-phosphorylated ERM family members was performed as described, except that 30 μg of protein was applied to the gel.

Confirmation of Inhibitory Effects of Y-27632 on Rho-Kinase Activity

Ten days after the beginning of treatment with l-NAME and intracisternal infusion of Y-27632, the animals were killed with an overdose of sodium pentobarbital. The tissues of the brainstem were obtained and Western blot analysis for phosphorylation of the ERM family members was performed as described, except that 30 μg of protein was applied to the gel.

Statistical Analysis

All values are expressed as mean±SEM. ANOVA was used to compare SBP between rats co-treated with Y-27632 and vehicle. Any two mean values were compared by application of the Bonferroni procedure. An unpaired t test was used to compare the baseline values and the effects of each intervention between the groups. Differences were considered to be statistically significant when P<0.05.
Results

Effect of Microinjection of Y-27632 Into the NTS
Baseline SBP (175±11 versus 120±11 mm Hg; \( P<0.01 \); \( n=9 \) for each) was significantly higher in L-NAME-treated rats than in control rats, and HR (278±8 versus 281±3 bpm; \( n=9 \) for each) did not differ between the two groups. Baseline RSNA did not differ between the two groups (110±15 versus 86±12 spikes/sec; \( n=5 \) for each). Microinjection of Y-27632 into the NTS elicited a dose-dependent decrease in SBP and HR in both groups (Figure 1A). The magnitude of the decrease in SBP and HR was significantly greater in L-NAME-treated rats than in control rats (Figure 1B). Furthermore, in other experiments in which RSNA was recorded, microinjection of Y-27632 into the NTS elicited decreases in SBP, HR, and RSNA in both groups (Figure 2A). The magnitude of the decreases in these variables was also significantly greater in L-NAME-treated rats than in control rats (Figure 2B). Moreover, the percent reduction of SBP (19±3 versus 10±3%; \( P<0.05 \); \( n=5 \) for each) or HR (15±2 versus 6±2%; \( P<0.01 \); \( n=5 \) for each) was significantly greater in L-NAME-treated rats than in control rats.

Effects of Repetitive Injection of Y-27632 Into the NTS
A similar depressor response was observed (40 pmol/site×2 sites/side, bilaterally; injection volume: 80 nL/site) if there was a 20-minute interval between injections (\( \Delta \text{SBP}: -32±3 \) versus \( -30±2 \) mm Hg; \( \Delta \text{HR}: -58±6 \) versus \( -56±7 \) bpm;
n=3 for each), although the magnitude of the decrease in SBP was smaller at 10 minutes after the injection than that of the first injection (SBP: 12±4 versus 29±3 mm Hg; HR: 20±3 versus 52±5 bpm; P<0.01; n=3 for each).

Effect of Microinjection of Hydralazine Into the NTS of l-NAME–Treated Rats
There was no significant change in SBP or HR after microinjection of hydralazine (50 pmol/site 1 site/side; SBP: 179±5 versus 177±4 mm Hg; HR: 280±3 versus 284±2 bpm; n=3 for each; or 500 pmol/site×1 site/side; SBP: 180±14 versus 177±11 mm Hg; HR: 288±9 versus 284±6 bpm; n=3 for each).

Intravenous Infusion of Rho-Kinase Inhibitor to l-NAME-Treated Rats
Intravenous infusion of Y-27632 (200 pmol or 1000 pmol/0.2 mL injection over 50-second period with infusion pump) elicited no significant changes in SBP or HR (200 pmol; SBP: 175±3 versus 176±5 mm Hg; HR: 278±5 versus 279±5 bpm; n=3 for each; or 1000 pmol; SBP: 177±12 versus 176±16 mm Hg; HR: 276±11 versus 274±66 bpm; n=3 for each).

Blood Pressure Changes After the Beginning of l-NAME Treatment
Figure 3A and 3B show the time course of SBP after treatment with l-NAME and intracisternal infusion of Y-27632. SBP was increased in the l-NAME-VEH and l-NAME-Y-27632 groups. Y-27632, however, significantly attenuated the increase in SBP. After discontinuing treatment with Y-27632, SBP increased to a level similar to that in the l-NAME-VEH rats (Figure 3A). There was a small transient decrease in SBP from day 7 to day 11 compared with before treatment (day 0) in the control-Y-27632 group.

Inhibitory Effects of Y27632 on Rho-Kinase Activity
To confirm the specific inhibitory effects of Y-27632 on Rho-kinase activity, we examined phosphorylation of the ERM family members, substrates of Rho-kinase, in the brainstem containing the NTS of l-NAME-treated rats. Data are expressed as the relative ratio to control rats, which was assigned a value of 1. Phosphorylation of the ERM family members was significantly increased in l-NAME-treated rats, and significantly reduced in the Y-27632-treated rats (n=4 for each). *P<0.05 vs l-NAME with vehicle. ##P<0.01 versus control rats. D, Western blot analysis for phosphorylated ERM family members in the brainstem containing the NTS of control rats. Data are expressed as relative ratio to control rats, which was assigned a value of 1. The expression level of phosphorylation of the ERM family members was slightly reduced in control-Y-27632 rats compared with control rats (n=3 for each). #P<0.05 vs control rats.

RhoA and Rho-Kinase Activity in the NTS
The expression level of RhoA in membranes, which represents RhoA activity, was greater in the l-NAME–treated rats than in control rats (Figure 4A). Furthermore, the extent of...
phosphorylation of the ERM family members, which represents Rho-kinase activity, was greater in l-NAME-treated rats than in control rats (Figure 4B).

**Discussion**

The present study provides the first direct evidence that activation of the Rho/Rho-kinase pathway in the brainstem plays an important role in hypertension caused by chronic NO synthase inhibition. We demonstrated that: (1) microinjection of Rho-kinase inhibitor into the NTS significantly decreased SBP, HR, and RSNA in the l-NAME-treated rats than in control rats; (2) chronic intracisternal infusion of Rho-kinase inhibitor suppressed hypertension caused by chronic NO synthase inhibition; and (3) membrane translocation of RhoA and phosphorylation of the ERM family members were increased in the NTS of l-NAME-treated rats.

The results of the present study demonstrate that the membrane expression level of RhoA in the NTS was significantly greater in l-NAME-treated rats than in control rats, and microinjection of Y-27632 elicited a dose-dependent decrease of SBP and HR in l-NAME-treated rats and control rats. The magnitude of the decrease in SBP, HR, and RSNA was greater in l-NAME-treated rats than in control rats. In the microinjection study, we reproduced the responses to injections of Y-27632 into the bilateral NTS by repeating the injection after 20 minutes, although it was expected that enzymatic inhibition of Rho-kinase evoked by Y-27632 could be effective for a much longer period. Thus, we cannot exclude the possibility that Y-27632, a pyridine derivative, might elicit an action like that of an agonist, or short-lived antagonist, at the specific receptors in the NTS. Furthermore, the extent of the phosphorylation of ERM family members in the NTS was greater in l-NAME-treated rats than in control rats. Previous studies demonstrated that the Rho/Rho-kinase pathway is activated in blood vessels of l-NAME-treated rats. Thus, in the peripheral circulation, Rho-kinase is apparently involved in the mechanisms of l-NAME-induced hypertension. There is evidence that l-NAME crosses the blood–brain barrier when administered orally and the sympathetic nervous system is involved primarily in the maintenance, rather than initiation, of l-NAME-induced hypertension. Furthermore, activation of the Rho/Rho-kinase pathway in the NTS plays an important role in the maintenance of the basal arterial blood pressure via the sympathetic nervous system. In addition, activation of the pathway might be involved in the central nervous system mechanisms of hypertension in spontaneously hypertensive rats. Our results indicate that the Rho/Rho-kinase pathway is activated in the NTS of l-NAME-treated rats and contributes, at least in part, to the central nervous system mechanisms of hypertension caused by chronic NO synthase inhibition.

The Rho/Rho-kinase pathway plays an important role in vascular tone regulation. Because the NTS is rich in blood vessels, it is possible that inhibition of Rho-kinase activity in the NTS changes blood pressure by increasing local blood flow. Therefore, we microinjected another vasodilator, hydralazine, bilaterally into the NTS of l-NAME-treated rats and confirmed that hydralazine did not alter blood pressure. Thus, the effects of the Rho-kinase inhibitor in the NTS on blood pressure regulation were not caused by its local vasodilator effect.

Our findings suggest that endogenous Rho-kinase in the brainstem plays an important role in l-NAME-induced hypertension. As demonstrated by the chronic experiments, the l-NAME-induced increase in blood pressure was significantly suppressed during intracisternal Y-27632 infusion. Nonspecific effects caused by the surgical procedure are unlikely because continuous intracisternal infusion of vehicle using the same devices did not suppress the SBP elevation, and after discontinuing the Y-27632 infusion, SBP increased to a level similar to that in l-NAME-VEH rats. In addition, phosphorylation of ERM family members in the brainstem was significantly increased in l-NAME-VEH rats and reduced in the l-NAME-Y-27632 rats, which strongly suggests that the Rho/Rho-kinase pathway in the brainstem of l-NAME-treated rats activates Rho-kinase activity in vivo and Y27632 suppresses it.

In the present study, intracisternal infusion of Y-27632 prevented the increase in SBP on the first day of l-NAME treatment, which is not consistent with the reports that the
initial increase in SBP with \( L \)-NAME is caused by the inhibition of vascular NO.\(^3\) The latency of the onset of the sympathectomy-sensitive component of intravenous infusion of \( L \)-NAME–induced hypertension, however, was 1 to 2 hours.\(^24\) The latency of onset of neural component after oral administration of \( L \)-NAME might be longer than intravenous infusion; 24 hours is when the neural component of \( L \)-NAME–induced blood pressure elevation becomes apparent. Furthermore, the effect of intracisternal infusion of Y-27632 on SBP was expected to begin much faster than that of oral administration of \( L \)-NAME. Therefore, intracisternal infusion of Y-27632 might prevent \( L \)-NAME–induced blood pressure elevation.

To exclude the possibility of vascular action of intracisternal infusion of Y-27632, we intravenously infused Y-27632 (1000 pmol/0.2 mL injection over 50-second period with an infusion pump) into \( L \)-NAME–treated rats. This amount of Y-27632 was the same as that of an 8-hour intracisternal infusion dose with the pump used in the present study and elicited no significant change in SBP or HR. Furthermore, Y-27632 was intracisternally infused into the control rats. In the control rats, Y-27632 induced a small transient decrease in SBP from day 7 to day 11 compared with before treatment (day 0). In addition, the expression level of phosphorylation of the ERM family members in the brainstem was slightly reduced in control-Y-27632 rats compared with control-VEH rats. SBP and the extent of phosphorylation of the ERM family members was significantly decreased in control-Y27632 rats, although the magnitudes were small because of indirect effects of Y-27632 in the NTS and other brain areas. These results suggest that the Rho/Rho-kinase pathway in the brainstem of normotensive rats contributes somewhat to blood pressure regulation.\(^17\) This result is also consistent with the results of our previous study, demonstrating transfection of adenovirus encoding dominant-negative Rho-kinase into the NTS of \( L \)-NAME–treated rats, although the magnitudes were small because of differences in Rho-kinase activity in \( L \)-NAME–treated rats. We demonstrated, however, that a greater depressor effect of Y-27632 on blood pressure and/or RSNA in \( L \)-NAME–treated rats than in control rats in microinjection experiments. In addition, RhoA and Rho-kinase activity was enhanced in \( L \)-NAME–treated rats compared with control rats. Thus, we believe that the effect of Y-27632 is at least partly mediated by inhibition of Rho-kinase activity in the NTS. Further studies are needed to determine whether other cardiovascular centers of the brainstem are affected by chronic NO synthesis inhibition or intracisternal infusion of Y-27632.

In conclusion, we demonstrated that the Rho/Rho-kinase pathway in the NTS is activated in \( L \)-NAME–treated rats as compared with control rats. Activation of the Rho/Rho-kinase pathway plays an important role in \( L \)-NAME–induced hypertension.

**Perspectives**

We do not know the mechanisms by which activation of the Rho/Rho-kinase pathway in the NTS alters arterial blood pressure via the sympathetic nervous system or the cause of the greater activation of the Rho/Rho-kinase pathway in \( L \)-NAME–treated rats. A recent study demonstrated that activity of the renin-angiotensin system in the NTS is increased by chronic NO synthase inhibition.\(^8\) Accumulating evidence suggests that Rho-kinase is substantially involved in the signal transduction initiated by many vasoactive substances, such as angiotensin II.\(^28,29\)

Indeed, a considerable amount of evidence suggests that angiotensin II in the central nervous system contributes to the neural mechanisms of hypertension.\(^37\) Therefore, activation of the Rho/Rho-kinase pathway in the NTS of \( L \)-NAME–treated rats might be elicited by activation of the renin–angiotensin system in the NTS. Furthermore, the neuronal Rho/Rho-kinase pathway contributes to dendritic spine formation.\(^30,31\) Dendritic spines form postsynaptic sites in excitatory synapses in the central nervous system. Recent studies suggest that morphological changes in dendritic spines occur rapidly\(^32\) and are associated with glutamate sensitivity.\(^33\) Indeed, there are structural differences of dendritic spines in the NTS between WKY rats and SHR.\(^34\) Therefore, the Rho/Rho-kinase pathway affects synaptic transmission in the NTS and might also be involved in baroreflex function. Further studies are needed to clarify the mechanisms responsible for our observations.

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