Inhibition of Rho-Kinase in the Nucleus Tractus Solitarius Enhances Glutamate Sensitivity in Rats

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Abstract—The Rho/Rho–kinase pathway in the central nervous system is involved in the maintenance of dendritic spines, which form the postsynaptic contact sites of excitatory synapses. Inhibition of the Rho–kinase pathway in neuron promotes dendritic spines or branches. In contrast, activation of the Rho/Rho–kinase pathway reduces dendritic spines or branches. Recent studies suggest that morphological changes of dendritic spines occur rapidly, and spine morphology is associated with glutamate sensitivity. The aim of the present study was to determine whether Rho-kinase activity affects glutamate sensitivity in the nucleus tractus solitarii (NTS) of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). We first examined the effects of unilateral glutamate injection in the NTS. There was a significantly smaller decrease in arterial pressure in SHR than in WKY. We then examined the depressor responses evoked by unilateral glutamate injection into the NTS after preinjection of Y-27632, a specific Rho-kinase inhibitor. Preinjection of Y-27632 enhanced the glutamate response in both strains. However, the magnitude of the augmentation was significantly greater in SHR than in WKY. Furthermore, we recorded single-unit activity of NTS neurons from medulla brain slice preparations. *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) was applied iontophoretically to the recorded neurons, and neuronal activity was recorded before and after Y-27632 perfusion. Y-27632 perfusion increased the response to NMDA and AMPA. These results suggest that inhibition of Rho-kinase activity in the NTS enhances glutamate sensitivity in WKY and SHR and might improve impaired glutamate sensitivity in SHR. **(*Hypertension*. 2005;46:360-365.)**

Key Words: blood pressure ■ autonomic nervous system ■ amino acid ■ rats, spontaneously hypertensive ■ central nervous system

The Rho/Rho–kinase pathway regulates myosin light chain phosphorylation and contributes to smooth muscle contraction.[1–3] Y-27632, a selective Rho-kinase inhibitor, reduces arterial pressure in rat models of hypertension,[4] and Rho-kinase activity is enhanced in hypertensive blood vessels.[5,6] Thus, the Rho/Rho–kinase pathway is involved in the peripheral mechanisms of hypertension. We reported previously that Rho-kinase is present in the brain stem and maintains arterial pressure via the sympathetic nervous system, and that activation of the Rho/Rho–kinase pathway in the brain stem might contribute to the central mechanisms of hypertension.[7,8] Furthermore, inhibition of Rho-kinase in the nucleus tractus solitarii (NTS) enhances baroreflex control of heart rate (HR) in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR), probably because of a cardiac sympathoinhibitory effect.[9]

The Rho/Rho–kinase pathway in the central nervous system is involved in the maintenance of dendritic spines.[10] Dendritic spines form the postsynaptic contact sites of excitatory synapses in the central nervous system.[11] Inhibition of the Rho-kinase pathway in neuron promotes dendritic spines or branches. In contrast, activation of the Rho/Rho–kinase pathway reduces dendritic spines or branches.[10,12] Recent studies suggest that morphological changes of dendritic spines occur rapidly,[13] and spine morphology is associated with glutamate sensitivity.[14] Furthermore, GTPase-activating protein p250GAP, which is highly expressed in the brain, coexists with RhoA in dendritic spines and is involved in *N*-methyl-D-aspartate (NMDA) glutamate receptor activity-dependent actin reorganization in dendritic spines.[15] Furthermore, Rho-kinase in the brain stem contributes to arterial blood pressure regulation and baroreflex function.[7–9] The physiological role of Rho-kinase in neurons has not been clarified; however, these findings led to the hypothesis that inhibition of the Rho/Rho–kinase pathway in the NTS affects synaptic transmission, particularly in the excitatory synapses, via an enhanced response to glutamate. Therefore, the aim of the present study was to determine whether the Rho/Rho–kinase pathway in the NTS affects glutamate sensitivity in the NTS. For this purpose, we examined depressor responses

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evoked by microinjection of glutamate with or without preinjection of Y-27632 into the NTS of rats. Furthermore, in medulla slice preparations, we recorded single-unit activity of NTS neurons evoked by extracellular iontophoretic application of NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) before and after Y-27632 perfusion.

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. Male WKY or SHR (280 to 340 g; 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).

Microinjection Study

Animals were anesthetized with sodium pentobarbital (50 mg/kg IP; followed by 10 to 20 mg/kg per hour IV), and a catheter was inserted into the right femoral artery for measurement of arterial pressure and HR and into the 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 sites were defined according to a rat brain atlas:[16] the coordinates for the NTS were 0.6 mm rostral and 0.6 mm lateral to the calamus scriptorius and 0.5 mm below the dorsal surface of the medulla, as described previously.[17] Microinjection was performed with a micropipette connected to a Hamilton microsyringe. We used 3 doses of glutamate (2 pmol, 20 pmol, and 200 pmol; 0.1, 1.0, and 10 mmol/L in 20 nL, injected over a 5-s period) and tested 1 or 2 doses of glutamate in each rat. To test 2 doses of glutamate in 1 rat, we microinjected the lower dose first. More than 30 minutes after the microinjection of lower dose of glutamate, we replaced the pipette using the same coordinates, and then the higher dose of glutamate was microinjected into the NTS. In a preliminary study, we observed a similar depressor response to glutamate microinjected into the NTS using 2 separate pipettes with equal doses of glutamate. Care was taken to maintain stable arterial blood pressure and HR during the experiment. In the Y-27632 coinjection study, we used a 2-barrel micropipette. One side of the pipette was filled with glutamate and the other side with Y-27632 (40 pmol; 0.5 mmol/L in 80 nL, injected over a 20-s period) or vehicle (artificial cerebrospinal fluid [a-CSF]; 80 nL, injected over a 20-s period). First, we microinjected only glutamate and waited ≥30 minutes as in the single-barrel pipette experiments, and then Y-27632 (or a-CSF) and glutamate were injected. Because the glutamate response was rapid and of short duration, we microinjected glutamate into the NTS unilaterally 60 s after the Y-27632 or a-CSF injection. In addition, to avoid the possibility of differences in the amount of drug spread, a microdialysis probe with an external injection line (MI-A-I-12-01; Eicom) connected to a syringe pump was placed unilaterally into the NTS. We confirmed previously that the dialysis probe was permeable to NMDA.[18] Therefore, NMDA (0.5 mmol/L; infusion speed 2 μL/min for 5 minutes) was infused through a microdialysis probe and Y-27632 (5 mmol/L; injection speed 0.02 μL/min for 5 minutes) was injected through the injection line with syringe pumps.[18] We selected the dose of NMDA or Y-27632 to produce an arterial pressure reduction of the same magnitude as that produced by 2.0 to 20 pmol glutamate microinjection or 0.5 mmol/L Y-27632 microinjection into the NTS.

Single-Unit Recordings of NTS Neurons

We performed single-unit recordings of NTS neurons as described previously.[19–21] Under ether anesthesia, the rat was killed by cervical dislocation, and the brain stem was rapidly removed and placed in cold Krebs–Ringer solution containing 126 mmol/L NaCl, 5 mmol/L KCl, 2.4 mmol/L $CaCl_2$, 1.3 mmol/L $MgSO_4$, 1.26 mmol/L KH_2PO_4, 26 mmol/L $NaHCO_3$, and 10 mmol/L D-glucose, saturated with 95% O_2 and 5% CO_2. A horizontal brain stem slice (400-μm thick) containing the area postrema and NTS was obtained using a vibratome (DTK-1000; Dosaka). The slice was incubated for ≥2 hours in Krebs–Ringer solution bubbled with 95% O_2 and 5%CO_2 before starting the experiment. The recording chamber was perfused with oxygenated Krebs–Ringer solution at 34°C. Slices were placed on a Plexiglas mesh in a submerged recording chamber and covered with nylon mesh and a silver ring to support the tissue. The recording chamber was perfused with oxygenated Krebs–Ringer solution at a flow rate of 3 mL/min. Using a microscope, the NTS was visualized as a translucent area in the slice, and the electrode was advanced into the NTS until an action potential was recorded. The spikes were amplified (MWZ-7200 and MEG-1200; Nihon-Koden), and the raw neurogram and firing rate were displayed on an oscilloscope (DS-8605; Iwatsu) and recorded; the output was fed into a computer program Chart 4 (AD Instruments) to calculate the numbers of spikes per second. NMDA or AMPA was injected using a 2-barrel glass electrode, which was independent of the recording electrode. The most effective infusion field was a circular area ≈50 μm in diameter.[22] The iontophoretic system was a Neurophore model BH-2 control unit (Medical Systems), and the chemicals were prepared as follows: NMDA (50 mmol/L in distilled water, pH 7.5) and AMPA (10 mmol/L in 150 mmol/L NaCl, pH 7.5). The current used (1000-ms duration) was −5 to −30 nA for the NMDA pipette and −5 to −20 nA for the AMPA pipette. Retention current was not routinely applied. For NMDA and AMPA, the current was varied for each neuron because of the variations in electrodes and ejection sites, and then currents were adjusted to produce almost identical responses for NMDA and AMPA. The control firing rate in spikes per second (Hz) induced by NMDA or AMPA was recorded before and after Y-27632 perfusion. Y-27632 (50 μmol/L) was dissolved in oxygenated Krebs–Ringer solution and perfused at a flow rate of 3 mL/min for 6 minutes. The effects of Y-27632 were defined as the peak changes in spikes per second between before and after Y-27632 perfusion.

Statistical Analysis

All values are expressed as mean±SE. Two-way ANOVA was used to compare the responses of glutamate injection in each dose between WKY and SHR and the effects of Y-27632 on the responses to glutamate injection for each dose. Any 2 mean values were compared by application of the Bonferroni correction for multiple comparisons. Differences were considered to be statistically significant at a P value of <0.05.

Results

Baseline Characteristics

Baseline mean arterial pressure (MAP) and HR in each group are shown in the Table. MAP and HR were significantly higher in SHR than in WKY. Unilateral injection of Y-27632 induced a decrease in MAP and HR in SHR but not in WKY (Table).

Effects of Rho-Kinase Inhibition on Glutamate Sensitivity in the NTS

Unilateral microinjection of glutamate into the NTS decreased MAP in a dose-dependent manner in WKY and SHR (Figure 1). When lower doses of glutamate were microinjected, the magnitude of the decrease in MAP was significantly reduced in SHR compared with WKY (Figure 1). The percent change in MAP was significantly greater in WKY than in SHR for all doses of glutamate examined (Figure 1). The magnitude of the decrease in HR induced by glutamate injection did not differ between WKY and SHR (Figure 1). The magnitude of the MAP decrease evoked by unilateral glutamate injection after Y-27632 injection into the NTS was

Baseline MAP and HR Values (n=5 for each)

WKY-MAP					
Glu concentration	Control	After Glu	Control	After Y	After Glu
2.0 pmol	101±2	94±2	104±2	98±1	86±1
20 pmol	94±3	81±3	98±2	93±2	70±1
200 pmol	91±2	60±4	100±2	94±1	53±2
WKY-HR					
Glu concentration	Control	After Gill	Control	After Y	After Glu
2.0 pmol	309±7	304±6	304±7	301±6	292±7
20 pmol	301±6	294±6	295±7	291±8	274±10
200 pmol	299±8	276±9	305±7	300±8	269±10
SHR-MAP					
Glu concentration	Control	After Glu	Control	After Y	After Glu
2.0 pmol	163±2	159±2	163±2	146±4*	134±4
20 pmol	161±7	152±7	169±5	150±4†	122±4
200 pmol	167±3	141±4	166±3	151±6*	116±6
SHR-HR					
Glu concentration	Control	After Glu	Control	After Y	After Glu
2.0 pmol	320±4	314±3	325±3	308±6*	299±5
20 pmol	324±7	312±8	315±6	302±4*	279±4
200 pmol	313±4	276±6	317±5	295±7*	249±10

*$P<0.05$; †$P<0.01$ vs control.
Glu indicates glutamate; Y, Y-27632 (40 pmol;0.5 mmol/L in 80 nL).

significantly greater compared with glutamate injection alone in both strains (Figures 2 and 3). However, the magnitude of the augmentation was significantly greater in SHR than in WKY (glutamate dose 2 pmol: 1.8±0.2 versus 4.0±0.3; 20 pmol: 1.7±0.3 versus 3.1±0.3; 200 pmol: 1.2±0.1 versus 1.4±0.2; data are expressed as the relative ratio of the percent change compared with only glutamate injection, which was assigned a value of 1; $P<0.05$; n=5 for each). The magnitude of the HR decrease evoked by unilateral glutamate injection after Y-27632 injection into the NTS was significantly greater compared with only glutamate injection in both strains (Figures 2 and 3). Preinjection of a-CSF did not change the magnitude of the decrease in MAP induced by glutamate injection into the NTS (Figure 4A). The perfusion of NMDA unilaterally into the NTS through a dialysis probe decreased MAP, as reported previously.[18] The magnitude of the decrease in MAP induced by NMDA with Y-27632 injection was significantly greater than that by NMDA perfusion alone (Figure 4B).

Effects of Rho-Kinase Inhibition on Neuronal Activity in the NTS

Twelve neurons responded to iontophoretically applied NMDA and AMPA. Iontophoretic application of NMDA and AMPA transiently increased neuronal activity (Figure 5). Perfusion of Y-27632 increased neuronal activity evoked by NMDA and AMPA (NMDA 1.22±0.19 versus 1.78±0.19 spikes/s; $P<0.05$; AMPA 0.98±0.09 versus 1.33±0.11 spikes/s; n=12 for each; $P<0.05$).

Discussion

The present study demonstrated that inhibition of Rho-kinase activity in the NTS enhances glutamate sensitivity in WKY and SHR, and might improve the impaired glutamate sensitivity in SHR. The present observation might elucidate, at least in part, the mechanisms underlying differences in glutamate sensitivity in the NTS between WKY and SHR reported previously.[23] The glutamate concentration in the NTS is greater in SHR than in WKY.[24] These findings suggest that glutamate sensitivity in the NTS is decreased in SHR compared with WKY.

There is extensive literature regarding glutamate injections into the NTS of SHR and WKY.[25–27] Most of these studies report a similar depressor response to glutamate injected into the NTS between WKY and SHR. We reported a similar result in the previous study.[7] When we examined the dose response to glutamate injections in the present study; how-

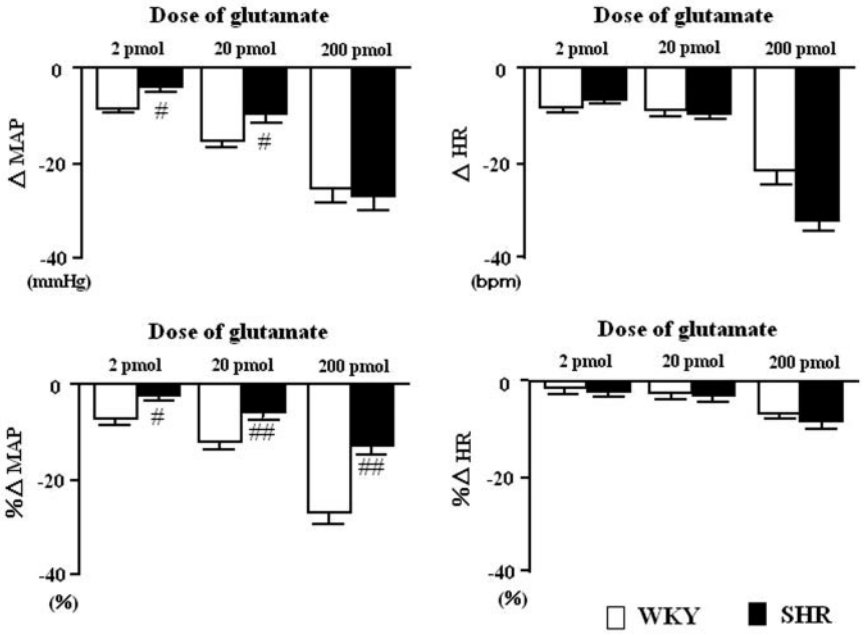

Figure 1. Effect of unilateral microinjection of glutamate into the NTS of WKY and SHR. Grouped data of responses evoked by unilateral injection of glutamate into the NTS (n=8 for each). #$P<0.05$; ##$P<0.01$ vs WKY.

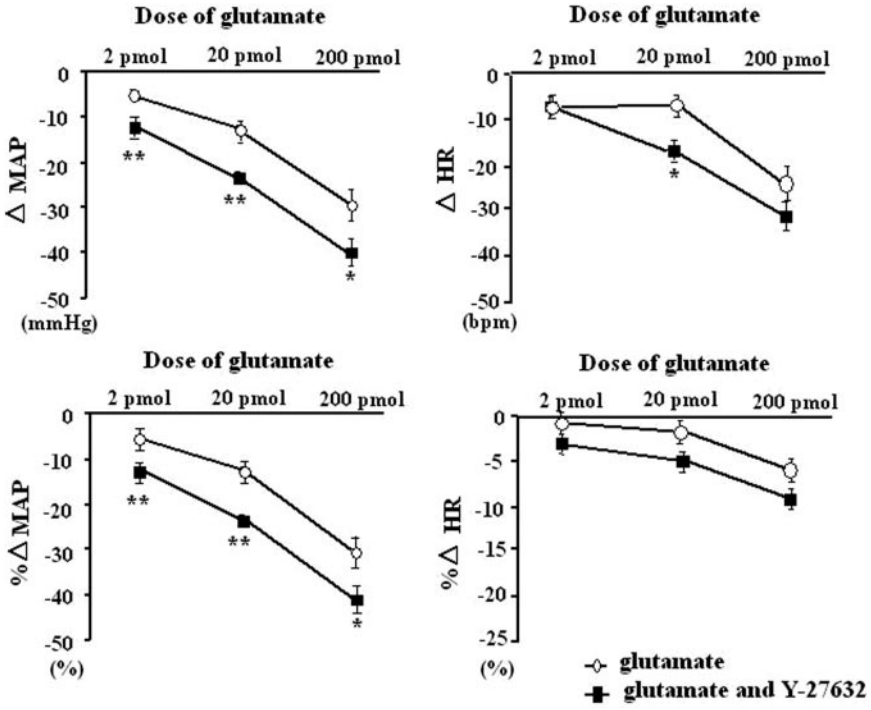

Figure 2. Effect of Rho-kinase inhibition on glutamate sensitivity in the NTS of WKY (n=5 for each). *$P<0.05$; **$P<0.01$ vs injection of only glutamate.

ever, there was less MAP reduction, particularly at lower doses, in SHR than in WKY. This result is consistent with that reported by Talman and Lewis.[23] However, it is difficult to compare the changes in MAP because there are baseline differences between the 2 groups. Talman and Lewis analyzed the data using repeated-measures ANOVA and covariate analysis to address the different MAP baseline values.[23] In the present study, we analyzed the data using a 2-way ANOVA with multiple comparison. Furthermore, we also compare the depressor response by percent change, as used previously.[28] In addition, there are structural vascular changes in SHR than in WKY.[29] Therefore, a greater depressor response is expected in SHR than in WKY. Nonetheless, a depressor response was demonstrated, suggesting an attenuated response to glutamate in SHR.

In the present study, we examined the effects of a Rho-kinase inhibitor on the decrease in arterial pressure evoked by unilateral glutamate injection into the NTS. The decrease in arterial pressure caused by glutamate injection after injection of Y-27632 was significantly greater than that by glutamate injec-

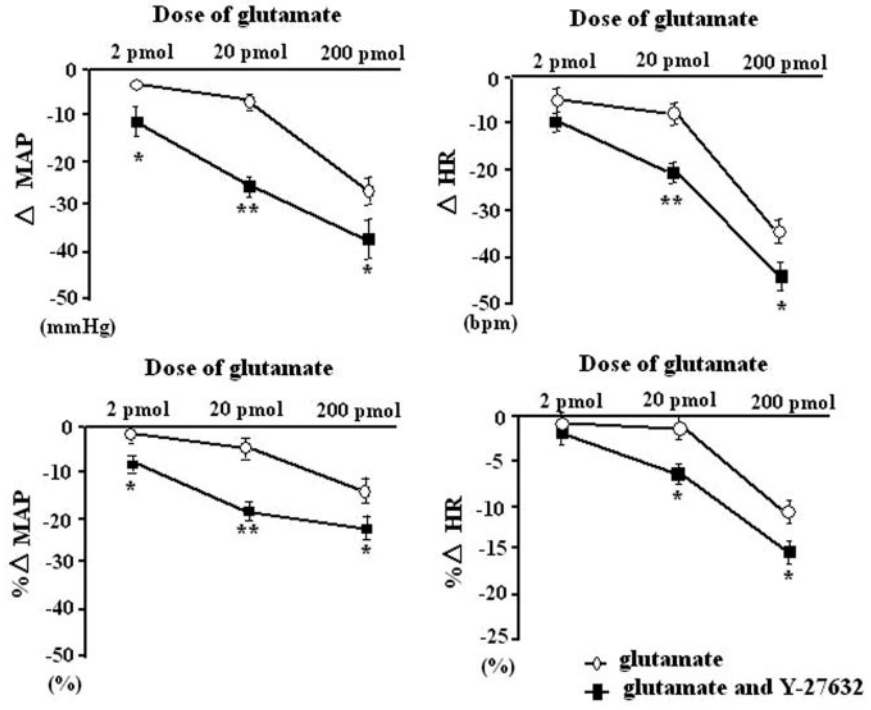

Figure 3. Effect of Rho-kinase inhibition on glutamate sensitivity in the NTS of SHR (n=5 for each). *$P<0.05$; **$P<0.01$ vs injection of only glutamate.

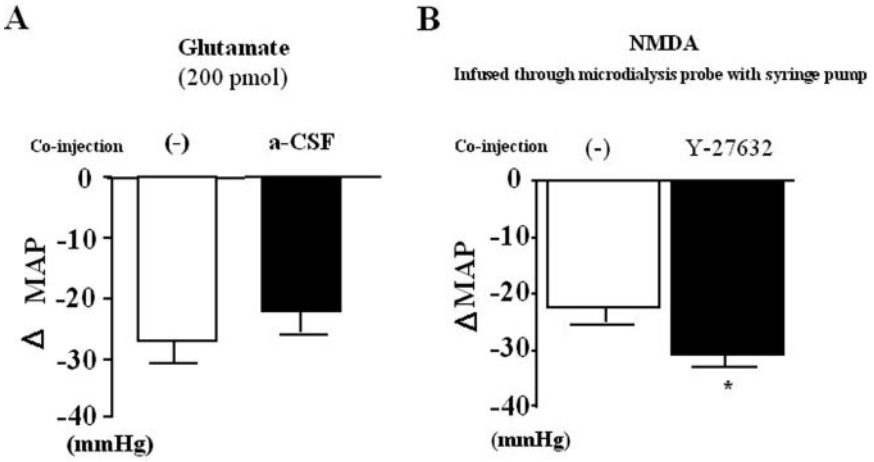

Figure 4. A, Effect of a-CSF on glutamate sensitivity in the NTS of WKY (n=3 for each). B, Effect of Rho-kinase inhibition on NMDA sensitivity in the NTS of WKY. NMDA and Y-27632 were applied using a microdialysis probe with a syringe pump (n=6 for each). *$P<0.05$ vs infusion of only NMDA.

tion alone in both strains. However, the magnitude of the augmentation was significantly greater in SHR than that in WKY, suggesting that inhibition of Rho-kinase in the NTS improves the impaired glutamate sensitivity in SHR. The HR responses to glutamate injection into the NTS did not differ between WKY and SHR (Figure 1), probably because this experiment was performed under anesthesia. However, the magnitude of HR reduction caused by glutamate was significantly augmented by preinjection of Y-27632 (Figures 2 and 3).

We took special care with the microinjection of glutamate to minimize differences in the amount of drug spread. However, differential drug spread is still possible, so we also used a microdialysis probe with an injection line that was connected to a syringe pump. Y-27632 was injected directly through the injection line, and NMDA was infused through the microdialysis probe, as described previously.[18] Infusion of only NMDA into the NTS decreased arterial pressure, as reported previously,[18] and infusion of NMDA with Y-27632 injection also decreased arterial pressure. However, the magnitude of the decrease in arterial pressure was significantly greater after infusion of NMDA with Y-27632 than that with NMDA alone. Therefore, the augmentation of the response to glutamate injection into the NTS is not likely to be attributable to differences in the amount of drug spread.

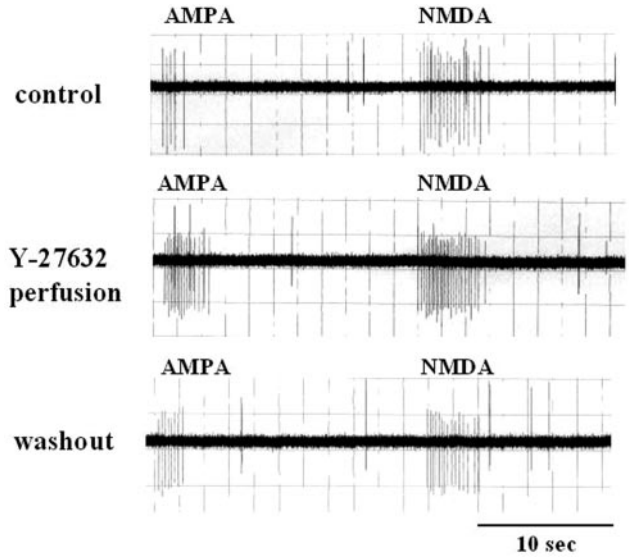

Figure 5. Effects of Y-27632 on neuronal activity in the NTS evoked by iontophoretically applied NMDA or AMPA. Example of raw neurograms indicating the increased neuronal activity after Y-27632 perfusion.

In the microinjection study, the effect of Rho-kinase inhibition on glutamate sensitivity was demonstrated indirectly. Therefore, we then recorded single-unit activity of NTS neurons to examine the direct effects of Rho-kinase inhibition. In the single-unit recording study, Rho-kinase inhibition increased the response of the recorded neurons to NMDA or AMPA. The magnitude of the augmentation differed in each recorded neuron. Because the NTS contains heterogenous neurons, including neurons related to cardiovascular control, it is possible that some of the recorded neurons did not contribute to baroreflex function, which might account for the different effects of Rho-kinase inhibition on the neurons.

We confirmed previously that the Rho/Rho–kinase pathway is activated in the NTS of SHR using Western blot analysis for membranous RhoA (translocation) or the phosphorylated ERM family (ezrin, radixin, moesin; target proteins of Rho-kinase).[7] These results suggest that activation of the Rho/Rho–kinase pathway is related to impaired glutamate sensitivity of the NTS neurons in SHR. As reported previously,[5] the Rho/Rho–kinase pathway plays an important role in regulating vascular tone. Therefore, it is possible that inhibition of Rho-kinase activity increases local blood flow and affects neuronal activity in the NTS. We consider it highly unlikely that the effects of Rho-kinase inhibition on arterial pressure regulation or neuronal activity in the NTS were caused by a local vasodilator effect because microinjection of another vasodilator, hydralazine, does not alter arterial pressure in either WKY or SHR.[7] In addition, the results using the brain slice preparation are independent from the vascular system.

In conclusion, inhibition of Rho-kinase in the NTS enhances glutamate sensitivity in the NTS. The Rho/Rho–kinase pathway in the NTS might be related to mechanism(s) underlying the resetting of baroreflex function in SHR via impaired glutamate sensitivity.

Perspectives

The precise mechanisms by which Rho-kinase inhibition in the NTS increases glutamate sensitivity cannot be elucidated

from the results of the present study. The small GTPase Rho and its downstream effector Rho-kinase are involved in many cellular functions.[30,31] The neuronal Rho/Rho–kinase pathway contributes to dendritic spine formation,[10] which forms the postsynaptic contact site for the majority of excitatory synapses.[11] Morphological changes in dendritic spines occur rapidly[13] and are associated with glutamate sensitivity.[14] Recently, it was demonstrated that there are structural differences in dendritic spines in the NTS between WKY and SHR.[32] However, that study also demonstrated that there were more GluR1-containig dendritic spines in the NTS of SHR compared with WKY, which was attributed to an increase in the proportion of dendritic spines containing GluR1 as well as an increase in the total number of dendritic spines.[32] Thus, it is unlikely that our observations are attributable to the morphological changes resulting from inhibition of the Rho/Rho–kinase pathway. Therefore, the effects of Rho-kinase inhibition might be produced not by the morphological changes in the dendritic spines (ie, an increase in the number of dendritic spines) but rather by a functional change in dendritic spines or other unknown mechanisms. Further studies are needed to clarify the mechanisms underlying our observations.

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