Overexpression of eNOS in the RVLM Causes Hypotension and Bradycardia Via GABA Release

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Abstract—In this study, we examine the role of NO located in the rostral ventrolateral medulla (RVLM) in the control of blood pressure and the activity of the sympathetic nervous system. To determine the effect of an increase in NO production in the RVLM on blood pressure in conscious rats, adenovirus vectors encoding either endothelial NO synthase (AdenOS) or β-galactosidase (Adβgal) were transfected into the bilateral RVLM. The local expression of endothelial NO synthase (eNOS) protein in the RVLM was confirmed by immunohistochemical staining for the eNOS protein and by Western blot analysis. Mean arterial blood pressure (MAP) and heart rate, which were monitored using a radio-telemetry system, were significantly decreased in the AdeNOS-treated group from day 5 to day 10 after the gene transfer. Urinary norepinephrine excretion was decreased on day 7 after the gene transfer in the AdeNOS-treated group. Microinjection of either NG-monomethyl-L-arginine (L-NMMA) or bicuculline, a γ-amino butyric acid (GABA) receptor antagonist, into the RVLM at day 7 after the gene transfer increased MAP to significantly greater levels in the AdeNOS-treated group. However, microinjection of kynurenic acid into the RVLM on day 7 after the gene transfer did not alter MAP levels in either group. GABA and glutamate levels in the RVLM, when measured by in vivo microdialysis, were significantly increased in the AdeNOS-treated group. These results suggest that the increase in NO production caused by the overexpression of eNOS in the bilateral RVLM decreases blood pressure, heart rate, and sympathetic nerve activity in conscious rats. Furthermore, these responses may be mediated by an increased release of GABA in the RVLM. (Hypertension. 2001;38:896-901.)

Key Words: genes ■ nitric oxide ■ cardiovascular regulation ■ brain ■ sympathetic nervous system.

Nitric oxide is synthesized from L-arginine through the activity of the synthetic enzyme, NO synthase (NOS). Studies using immunohistochemistry, NADPH-diaphorase staining, or autoradiography have demonstrated the presence of neuronal NO synthase (nNOS) at a high concentration in regions of the brain stem, especially in the nucleus tractus solitarii (NTS) and rostral ventrolateral medulla (RVLM).1,2 In addition, considerable evidence suggests that NO in the brain affects sympathetic nerve activity and modulates blood pressure and heart rate (HR).3–11 The role of NO in cardiovascular regulation has been investigated in specific areas of the central nervous system, including the NTS,3,4 RVLM,5–9 and paraventricular nucleus of the hypothalamus (PVN).10 The RVLM contains sympathetic premotor neurons responsible for maintaining the tonic excitation of sympathetic preganglionic neurons involved in cardiovascular regulation, and the functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone.11 However, previous studies investigating the role of NO in cardiovascular regulation by the RVLM have reported controversial results.3,5–9 In some of those studies, a precursor of NO, L-arginine,3,8 and NO donors, such as Et,N[N(O)NO]Na (NOC 18)8 and sodium nitroprusside (SNP),3,5 caused a depressor response. In contrast, NOS inhibitors, such as Nω-nitro-L-arginine (L-NNA),6 nitro-L-arginine methyl ester (L-NAME),8 and Nω-monomethyl-L-arginine (L-NMMA),3,5 were found to cause a pressor response in anesthetized animals. Contrary to the studies described above, both SNP and L-NAME were reported to result in a pressor response in denervated anesthetized rabbits.7 In conscious rats, Martins-Pinge et al9 reported that L-arginine, SNP, and 5-nitroso-N-acetylpencillamine (SNAP) caused a pressor response; however, they monitored the response for only 60 minutes after the injection. These conflicting reports indicate that the chronic effects of NO in the RVLM on cardiovascular regulation in conscious animals need to be clarified.

Replicant-deficient recombinant adenovirus vectors are currently used in gene transfer techniques.12,13 The transfection of replication-defective adenoviral vectors encoding the cDNA of endothelial NO synthase (eNOS) into the brain may provide a method to study the effects of an increase in NO production in the brain on the regulation of blood pressure and sympathetic nerve activity in conscious animals for a much longer period. In a previous study,14 we developed a technique of eNOS gene transfer into the NTS of rats in vivo, and we found that NO production was increased locally in the NTS.
for several days in conscious rats. We examined the role of NO in the NTS in cardiovascular regulation in conscious states and demonstrated that an increase in NO production in the NTS resulted in a decrease in blood pressure, HR, and sympathetic nerve activity in conscious rats.14 In this study, we intended to determine the effects of a sustained increase in NO production by eNOS overexpression in the RVLM on blood pressure, HR, and sympathetic nerve activity in conscious rats. We transfected adenovirus vectors encoding either eNOS (AdeNOS) or β-galactosidase (Adβgal) into the RVLM of rats in vivo. Furthermore, to explore the mechanisms by which eNOS overexpression in the RVLM affects the mean arterial blood pressure (MAP) and HR, we examined the effect of γ-amino butyric acid (GABA) and glutamate in the RVLM by the microinjection of either bicuculine (a GABA receptor antagonist) or kynurenic acid (a glutamate receptor antagonist) into the bilateral RVLM and measured GABA and glutamate levels in the RVLM by in vivo microdialysis.

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

We used adenoviral vectors encoding either the bacterial β-galactosidase gene or the bovine eNOS gene as described previously.14 These adenoviral vectors were constructed in the Gene Transfer Core Laboratory at the University of Iowa (Iowa City).12,13 Adult (16 to 20 weeks old) male Wister-Kyoto rats, weighing 280 to 340 g, were used for the in vivo gene transfer experiments. The rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and placed in a stereotaxic frame, with the head flexed downward at a 45° angle. An incision was made on the skin at the midline of the head, and the muscles were dissected to expose the cisterna magna. The atlanto-occipital membrane was incised, and the dorsal surface of the medulla was visualized. A glass micropipette (5-µm OD) was filled with PBS containing Adβgal or AdeNOS. The pipette was placed in a micromanipulator and positioned at the injection site. A microinjection was made at the bilateral RVLM through a polyvinyl tube connected to the microinjection pipette. The sites of microinjection were defined, according to an atlas of the rat18 with stereotaxic coordinates, as an anteroposterior angle 18°, 1.8 mm lateral, 3.5 mm deeper to calamus scriptorius. Before the microinjection of vectors, the RVLM was identified by monitoring the mean arterial pressure (MAP) after an injection of a small dose of L-glutamate. Identification of the RVLM was confirmed according to the following criteria: (1) an increase in MAP occurred immediately after the injection of L-glutamate, (2) the response plateau occurred within 20 seconds of the injection, and (3) the change in MAP was greater than 20 mm Hg.19 An adenoviral suspension containing 1×109 plaque-forming units (pfu)/mL was injected into each injection site for over 15 minutes (800 nL for each site). The pipette was removed, and the incision of the atlanto-occipital membrane and the skin were closed. After the injection, all rats recovered from anesthesia and were unrestrained and free to move about their cages.

Analysis of Gene Expression for β-Galactosidase or eNOS

At day 7 after gene transfer, β-galactosidase expression was confirmed by histostaining with X-Gal in PBS as described previously.14 We performed double-immunohistochemical staining for eNOS and phenylethanolamine-N-methyltransferase (PNMT), an enzyme that catalyzes the final step of epinephrine synthesis and is identified specifically in C1 neurons of RVLM. We used a mouse IgG monoclonal antibody to eNOS (Transduction Laboratories) and a rabbit PNMT polyclonal antibody (Chemicon International). At 7, 14, 21, or 30 days after the gene transfer, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. The brain was removed, and coronal sections (50 µm) of the medulla were cut using a vibratome. The sections were rinsed for 30 minutes in PBS. After incubation overnight in 1% BSA in PBS, the sections were immunohistochemically stained using mouse IgG monoclonal antibody to human eNOS (1:200) as previously described.14 For double immunohistochemical staining, the sections were incubated in rabbit anti-PNMT antibody (1:200) at 4°C for 48 hours. After rinsing 3 times in PBS, the sections were incubated for 2 hours at room temperature in rhodamine-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories). The sections were rinsed 3 times in PBS and mounted in Vectashield (Vector Laboratories). Double-stained sections with eNOS and PNMT antibody were photographed with a confocal laser scanning microscope as described previously.14 Confocal images were then transferred to a personal computer using the image analysis software package NIH Image.16 To confirm the local overexpression of eNOS in the RVLM, Western blot analysis for eNOS protein from the coronal section of the brain stem containing the injected sites of the RVLM was performed at day 7 after the gene transfer as described previously.14 The densitometric average was normalized to the values obtained from the nontreated rats.

Radio-Telemetry Monitoring of Blood Pressure and Heart Rate

The UA-10 telemetry system (Data Sciences International) was used to measure MAP and HR.14,17 The surgical procedure has been described previously.14 MAP and HR were recorded continuously for 10 minutes every day between 10:00 AM and 11:00 AM by a multichannel amplifier and signal converter. Previous studies have shown that blood pressure and HR becomes stabilized at day 7 after injection of the adenovirus vectors. Therefore, microinjection of further agents into the RVLM was performed at day 7 after the viral injection.

Measurement of Urinary Norepinephrine Excretion

As described previously, we measured the urinary norepinephrine concentration before the gene transfer and at day 7 after the gene transfer by high-performance liquid chromatography (HPLC), and calculated the urinary norepinephrine excretion for 24 hours.14

Microinjections Into the RVLM

To confirm that changes of MAP and HR caused by the AdeNOS transfection were the result of an increase in NO production, we microinjected L-NMMA (100 nmol) into the bilateral RVLM at day 7 after transfection with Adβgal or AdeNOS. This dose of L-NMMA was chosen from the results of the previous study.3 The rats were anesthetized with sodium pentobarbital (50 mg/kg IP followed by 20 mg · kg⁻¹ · h⁻¹ IV). Catheters, constructed from PE-50 tubing and filled with heparinized saline, were inserted into the femoral artery for recording arterial blood pressure. A tracheal cannula was instilled and connected to a ventilator. The rats were artificially ventilated, and arterial blood pressure was recorded with a P23XL transducer connected to an RS3000 recorder. To explore the mechanisms by which the increase in NO production, resulting from the overexpression of eNOS in the RVLM, affects MAP and HR, we microinjected either bicuculine (200 pmol), a GABA receptor antagonist, or kynurenic acid (2.7 nmol), a glutamate receptor antagonist, into the bilateral RVLM at day 7 after transfection with Adβgal or AdeNOS. The surgical and recording procedures used were identical with that used for the microinjection of L-NMMA into the RVLM.

Measurement of GABA and Glutamate Levels by In Vivo Microdialysis

We measured GABA and glutamate levels in the RVLM of AdeNOS-treated and Adβgal-treated rats by in vivo microdialysis at...
day 7 after the gene transfer. The mobile phase for GABA and glutamate detection consisted of 75% 0.1 mol/L PBS and 25% methanol. The separation and reduction columns and the reaction coil were placed in a column oven that was set at 30°C. To measure GABA and glutamate levels in the dialysates, the perfused dialysates were collected every 10 minutes, and GABA and glutamate levels were measured by an HPLC system with an electrochemical detector (ECD-300, Eicom). GABA and glutamate levels were quantitated by averaging 3 consecutive stable dialysate samples, which were obtained at approximately ±1 hour after starting the brain perfusion with Ringer’s solution.

**Statistical Analysis**

All values are expressed as mean±SEM. Two-way ANOVA was used to compare the MAP, HR, and the GABA and glutamate levels between the Adβgal-treated and AdeNOS-treated groups. Comparisons between any 2 mean values were performed with the application of Bonferroni’s procedure. Paired t-test was used to compare the 24 hours excretion of urinary norepinephrine before and 7 days after the gene transfer. Differences were considered to be significant when P<0.05.

**Results**

**Analysis of Gene Expression for β-Galactosidase or eNOS**

Figure 1A shows the β-galactosidase staining in a section of the rat brain medulla at day 7 after the gene transfer. The presence of β-galactosidase staining was noted in the RVLM, where Adβgal had been microinjected. No X-Gal-positive cells were found in the other regions of the brain. In the AdeNOS-treated rats, the expression of eNOS protein was observed locally in the RVLM, where the AdeNOS had been transfected. No eNOS-positive neurons were observed in other areas of the brain, such as the NTS, caudal ventrolateral medulla (CVLM), and hypothalamus. The expression of eNOS peaked at day 7 after the gene transfer and, thereafter, declined over time as detected by immunohistochemistry. At day 21 or day 30 after the eNOS gene transfer, we were not able to detect eNOS-positive cells. Some of the C1 neurons labeled with the PNMT antibody were also detected with the anti-eNOS antibody (Figure 1B through 1E). Western blot analysis revealed that the expression of eNOS was significantly increased in tissue containing the RVLM of AdeNOS-treated rats when compared with that of Adβgal-treated and nontreated rats at day 7 after gene transfer (Figure 1F). The intensity quantified by densitometric analysis was significantly greater in AdeNOS-treated rats than in Adβgal-treated rats (3.2±0.1 versus 1.0±0.1, respectively, n=5, P<0.01).

**Blood Pressure, HR, and Urinary Norepinephrine Excretion**

Figure 2A and 2B shows the changes in the MAP and HR before and after the gene transfer into the RVLM. The MAP and HR decreased significantly in the AdeNOS-treated rats between days 5 and 10 after the gene transfer. In contrast, these variables did not change in the Adβgal-treated or aCSF-treated rats. As shown in Figure 2C, urinary norepinephrine excretion measured at day 7 after the gene transfer was significantly decreased in the AdeNOS-treated rats relative to that measured before the gene transfer. However, in the Adβgal-treated rats, urinary norepinephrine excretion was not decreased.

**Microinjection Into the RVLM**

The microinjection of L-NMMA into the RVLM at day 7 after the gene transfer produced a gradual increase in MAP in both the AdeNOS-treated and the Adβgal-treated rats. As shown in Figure 3A, the increase in MAP caused by L-NMMA was significantly greater in the AdeNOS-treated rats (from 81±5 to 96±6 mm Hg) than in the Adβgal-treated (from 94±4 to 99±3 mm Hg) and nontreated rats (from 95±2 to 100±3 mm Hg). Similarly, the microinjection of bicuculline into the RVLM at day 7 after gene transfer also produced a gradual increase in MAP. The increase in MAP...
was significantly greater in AdeNOS-treated rats than in Adβgal-treated and nontreated rats (Figure 3B). In contrast to the effect of L-NMMA and bicuculine, the microinjection of kynurenic acid into the RVLM at day 7 after gene transfer did not alter MAP in AdeNOS-treated or Adβgal-treated rats (data not shown).

**Measurement of GABA and Glutamate Levels by In Vivo Microdialysis**

GABA levels in the RVLM were significantly greater in AdeNOS-treated rats (37.2 ± 4.9 pmol/20 μL, n = 8) than in Adβgal-treated (13.6 ± 4.5 pmol/20 μL, n = 8) and nontreated rats (15.3 ± 2.7 pmol/20 μL, n = 8) (Figure 3C). Glutamate levels in the RVLM were also significantly greater in AdeNOS-treated rats (50.8 ± 2.7 pmol/20 μL, n = 8) than in Adβgal-treated (31.9 ± 4.2 pmol/20 μL, n = 5) and nontreated rats (29.7 ± 3.0 pmol/20 μL, n = 5).

**Discussion**

In this study, we demonstrate that the overexpression of eNOS in the RVLM results in a local increase in NO production that persists for several days. The increased NO in the RVLM caused a decrease in MAP, HR, and sympathetic nerve activity in conscious rats. Further, we found that these effects were mediated, at least in part, by the increased release of GABA in the RVLM. Together, these findings suggest an important role of NO in the RVLM in the regulation of sympathetic nerve activity in conscious condition.

We transfected adenovirus vectors encoding either the β-galactosidase gene or the eNOS gene into the RVLM of rats in vivo. The successful gene transfer into the RVLM was confirmed by several methods. First, β-galactosidase protein expression was confirmed by histochemical staining for β-galactosidase in the RVLM. Second, eNOS protein expression in the RVLM was confirmed by immunohistochemical staining for eNOS protein and by Western blot analysis. The expression of eNOS protein was not observed in other regions of the brain, including the CVLM. For confirmation of the transfection site in the RVLM, we identified the area functionally through the prior injection of l-glutamate and anatomically by immunohistochemical staining for PNMT, which indicates the C1 area where the RVLM neurons are...
located.\textsuperscript{11} As previously reported, we did not find any increase in the nitrite and nitrate level at a site 3 mm distant from the NTS when we transfected eNOS gene into the NTS.\textsuperscript{14} Thus, we consider that the transfected gene as site-specific in the RVLM in this study. The time course of expression of the transfected eNOS protein observed in this study was compatible with the result of a previous study using adenovirus vectors.\textsuperscript{14} Finally, the changes in MAP and HR resulting from bilateral microinjections of L-NMMA were greater in AdeNOS-treated rats than in Ad\textbeta gal-treated rats. This result suggests that the production of NO was increased in the RVLM of AdeNOS-treated rats and that the responses that occurred following the eNOS gene transfer were indeed mediated by NO.

Several factors influenced our decision to use eNOS in our experiments instead of nNOS, which is normally present in the central nervous system. First, the purpose of this study was to increase the NO production locally in the RVLM by overexpression of NO. Therefore, eNOS is as useful in increasing NO production as nNOS. Second, to confirm the local overexpression of NOS resulting from the gene transfer, eNOS is more useful in discriminating de novo production from endogenous production than nNOS, which is present at greater levels than eNOS in the RVLM. As reported previously,\textsuperscript{14} eNOS is expressed in neurons, glia, and other tissues of the RVLM in the rats transfected with AdeNOS.

Inflammation and cytotoxicity caused by the adenovirus infection may have affected the present findings. However, we reported previously that the extent of ED-1–positive cell infiltration, a marker of inflammation, did not differ significantly between animals treated with Ad\textbeta gal and those treated with AdeNOS in a study of gene transfer into the NTS using an adenovirus vector.\textsuperscript{14} Moreover, the \textbeta -galactosidase gene transfer did not alter MAP, HR, and urinary norepinephrine excretion. Finally, the increase in MAP caused by the microinjection of L-NMMA was greater in AdeNOS-treated rats than in Ad\textbeta gal-treated rats. These results suggest that the change in these variables in the AdeNOS-treated rats did not result from either inflammation or cytotoxicity but were mediated by the increase in NO production in the RVLM.

An important finding of our study is that transfer of the eNOS gene into the RVLM decreased MAP and HR in conscious rats. The time course of the MAP and HR changes were similar to those produced by transgene expression with AdeNOS. Urinary norepinephrine excretion for 24 hours was decreased at day 7 after gene transfer into the RVLM, which strongly suggests that the increase in NO production caused by eNOS gene transfer into the RVLM decreased sympathetic nerve activity in conscious rats. The \textbeta -galactosidase gene transfer into the RVLM had no effects on MAP, HR, and urinary norepinephrine excretion.

Another important finding of our study is that the increase in NO production in the RVLM caused by the overexpression of eNOS increased the release of GABA and glutamate in the RVLM. Because both excitatory and inhibitory neurotransmitters were found to be increased in this study, the mechanisms by which an increase of NO production in the RVLM decreased MAP, HR, and urinary norepinephrine excretion remain to be clarified. There is considerable evidence that in general, excitatory amino acid neurotransmitters, such as glutamate and aspartate, cause pressor and tachycardic responses, whereas inhibitory amino acid neurotransmitters, such as GABA and glycine, cause depressor and bradycardic responses.\textsuperscript{11} GABA is one of the major inhibitory neurotransmitters in the RVLM,\textsuperscript{19,20} and the presence of GABA receptors in the RVLM has been demonstrated elsewhere.\textsuperscript{21} We suggest that NO in the RVLM increases the release of both GABA and glutamate in conscious rats and that the increased release of GABA in the RVLM influenced sympathetic nerve activity to a greater extent than glutamate. The RVLM is known to receive both excitatory and inhibitory inputs.\textsuperscript{11,19,22} However, at resting condition GABA-ergic input for inhibiting sympathetic nerve activity may be more important. NO has been shown to increase not only the release of excitatory amino acids, but also that of inhibitory amino acids in the PVN, although blood pressure was decreased by NO.\textsuperscript{10} In addition, GABA has been reported to mediate the inhibitory effect of endogenous NO within the PVN on renal sympathetic nerve activity.\textsuperscript{23} A recent study suggests that NO mediates GABA-ergic inhibition elicited by the activation of N-methyl-D-aspartate receptors and thus contributes to the regulation of neuronal excitability in the PVN.\textsuperscript{24} Other studies also report that an inhibitory component is mediated by activating a GABA\textsubscript{A} receptor on the RVLM cardiovascular neurons.\textsuperscript{20,25} In our study, the increase in MAP and HR caused by the microinjection of bicuculine and the increase in GABA levels in the RVLM measured by in vivo microdialysis were significantly greater in AdeNOS-treated rats than in Ad\textbeta gal-treated rats. However, the microinjection of kynurenic acid into the RVLM did not change blood pressure in either AdeNOS-treated rats or Ad\textbeta gal-treated rats. The dose of kynurenic acid used in this study has been demonstrated previously to effectively and selectively block ionotropic excitatory amino acid receptors in the RVLM.\textsuperscript{26} Together, these results suggest that the depressor and bradycardic effects of the increase in NO production in the RVLM caused by eNOS gene transfer may be mediated by an increase in GABA levels. Recently, we have shown that the facilitatory release of glutamate evoked by NO enhances the depressor and bradycardic responses in the NTS.\textsuperscript{27} Thus, we speculate that the role of NO in cardiovascular responses is determined by the balance of the increased release of neurotransmitters evoked by NO production, and by the site in the brain influenced by such neurotransmitters. The other possibility is that even the release of glutamate in the RVLM may contribute to the hypotensive response evoked by NO, an idea that has been recently postulated by Ito and Sved.\textsuperscript{28}

In summary, we have developed a technique of eNOS gene transfer into the RVLM of rats in vivo. The persistent increase in NO production resulting from the overexpression of eNOS in the RVLM led to decreases in MAP, HR and urinary norepinephrine excretion, and increased GABA levels in the RVLM in conscious rats. Together, our results suggest that NO in the RVLM exerts an inhibitory effect on sympathetic nerve activity in vivo. Furthermore, these responses may be mediated, in part, by the increased release of GABA caused by NO production in the RVLM.
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