Overexpression of eNOS in RVLM Improves Impaired Baroreflex Control of Heart Rate in SHRSP

Takuya Kishi, Yoshitaka Hirooka, Yoshikuni Kimura, Koji Sakai, Koji Ito, Hiroaki Shimokawa, Akira Takeshita

Abstract—We previously demonstrated that the overexpression of endothelial nitric oxide synthase (eNOS) in the rostral ventrolateral medulla (RVLM) decreases blood pressure, heart rate (HR), and sympathetic nerve activity and that these effects are enhanced in stroke-prone spontaneously hypertensive rats (SHRSP). The aim of this study was to determine if an increase in NO production in the RVLM caused by the overexpression of eNOS improves the impaired baroreflex control of HR in SHRSP. We transfected adenovirus vectors encoding eNOS (AdeNOS) into the RVLM of SHRSP or Wistar-Kyoto rats (WKY). Mean arterial pressure and HR were measured by a radio-telemetry system in the conscious state. Reflex changes in HR were elicited by intravenous infusion of either phenylephrine, sodium nitroprusside, or hydralazine at day 7 after the gene transfer. The maximum gain of the baroreflex control of HR was significantly decreased in SHRSP compared with WKY. Overexpression of eNOS in the RVLM of SHRSP improved the impaired maximum gain of the baroreflex control of HR. After treatment with atropine, the maximum gain was still significantly greater in SHRSP in the AdeNOS-transfected group than in the nontransfected group, although it was decreased in both groups. In contrast, after treatment with metoprolol, the maximum gain did not differ between the two groups. These results indicate that an increase in NO production in the RVLM improves the impaired baroreflex control of HR in SHRSP and that these effects may have resulted from a cardiac sympathoinhibitory effect of NO in the RVLM of SHRSP. (Hypertension. 2003;41:255-260.)

Key Words: genes ■ nitric oxide ■ sympathetic nervous system ■ brain ■ baroreflex

It is well established that the central nervous system plays an important role in controlling arterial baroreflex function.1-2 The major baroreceptor reflex pathway exits in the brain stem, such as the nucleus tractus solitarii (NTS), the caudal ventrolateral medulla (CVLM), and the rostral ventrolateral medulla (RVLM).1,2 In particular, the RVLM contains sympathetic premotor neurons responsible for maintaining sympathetic outflow.3 It is also well known that the baroreceptor reflex control of heart rate is impaired in various animal models of hypertension4-9 and in patients with hypertension.10 Reduced arterial baroreceptor sensitivity has been shown to be responsible for this impaired baroreflex function.4-8 However, recent studies have suggested that central nervous mechanisms may also be involved in the impaired baroreceptor reflex function in hypertension.9,11

There is considerable evidence that neuronal nitric oxide synthase (nNOS) exists within the brain stem, including the RVLM and NTS,12 and plays an important role in regulating sympathetic nerve activity.13-16 At resting conditions, microinjection studies into the RVLM with NO donors or NOS inhibitors have produced conflicting results, including both pressor or depressor responses, and explanation of these results remains to be elucidated.16-22 It is possible that anesthesia might affect the results, and it is also possible that different levels of NO may have opposite effects on the neuronal activity of the RVLM neurons.22,23 Furthermore, previous studies on the modulatory effect of NO on the baroreflex control of heart rate (HR) and sympathetic nerve activity have also yielded conflicting results in normotensive animals. Some studies reported that baroreflex control of HR and/or sympathetic nerve activity were not changed in response to NO.9,24,25 Other studies reported that baroreceptor reflex gain was increased by NO in the bradycardic component in the conscious state, although these studies were performed to examine the role of NO on baroreflex function by systemic administration of NOS inhibitors.26,27

It has also been shown that there is a disorder of the L-arginine–NO pathway in stroke-prone spontaneously hypertensive rats (SHRSP).15 Recently, we developed a technique of endothelial NOS (eNOS) gene transfer into the RVLM14,15 or NTS13,28 of rats in vivo, demonstrating that an increase in NO production in the RVLM14,15 or NTS13,28 decreases blood pressure, HR, and sympathetic nerve activity in the conscious state. Using these methods, we were able to increase NO production locally in the RVLM or NTS for several days and to measure blood pressure and HR in the

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conscious state. More importantly, we have shown that the magnitude of these responses is greater in SHRSP. However, there are no studies examining effects of NO in the RVLM on baroreflex function in SHRSP as well as Wistar-Kyoto rats (WKY). This is important because RVLM neurons regulate sympathetic nerve activity by both baroreflex-dependent and baroreflex-independent mechanisms.

The aim of this study was thus to determine if an increase in NO production in the RVLM improves the impaired baroreceptor reflex control of HR in SHRSP, and, if so, to determine if a cardiac sympathetic component is responsible for the improvement. For this purpose, we transfected adenovirus vectors encoding either eNOS (AdeNOS) or β-galactosidase (Adβgal) into the RVLM or NTS of rats in vivo and constructed HR–mean arterial pressure (MAP) curves by using an intravenous infusion of phenylephrine, sodium nitroprusside, or hydralazine to examine the baroreflex function. Moreover, we examined the effects of atropine or metoprolol on the relation of MAP-HR in rats transfected with AdeNOS into the RVLM.

Methods
This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University.

In Vivo Gene Transfer Into the RVLM or NTS
Adenoviral vectors encoding either the bacterial β-galactosidase gene or the eNOS gene were used as described previously. These adenoviral vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa, Iowa City. Adult (16 to 20 weeks old) male WKY and SHRSP/Izm (Japan SLC, Hamamatsu, Japan), weighing 240 to 300 g, were used. Before the microinjection of vectors into the RVLM or NTS, the sites were identified by monitoring blood pressure after local injection of a small dose of l-glutamate. We performed X-Gal staining for β-galactosidase and immunohistochemical staining for eNOS as described previously.

Analysis of Baroreflex Control of HR
The UA-10 telemetry system (Data Sciences International) was used to measure MAP and HR in a conscious state, as described previously. Measurement of the sensitivity of the baroreflex control of HR was performed at day 7 after the gene transfer in the conscious state. The peak changes in MAP and HR were at day 7 after gene transfer, as described previously. At the day of the gene transfer, under the anesthetized condition, the catheter was inserted into the femoral vein, and at day 7 after gene transfer, the catheter was connected to the infusion pump and progressive infusion of sodium nitroprusside (SNP) (5 to 10 μg/kg per minute) was performed at flow rates of 0.007 to 0.013 ml/min for 1 minute to induce a decrease in MAP between 40 and 50 mm Hg. Phenylephrine hydrochloride (2 to 32 μg/kg per minute) was infused at flow rates of 0.008 to 0.13 ml/min for 1 minute to induce an increase in MAP between 40 and 50 mm Hg. All rats were infused finally with hydralazine (1 mg/kg per minute) to decrease MAP between 40 and 50 mm Hg, since sodium nitroprusside may affect the results by acting on an NO donor in the central nervous system. The speed of an increase or a decrease in mean blood pressure was 0.6 to 0.8 mm Hg per second. At least 20 minutes elapsed between infusion of each vasoactive agent to allow MAP and HR to return to baseline values.

Effects of Autonomic Blockade
In the groups of nontransfected SHRSP and those in which AdeNOS was transfected into the RVLM, to examine the sympathetic and parasympathetic component of the interaction between NO in the RVLM and autonomic innervation of the sinoatrial node, metoprol

bitartrate (a selective β1-receptor blocker, 2 mg/kg IV, supplemented by 0.2 mg/kg IV every 30 minutes) or atropine methyl bromide (0.2 mg/kg IV, supplemented by 0.02 mg/kg IV every 30 minutes) was injected, and the measurement of the gain of the baroreflex control of HR was then performed as described previously.

Data Analysis
To implement analysis of the baroreflex control of HR, heart rate and blood pressure data were taken every 2 seconds from the threshold to the saturation point; the relation between MAP and HR was determined by fitting pairs of data points to a logistic function, using a computer program (Igor Pro, Wave Metrics) run on a computer as described previously. To construct each curve, we used ~80 to 100 points. The logistic function used for data analysis conformed to the mathematical expression \( HR = P_1/[1 + \exp(P_2(MAP - P_3))] + P_4 \). In this equation, \( P_1 \) is the range of responses of HR, \( P_2 \) is the slope coefficient, \( P_3 \) is the MAP at the midpoint of the range for HR, and \( P_4 \) is the minimum HR. The control values of MAP and HR were taken as their 3-minute average before infusion of SNP, hydralazine, or phenylephrine. Values of HR were averaged at 5-mm Hg changes of MAP from baseline levels. The maximum gain of the baroreflex control of HR was expressed as \( -P_2/P_1 \times P_4/4 \) 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, Adβgal-transfected, and AdeNOS-transfected groups from day 0 to day 7 after the gene transfer. Comparisons between any two mean values were performed with the application of the Bonferroni procedure. An unpaired t test was used to compare the maximum gain of the baroreceptor reflex control of HR between groups. Differences were considered statistically significant at a value of \( P<0.05 \).

Results
Analysis of Gene Expression of β-Galactosidase or eNOS
In a section of the rat brain medulla at day 7 after the gene transfer, the presence of β-galactosidase staining was verified histochemically at the site of injection. Similarly, in the AdeNOS-transfected rats, the expression of eNOS protein was verified by immunohistochemistry at the site of injection (Figure 1, A and B).

Blood Pressure and Heart Rate
MAP and HR significantly decreased at day 7 after the eNOS gene transfer in both SHRSP and WKY. Time courses of changes of blood pressure and HR were similar to the previous experiments. In the RVLM-transfected groups, the magnitude of the decreases in MAP and HR was significantly greater in SHRSP than in WKY (MAP, −39±4 mm Hg versus −20±5 mm Hg; HR, −94±4 bpm versus −79±8 bpm, n=5 for each, \( P<0.01 \) for each). In the NTS-transfected group, the magnitudes of the decrease in MAP and HR were also significantly greater in SHRSP than in WKY (MAP, −20±2 mm Hg versus −12±4 mm Hg; HR, −72±4 bpm versus −59±6 bpm, n=5 for each, \( P<0.01 \) for each). In contrast, in the nontransfected and Adβgal-transfected rats, these variables did not change in either strain.

Effects of Overexpression of eNOS in the RVLM on Baroreflex Control of HR
The parameters of the logistic function analyses are shown in the Table. In the nontransfected group, the baroreflex curve
shifted to the right and the maximum gain of HR was significantly decreased in SHRSP compared with WKY (\(-0.4\pm0.1\) versus \(-1.4\pm0.1\), \(n=5\) for each, \(P<0.01\)) (Table and Figure 2, A through D). In the Ad\(\beta\)gal-transfected group, none of the parameters were significantly different compared with those of the nontransfected group. In the group of SHRSP in which AdeNOS was transfected into the RVLM, the maximum gain of HR was increased significantly compared with those in nontransfected SHRSP (\(-0.8\pm0.2\) versus \(-0.4\pm0.1\), \(n=5\) for each, \(P<0.01\)) (Table and Figure 2, A and B). However, in the WKY in which AdeNOS was transfected into the RVLM, the maximum gain of HR was not significantly changed compared with those in nontransfected rats (Table and Figures 2A and 2B). In all groups, hydralazine did not alter the parameters or maximum gain of baroreflex control of HR compared with the experiments that used SNP.

### Parameters and Maximum Gain of Baroreflex Control of HR Using Phenylephrine and SNP

<table>
<thead>
<tr>
<th></th>
<th>P1, bpm</th>
<th>P2, bpm/mm Hg</th>
<th>P3, mm Hg</th>
<th>P4, bpm</th>
<th>Gmax, bpm/mm Hg</th>
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<tr>
<td>Control</td>
<td>110±6</td>
<td>0.05±0.01</td>
<td>105±4</td>
<td>263±12</td>
<td>(-1.4\pm0.1)</td>
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<tr>
<td>AdeNOS</td>
<td></td>
<td></td>
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<tr>
<td>NTS</td>
<td>127±7*</td>
<td>0.05±0.01</td>
<td>94±5*</td>
<td>257±13*</td>
<td>(-1.4\pm0.1)</td>
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<tr>
<td>RVLM</td>
<td>128±7*</td>
<td>0.05±0.01</td>
<td>88±6*</td>
<td>263±12</td>
<td>(-1.5\pm0.1)</td>
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<tr>
<td><strong>SHRSP</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>50±4</td>
<td>0.03±0.01</td>
<td>151±9</td>
<td>354±11</td>
<td>(-0.4\pm0.1)</td>
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<td>Atropine</td>
<td>12±4*</td>
<td>0.03±0.01</td>
<td>155±6</td>
<td>388±9*</td>
<td>(-0.1\pm0.1)</td>
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<td>Metoprolol</td>
<td>25±6*</td>
<td>0.06±0.01*</td>
<td>152±6</td>
<td>356±6</td>
<td>(-0.4\pm0.1)</td>
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<tr>
<td><strong>AdeNOS</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NTS</td>
<td>54±4</td>
<td>0.03±0.01</td>
<td>135±5*</td>
<td>301±11*</td>
<td>(-0.4\pm0.1)</td>
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<tr>
<td>RVLM</td>
<td>72±6*</td>
<td>0.05±0.01</td>
<td>105±4*</td>
<td>273±12*</td>
<td>(-0.8\pm0.2)</td>
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<tr>
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<td>121±8*</td>
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<td>(-0.4\pm0.1)</td>
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<td>0.03±0.01</td>
<td>112±7*</td>
<td>256±8</td>
<td>(-0.4\pm0.1)</td>
</tr>
</tbody>
</table>

*\(P<0.05\) vs control of each group.
Effects of Overexpression of eNOS in the NTS on Baroreceptor Reflex Control of HR

The parameters of the logistic function analyses are shown in Table. In the Adβgal-transfected group, none of the parameters were significantly different compared with those of the nontransfected group. In the group of SHRSP in which AdeNOS was transfected into the NTS, the maximum gain of HR was not increased compared with those in nontransfected SHRSP (Table and Figures 2C and 2D). Moreover, in the group of WKY in which AdeNOS was transfected into the NTS, the maximum gain of HR was not significantly changed compared with those in nontransfected rats (Table and Figures 2C and 2D). In all groups, hydralazine did not alter the maximum gain of HR compared with the experiments that used SNP.

Effects of Overexpression of eNOS in the RVLM of SHRSP on Baroreflex Control of HR After Autonomic Blockade

In the nontransfected SHRSP, atropine significantly increased the baseline HR (372±8 bpm to 398±7 bpm, n=5 for each, P<0.05) and the range of HR (50±4 bpm to 25±6 bpm, n=5 for each, P<0.05) but did not significantly change the minimum HR, maximum gain of HR, or baseline MAP (Table and Figures 3C and 3D). In the SHRSP in which AdeNOS was transfected into the RVLM, atropine significantly increased baseline HR (332±8 bpm to 352±7 bpm, n=5 for each, P<0.01) and minimum HR (273±12 to 352±15 bpm, n=5 for each, P<0.01) and decreased the range of HR (72±6 bpm to 28±4 bpm, n=5 for each, P<0.01) and the maximum gain of HR (−0.8±0.2 to −0.4±0.1, n=5 for each, P<0.05) (Table and Figures 3C and 3D). Metoprolol also significantly decreased the range of HR (72±6 bpm to 40±5 bpm, n=5 for each, P<0.05) and the maximum gain of HR (−0.8±0.2 to −0.4±0.1, n=5 for each, P<0.05) but did not significantly change the minimum HR (Table and Figures 3C and 3D). After treatment with atropine, the maximum gain of HR was significantly greater in the SHRSP in which AdeNOS was transfected into the RVLM than in nontransfected SHRSP (−0.4±0.1 versus −0.1±0.1, n=5 for each, P<0.05). However, after treatment with metoprolol, the maximum gain of HR was not different between the SHRSP in which AdeNOS was transfected into the RVLM and in the nontransfected SHRSP.

Discussion

The present study shows that overexpression of eNOS in the RVLM results in significant shifts in the MAP-HR relation in both WKY and SHRSP, but there is a significant change in the calculated maximum gain only in the case of overexpression of eNOS in the RVLM of SHRSP. Furthermore, the major effect of NO in the RVLM of SHRSP on the baroreflex control of HR appears to be due to inhibition of the cardiac sympathetic component. In addition, the overexpression of eNOS in the NTS of SHRSP and that in the NTS or RVLM of WKY did not increase the maximum gain of the baroreflex control of HR in the conscious state.

In the nontransfected SHRSP, the maximum gain of the baroreflex control of HR was significantly decreased compared with WKY as expected. In SHRSP, which is a model of chronic hypertension with an increase in sympathetic nerve activity, others have reported that the gain of the baroreflex control of HR is decreased. We showed that after treatment with atropine, the maximum gain of baroreflex control of HR was significantly decreased in both nontransfected SHRSP and in SHRSP in which AdeNOS was transfected into the RVLM. However, there was still a significant difference between the AdeNOS-transfected and nontransfected SHRSP. Conversely, after treatment with metoprolol, the maximum gain of baroreflex control of HR was significantly decreased only in SHRSP in which AdeNOS was transfected into the RVLM, and there was no difference in gain between the transfected and nontransfected SHRSP. These results suggest that overexpression of eNOS in the RVLM of SHRSP improves the sympathetic component of baroreflex function. The maximum gain is influenced by both reflex range and the slope coefficient, and the increase in NO production in the RVLM of SHRSP increased both these component.
results suggest that the increase in the gain of HR is not only due to the increase in the range of HR. Taken together, our results suggest that NO in the RVLM improves the impaired baroreflex control of HR in SHRSP and that these effects are mainly mediated by the improvement of the sympathetic component.

The transfected eNOS is expressed in neurons, glia, and vasculature. However, the aim of our study was to increase the NO production locally at the transfected site for a longer period in the conscious state. The transfected eNOS in the brain produces NO by the stimulation of the \( N \)-methyl-D-aspartate (NMDA) receptor, the same mechanism as nNOS. We need to consider the possibility that the depressor effect was due to the inflammation or cytotoxicity caused by transfection of adenovirus vectors or high levels of NO. However, this possibility is unlikely, based on the following reasons in our experiments. First, in our previous study, we examined the extent of ED-1–positive cell infiltration, a marker of inflammation, which did not differ significantly between rats transfected with Ad\( \beta \)gal and those transfected with AdeNOS in the NTS. Second, the \( \beta \)-galactosidase gene transfer did not alter blood pressure, heart rate, urinary norepinephrine excretion, or NOx production. Third, magnitude of the increases in blood pressure and heart rate evoked by \( N \)-monomethyl-L-arginine (L-NMMA) were greater in the AdeNOS-transfected than in the Ad\( \beta \)gal-transfected rats. These results suggest that changes in these variables in the rats with eNOS gene transfer did not result from inflammation or cytotoxicity but were mediated by the increase in NO production in the NTS or the RVLM. Furthermore, if temporary damage of presympathetic cells occurred, blood pressure would be expected to be increased in the case of NTS. Although produced NO might spread and affect some neurons in the respiratory center or small parts of the caudal ventrolateral medulla neurons, we believe that our technique of eNOS gene transfer used in this study is specific and has an advantage for examining the effects of NO at local sites of the brain for a much longer period compared with microinjection or intracerebroventricular injection techniques.

It is necessary to consider the possibility that the intravenous infusion of SNP itself affects NO in the brain and thereby the baroreflex control of HR. However, this possibility is unlikely. In our study, we used not only SNP but also hydralazine to decrease the blood pressure, and the maximum gain of the baroreflex control of HR was not different between experiments that used SNP and hydralazine. Thus, we conclude that the intravenous infusion of SNP for 4 or 5 minutes does not affect NO in the RVLM or NTS.

In this study, the overexpression of eNOS in the NTS of WKY or SHRSP did not alter the baroreflex control of HR. We do not have a clear answer for this observation. However, it was reported that the gain of arterial baroreflex control of renal sympathetic nerve activity did not differ before and after microinjection of L-NMMA into the NTS. Furthermore, the changes of blood pressure caused by the overexpression of eNOS in the NTS of WKY or SHRSP was smaller than those caused by the overexpression of eNOS in the RVLM of WKY or SHRSP, which might affect the results.

The mechanisms by which NO in the RVLM improved the impaired baroreflex control of HR in SHRSP cannot be elucidated from the results of our study. Although increased eNOS expression in the RVLM clearly increased baroreflex gain in SHRSP, this change could be due to the decrease in pressure. It was reported that some \( \gamma \)-amino butyric acid (GABA)ergic CVLM neurons are tonically activated by inputs independent of arterial baroreceptors or the NTS. Providing a GABAergic-mediated inhibition of the sympathetically RVLM neurons that is autonomous of baroreceptor inputs. Furthermore, we previously showed that the increase in NO production evoked by the overexpression of eNOS in the RVLM causes greater depressor and sympathoinhibitory responses in SHRSP than in WKY by improving an inhibitory action of GABA on the RVLM neurons. Therefore, we suggest that the impaired GABAergic input to the RVLM neurons from the CVLM neurons was improved by the action of NO. Regardless of the precise mechanism(s), our results suggest that the improvement of the impaired baroreflex control of HR in SHRSP by NO in the RVLM is mainly mediated by the improvement of the sympathetic component through the inhibition of sympathetic nerve activity. However, we cannot exclude the possibility that blood pressure reduction itself influenced our observation.

In conclusion, we have shown that in the conscious state, the increase in NO production caused by the overexpression of eNOS in the RVLM of SHRSP increased the maximum gain of the baroreflex control of HR, which is significantly decreased in SHRSP compared with WKY. These results suggest that NO in the RVLM of SHRSP improves the impaired baroreflex control of HR and that this effect may have resulted from a cardiac sympathoinhibitory effect of NO.

**Perspectives**

The present study was designed to elucidate the role of NO in the RVLM in baroreflex control of HR in SHRSP by overexpression of eNOS. The results of our study indicate that an increase in NO production in the SHRSP improves this function. However, it is unclear whether endogenous NO production in the RVLM is impaired or insufficient to compensate for the abnormal baroreflex function in hypertension, although we previously reported that nNOS expression in the RVLM did not differ between SHRSP and WKY. A functional abnormality of the expressed nNOS may exist in SHRSP. In addition, the RVLM neurons receive a tonic GABAergic input that is partly dependent on baroreceptor inputs and partly independent. We previously examined the effects of microinjection of bicuculline, a GABA-A receptor antagonist, into the RVLM of WKY and SHRSP and found that the pressor response evoked by bicuculline into the RVLM was smaller in SHRSP than in WKY and that it was normalized after transfection of eNOS into the RVLM. From these results, we suggest the role of GABA in the improvement of reflex control of HR as one of the possibilities. Further studies will be needed to clarify these questions.
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