Inhibition of Rho-Kinase in the Brainstem Augments Baroreflex Control of Heart Rate in Rats

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Abstract—The Rho/Rho-kinase pathway in the nucleus tractus solitarii (NTS) of the brain stem contributes to blood pressure regulation. Activation of this pathway might be involved in the central nervous system mechanisms of hypertension. The aim of the present study was to determine whether baroreflex control of heart rate is altered by inhibition of Rho-kinase in the NTS. Adenovirus vectors encoding dominant-negative Rho-kinase or β-galactosidase were transfected into the nucleus tractus solitarii of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Baroreflex control of heart rate was examined by changing arterial pressure with an intravenous infusion of phenylephrine or sodium nitroprusside. The maximum gain of baroreflex control of heart rate was attenuated in SHR compared with WKY before the gene transfer. Transfection of adenovirus vectors encoding dominant-negative Rho-kinase significantly augmented the maximum gain in both WKY and SHR. The extent of this augmentation, however, was greater in SHR than in WKY. After treatment with metoprolol, the maximum gain was significantly decreased in rats transfected with adenovirus vectors encoding dominant-negative Rho-kinase, but not in nontransfected rats. In contrast, after treatment with atropine, the maximum gain was greater in rats transfected with adenovirus vectors encoding dominant-negative Rho-kinase compared with nontransfected rats, although it was decreased in both groups. These results suggest that inhibition of Rho-kinase in the NTS augments baroreflex control of heart rate, in both WKY and SHR, probably because of a cardiac sympathoinhibitory effect. (Hypertension. 2004;44:478-483.)

Key Words: blood pressure ■ heart rate ■ rho ■ hypertension ■ brain

It is well established that the arterial baroreceptor reflex is an important determinant of cardiovascular homeostasis.1,2 The major baroreflex pathway is in the brain stem, and baroreceptor afferent inputs are initially integrated by neurons in the nucleus tractus solitarii (NTS).3,4 Baroreflex control of heart rate (HR) is impaired in various animal models of hypertension as well as in hypertensive humans.5–7 Furthermore, evidence suggests that many vasoactive substances, such as angiotensin II, affect sympathetic nerve activity and modulate the baroreflex control of HR and sympathetic nerve activity.8,9

The small GTPase, Rho, and its effector, Rho-kinase, are involved in various cellular functions, including myosin light chain phosphorylation and smooth muscle contraction.10–12 Activation of this pathway contributes to the pathophysiology of hypertension.13–15 We previously reported that Rho/Rho-kinase is present in the NTS and maintains arterial pressure via the sympathetic nervous system, and that activation of the Rho/Rho-kinase pathway in the NTS might contribute to the hypertensive mechanisms.16,17 It is not known, however, whether Rho/Rho-kinase in the NTS is involved in baroreflex function. The Rho/Rho-kinase pathway in the central nervous system is involved in the maintenance of dendritic spines.18 Dendritic spines form the postsynaptic contact sites of excitatory synapses in the central nervous system.19,20 Therefore, the aim of the present study was to elucidate the role of the Rho/Rho-kinase pathway in the NTS in baroreflex control of HR in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). For this purpose, adenovirus vectors encoding either dominant-negative Rho-kinase (AdDNRhoK) or β-galactosidase (Adβgal) were transfected into the NTS in vivo and mean arterial pressure (MAP)–HR curves were constructed using an intravenous infusion of phenylephrine or sodium nitroprusside to examine baroreflex function in WKY and SHR.

Methods
This study was approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University. Male WKY or SHR (280 to 340 grams, 16 to 20 weeks old) were used in the present study. Rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine (Fukuoka, Japan).13,17
In Vivo Gene Transfer Experiments

Adenoviral vectors encoding either the β-galactosidase gene or the DNRhoK (Rho-binding domain of Rho-kinase) gene were used as described previously. Systolic blood pressure (SBP) was measured before and at day 7 after gene transfer using the tail-cuff method. At day 7 after the gene transfer, we performed immunohistochemistry for β-gal, an AdDNRhoK tag protein, as described previously.

Western Blot Analysis

We performed Western blot analysis for phosphorylated ERM (p-ERM) or total ERM family members (ezrin, radixin, moesin), which are target proteins of Rho-kinase and membranous or total RhoA (1:1000; Santa Cruz Biotechnology) in the NTS of nontransfected rats as described previously. Furthermore, we performed Western blot analysis for c-myc and p-ERM of brain tissue containing the injection sites in the NTS of AdDNRhoK-transfected rats before and at day 7 after gene transfer.

Analysis of Baroreflex Control of HR

In AdDNRhoK-transfected rats or Adβgal-transfected rats, measurement of the sensitivity of baroreflex control of HR was performed on day 7 after the gene transfer. Under pentobarbital anesthesia (40 mg/kg IP), catheters were inserted into the right femoral artery for measurement of blood pressure and HR, and into the femoral vein for infusion of phenylephrine or sodium nitroprusside. After animals awoke from the anesthesia, analysis of baroreflex control of HR was performed with animals in the conscious state. The femoral vein catheter was connected to an infusion pump and progressive infusion of phenylephrine (2 to 32 μg/kg per minute) at flow rates of 0.008 to 0.13 mL/min or sodium nitroprusside (5 to 10 μg/kg per minute) at flow rates of 0.007 to 0.015 mL/min for 1 minute was performed to induce changes in MAP between 40 and 50 mm Hg. The speed of the increase or decrease in MAP was ~0.8 mm Hg per second.

Some animals (hydralazine-treated WKY or SHR, n=5 for each) were treated with hydralazine hydrochloride in their drinking water (0.6 mg/mL) for 7 days. On day 7 after treatment with hydralazine, baroreflex control of HR was analyzed as described.

Effects of Autonomic Blockade

To examine the sympathetic and parasympathetic component of the interaction between Rho-kinase activity in the NTS and autonomic innervation of the sinoatrial node, metoprolol (a selective β1-receptor blocker, 2 mg/kg IV, supplemented with 0.2 mg/kg IV every 30 minutes) or atropine methyl bromide22 (0.2 mg/kg IV, supplemented with 0.02 mg/kg IV every 30 minutes) was injected in the NTS of nontransfected and AdDNRhoK-transfected rats. The measurement of the gain of baroreflex control of HR was performed as described previously.

Data Analysis of Baroreflex Control of HR

To analyze the baroreflex control of HR, HR and MAP data were obtained every 2 seconds, and the relation between MAP and HR was determined by fitting pairs of data points to a logistic function using the computer program Igor Pro (Wave Metrics) as described previously. The logistic function used for data analysis conformed to the mathematical expression HR = P1/[1 + exp[P2(MAP – P3)] + P4], where P1 is the range of responses of the HR, P2 is the slope coefficient, P3 is the MAP at the midpoint of the range for the HR, and P4 is the minimum HR. The maximum gain of baroreflex control of HR was expressed as −P1 × P2/4 of the logistic function curve.

Statistical Analysis

All values are expressed as mean±SEM. Two-way ANOVA was used to compare the MAP and HR between the nontransfected and AdDNRhoK-transfected rats. Comparisons between any 2 mean values were performed using Bonferroni correction for multiple comparisons. An unpaired t test was used to compare the maximum gain of baroreflex control of HR between groups. Differences were considered statistically significant at P<0.05.

Results

Analysis of AdDNRhoK Expression in the NTS

Immunohistochemistry after AdDNRhoK transfection revealed c-myc expression locally in the NTS, where AdDNRhoK was microinjected (Figure 1A). In addition, Western blot analysis revealed that c-myc expression was significantly increased at day 7 after the transfection of AdDNRhoK in WKY and SHR (Figure 1B). The magnitude of the increase in c-myc expression did not differ between WKY and SHR (Figure 1B).

Effects of AdDNRhoK Transfection on Blood Pressure and HR

SBP and HR were significantly decreased on day 7 after the gene transfer of AdDNRhoK in SHR and WKY. The magnitude of the decrease in SBP and HR, however, was significantly greater in SHR than in WKY (ΔSBP: −39±4 versus −24±3 mm Hg; ΔHR: −98±8 versus −68±4 bpm; n=6 for each; P<0.05). In contrast, these variables did not change in either strain in nontransfected or Adβgal-transfected rats. To confirm the specific inhibitory effects of AdDNRhoK transfection on Rho-kinase activity, we examined phosphorylation of the ERM family in rats transfected with AdDNRhoK into the NTS. Phosphorylation of the ERM family was significantly reduced in AdDNRhoK-transfected animals (Figure 1C).

Effects of Rho-Kinase Inhibition in the NTS on Baroreflex Control of HR

In the nontransfected group, the baroreflex curve shifted to the right and the maximum gain of baroreflex control of HR was significantly decreased in SHR compared with WKY (−0.8±0.1 versus −1.8±0.2; n=6 for each; P<0.01; Figure 2A and 2B). In AdDNRhoK-transfected rats, the baroreflex curve shifted to the left and maximum gain of baroreflex control of HR was significantly increased compared with controls in both WKY (−2.7±0.3 versus −1.8±0.2; n=6 for each; P<0.05) and SHR (−2.0±0.2 versus −0.8±0.1; n=6 for each; P<0.01; Figure 2A and 2B). The magnitude of the augmentation of the maximum gain of baroreflex control of HR was significantly greater in SHR than in WKY (1.5±0.2 versus 2.5±0.1; data are expressed as relative ratio to the control group of each strain, which was assigned a value of 1). In Adβgal-transfected rats, none of the parameters was significantly different compared with those of nontransfected rats (data not shown).

Effects of Blood Pressure Reduction on Baroreflex Control of HR

In hydralazine-treated WKY (n=5), baseline MAP was significantly decreased compared with nontreated WKY (96±3 versus 116±3 mm Hg, P<0.01), and baseline HR was significantly increased (348±7 versus 323±7 bpm, P<0.05). There was no difference in the maximum gain of baroreflex control of HR between hydralazine-treated WKY and nontreated WKY (−1.7±0.2 versus −1.8±0.2; Figure 2C and 2D). In hydralazine-treated SHR (n=5), baseline MAP was significantly decreased compared with nontreated SHR (n=6) (123±5 versus 160±3 mm Hg; P<0.01), and baseline
HR was significantly increased (362 ± 6 versus 339 ± 4 bpm; P < 0.01). The maximum gain of baroreflex control of HR was significantly increased in hydralazine-treated SHR compared with nontreated SHR (−1.4 ± 0.3 versus −1.7 ± 0.2; P < 0.01; Figure 2C and 2D). Although hydralazine treatment produced a decrease in baseline blood pressure as did AdDNRhoK-transfection in SHR (baseline MAP 135 ± 2 versus 123 ± 5 mm Hg), the improvement in baroreflex control of HR was smaller in the hydralazine-treated SHR than in AdDNRhoK-transfected SHR.

Effects of Inhibition of Rho-Kinase in the NTS on Baroreflex Control of HR After Autonomic Blockade

In nontransfected WKY, atropine significantly increased the baseline HR (317 ± 8 to 335 ± 5 bpm; n = 6 for each; P < 0.05) and decreased the range of HR (113 ± 13 to 53 ± 8 bpm; P < 0.01) and maximum gain of baroreflex control of HR (−1.8 ± 0.2 to −0.9 ± 0.1; P < 0.01), but did not significantly change the baseline MAP (104 ± 5 to 106 ± 4 mm Hg; Figure 3A and 3B). Metoprolol significantly decreased the baseline HR (304 ± 2 to 272 ± 4 bpm; n = 6 for each; P < 0.01) and range of HR (113 ± 13 to 32 ± 4 bpm; P < 0.01), and increased the slope coefficient (−0.07 ± 0.02 to −0.23 ± 0.03 bpm/mm Hg; P < 0.01), but did not change the maximum gain of baroreflex control of HR (−2.7 ± 0.3 to −1.5 ± 0.1; P < 0.01), but did not alter the baseline MAP (89 ± 4 to 89 ± 4 mm Hg; n = 6 for each). Baseline HR increased to 250 ± 7 from 241 ± 6 bpm, but this increase was not statistically significant (Figure 3A and 3B). Metoprolol significantly decreased baseline HR (243 ± 2 to 220 ± 4 bpm; P < 0.01), the range of HR (102 ± 10 to 60 ± 10 bpm/min; P < 0.05), and the maximum gain of baroreflex control of HR (−2.7 ± 0.3 to −1.5 ± 0.1; P < 0.01), but did not alter the baseline MAP (90 ± 2 to 86 ± 2 mm Hg; Figure 3C and 3D). After treatment with atropine, the maximum gain of baroreflex control of HR was significantly greater in AdDNRhoK-transfected WKY than in nontransfected WKY (−1.4 ± 0.3...
versus −0.9±0.1; P<0.05; Figure 3A and 3B). After treatment with metoprolol, however, the maximum gain of baroreflex control of HR was not different between AdDNRhoK-transfected and nontransfected WKY (1.5±0.1 versus 1.7±0.2; Figure 3C and 3D).

Rho/Rho-Kinase Activity in the NTS
Expression of membranous RhoA, which represents RhoA activity,23 was greater in SHR than in WKY (Figure 4 A). The expression level of membranous RhoA in hydralazine-treated SHR tended to decrease, but was not statistically different from that in nontreated SHR (Figure 4A). The level of p-ERM, which represents Rho-kinase activity,21 was greater in SHR than in WKY (Figure 4 B). Furthermore, p-ERM family levels in hydralazine-treated SHR were significantly decreased compared with nontreated SHR, but significantly increased compared with nontreated WKY (Figure 4B).

Discussion
The present study demonstrated that inhibition of Rho-kinase in the NTS augments the baroreflex control of HR in WKY and SHR and improves the impaired baroreflex function in SHR. The major effect of Rho-kinase inhibition in the NTS on baroreflex control of HR appears to be caused by inhibition of the cardiac-sympathetic component.

In nontransfected SHR, the maximum gain of baroreflex control of HR was significantly decreased compared with nontransfected WKY; consistent with previous reports.6,7 Inhibition of Rho-kinase activity in the NTS by AdDNRhoK increased the maximum gain in WKY and SHR; however, the magnitude of the augmentation of the maximum gain was significantly greater in SHR than in WKY. These results indicate that Rho-kinase in the NTS contributes to maintain baroreflex control of HR in both strains, and activation of this pathway contributes to impaired baroreflex control of HR in SHR. Consistent with this suggestion, RhoA and Rho-kinase activities were enhanced in the NTS of SHR compared with WKY, as shown in Figure 4. After treatment with atropine, the maximum gain was significantly decreased in both nontransfected WKY and AdDNRhoK-transfected WKY. Conversely, after treatment with metoprolol, the maximum gain was significantly decreased in only AdDNRhoK-transfected WKY. Furthermore, after treatment with atropine, the maximum gain was significantly greater in AdDNRhoK-transfected WKY than in nontransfected WKY, but after...
treatment with metoprolol, the maximum gain was not different between AdDNRhoK-transfected and nontransfected WKY. In the present study, we used adenovirus vectors, which might cause an inflammatory response. There is, however, no evidence that inflammation affects cardiovascular regulation.\(^1\)\(^6\)\(^-\)\(^2\)\(^4\) These results suggest that gene transfer of AdDNRhoK into the NTS affects the cardiac sympathetic component of baroreflex function.\(^7\)

There is a possibility that changes in arterial pressure by intravenous infusion of phenylephrine or sodium nitroprusside affect the cardiopulmonary receptors. The activity of the baroreceptors to pressure changes is decreased in animal models of hypertension.\(^2\)\(^5\) Therefore, another experimental design, such as stimulation of the aortic depressor nerve, might be preferable. It is difficult to perform such an experiment in the awake rat, however, because the stimulating electrode must be implanted for a long period of time. However, the evaluation of the baroreflex function between normotensive and hypertensive models using phenylephrine or sodium nitroprusside has been widely used in previous studies.\(^2\)\(^6\)\^-\(^2\)\(^8\)

Rho-kinase inhibition in the NTS decreased arterial pressure, consistent with our previous report.\(^1\)\(^2\) The decrease in blood pressure alone might affect baroreflex control of HR. Therefore, we examined the baroreflex control of HR in hydralazine-treated rats. In hydralazine-treated WKY, baseline MAP significantly decreased, but the maximum gain did not change compared with nontreated WKY, despite the fact that blood pressure decreased to a similar extent as in AdDNRhoK-transfected rats. In hydralazine-treated SHR, baseline MAP significantly decreased and the maximum gain was significantly increased compared with nontreated SHR, although the value of the maximum gain of hydralazine-treated SHR was smaller than in AdDNRhoK-transfected SHR, despite the fact that blood pressure decreased to a similar level as in AdDNRhoK-transfected SHR. These results indicate that the effects of Rho-kinase inhibition in the NTS on baroreflex function are not simply caused by the change in systemic blood pressure.

In conclusion, inhibition of endogenous Rho-kinase in the NTS augments the baroreflex control of HR in WKY and SHR and improves the impaired baroreflex function in SHR. The major effect of Rho-kinase inhibition in the NTS on baroreflex control of HR was probably caused by a cardiac sympatho-inhibitory effect.

**Perspectives**

The precise mechanisms by which Rho-kinase inhibition in the NTS augments the baroreflex control of HR cannot be determined from the results of our study. With regard to cardiovascular disease, the Rho/Rho-kinase pathway contributes to hypertension\(^1\)\(^3\)\^-\(^1\)\(^5\)\^-\(^2\)\(^9\)\^-\(^2\)\(^0\)^\(^-\)\(^2\)\(^3\) and endothelial dysfunction.\(^3\)\(^0\)\^-\(^3\)\(^1\) The neuronal Rho/Rho-kinase pathway contributes to dendritic spine formation, which forms the postsynaptic contact site for the majority of excitatory synapses.\(^1\)\(^9\)\^-\(^2\)\(^0\)\^-\(^2\)\(^3\) Recent studies demonstrate that morphological changes in dendritic spines occur rapidly and are associated with glutamate sensitivity.\(^3\)\(^4\) Furthermore, there are structural differences in dendritic spines in the NTS between WKY and SHR.\(^3\)\(^5\) Therefore, the Rho/Rho-kinase pathway might affect synaptic transmission in the NTS. Further studies are needed to clarify the mechanisms underlying our observations.

**Acknowledgments**

This study was supported by grants-in-aid for Scientific Research (C13670721, C15590757) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by a grant for research on the autonomic nervous system and hypertension from Kimura Memorial Heart Foundation/Pfizer Pharmaceuticals, Inc.

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Hypertension. published online September 7, 2004;
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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