

Overexpression of Inducible Nitric Oxide Synthase in Rostral Ventrolateral Medulla Causes Hypertension and Sympathoexcitation via an Increase in Oxidative Stress

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Abstract—The present study examined the role of inducible nitric oxide synthase (iNOS) in the rostral ventrolateral medulla (RVLM) of the brain stem, where the vasomotor center is located, in the control of blood pressure and sympathetic nerve activity. Adenovirus vectors encoding iNOS (AdiNOS) or β -galactosidase (Ad β gal) were transfected into the RVLM in Wistar-Kyoto (WKY) rats. Blood pressure and heart rate were monitored using a radiotelemetry system. iNOS expression in the RVLM was confirmed by immunohistochemical staining or Western blot analysis. Mean arterial pressure significantly increased from day 6 to day 11 after AdiNOS transfection, but did not change after Ad β gal transfection. Urinary norepinephrine excretion was significantly higher in AdiNOS-transfected rats than in Ad β gal-transfected rats. Microinjection of aminoguanidine or *S*-methylisothiourea, iNOS inhibitors, or tempol, an antioxidant, significantly attenuated the pressor response evoked by iNOS gene transfer. The levels of thiobarbituric acid-reactive substances, a marker of oxidative stress, were significantly greater in AdiNOS-transfected rats than in Ad β gal-transfected rats. Dihydroethidium fluorescence in the RVLM was increased in AdiNOS-transfected rats. In addition, nitrotyrosine-positive cells were observed in the RVLM only in AdiNOS-transfected rats. Intracisternal infusion of tempol significantly attenuated the pressor response and the increase in the levels of thiobarbituric acid-reactive substances induced by AdiNOS transfection. These results suggest that overexpression of iNOS in the RVLM increases blood pressure via activation of the sympathetic nervous system, which is mediated by an increase in oxidative stress. (*Circ Res.* 2005;96:252-260.)

Key Words: nitric oxide synthase ■ blood pressure ■ sympathetic nervous system ■ oxidative stress ■ gene transfer

Nitric oxide (NO) in the central nervous system (CNS), including the brain stem and hypothalamus, plays an important role in the regulation of blood pressure via the sympathetic nervous system.¹⁻⁷ In general, NO in the CNS inhibits sympathetic nerve activity, thereby reducing blood pressure.²⁻⁴ The rostral ventrolateral medulla (RVLM) in the brain stem contains sympathetic premotor neurons responsible for maintaining the tonic excitation of sympathetic preganglionic neurons involved in cardiovascular regulation.⁸⁻¹⁰ The functional integrity of the RVLM is essential for the maintenance of basal vasomotor tone, and RVLM abnormalities might be related to the pathophysiology of hypertension¹¹⁻¹⁴ and heart failure.^{15,16}

Recently, we developed a technique for adenovirus-mediated endothelial NO synthase (eNOS) gene transfer into the RVLM^{11,14,17-19} or the nucleus tractus solitarius (NTS)^{20,21} in vivo. An increase in NO production in the RVLM induced by eNOS overexpression decreases blood pressure and heart rate (HR) by inhibiting the sympathetic nervous system.^{11,14,19} In that series of studies, we used eNOS instead of neuronal

NO synthase (nNOS), which is normally abundant in the CNS, because the purpose of the study was to examine the effect of an increase in NO production in the RVLM on cardiovascular function. There are three types of NOS: eNOS, nNOS, and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed, but iNOS is expressed only during pathophysiological states such as hypertension, heart failure, and endotoxin shock, and in aging.²²⁻²⁸

The aim of the present study was to examine the effect of iNOS overexpression in the RVLM on blood pressure in vivo and to determine whether an increase in oxidative stress in the RVLM is involved in blood pressure changes. For this purpose, we transfected adenovirus encoding the iNOS gene (AdiNOS) into the RVLM and monitored mean arterial pressure (MAP) and HR using a radiotelemetry system in awake rats. NO activity is determined by the balance of NO and reactive oxygen species production.²³ Therefore, thiobarbituric acid-reactive substances (TBARS) in the RVLM were measured as an indirect marker of oxidative stress,^{29,30} and tempol, a superoxide dismutase mimetic,^{29,30} was microin-

Original received July 26, 2004; revision received November 9, 2004; accepted November 29, 2004.

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DOI: 10.1161/01.RES.0000152965.75127.9d

jected bilaterally into the RVLM after transfection of Ad*iNOS*.

Materials and Methods

General Procedures and In Vivo Gene Transfer Into the RVLM

The present study was approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, and conducted according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University. Male Wistar-Kyoto (WKY) rats (280 to 300 g, 16 to 20 weeks old) were used. Rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine (Fukuoka, Japan). Details of the general procedures of transfection of adenovirus vectors are available in the online data supplement at <http://circres.ahajournals.org>.

Construction of Adenovirus Vectors

We used adenoviral vectors encoding the bacterial β -galactosidase gene, mouse *iNOS* gene,^{31,32} or bovine endothelial NOS (eNOS) gene (see online data supplement for details).^{19,33}

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

At day 7 after gene transfer, β -galactosidase expression was confirmed by staining with X-Gal in phosphate buffered saline as described previously.²¹ We performed double-immunohistochemical staining for *iNOS* and phenylethanolamine-*N*-methyltransferase (PNMT)¹⁹ or nitrotyrosine. Details of the methods of immunohistochemistry are available in the online data supplement.

Western Blot Analysis for *iNOS*

To confirm the local overexpression of *iNOS* in the RVLM, Western blot analysis for *iNOS* protein from tissue containing the injection sites of the RVLM obtained using the micropunch technique¹⁹ was performed at day 0, 3, 5, 7, 9, 11, or 14 after the gene transfer. The procedure for Western blot analysis of RVLM tissues was described previously (see online data supplement for details).^{11,19}

Microinjection Into the RVLM

To confirm that changes in MAP and HR induced by Ad*iNOS* transfection were the result of an increase in *iNOS* protein, we microinjected aminoguanidine (2.5 mmol/L, 50 nL per site, 250 pmol) or *S*-methylisothiourea (SMT; 2.5 mmol/L, 50 nL per site, 250 pmol) bilaterally into the RVLM at day 7 after transfection with Ad β gal or Ad*iNOS*. All injections were performed in rats anesthetized with sodium pentobarbital (50 mg/kg, IP followed by 20 mg/kg per hour, IV). A nonselective NOS inhibitor, *N*^G-monomethyl-L-arginine (L-NMMA), was also microinjected bilaterally into the RVLM. We microinjected L-arginine, a precursor of NO, (70 mmol/L, 50 nL per site, 7 nmol) bilaterally into the RVLM at day 7 after transfection with Ad β gal or Ad*iNOS*. To examine whether the generation of superoxide anions is involved in blood pressure alteration induced by Ad*iNOS* transfection, microinjection of tempol, a superoxide dismutase mimetic, was performed bilaterally into the RVLM (see online data supplement for details).

Microdialysis and Measurement of NO Metabolites

We measured NO production in the RVLM as nitrite/nitrate (NO_x) with in vivo microdialysis before and at day 7 after gene transfer, as described previously (see online data supplement for details).^{21,34,35}

Measurement of MAP, HR, and Urinary Norepinephrine Excretion

A UA-10 telemetry system (Data Sciences International) was used to measure MAP and HR. We measured urinary norepinephrine excretion for 24 hours before the gene transfer and at day 7 after the gene transfer (see online data supplement for details).

Evaluation of Oxidative Stress in the RVLM

The RVLM tissues were homogenized in 1.15% KCl (pH 7.4), and 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH, and 0.3% thiobarbituric acid were added to the homogenate. The amount of TBARS was determined by absorbance with a molecular extinction coefficient of 156 000 and expressed as μ mol/g wet weight, as described previously (see online data supplement for details).³⁰ Brain superoxide anion levels were estimated in two groups of rats (Ad*iNOS*-transfected rat, *n*=5; nontreated, *n*=5) using dihydroethidium (DHE) staining following procedures used in previous studies (see online data supplement for details).^{36,37}

Continuous Intracisternal Infusion Experiments With Tempol

The rats were randomly divided into four groups. Two of the groups were transfected with Ad*iNOS* and two of the groups with Ad β gal. Either vehicle (artificial cerebrospinal fluid, aCSF) or tempol (12 μ mol/d) were continuously infused intracisternally (0.25 μ L/h) for 1 week with an osmotic minipump (Alzet model 1002; DURECT Corporation), as described previously (see online data supplement for details).^{38,39} Half of the animals in each transfection group were infused with vehicle and the other half were infused with tempol, producing four groups of animals: Ad*iNOS*-VEH, Ad*iNOS*-tempol, Ad β gal-VEH, and Ad β gal-tempol.

Statistical Analysis

All values are expressed as mean \pm SEM. Two-way ANOVA was used to compare MAP, HR, and NO_x levels between the Ad*iNOS*-treated group and the other groups. Comparisons between any two mean values were performed using Bonferroni's correction for multiple comparisons. A paired *t* test was used to compare 24-hour urinary norepinephrine excretion before and at day 7 after the gene transfer. A level of *P* < 0.05 was considered to be significant.

Results

Analysis of β -Galactosidase, *iNOS*, or Nitrotyrosine Expression

Figure 1B shows the β -galactosidase staining in a section of the rat brain medulla at day 7 after the gene transfer. A schematic representing injection site is shown in Figure 1A. β -galactosidase staining was noted in the RVLM, where Ad β gal had been microinjected. There were no X-Gal-positive cells in the adjacent brain regions. In the Ad*iNOS*-transfected rats, the expression of *iNOS* protein was observed locally in the RVLM, where the Ad*iNOS* had been transfected. Figures 1D, 1E, and 1F show the expression of *iNOS* in the RVLM at day 7 after the gene transfer by immunohistochemistry. Some of the C1 neurons labeled with the PNMT antibody were also detected with the anti-*iNOS* antibody (Figure 1C). The expression level of *iNOS* peaked at day 7 after the gene transfer and thereafter declined over time as detected by Western blot analysis (Figure 2).

Microdialysis and Measurement of NO Metabolites

We measured the production of NO in the RVLM as NO_x using in vivo microdialysis before and after gene transfer. The level of NO_x was significantly higher in rats transfected with Ad*iNOS* or Ad*eNOS* at day 7 (Ad*iNOS*, 58.8 \pm 1.2 or Ad*eNOS*, 29.4 \pm 1.0 pmol/20 μ L, *n*=6 for each) than in Ad β gal-treated rats (8.2 \pm 0.4 pmol/20 μ L, *n*=6; Figure 3). NO_x levels in Ad*iNOS*-transfected rats were also significantly higher than in Ad*eNOS*-transfected rats (*P* < 0.05).

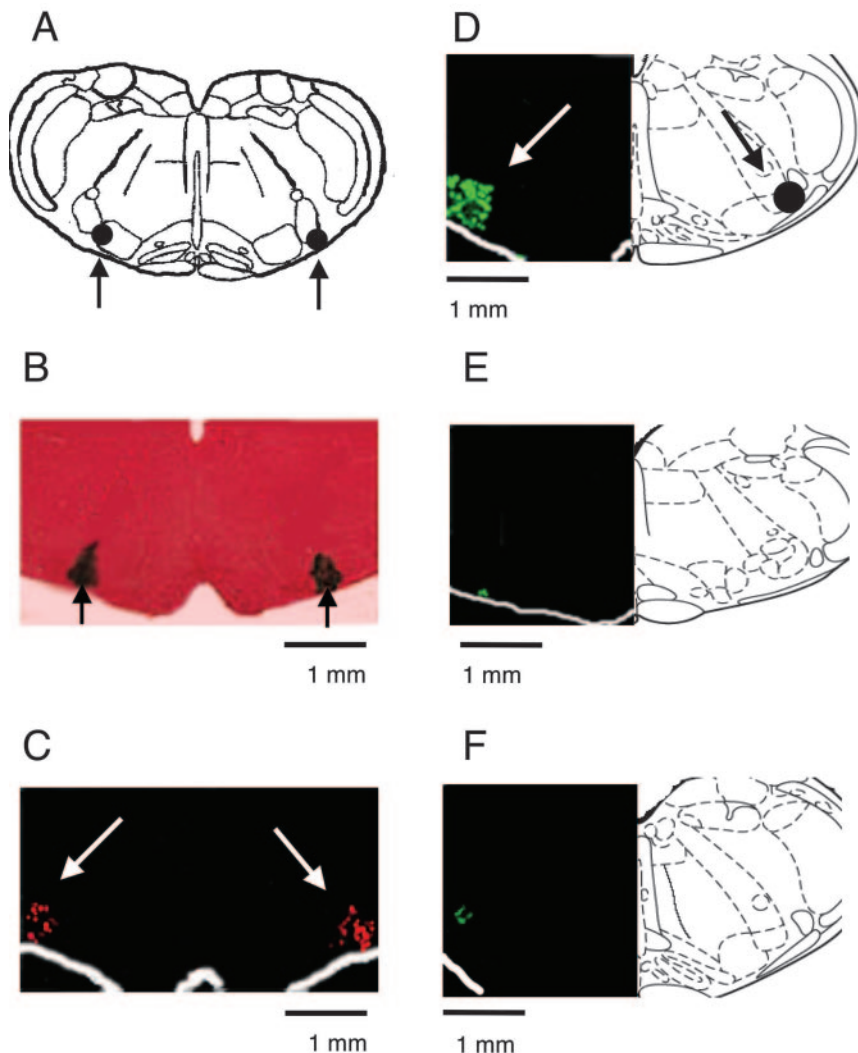


Figure 1. A, Schematic drawing of a section that includes the RVLM. Arrows indicate the RVLM. B, Site-specific expression of β -galactosidase by X-Gal staining at day 7 after gene transfer. C, Site-specific expression of PNMT by immunohistochemistry. D, Site-specific expression of iNOS protein by immunohistochemistry at day 7 after gene transfer. E, 0.5-mm rostral from the section as shown in D; F, 0.5-mm caudal from the section as shown in D. Arrows indicate the RVLM. Immunohistochemical staining for iNOS (green, visualized with fluorescein isothiocyanate-conjugated fluoroprobe) (D, E, and F) and PNMT (red, visualized with rhodamine-conjugated fluoroprobe) (C).

MAP, HR, and Urinary Norepinephrine Excretion

Figure 4A and 4B show the changes in MAP and HR before and after the gene transfer into the RVLM. MAP was significantly increased in the AdiNOS-transfected rats between days 6 and 11 after the gene transfer ($+56 \pm 14$ mm Hg at day 7 after the gene transfer; $P < 0.05$, $n = 6$). In contrast, MAP did not change in the Ad β gal-transfected rats. Injection

of AdiNOS 1 mm caudal to the RVLM also did not alter MAP. HR was not altered in either group (Figure 4B). Urinary norepinephrine excretion measured at day 7 after the gene transfer was significantly increased in the AdiNOS-transfected rats relative to that measured before gene transfer (Figure 4C). Urinary norepinephrine did not change in the Ad β gal-transfected rats (Figure 4C).

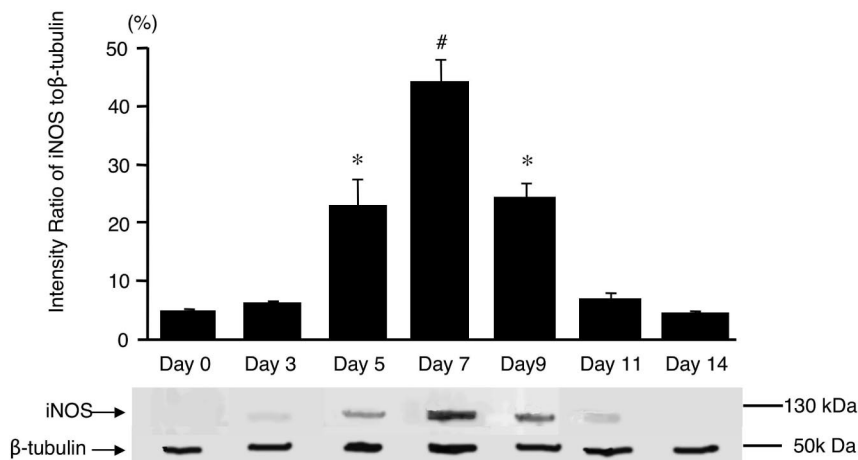


Figure 2. Representative Western blot analysis demonstrating the expression of iNOS protein, in the medulla containing the RVLM. Densitometric average was normalized to the values obtained from the analysis of β -tubulin ($n = 5$ for each). * $P < 0.05$ vs day 0. # $P < 0.01$ vs day 0.

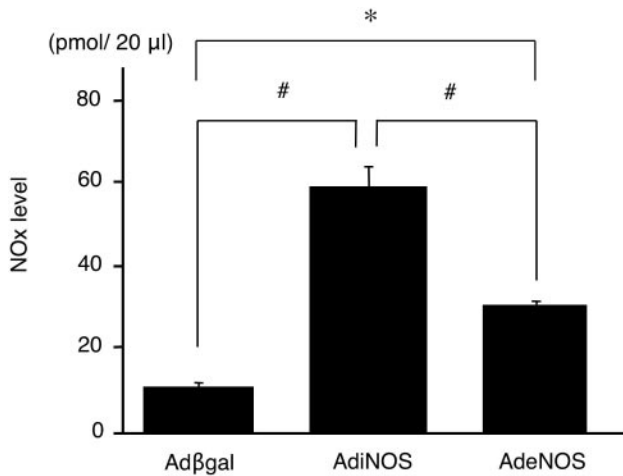


Figure 3. Effects of transfection of Adβgal (n=6), AdiNOS (n=6), and AdeNOS (n=6) into RVLM on NOx release in dialysate of the RVLM. Basal NOx levels in AdiNOS-transfected rats were significantly higher than those in Adβgal- or AdeNOS-transfected rats. Basal NOx levels in AdeNOS-transfected rats were also significantly higher than in Adβgal-transfected rats. All data are expressed as amount of NOx in 20 µL dialysate of RVLM. #*P*<0.01 compared with values of AdiNOS-transfected rats. **P*<0.05 compared with values of Adβgal-transfected rats.

Microinjection of NOS Inhibitors Into the RVLM

Microinjection of aminoguanidine into the RVLM at day 7 after the gene transfer produced a gradual decrease in MAP in the AdiNOS-transfected rats (Figure 5A). The maximum decrease in MAP evoked by aminoguanidine was -38 ± 12 mm Hg (*P*<0.05, n=5). In contrast, microinjection of aminoguanidine did not alter MAP in the Adβgal-transfected rats (6 ± 4 mm Hg, *P*<0.05, n=5). Microinjection of SMT also decreased MAP in the AdiNOS-transfected rats (-42 ± 12 mm Hg, n=5). Microinjection of L-NMMA also decreased MAP in AdiNOS-transfected rats (Figure 5B), but the change was smaller than that evoked by microinjection of aminoguanidine or SMT. In contrast, L-NMMA elicited a small but significant increase in MAP in Adβgal-transfected rats (*P*<0.05, n=3; Figure 5B).

Microinjection of L-Arginine Into the RVLM

Microinjection of L-arginine into the RVLM at day 7 after the gene transfer produced a gradual decrease in MAP in the AdiNOS-transfected rats. The maximum decrease in MAP evoked by L-arginine was -35 ± 6 mm Hg (n=5).

Oxidative Stress in the RVLM After Gene Transfer

TBARS levels were significantly higher in the RVLM of AdiNOS-transfected rats than in Adβgal-transfected rats (Figure 6A). In AdeNOS-transfected rats, TBARS levels did not differ from those of Adβgal-transfected rats (AdeNOS, 0.32 ± 0.03 versus Adβgal, 0.29 ± 0.05 µmol/g, n=5 for each). Figure 6B and 6C show representative images of DHE-treated brain slices from the RVLM. Increased fluorescence, representing higher superoxide anion levels, was present in the brain slices from AdiNOS-transfected rats (Figure 6B) compared with nontreated rats (Figure 6C). Some of the iNOS-positive cells were also detected with the

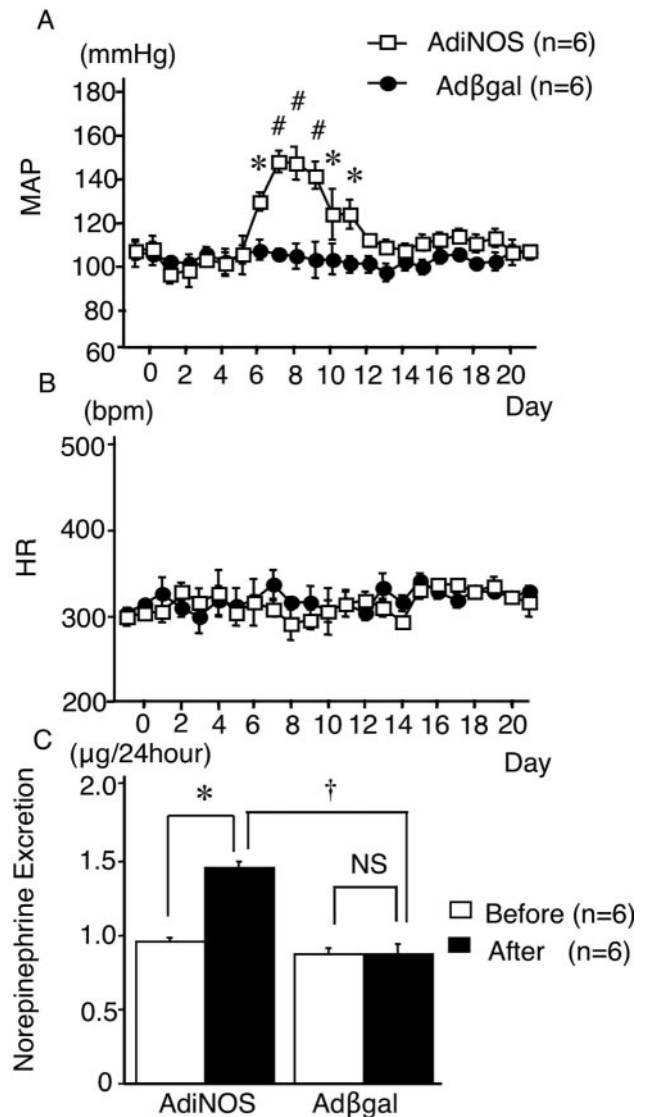


Figure 4. A and B, Time course of MAP (A, in mm Hg) and HR (B, in bpm) in Adβgal-transfected rats and AdiNOS-transfected rats before and after gene transfer. **P*<0.05 vs the values between the two groups. #*P*<0.01 vs the values between the two groups. C, Urinary norepinephrine excretion for 24 hours (µg) before and at day 7 after the gene transfer in AdiNOS-transfected rats and Adβgal-transfected rats. **P*<0.05, #*P*<0.01 vs the values before the gene transfer. †*P*<0.05 vs Adβgal-transfected rats. Data are shown as mean±SEM (n=6 per group).

anti-nitrotyrosine antibody (Figure 6D and 6E). Microinjection of tempol elicited a depressor response in the AdiNOS-transfected rats, but not in the Adβgal-transfected rats (Figure 7).

Effect of Continuous Intracisternal Infusion With Tempol

Figure 8A shows the changes in MAP after intracisternal infusion of tempol for 1 week. Tempol significantly attenuated the increase in MAP in AdiNOS-transfected rats (Figure 8A). Urinary norepinephrine excretion measured at day 7 after the gene transfer was significantly increased in the AdiNOS-transfected rats treated with tempol relative to the AdiNOS-transfected rats treated with aCSF (Figure 8B).

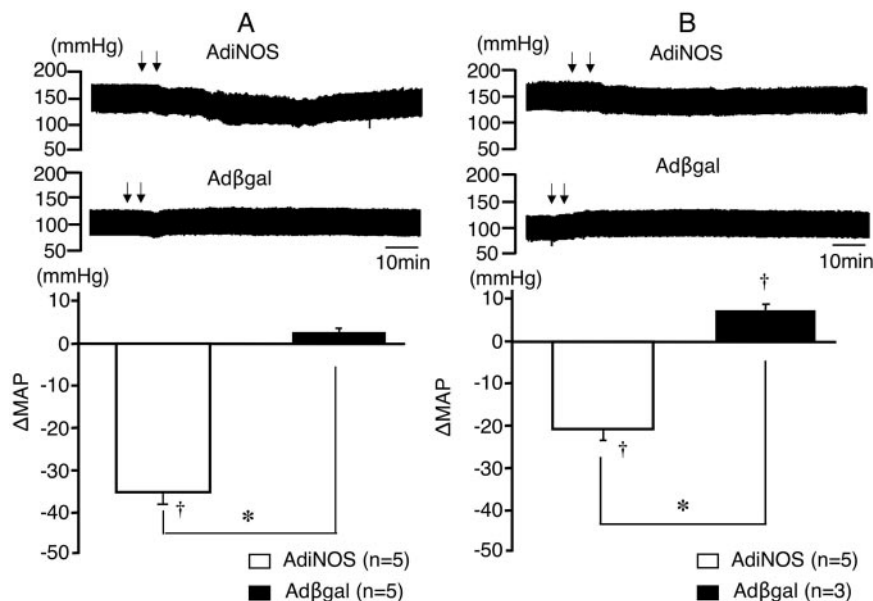


Figure 5. Changes in MAP caused by microinjection of either aminoguanidine (250 pmol) (A, $n=5$ for each) or L-NMMA (100 nmol) (B, $n=5$ for AdiNOS-transfected, $n=3$ for Adβgal-transfected) into the RVLM at day 7 after the gene transfer (Adβgal or AdiNOS) in anesthetized rats. Representative recordings showing blood pressure response to microinjection of aminoguanidine or L-NMMA into the bilateral RVLM at day 7 after the gene transfer. * $P<0.05$ vs the values between the two groups. † $P<0.05$ from the baseline values.

Urinary norepinephrine did not change in the Adβgal-transfected rats (Figure 8B). TBARS levels in AdiNOS-transfected rats treated with intracisternal infusion of tempol were significantly lower than in those treated with intracisternal infusion of aCSF (0.68 ± 0.03 versus 0.52 ± 0.03 $\mu\text{mol/g}$ wet tissue; $P<0.05$, $n=5$ for each).

Discussion

The present study demonstrated that overexpression of iNOS in the RVLM elicits a pressor response in awake normotensive WKY rats in vivo, and that an increase in oxidative stress in the RVLM is likely to be responsible for this response. Urinary norepinephrine excretion was higher in AdiNOS-transfected rats than in Adβgal-transfected rats, indicating that it was mediated by activation of the sympathetic nervous system. In addition, the pressor response was mediated by iNOS, because aminoguanidine or SMT inhibited the response. Taken together, these results suggest that overexpres-

sion of iNOS in the RVLM causes a pressor response via activation of the sympathetic nervous system, probably attributable to an increase in oxidative stress.

Expression of iNOS protein in the RVLM was confirmed by immunohistochemistry and Western blot analysis, as shown in Figures 1 and 2. The expression level of iNOS protein after AdiNOS transfection gradually increased, peaked at day 7, and then gradually declined over time. The time course of transfected gene expression was consistent with transfected eNOS expression reported in previous studies.^{11,19} To confirm the transfection site in the pressor areas in the RVLM, we identified the site functionally by prior injection of L-glutamate and anatomically by immunohistochemical staining for PNMT, which indicates the C1 area where the RVLM neurons are located.^{8,19} We did not detect iNOS-positive neurons in other areas of the brain, such as the NTS, caudal ventrolateral medulla, and hypothalamus. Because of the possibility of significant diffusion to the

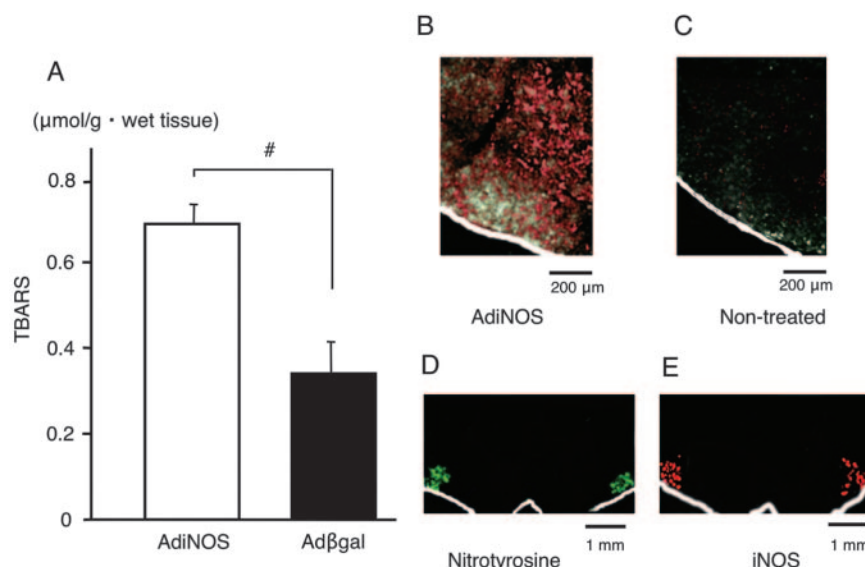


Figure 6. A, Lipid peroxidation as indicated by TBARS levels in RVLM tissues from AdiNOS-transfected rats ($n=10$) and Adβgal-transfected rats ($n=5$). # $P<0.01$. B and C, Images of dihydroethidium-treated brain slices from the RVLM from AdiNOS-transfected rats (B) and nontreated rat (C). D and E, Images of expression of nitrotyrosine (green, visualized with fluorescein isothiocyanate-conjugated fluorophore) (D) or iNOS protein (red, visualized with rhodamine-conjugated fluorophore) (E) in the RVLM.

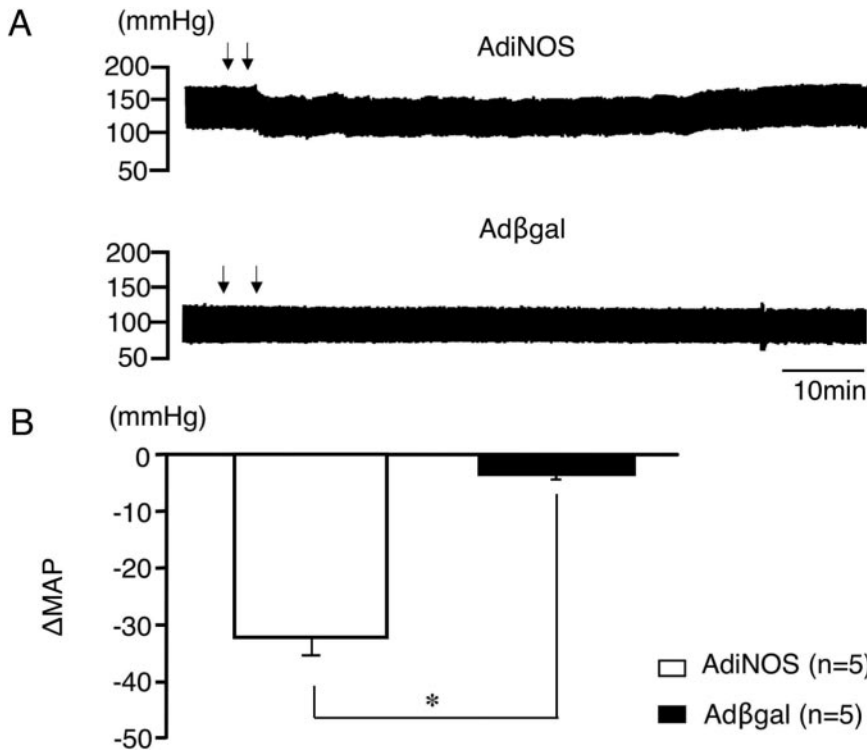


Figure 7. A, Representative recordings from AdiNOS-transfected rats and Adβgal-transfected rats showing the arterial blood pressure response to bilateral microinjections of tempol into the RVLM. Arrows indicate timing of the microinjection. B, Grouped data of MAP and HR responses evoked by microinjection of tempol into the RVLM (n=5 for each). * $P < 0.05$ vs Adβgal-transfected rats.

caudal ventrolateral medulla region, which is adjacent to the RVLM, we slowly injected the adenovirus 50 nL/min with the total volume injected over 15 minutes. Thus, as shown in Figure 1D through 1F, expression of the transfected adenovirus was observed within a 1-mm wide region in the rostrocaudal direction, and there was no significant staining observed in the caudal ventrolateral medulla. Furthermore, when we injected AdiNOS 1 mm caudal to the RVLM, there were no changes in blood pressure (data not shown). There might be some cells that express iNOS, because Western blot analysis revealed a small amount of iNOS protein in the brain of WKY rats.¹³ In fact, there is iNOS expression in the cerebral blood vessels (vascular smooth muscle cells) and glia (microglia and astrocytes),⁴⁰ although iNOS is normally induced by inflammatory stimuli.^{40,41}

In a previous study, we reported that overexpression of eNOS in the RVLM decreases blood pressure and HR by

inhibiting the sympathetic nervous system.¹⁹ The different cardiovascular responses induced by overexpression of iNOS and eNOS might be attributable to differences in the amount of NO production.^{42,43} Large amounts of NO production might consume L-arginine, a precursor of NO, thereby inducing chronic L-arginine depletion. In such conditions, iNOS produces superoxide anions instead of NO.^{44,45} In the present study, the NO production measured as NOx was approximately 4.5-fold higher in AdiNOS-transfected rats than in Adβgal-transfected rats. In contrast, NOx levels in the RVLM of Adβgal-transfected rats were approximately 2-fold higher than in Adβgal-transfected rats. This increase in basal NO production is consistent with the results of a previous study in which eNOS was transfected into the NTS²¹ and of another in vivo study.⁴⁶ It is difficult, however, to explain the different effects of eNOS and iNOS overexpression in the RVLM on blood pressure based on differences in

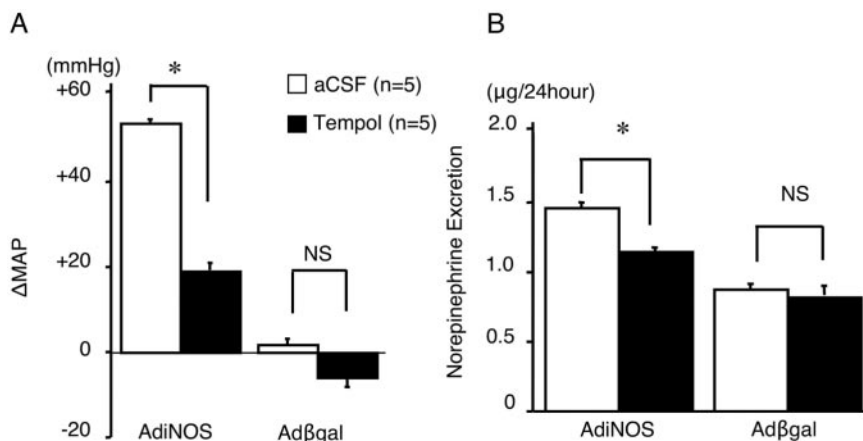


Figure 8. A, Changes in MAP caused by continuous intracisternal (i.c.) infusion with tempol (2 mol/L, 0.25 μL/h) or aCSF for 1 week (n=5 for each; Adβgal or AdiNOS). B, Urinary norepinephrine excretion for 24 hours (μg) before and at day 7 after the gene transfer in AdiNOS-transfected rats and Adβgal-transfected rats treated with tempol or aCSF (n=5 for each). * $P < 0.05$ vs AdiNOS-transfected rats with aCSF.

the amount of NO release. The levels of TBARS, an indirect marker of oxidative stress in the RVLM, were higher in the AdiNOS group than in the Ad β gal group. DHE staining, an oxidative fluorescent dye, detects in situ superoxide in the RVLM, and the intensity of the staining was greater in AdiNOS-transfected rats than in Ad β gal-transfected rats. In addition, microinjection of tempol decreased blood pressure in the AdiNOS group, but not in the nontreated group. Furthermore, intracisternal infusion of tempol markedly attenuated the pressor response induced by AdiNOS transfection. In addition, the increased TBARS levels after AdiNOS transfection were significantly attenuated. Taken together, these results suggest that oxidative stress in the RVLM is increased in AdiNOS-transfected rats, and this increase might contribute to the pressor response evoked by iNOS transfection. NO might be trapped by superoxide anions. In support of this idea, we recently reported that increased reactive oxygen species in the RVLM contribute to the neural mechanisms of hypertension.³⁰

An important finding of the present study was that blood pressure was increased after transfection of AdiNOS. The time course of the change in blood pressure was consistent with that of iNOS protein expression levels. This increase in blood pressure was nearly abolished by microinjection of aminoguanidine or SMT, a selective iNOS inhibitor, and partly inhibited by microinjection of L-NMMA, a nonselective NOS inhibitor. These results suggest that the pressor response that occurred after iNOS gene transfer was mediated by iNOS. If this is the case, then what caused the pressor response after iNOS production? We previously demonstrated that blood pressure decreased after transfection of AdeNOS into the RVLM.¹⁹ eNOS and nNOS are constitutive NOS. Microinjection of L-NMMA in rats transfected with Ad β gal elicited the pressor response, suggesting that NO produced by endogenous NOS in the RVLM, mainly nNOS, decreases blood pressure. In contrast, microinjection of aminoguanidine into the RVLM in Ad β gal-transfected rats did not alter blood pressure, suggesting that endogenous iNOS in the RVLM does not affect blood pressure, at least in normotensive rats. In support of this finding, we demonstrated that expression levels of iNOS protein in the brain of WKY are very low compared with the aorta and heart and with stroke-prone spontaneously hypertensive rats.¹³ HR did not change despite the fact that blood pressure was increased after iNOS gene transfection. This might be attributable to inhibition of the baroreflex control of HR. Blood pressure also returned to the control level after iNOS transfection into the RVLM, indicating that the cytotoxic effects of NO produced by iNOS in the present study are reversible. We transfected iNOS bilaterally into the RVLM. If RVLM neurons were irreversibly damaged, blood pressure would be expected to decrease to the level produced by spinal transection.⁸

The effects of NO in the RVLM on blood pressure regulation are controversial. NO in the RVLM is reported to reduce blood pressure by inhibiting sympathetic nerve activity;^{6,12,47,48} but opposite results have also been reported.^{49–51} In addition, it was reported that NO elicits a biphasic response that depends on the dose injected.⁴³ Most of these studies,

however, were performed in anesthetized animals, and only acute effects of NO donors or nonselective NO blockers were examined. It is possible that NO donors such as sodium nitroprusside produce reactive oxygen species. To exclude the above-mentioned limitations, we demonstrated that transfection of adenovirus encoding constitutive eNOS in the RVLM reduces blood pressure via inhibition of the sympathetic nervous system and this effect is probably attributable to an increase in γ -amino-butyric acid (GABA) in the RVLM in conscious rats.¹⁹ We used eNOS instead of nNOS, which is normally abundant in the CNS, because the purpose of that study was to increase NO production from constitutively expressed NOS. In support of these findings, a similar finding was obtained in the paraventricular nucleus of the hypothalamus⁵² and RVLM.¹⁶

Recently, the contribution of nNOS or iNOS in the RVLM to blood pressure regulation was examined in propofol-anesthetized rats.⁴² A selective inhibitor of nNOS, 7-nitroindazole, or selective antagonists of iNOS, aminoguanidine, *N*⁶-(L-iminoethyl)-L-lysine, or SMT, were used in that study. The nNOS inhibitor reduced blood pressure and iNOS antagonists increased blood pressure, suggesting that endogenous NO produced by nNOS increases blood pressure and that produced by iNOS decreases blood pressure.³⁴ In a subsequent study, they explained that the different blood pressure responses evoked by nNOS and iNOS were attributable to differences in the amount of release of an excitatory neurotransmitter, L-glutamate, and an inhibitory neurotransmitter, GABA.⁵³ We do not yet have a clear explanation for the differences between their results and ours. Therefore, in the present study, we performed iNOS gene transfer into the RVLM in awake rats to clarify the role of iNOS in the RVLM. Our results raise another possibility that NO produced by iNOS enhances the production of reactive oxygen species, which influences the neuronal activity of the RVLM neurons.^{30,53} Increased and sustained NO levels might lead to the formation of superoxide anions that react with NO to form peroxynitrite.⁵⁴ In support of this suggestion, nitrotyrosine staining in the RVLM was observed after transfection of AdiNOS as a peroxynitrite footprint. Indeed, lipopolysaccharide-induced NO generation results in an increase in oxidative stress in the rat liver and kidney and is inhibited by iNOS inhibitors.⁵⁵

In summary, the present studies demonstrate that overexpression of iNOS in the RVLM elicits hypertension by activating the sympathetic nervous system, and these effects might be mediated by an increase in oxidative stress in the RVLM. An increase in iNOS expression levels occurs in some pathophysiological states, such as hypertension, heart failure, and endotoxin shock, and in aging.^{22–28,41} Thus, it is conceivable that the increase in iNOS expression levels in the brain, particularly in the RVLM, occurs in those conditions, thereby modulating central sympathetic outflow resulting in blood pressure changes.

Acknowledgments

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture (C13670721, C15590757), and by grants for research on auto-

onomic nervous system and hypertension from Kimura Memorial Heart Foundation/Pfizer Japan, Inc. We thank Drs Donald D. Heistad and Beverly L. Davidson (The University of Iowa Gene Transfer Vector Core, supported by the National Institutes of Health Grants and the Carver Foundation) for vector preparation.

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Expanded Materials and Methods

General Procedures and In Vivo Gene Transfer into the RVLM

Rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine (Fukuoka, Japan).¹ The rats were anesthetized with sodium pentobarbital (50mg/kg, IP), placed in a stereotaxic frame, and the dorsal surface of the medulla was exposed. A glass micropipette was filled with artificial cerebrospinal fluid (aCSF) or phosphate buffered saline (PBS) containing Ad β gal, AdiNOS, or AdeNOS. A microinjection was made bilaterally into the RVLM through a polyvinyl tube connected to the microinjection pipette. The microinjection sites were defined, according to a rat brain atlas² with stereotaxic coordinates, with an anteroposterior angle of 18°, 1.8 mm lateral, 3.5 mm below the calamus scriptorius. Before microinjectioning the vectors, the pressor region of the RVLM was identified by monitoring the mean arterial pressure (MAP) after an injection of a small amount of L-glutamate.³ An adenoviral suspension containing 2×10^9 plaque forming units (pfu)/mL was injected into each injection site over the course of 15 min (500 nL per site). The pipette was removed, and the incision was closed. After the injection, all rats

recovered from the anesthesia and were unrestrained and free to move about their cages.

Adenovirus Vector Construction

We used adenoviral vectors encoding either the bacterial β -galactosidase gene, mouse iNOS gene, or bovine eNOS gene.^{4,5} The adenoviral vectors were constructed by Dr. Yi Chu, with assistance from the Gene Transfer Vector Core Laboratory at the University of Iowa.^{6,7} 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 used.

Analysis of Gene Expression for iNOS

We performed double-immunohistochemical staining for iNOS and phenylethanolamine-*N*-methyltransferase (PNMT)³ or nitrotyrosine. We used a mouse IgG monoclonal antibody to iNOS (Transduction Laboratories), a rabbit polyclonal antibody to PNMT, and a rabbit IgG polyclonal antibody (Chemicon International) to nitrotyrosine (Upstate Cell Signaling Solutions). At day 7 after the gene transfer, the rats were deeply anesthetized with sodium pentobarbital (100mg/kg, IP) and perfused transcardially with PBS,

followed by 4% paraformaldehyde in PBS. The brain was removed and coronal sections (50 μ m) of the medulla were incubated in mouse IgG monoclonal antibody to mouse iNOS (1:100) (Transduction Laboratories) at 4°C for 48 hours. After incubation in biotinylated horse anti-mouse IgG (1:1000, Vector Laboratories) for 4 hours, the sections were incubated for 2 hours in a mixture of streptavidin-conjugated fluorescein isothiocyanate (1:100, Vector Laboratories). For double immunohistochemical staining, the sections were incubated in rabbit anti-PNMT antibody (1:200) or rabbit anti-nitrotyrosine antibody (1:200). The sections were incubated in rhodamine-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories). Double-stained sections with iNOS and PNMT or nitrotyrosine antibodies were photographed with a confocal laser scanning microscope.

Western Blot Analysis for iNOS

To confirm the local overexpression of iNOS in the RVLM, Western blot analysis for iNOS protein from tissue containing the injection sites of the RVLM obtained using the micropunch technique³ was performed at day 0, 3, 5, 7, 9, 11, or 14 after the gene transfer. The procedure for Western blot analysis for RVLM tissues was described previously.³ In the Western blot analysis for iNOS protein, we used mouse IgG monoclonal antibody to iNOS (1:1000, Santa

Cruz Biotechnology). Rabbit IgG polyclonal antibody to β -tubulin (1:5000, Santa Cruz

Biotechnology) for the brain tissues was used as an internal control. The densitometric

average was normalized to the values obtained from the analysis of β -tubulin protein.⁸

Microinjection of NOS Inhibitors into the RVLM

To confirm that changes in MAP and HR induced by AdiNOS transfection were the result of

an increase in iNOS protein, we microinjected aminoguanidine (2.5mM, 50nl) or

S-methylisothiourrea (SMT) (2.5mM, 50nl) into the bilateral RVLM at day 7 after transfection

with Ad β gal or AdiNOS. This dose of aminoguanidine and SMT was chosen according to the

results of a previous study.⁹ A nonselective NOS inhibitor, *N*^G-monomethyl-L-arginine

(L-NMMA), was also bilaterally microinjected into the RVLM. The rats were anesthetized

with sodium pentobarbital (50 mg/kg, IP, followed by 20 mg/kg/h, IV). The rats were

artificially ventilated, and arterial blood pressure was recorded with a P23XL transducer

connected to an RS3400 recorder.

Microinjection of L-Arginine into the RVLM

We microinjected L-arginine, a precursor of NO (70mM, 50nl), bilaterally into the RVLM at

day 7 after transfection with Ad β gal or AdiNOS. This dose of L-arginine was chosen according to the results of a previous study.¹⁰

Microinjection of Tempol into the RVLM

To examine whether the generation of superoxide anions is involved in blood pressure changes induced by AdiNOS transfection, tempol, a superoxide dismutase mimetic, was microinjected bilaterally into the RVLM.

Microdialysis and Measurement of NO Metabolites

We measured the production of NO in the RVLM as nitrite/nitrate (NO_x) by in vivo microdialysis before and at day 7 after gene transfer, as described previously.^{5,11,12} A microdialysis probe (A-I-12-01, 1-mm length, Eicom) was inserted into the RVLM and perfused with Ringer's solution at a constant flow rate of 2 μ L/min. The perfused dialysates were collected every 10 min in a sample loop of an automated sample injector connected to an automated NO detector HPLC system (ENO-10, Eicom), based on the Griess reaction. The basal NO_x levels were measured by averaging three consecutive stable dialysate samples, which were obtained at least 1 hour after starting brain perfusion with Ringer's solution.

Measurement of MAP, HR, and Urinary Norepinephrine Excretion

A UA-10 telemetry system (Data Sciences International) was used to measure MAP and HR.

The surgical procedure was described previously.^{3,5} MAP and HR were recorded continuously for 10 min daily between 10:00 AM and 11:00 AM using a multichannel amplifier and signal converter. We measured urinary norepinephrine concentrations before the gene transfer and at day 7 after the gene transfer by HPLC, and calculated the urinary norepinephrine excretion for 24 hours.^{1,3,5}

Dihydroethidium (DHE) Staining

Brain superoxide anion levels were estimated in two groups of rats (AdiNOS-transfected rats, n=5; non-treated, n=5) using DHE staining based on previous studies studies.^{13,14} On day 7, brains were removed, quickly frozen, and unfixed frozen regions of the RVLM were cut into 50- μ m sections using a cryostat and transferred to glass slides. Sections were thawed at room temperature, rehydrated with PBS, and incubated for 5 min in the dark with the O^{2•-}-specific fluorogenic probe DHE (1 μ mol/L). After washing with phosphate-buffered saline, DHE fluorescence was visualized by confocal microscopy using an excitation wavelength of 543

nm and a rhodamine emission filter.

Continuous Intracisternal Infusion Experiments with Tempol

The rats were randomly divided into four groups. Two of the groups were transfected with AdiNOS and two of the groups with Ad β gal. Either vehicle (artificial cerebrospinal fluid [aCSF]) or tempol (12 μ mol/day) were continuously infused intracisternally (0.25 μ L/h, 1 week) with an osmotic mini-pump (Alzet model 1002; DURECT Corporation), as described previously.¹⁵ Half of the animals in each transfection group were infused with vehicle and the other half were infused with tempol, producing four groups of animals: AdiNOS-VEH, AdiNOS-tempol, Ad β gal-VEH, and Ad β gal-tempol. This dose of tempol was chosen according to the results of a previous study.¹⁶ The rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). The osmotic mini-pump, filled with vehicle or tempol, was implanted subcutaneously in the back and connected to a polyethylene tube (PE-10), and the tip of the tube was placed intracisternally and fixed in the atlanto-occipital membrane with tissue adhesive. After full recovery from anesthesia, the rats were free to move about their cages. We measured urinary norepinephrine concentrations before the gene transfer and at day 7 after the gene transfer by HPLC, as described previously.^{1,3,5}

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Overexpression of Inducible Nitric Oxide Synthase in Rostral Ventrolateral Medulla Causes Hypertension and Sympathoexcitation via an Increase in Oxidative Stress

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Circ Res. 2005;96:252-260; originally published online December 9, 2004;
doi: 10.1161/01.RES.0000152965.75127.9d

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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