Overexpression of eNOS in NTS Causes Hypotension and Bradycardia In Vivo

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Abstract—The role of nitric oxide (NO) in the brain in the control of blood pressure and the sympathetic nervous system is debated. This study examined the effect of overexpression of endothelial NO synthase (eNOS) in the nucleus tractus solitarii (NTS) on blood pressure in conscious rats. Adenovirus vectors encoding either eNOS (AdeNOS) or β-galactosidase were transfected into the NTS in vivo. In the AdeNOS-treated rats, the local expression of eNOS in the NTS was confirmed by immunohistochemical staining and Western blot analysis for the eNOS protein and by increased production of nitrite/nitrate in the NTS measured by in vivo microdialysis. Blood pressure and heart rate, monitored by the use of a radiotelemetry system in a conscious state, were significantly decreased in the AdeNOS-treated group at day 5 to day 10 after the gene transfer. Urinary norepinephrine excretion also was decreased at day 7 after the gene transfer in the AdeNOS-treated group. Our results indicate that overexpression of eNOS in the NTS decreases blood pressure, heart rate, and sympathetic nerve activity in conscious rats. (Hypertension. 2000;36:1023-1028.)

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

There is considerable evidence that nitric oxide (NO) in the brain affects sympathetic nerve activity and modulates blood pressure and heart rate.1–6 Studies that used immunohistochemistry for neuronal NO synthase (nNOS), nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining, have demonstrated the presence of nNOS at a high concentration in the regions of the brain stem, such as the nucleus tractus solitarii (NTS) and the ventrolateral medulla (VLM), which plays an important role in regulation of sympathetic nerve activity.7,8 However, conflicting results have been obtained with regard to the effect of NO in the regulation of blood pressure and sympathetic nerve activity. Several studies have demonstrated that unilateral microinjection of the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) into the NTS produced the pressor effect.1,2 and L-arginine, the precursor of NO, decreased blood pressure, heart rate, and renal sympathetic nerve activity.2 On the contrary, the other study has shown that the microinjection of Nω-nitro-L-arginine methyl ester (L-NAME), another NOS inhibitor, into the NTS decreased blood pressure, heart rate, and renal sympathetic nerve activity.3 and the NO donor Et₃N[N(O)NO]Na (NOC 18) increased those variables.3 Similarly, conflicting results have also been reported as regard to the effects of NO in the rostral VLM.2,4,5 However, all of these studies were performed in anesthetized animals and examined only acute effects of NO or NOS inhibitors. Long-term effects of increased NO production in these regions on the regulation of blood pressure and sympathetic nerve activity in conscious animals remain to be clarified.

Replicant-deficient recombinant adenovirus is now widely used for gene transfer into the brain as well as into the blood vessel.9–12 Of the viral vectors currently used for gene transfer, adenovirus is considered to offer substantial advantages, including high capability of infecting a variety of differentiated cell types.9–12 Transfection of replication-defective adenoviral vectors encoding cDNA of endothelial NOS (eNOS) into the brain may provide a way to study the long-term effects of increased NO production in the brain on the regulation of blood pressure and sympathetic nerve activity in conscious animals.

The aim of this study was therefore to determine the effects of increased NO production for much longer periods caused by eNOS overexpression in the NTS on blood pressure, heart rate, and sympathetic nerve activity in conscious rats. For this purpose, we transfected adenovirus vectors encoding either eNOS (AdeNOS) or β-galactosidase (Adβgal) into the NTS of rats in vivo. Blood pressure and heart rate were continuously monitored with a radiotelemetry system in a conscious state.

Methods

General Procedures and In Vivo Gene Transfer Into NTS

This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University.
Text. Dark blue staining is found locally in NTS of eNOS immunostaining (E and F) were performed as described in NTS. At day 7 after gene transfer, X-Gal staining (C and D) and specific expression of β-galactosidase or eNOS protein within NTS. At day 7 after gene transfer, X-Gal staining (C and D) and eNOS immunostaining (E and F) were performed as described in text. Dark blue staining is found locally in NTS of β-galactosidase injected rats (C and D). Confocal laser scanning microscope images of section of medulla, double-stained with anti-eNOS antibody (green, visualized with FITC-conjugated fluoroprobe) and PI (red). Green staining is found locally in NTS of Ad-eNOS injected rats (E and F). White arrow indicates NTS; XII, hypoglossal nucleus.

and was conducted according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University. Male Wister-Kyoto rats, weighing 280 to 340 g, 16 to 20 weeks old, were used. The rat was anesthetized with sodium pentobarbital (50 mg/kg IP) and placed in a stereotaxic frame; then, the dorsal surface of the medulla was exposed. A glass micropipette (5 μm OD) was filled with artificial cerebrospinal fluid (CSF), PBS with 3% sucrose (vehicle), or PBS containing Adβgal or Ad-eNOS. A microinjection was made at 6 sites of the bilateral NTS, as shown in Figure 1A. The sites of microinjections were defined according to an atlas of the rat.13 An adenoviral suspension containing 1×10⁹ plaque-forming units per milliliter, CSF, or vehicle was injected into each injection site for 5 minutes (800 nL for each site, infusion rate =0.2 μL/min). After the injection, all rats recovered from anesthesia and were unrestrained and free to move in their cages.

**Construction and Purification of Recombinant Adenovirus**

We used adenoviral vectors encoding either bacterial β-galactosidase gene or bovine eNOS gene. These adenoviral vectors were constructed in the Gene Transfer Vector Core Laboratory at the University of Iowa.13,14 Each gene expression was driven by the cytomegalovirus early enhancer/promoter with a simian virus 40 polyadenylation sequence cloned downstream from this reporter. These vectors were suspended in PBS with 3% sucrose and stored at −80°C until they were used.

**Histochemical Analysis of Gene Expression for β-Galactosidase**

At day 7 after the gene transfer, the rat was 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 the coronal sections of the medulla were cut serially with a vibratome. Sections of the medulla (50 μm) were evaluated for β-galactosidase expression by X-Gal staining at 37°C for 4 hours.

**Immunohistochemistry for eNOS**

We performed immunohistochemistry as described previously.14 At day 3, 7, 14, 21, or 30 after gene transfer, serial sections of the medulla were obtained as described above. The sections were rinsed for 30 minutes in PBS. After incubation overnight in 1% BSA in PBS, the sections were incubated in mouse IgG monoclonal antibody to human eNOS (1:200) (Transduction Laboratories) at room temperature overnight and then rinsed 3 times in PBS. After incubation in biotinylated horse anti-mouse IgG (1:1000, Vector Laboratories) for 4 hours, the sections were rinsed 3 times in PBS and incubated for 2 hours in a mixture of streptavidin-conjugated fluorescein isothiocyanate (1:100, Vector Laboratories) and propidium iodide (PI, 10 μg/mL). After rinsing 3 times in PBS, the sections were mounted in Vectashield (Vector Laboratories). BSA (1%), Triton X-100 (0.3%), and sodium azide (0.05%) were added in each dilution buffer of the primary and secondary antibodies. Sections double-stained with eNOS antibody and PI were photographed with a confocal laser scanning microscope (Bio-Rad MRC 1000), with laser beams of 488 and 568 nm for excitation. Confocal images were then transferred to a computer with the image analysis software package NIH Image.14 As a marker of inflammation, the sections were stained immunohistochemically by antibody against ED1, as described previously.15 The number of cells positive for ED1 was counted and averaged per section.

**Western Blot Analysis for eNOS**

At day 7 after gene transfer, the coronal block of the brain containing the injected sites of the NTS were obtained. The NTS tissues were homogenized and then sonicated in a lysing buffer containing 40 mmol/L HEPES, 1% Triton X-100, 10% glycerol, and 1 mmol/L phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged at 6000 rpm for 5 minutes at 4°C with a microcentrifuge. The lysate was collected, and protein concentration was determined with a BCA protein assay kit (Pierce). An aliquot of 20 μg of protein from each sample was separated on 12% SDS-polyacrylamide gel. Proteins were subsequently transferred onto polyvinylidene difluoride membranes (Immobilon-P membrane; Millipore). Membranes were incubated for 2 hours with mouse IgG monoclonal antibody to eNOS (1:2500). Membranes were then washed and incubated with a horseradish peroxidase–conjugated horse anti-mouse IgG antibody (1:10000) for 40 minutes. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (plus Western blotting detection kit; Amersham).

**Microdialysis and Measurement of NO Metabolites**

We measured the production of NO in the NTS as nitrite/nitrate (NOx) by in vivo microdialysis, as previously described with some modifications,16 before and at day 7 after gene transfer. The rat was anesthetized with pentobarbital (50 mg/kg IP followed by 10 to 20 mg/kg per hour IV), mechanically ventilated with room air suplemented with oxygen, and placed in a stereotaxic frame; the dorsal surface of the medulla was then exposed. A microdialysis probe (A-I-12-01, 1-mm length, Eicom) was inserted into the NTS (0.6 mm rostral and 0.6 mm lateral to the calamus scriptorius and 1 mm below the dorsal surface of the medulla) and was perfused with Ringer’s solution at a constant flow rate of 2 μL/min. The perfused dialysates were collected every 10 minutes in a sample loop of an automated...
sample injector connected to an automated NO detector high-performance liquid chromatography system (ENO-10, Eicom), which was based on a Griess reaction. The basal NOX levels were measured by averaging 3 consecutive stable dialysate samples, which were obtained ∼1 hour or more after starting brain perfusion with Ringer’s solution. To augment NO production, we used 2 agents that were administered locally by microdialysis probes. The first agent was 1 mmol/L of N-methyl-d-aspartate (NMDA) and the second agent was 100 μmol/L of L-arginine. Each drug was dissolved in Ringer’s solution, and pH was adjusted to 7.4. NMDA or L-arginine was infused for 10 minutes into the NTS through the dialysis probe. After the infusion of NMDA or L-arginine, the microdialysis probe was again perfused with Ringer’s solution.

Radiotelemetry Monitoring of Blood Pressure and Heart Rate
The UA-10 telemetry system (Data Sciences International) was used to measure systolic blood pressure and heart rate. Briefly, the monitoring system consisted of a transmitter (radiofrequency transducer model TAI1PA-C40 or TL11 mol/L2-C50-PXT), receiver panel, consolidation matrix, and the computer with a MacLab System (AD Instruments). Before the device was implanted, calibrations were verified to be accurate within ±3 mmHg. Rats were anesthetized with sodium pentobarbital, and a flexible catheter of the transmitter was surgically secured in the abdominal aorta just below the renal arteries while pointing upstream (against the flow). Each rat was housed in an individual cage after the operation, which was placed over the receiver panel that was connected to the computer for data acquisition. The rats were unrestrained and free to move in their cages. Blood pressure and heart rate were recorded continuously for 10 minutes every day between 10 and 11 AM, with a multichannel amplifier and signal converter. Previous studies have shown that blood pressure and heart rate become stabilized 7 days after surgery. Therefore, microinjection of CSF, vehicle, or adenovirus vectors into the NTS was performed 7 days after surgery, and telemetry data were collected for a maximum of 30 days.

To confirm that changes of blood pressure and heart rate caused by AdeNOS transfection was due to the effect of increased NO production, we examined the effect of intracisternal injection of L-NMMA (1 μmol) on blood pressure and heart rate at day 7 after the transfection of Adβgal or AdeNOS into the NTS in rats anesthetized with pentobarbital (50 mg/kg IP followed by 10 to 20 mg/kg per hour IV). This approach and dose of L-NMMA were chosen on the basis of our previous study showing that intracisternal injection of a NOS inhibitor effectively demonstrates the role of endogenous NO in the rapid central adaptation of baroreflex control of sympathetic nerve activity.

Measurement of Urinary Norepinephrine Excretion
Urine was collected for 24 hours by means of a metabolic cage. We measured urinary norepinephrine concentration before and 7 days after the gene transfer by high-performance liquid chromatography and calculated urinary norepinephrine excretion.

Statistical Analysis
All values are expressed as mean±SEM. Two-way ANOVA was used to compare systolic blood pressure, heart rate, and NOX levels between the Adβgal-treated group and the other groups. Comparisons between any two mean values were performed with application of Bonferroni’s procedure. A paired t test was used to compare 24-hour urinary norepinephrine excretion before and at day 7 after the gene transfer. Differences were considered to be significant at the level of P<0.05.

Results
Histochemical Analysis of β-Galactosidase
Figure 1 (C and D) shows the staining for β-galactosidase in the section of the rat brain medulla at day 7 after gene transfer. Positive staining for β-galactosidase was noted in the NTS, where Adβgal had been microinjected. No X-Gal staining–positive cells were found in the other regions of the brain or the other organs.

Local Expression of eNOS Protein and NOX Production in NTS
Western blot analysis showed that the expression of eNOS was significantly increased in the NTS tissue transfected with AdeNOS at day 7 after gene transfer (Figure 1B). The distribution of eNOS expression after AdeNOS transfer into the NTS was examined by immunohistochemistry at day 3, 7, 14, 21, or 30 after the gene transfer. In the AdeNOS-treated rats, the expression of eNOS protein was observed locally in the NTS where AdeNOS had been microinjected (Figure 1, E and F). In the rats treated with AdeNOS, no eNOS-positive neurons were found in the VLM or the hypothalamus. The expression of eNOS protein peaked at day 7 and thereafter declined over time not only by immunohistochemistry but also by Western blot analysis (at day 0, 3, 5, 7, 14, 21, or 30; n=4 each, data not shown).

The number of ED1-positive cells per section did not differ between the rats treated with Adβgal and those with AdeNOS (24.8±5.9 versus 21.7±5.0, respectively, n=4 for each).

We also measured the production of NO in the NTS as NOX by in vivo microdialysis before and after gene transfer. The basal level of NOX was significantly higher in the rats treated with AdeNOS at day 7 (20.5±2.0 pmol/20 μL, n=10) than that in the untreated (10.8±1.0 pmol/20 μL, n=12), vehicle-treated (12.4±0.8 pmol/20 μL, n=3), or Adβgal-
Blood Pressure and Heart Rate Changes

Figure 3 shows the changes in systolic blood pressure and heart rate before and after microinjection of Adβgal or AdeNOS into the NTS, recorded with the radiotelemetry system in conscious rats. Systolic blood pressure and heart rate were decreased in the AdeNOS-treated animals at day 5 to day 10 after gene transfer. In contrast, these variables did not change in the Adβgal-treated or vehicle-treated animals (n=2; data not shown). We examined the effect of intracisternal injection of L-NMMA on blood pressure and heart rate at day 7 after the Adβgal or AdeNOS transfection. The magnitudes of the increases in systolic blood pressure and heart rate evoked by L-NMMA were significantly greater in the AdeNOS-treated than in the Adβgal-treated rats (16±4 versus 4±1 mm Hg, P<0.05; 19±4 versus 1±1 bpm, P<0.01; n=6, respectively).

Urinary Norepinephrine Excretion for 24 Hours (μg) Before and After Gene Transfer

<table>
<thead>
<tr>
<th>Group</th>
<th>Before</th>
<th>After</th>
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<tr>
<td>CSF-treated rats (n=6)</td>
<td>0.95±0.04</td>
<td>1.14±0.08</td>
</tr>
<tr>
<td>Adβgal-treated rats (n=12)</td>
<td>0.88±0.10</td>
<td>0.84±0.14</td>
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<tr>
<td>AdeNOS-treated rats (n=12)</td>
<td>1.09±0.14</td>
<td>0.69±0.05*</td>
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*P<0.05 compared with values before gene transfer.

Discussion

This study has demonstrated for the first time that long-term increased NO production in the NTS evoked by overexpression of eNOS in the NTS decreases blood pressure, heart rate, and urinary norepinephrine excretion in conscious rats, suggesting an important modulatory role of NO in the regulation of sympathetic nerve activity under conscious conditions.

We transfected adenovirus vectors encoding β-galactosidase gene or eNOS gene into the NTS in rats. The successful gene transfer into the NTS was confirmed by several methods. First, β-galactosidase protein expression was confirmed by the histochemical staining of β-galactosidase in the NTS. Second, eNOS protein expression in the NTS was confirmed by immunohistochemistry and Western blot analysis. The time course of the transfected eNOS protein expression observed in this study was compatible with that in the previous study with adenovirus vectors. Third, NOx production in the NTS assessed by in vivo microdialysis was increased at day 7 after eNOS gene transfer. Furthermore, NOx production in the NTS was augmented by NMDA or L-arginine after eNOS gene transfer into the NTS. Finally, changes in blood pressure and heart rate evoked by L-NMMA were greater in the AdeNOS-treated than in the Adβgal-treated rats, indicating that the responses that occurred after eNOS gene transfer were indeed mediated by NO.

We used eNOS, instead of nNOS, which normally exists in the nervous system. However, the purpose of this study was to increase the NO production locally in the NTS for a much longer period in the rats treated with AdeNOS in a conscious state. In the rats treated with AdeNOS, eNOS was expressed in neurons, glia, and other tissues in the NTS where we transfected genes in this study (Figure 1, E and F), as reported previously. It may be possible that AdeNOS might have been transported from the NTS to the other regions where the effects of NO might also be exerted. In the rats treated with AdeNOS, however, no eNOS-positive neurons were found immunohistochemically in the VLM or the hypothalamus, both of which are known to have direct projections from the NTS. Furthermore, no X-Gal staining–positive cells were found in the other region of the brain or the other organs. Thus, it is unlikely that adenovirus vector was transfected in regions other than the NTS. Moreover, the effects of the eNOS gene
transfer were site-specific, because we did not find any increase in the NOx level at the site 3 mm apart from the NTS. We therefore consider that we were able to transfer genes locally into the NTS in this study.

It is possible that inflammation and cytotoxicity caused by adenovirus infection might have affected the present findings. However, the extent of ED1-positive cell infiltration, a marker of inflammation, did not differ significantly between animals treated with Adβgal and those treated with AdeNOS. Moreover, the β-galactosidase gene transfer did not alter blood pressure, heart rate, urinary norepinephrine excretion, or NOx production. Finally, magnitudes of the increases in systolic blood pressure and heart rate evoked by L-NMMA were greater in the AdeNOS-treated than in the Adβgal-treated 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.

The major novel finding of this study is that eNOS gene transfer into the NTS decreased blood pressure and heart rate in conscious rats. Blood pressure and heart rate were significantly lowered at day 5 to day 10 and returned to the control levels by day 14 after gene transfer. The time course of blood pressure and heart rate was consistent with that of transgene expression with AdeNOS. The 24-hour urinary norepinephrine excretion was decreased at day 7 after eNOS gene transfer into the NTS, which strongly suggests that eNOS gene transfer into the NTS decreased sympathetic nerve activity in conscious rats. The β-galactosidase gene transfer into the NTS had no effects on blood pressure, heart rate, NOx production, or urinary norepinephrine excretion. These results indicate that the depressor and bradycardic effects of eNOS gene transfer as well as the decrease in urinary norepinephrine excretion were mediated by the action of NO in the NTS.

Previous studies performed under anesthesia have yielded conflicting results with regard to the modulatory role of NO in the NTS. It has been shown that acute injection of NO donor or its precursor L-arginine into the NTS decreases blood pressure and heart rate in anesthetized rats. On the other hand, acute injection of NOS inhibitor (eg, L-NAME or L-NMMA) into the NTS increased or decreased blood pressure and renal sympathetic nerve activity. Anesthesia is known to alter otherwise normal regulatory functions of the brain. In this study, we therefore evaluated the effect of increased NO production for a much longer time on blood pressure and heart rate in a conscious state by using a radiotelemetry monitoring system. Our results indicate that increased NO production in the NTS decreases blood pressure, heart rate, and urinary norepinephrine excretion. Changes in heart rate might have been due to NO production in the dorsal motor nucleus of vagus (DMV), which is located close to the NTS.

The mechanism(s) by which NO in the NTS decreases blood pressure, heart rate, and sympathetic nerve activity remains to be clarified. NO has been suggested to increase the release of excitatory amino acids in the dorsomedial medulla by cGMP-dependent processes. There is evidence that in hippocampal slices, NO may mediate the release of the excitatory amino acid through the activation of release-regulating NMDA receptors on presynaptic terminals. We have recently shown that NMDA receptor stimulation in the NTS induces NO release, which in turn facilitates glutamate release from presynaptic terminals and thus augments the depressor and bradycardic action of NMDA receptor activation. It is known that activation of primary baroreceptor afferents and primary chemoreceptor afferents terminating in the NTS results in depressor and pressor responses, respectively. There is evidence that in both cases, L-glutamate is the primary neurotransmitter. We do not know why eNOS transfection in the NTS caused a response similar to that evoked by baroreceptor afferent stimulation rather than chemoreceptor afferent stimulation from our study. However, it has been shown that microinjection of L-glutamate into the NTS produces depressor responses in the rat. Thus, we speculate that the relative contribution of L-glutamate in the NTS is greater as baroreceptor stimulation rather than as chemoreceptor stimulation. The other possibility is that eNOS was expressed much more in the baroreceptor afferent terminated area within the NTS because we transfected eNOS at 6 sites of the bilateral NTS. It has been demonstrated that the caudal part of the NTS is important for chemoreceptor stimulation. Further experiments will be needed to examine the relative contribution of NO in the NTS between baroreceptor stimulation and chemoreceptor stimulation.

In summary, we developed a technique of eNOS gene transfer into the NTS of rats in vivo. Using this technique, we were able to demonstrate that long-term overexpression of eNOS in the NTS decreases blood pressure, heart rate, and urinary norepinephrine excretion in conscious rats. Our results thus indicate that NO in the NTS exerts an inhibitory effect on sympathetic nerve activity in vivo.

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References


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