Rho/Rho-Kinase Pathway in Brain Stem Contributes to Blood Pressure Regulation via Sympathetic Nervous System Possible Involvement in Neural Mechanisms of Hypertension

Koji Ito, Yoshitaka Hirooka, Koji Sakai, Takuya Kishi, Kozo Kaibuchi, Hiroaki Shimokawa, Akira Takeshita

Abstract—Recent studies have demonstrated that the Rho/Rho-kinase pathway plays an important role in various cellular functions, including actin cytoskeleton organization and vascular smooth muscle contraction. This pathway is also present in the central nervous system and is involved in the maintenance of dendritic spines and axon outgrowth in the regulation of neurotransmitter release. However, its role in central blood pressure regulation is unknown. In the present study, blockade of the Rho/Rho-kinase pathway in the nucleus tractus solitarii (NTS) of the brain stem by microinjection of a specific Rho-kinase inhibitor decreased blood pressure, heart rate, and renal sympathetic nerve activity in both Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). However, the magnitude of decreases in these variables was greater in SHR than in WKY rats. In addition, an adenovirus vector encoding dominant-negative Rho-kinase decreased blood pressure, heart rate, and urinary norepinephrine excretion in both WKY rats and SHR in an awake and free-moving state. The magnitude of decreases in these variables was also greater in SHR than in WKY rats. Furthermore, membrane RhoA expression and Rho-kinase activity in the NTS were enhanced in SHR compared with WKY rats. These observations indicate that the Rho/Rho-kinase pathway in the NTS contributes to blood pressure regulation via the sympathetic nervous system in vivo and suggest that activation of this pathway is involved in the central mechanisms of hypertension. (Circ Res. 2003;92:1337-1343.)

Key Words: blood pressure ■ heart rate ■ sympathetic nervous system ■ hypertension ■ brain

The small GTPase, Rho, and its downstream effector, Rho-kinase, are implicated in various cellular functions, including myosin light chain phosphorylation and smooth muscle contraction.1–3 A recent study has reported that Y-27632, a specific Rho-kinase inhibitor, dramatically reduces hypertension in rat models of hypertension.4 In addition, Rho-kinase activity is augmented in hypertensive blood vessels,5 and inhibition of Rho-kinase induces preferential forearm vasodilatation in hypertensive patients but not in normal subjects.6 Thus, the Rho/Rho-kinase pathway plays a role in peripheral mechanisms of hypertension.3–6

RhoA and Rho-kinase are also distributed in the central nervous system (CNS).7,8 The Rho/Rho-kinase pathway is involved in the maintenance of dendritic spines,9 neurite remodeling,10 and axon outgrowth in vitro.11 These morphological changes are actin dependent and are regulated by the activation of RhoA and Rho-kinase.11,12 Dendritic spines form the postsynaptic contact sites for the majority of excitatory synapses in the CNS. Recent studies suggest that morphological changes in dendritic spines occur rapidly and are associated with synaptic transmission.13,14 In addition to contributing to the formation of these structures, the Rho/Rho-kinase pathway also regulates the exocytosis of neurotransmitters.15 Therefore, the Rho/Rho-kinase pathway might play important roles in the establishment and maintenance of synaptic transmission.

The role of the Rho/Rho-kinase pathway in the central mechanism of blood pressure regulation is unknown, especially in the nucleus tractus solitarii (NTS) of the brain stem, which has cardiovascular regulatory functions.16,17 The NTS receives signals through afferent fibers from arterial baroreceptors, chemoreceptors, cardiopulmonary receptors, and other visceral receptors.18 Thus, the NTS plays an important role in the integration of the cardiovascular system.16–19 The aim of the present study was to elucidate the role of the Rho/Rho-kinase pathway in the NTS in blood pressure regulation in vivo. For this purpose, a specific Rho-kinase inhibitor was microinjected into the rat NTS, and blood pressure, heart rate (HR), and renal sympathetic nerve activity (RSNA) were monitored with the animals under anesthesia. Furthermore, adenovirus vectors encoding either a dominant-negative Rho-kinase (AdDNRhoK) or β-galactosidase (Adβgal) were transfected into the NTS in vivo, and blood pressure and HR were continuously moni-
tored in awake free-moving rats using a radiotelemetry system. Finally, RhoA and Rho-kinase expression or Rho-kinase activity in the NTS was compared between Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), and the inhibitory effects of AdDNRhoK were examined.

**Materials and Methods**

The present 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 a Rho-Kinase Inhibitor**

Male SHR or WKY rats (280 to 340 g, 16 to 20 weeks old) were used. Rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine (Fukuoka, Japan). The animals were anesthetized with sodium pentobarbital (50 mg/kg IP), and a cannula was inserted into the right femoral vein for infusion of pentobarbital. The anesthetized animals were artificially ventilated and placed in a stereotaxic frame. The dorsal surface of the medulla was exposed, and the microinjection site was determined by checking for an L-glutamate (40 pmol)-induced depressor response. A specific Rho-kinase inhibitor, Y-27632 (0.4, 4, and 40 pmol/site at four sites; 80 nL injection over 30-second period), was microinjected. RSNA was measured in both SHR and WKY rats (n = 4 for each). After general procedures, the left renal nerve was exposed with a left retroperitoneal flank incision. A pair of stainless steel bipolar electrodes was placed beneath the renal nerve to record multifiber RSNA, as described previously.20 Background noise levels were determined by crushing an incision. A pair of stainless steel bipolar electrodes was placed bilaterally into the NTS (40 pmol/site at two sites) because of the difficulty in obtaining a stable noise-free RSNA recording.

**In Vivo Gene Transfer Experiments**

The Rho-binding domain,21 a dominant-negative Rho-kinase mutant driven by the cytomegalovirus promoter and containing a c-myc tag, was expressed through homologous recombination between cotransfected pJM17 and shuttle plasmids in 293 cells. Integration of the transgene into the adenoviral genome was determined by polymerase chain reaction and restriction analysis. Adβgal was used as a control.24–26 An adenoviral suspension containing 1×10⁹ plaque-forming units per milliliter was injected at four sites over 20 minutes. After the injection, all animals recovered from the anesthesia and were unrestrained and free to move in their cages.24 The UA-10 telemetry system (Date Sciences International) was used to measure blood pressure and HR.24,25 Twenty-four-hour urine norepinephrine excretion was measured before and at day 7 after the AdDNRhoK gene transfer, as described previously.24,25 We performed immunohistochemistry for c-myc, a marker of AdDNRhoK.26 At day 7 after the gene transfer, serial sections of the medulla were obtained. After incubation in 3% H₂O₂ in 80% methanol and 1% BSA in PBS, the sections were incubated in mouse IgG monoclonal antibody to c-myc (Neomarkers) at 4°C for 3 days and then rinsed in PBS. After incubation in biotinylated horse anti-mouse IgG (1:1000, Vector Laboratories) for 4 hours, the sections were rinsed in PBS and incubated for 3 hours in streptavidin-conjugated FITC (1:200, Vector Laboratories). The sections stained with c-myc antibody were photographed using a confocal laser scanning microscope.24,25

**Western Blot Analysis**

The animals were killed with an excessive dose of sodium pentobarbital, and the NTS tissues were obtained using the micropunch technique. The tissues were homogenized in a lysis buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, 1 mmol/L Na₂VO₄, and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged, and the supernatant was collected. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical). An aliquot of 15 or 50 (for adducin) μg protein from each sample was separated on a 10% SDS-polyacrylamide gel. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membranes, Millipore). Membranes were incubated with mouse IgG monoclonal antibody to Rho-kinase (1:1000, Transduction Laboratories) or with members of the rabbit antiphosphorylated ERM family: moesin (Thr564), ezrin (Thr567), and radixin (Thr564), which are target proteins of Rho-kinase.27 Membranes were then incubated with a horseradish peroxidase–conjugated horse anti-mouse IgG antibody (1:10 000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biototechnology), and film was analyzed using the NIH image software package. Membrane and cytosolic proteins were isolated as described previously,28 and Western blot analysis was performed as mentioned above using rabbit IgG polyclonal antibody to RhoA (1:1000, Santa Cruz Biototechnology) and horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (1:10 000). After the AdDNRhoK gene transfer, a block of brain tissue containing the injection sites was obtained, and Western blot analysis was performed as mentioned above using rabbit IgG polyclonal c-myc antibody (1:1000, Santa Cruz Biototechnology). At day 7 after the gene transfer, Western blot analysis was performed using rabbit antiphosphorylated ERM family members or rabbit antiphosphorylated α-adducin (Thr445), which represents Rho-kinase activity.29,29 In addition, Western blot analysis was performed using the mouse anti-neuronal NO synthase (anti-nNOS) antibody (1:2000, Transduction Laboratories) or mouse anti-endothelial NO synthase (anti-eNOS) antibody (1:2000, Transduction Laboratories) in WKY rats before and after the gene transfer.30

**Statistical Analysis**

All values are expressed as mean±SEM. ANOVA was used to compare mean blood pressure (MBP) and HR between the Adβgal-transfected and AdDNRhoK-transfected groups and to compare urinary norepinephrine excretion between SHR and WKY rats. Comparisons between any two mean values were performed by application of the Bonferroni procedure. A paired t test was used to compare urinary norepinephrine excretion before and after the gene transfer. An unpaired t test was used to compare changes in urinary norepinephrine excretion between SHR and WKY rats. Differences were considered to be statistically significant at P<0.05.

**Results**

**Effect of Microinjection of Y-27632 Into the NTS**

Baseline MBP (160±3 versus 95±2 mm Hg, P<0.01; n=19 for each) and HR (327±6 versus 292±5 bpm, P<0.01; n=19 for each) were significantly greater in SHR than in WKY rats. Baseline RSNA did not differ between the two strains (112±25 versus 84±40 spikes/s, n=4 for each). Microinjection of Y-27632 into the NTS elicited a dose-dependent decrease in MBP and HR in both SHR and WKY rats (Figure 1). The magnitude of the decreases in MBP and HR was significantly greater in SHR than in WKY rats (Figure 1B). Furthermore, in another series of experiments in which RSNA was recorded, microinjection of Y-27632 into the NTS decreased MBP, HR, and RSNA in both SHR and WKY rats (Figure 2). The magnitude of the decreases in these variables was significantly greater in SHR than in WKY rats (Figure 2).
Moreover, in these experiments, the percent reduction of MBP (26% versus 11%, P < 0.05; n = 4 for each) or HR (11% versus 7%, P < 0.05; n = 4 for each) was significantly greater in SHR than in WKY rats, respectively.

**MBP and HR Changes After Gene Transfer**

Figures 3A and 3B show the time course of MBP and HR before and after the adenovirus-mediated in vivo gene transfer of either Adβgal or AdDNRhoK into the bilateral NTS. MBP and HR responses evoked by bilateral microinjection of Y-27632 into the NTS (4 injections) (n = 5 for each). *P < 0.05 and **P < 0.01 vs WKY rats.

2B). Moreover, in these experiments, the percent reduction of MBP (26±2% versus 11±3%, P < 0.05; n = 4 for each) or HR (11±3% versus 7±1%, P < 0.05; n = 4 for each) was significantly greater in SHR than in WKY rats, respectively.

**Urinary Norepinephrine Excretion**

Urinary norepinephrine excretion measured at day 7 after the gene transfer was significantly decreased after AdDNRhoK transfection (Figure 3C). Moreover, urinary norepinephrine excretion before transfection was significantly higher in SHR than in WKY rats, and the magnitude of the AdDNRhoK transfection-induced decrease was greater in SHR than in WKY rats (−0.80±0.12 versus −0.48±0.07 μg/d, respectively; P < 0.05).

**Expression of AdDNRhoK in the NTS**

Immunohistochemistry after AdDNRhoK transfection revealed that the expression of c-myc, a marker of AdDNRhoK, was observed only in the NTS, where AdDNRhoK was microinjected (Figure 4). In addition, Western blot analysis revealed that c-myc expression was significantly increased and peaked at day 7 after AdDNRhoK transfection in both SHR and WKY rats (Figure 5A). The magnitude of an increase in c-myc expression did not differ between SHR and WKY rats (Figure 5A).

**Inhibitory Effects of AdDNRhoK Transfection on Rho-Kinase Activity**

To confirm the specific inhibitory effects of AdDNRhoK transfection on Rho-kinase activity, we examined the phosphorylation of α-adducin or the ERM family members (ezrin, radixin, and moesin), which are target proteins of Rho-kinase, in rats transected with AdDNRhoK into the NTS. Phosphorylation of α-adducin and of ERM was significantly reduced in both the WKY and SHR AdDNRhoK-transfected animals. Furthermore, the extent of phosphorylation of these proteins in the control animals was greater in SHR than in WKY rats (Figure 5B).
Expression of RhoA/Rho-Kinase and Rho-Kinase Activity in the NTS

RhoA and Rho-kinase proteins were expressed in the NTS of both SHR and WKY rats. The RhoA expression levels in membrane fraction, which represent RhoA activity, were greater in SHR than in WKY rats (Figure 6A). In contrast, there was less cytosol RhoA expression in SHR than in WKY rats (Figure 6A). The Rho-kinase protein expression level was comparable between the two strains (Figure 6B). Furthermore, the extent of phosphorylation of the ERM family, which represents Rho-kinase activity, was greater in SHR than in WKY rats (Figure 6C).

Effects of AdDNRhoK Transfection on NOS Expression

To investigate the effects of AdDNRhoK transfection on NO synthase (NOS) expression, we performed Western blot analysis for nNOS and eNOS. nNOS and eNOS expression levels were significantly decreased in AdDNRhoK-transfected rats (Figure 7).

Discussion

The present study provides the first direct evidence that activation of the Rho/Rho-kinase pathway in the NTS plays an important role in the maintenance of basal arterial blood pressure via the sympathetic nervous system in vivo. In addition, the results suggest that activation of the Rho/Rho-kinase pathway is involved, at least in part, in the central mechanisms of hypertension in SHR.

RhoA gene expression in the brain stem has been demonstrated by in situ hybridization and has been reported in bovine cerebral cortex, hippocampus, and cerebellum. However, the present study is the first to demonstrate Rho-kinase expression in the brain stem. Western blot analysis for RhoA and Rho-kinase confirmed expression of those proteins in the NTS.

Our findings suggest that inhibition of endogenous Rho-kinase in the NTS of anesthetized and conscious WKY rats decreases blood pressure and HR through inhibition of the sympathetic nervous system. When Y-27632 was microinjected into the brain stem 1 mm away from the NTS, blood pressure did not change (data not shown). As shown in the gene-transfer study, blood pressure and HR were significantly decreased on days 5 to 7 after AdDNRhoK gene transfer but returned to control levels by day 10. The time course of the
changes in blood pressure and HR corresponded to the time course of c-myc expression. Urinary norepinephrine excretion was also decreased at day 7 after AdDNRhoK transfection into the NTS in WKY rats. Importantly, the phosphorylation of α-adducin and ERM was significantly reduced in the AdDNRhoK-transfected animals, which strongly suggests specific suppression of Rho-kinase activity in vivo. Using adenovirus vectors might cause inflammatory responses in the NTS,32 and this possibility must be considered. We used adenovirus vectors might cause inflammatory responses in the NTS,32 and this possibility must be considered. We used adenovirus vectors.24,25 We previously reported a similar macrophage/mast cell infiltration, a marker of inflammation, between animals transfected with Adβgal and those transfected with an adenovirus vector encoding endothelial nitric oxide synthase. In addition, Adβgal transfection into the NTS had no inhibitory effect on blood pressure, HR, or urinary norepinephrine excretion. Taken together, our findings indicate that Rho-kinase in the NTS is substantially involved in the maintenance of the basal blood pressure via sympathetic nerve activity in normotensive rats.

The NTS is a relatively large nucleus involved in many physiological processes. However, it is very difficult to discriminate between the cardiovascular and other functional portions. We microinjected the drugs into the NTS, where a depressor response was evoked by L-glutamate. Regarding the volume injected, we confirmed that the distribution of the injection was restricted to the NTS by microinjection of artificial cerebrospinal fluid, including dye (Evans blue) in a previous study.31 We took special care to inject slowly so as not to increase the amount of spread. In addition, other studies have used a much larger volume of injection (100 nL).23 Y-27632 was also microinjected into noncardiovascular areas in the brain stem and did not affect blood pressure (data not shown). However, it is difficult to microinject only the noncardiovascular areas within the NTS.

There is a possibility that the differences in baseline blood pressure affected the changes in blood pressure between SHR and WKY rats, although microinjection of Y-27632 or AdDNRhoK elicited a greater reduction in arterial blood pressure and HR in SHR than in WKY rats. This possibility is unlikely, however, because in the RSNA measurement study in which we microinjected Y-27632 at two sites in the NTS (Figure 2), the percent reduction of MBP and HR was significantly greater in SHR than in WKY rats (MBP 26% vs 21%, respectively [P<0.01]; HR 11% vs 7%, respectively [P<0.01]; n=4 for each). In addition, the magnitude of the decreases in RSNA and urinary norepinephrine excretion was greater in SHR than in WKY rats, supporting the idea that the decrease in blood pressure via inhibition of sympathetic nerve activity is greater in SHR than in WKY rats. Finally, prior injection of L-glutamate...
produced similar depressor responses in the two groups (for WKY rats, \(-35 \pm 3\) mm Hg; for SHR, \(-36 \pm 4\) mm Hg).

The membrane RhoA expression level in the NTS was significantly greater in SHR than in WKY rats. Furthermore, the extent of the phosphorylation of ERM was greater in SHR than in WKY rats. Although there was no difference in the Rho-kinase expression level,

\[ P < 0.05 \] and \[ P < 0.01 \] vs Ad gal or control.

In conclusion, inhibition of Rho-kinase in the NTS decreases arterial blood pressure, HR, and sympathetic nerve activity. Furthermore, the Rho/Rho-kinase pathway is activated to a greater extent in SHR than in WKY rats.

Acknowledgments
This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (A13307024, B12470158, and C13670721) and by a Grant for Research on Autonomic Nervous System and Hypertension from Kimura Memorial Heart Foundation/Pfizer Pharmaceuticals, Inc. We thank the Mitsubishi Pharma Corporation for the generous gift of Y-27632.

References


Rho/Rho-Kinase Pathway in Brain Stem Contributes to Blood Pressure Regulation via Sympathetic Nervous System: Possible Involvement in Neural Mechanisms of Hypertension

Koji Ito, Yoshitaka Hirooka, Koji Sakai, Takuya Kishi, Kozo Kaibuchi, Hiroaki Shimokawa and Akira Takeshita

_Circ Res._ 2003;92:1337-1343; originally published online June 5, 2003; doi: 10.1161/01.RES.0000079941.59846.D4

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/92/12/1337