Downregulation of Basal iNOS at the Rostral Ventrolateral Medulla Is Innate in SHR


Abstract—We demonstrated recently that a significant reduction in both the molecular synthesis and functional expression of inducible nitric oxide synthase (iNOS) in the rostral ventrolateral medulla (RVLM), the medullary origin of sympathetic vasomotor outflow, underlies the augmented sympathetic vasomotor tone during hypertension. This study further evaluated the hypothesis that this downregulation of basal iNOS at the RVLM during hypertension is innate. In adult spontaneously hypertensive rats (SHR) treated for 4 weeks with the antihypertensive captopril to normalize elevated blood pressure or in young prehypertensive SHR, the significantly lower iNOS mRNA and protein levels at the ventrolateral medulla under basal conditions or on activation by microinjection bilaterally into the RVLM of lipopolysaccharide (10 ng) remained unaltered. The retarded efficacy of lipopolysaccharide (10 ng) to elicit cardiovascular depression (hypotension, bradycardia, and reduction in sympathetic vasomotor tone) also persevered in captopril-treated adult or young normotensive SHR. On the other hand, compared with Wistar-Kyoto normotensive rats, the magnitude of cardiovascular depression induced in adult SHR by local administration into the RVLM of the NO precursor L-arginine (40 nmol) was significantly smaller. In addition, microinjection bilaterally into the RVLM of a selective iNOS inhibitor, aminoguanidine (125 or 250 pmol), was discernibly less efficacious in unmasking hypertension, tachycardia, and the increase in sympathetic vasomotor tone in adult SHR. We conclude that a predisposed reduction in molecular synthesis and functional expression of basal iNOS in the RVLM is associated with the sympathetic vasomotor overactivity during hypertension. (Hypertension. 2003;41:563-570.)

Key Words: nitric oxide synthase ■ hypertension, essential ■ nitric oxide ■ blood pressure ■ heart rate ■ nervous system, autonomic

Whereas the notion that hypertension in human patients and animal models is attributable to an augmented central sympathetic outflow to the heart or peripheral vasculature is well accepted, the mechanism that underlies sympathetic overactivity during hypertension requires further delineation. Recent studies implicate an abnormality of the nitric oxide (NO) system in the brain in hypertension. Originally identified as an endothelium-derived vascular relaxing factor, NO is now known to play an active role in central cardiovascular regulation. In the rostral ventrolateral medulla (RVLM), where premotor neurons that provide the tonic sympathetic vasomotor drive are located, recently that whereas small amounts of NO generated by neuronal NO synthase (nNOS) in the RVLM promote sympathoexcitation, large amounts of NO produced by inducible NOS (iNOS) elicit sympathoinhibition. Under physiological conditions, the prevalence of nNOS over iNOS activity and the associated dominance of sympathoexcitation over sympathoinhibition are responsible for the maintenance of neurogenic vasomotor tone and stable arterial pressure elicited by endogenous NO at the RVLM. We proposed that further shifts in the balance toward nNOS by a significant reduction in both the synthesis and activity of iNOS at the RVLM underlie the augmented sympathetic vasomotor tone during hypertension.

Whether the proposed downregulation of iNOS at the RVLM during hypertension is a consequence of the elevated arterial pressure is essentially unknown. The present study was undertaken to address this issue. We conclude that a predisposed reduction in molecular synthesis and functional expression of basal iNOS in the RVLM is associated with sympathetic vasomotor overactivity during hypertension.

Methods

Animals

Experiments were carried out in accordance with the guidelines for animal experimentation endorsed by our institutional animal care committee. Male adult (8-week-old, 175 to 185 g; n=167) or young (4-week-old, 100 to 110 g; n=60) spontaneously hypertensive rats (SHR) or normotensive Wistar-Kyoto (WKY; 8-week-old, 175 to 185 g, n=168; or 4-week-old, 105 to 115 g, n=60) rats purchased from the Experimental Animal Center, National Science Council, Taiwan, were used.

Antihypertensive Treatment

After an initial measurement of baseline mean systemic arterial pressure (MSAP) noninvasively by tail-cuff plethysmography (Softron, Japan) in
Expression of nNOS mRNA or Protein in the Ventrolateral Medulla of Adult SHR or WKY Rats

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Animals received microinjection bilaterally into the RVLM of aCSF or LPS (10 ng) after they were subjected to either captopril (Cap) or water (H₂O) treatment for 4 weeks. Denoted is the amount of nNOS mRNA or protein expressed as a ratio (%) to β-actin mRNA or β-tubulin protein. Values are mean±SEM of quadruplicate analyses on samples pooled from 7 or 8 animals in each group. No significant difference exists among groups in ANOVA analysis.

General Preparation

The femoral artery and both femoral veins were cannulated for measurement of SAP and drug administration11,14,15 under pentobarbital sodium anesthesia (50 mg/kg IP). Animals thereafter received an intravenous infusion of propofol (Zeneca)11,12,14 at 30 mg/kg per hour, which commenced routinely 50 to 60 minutes after pentobarbital induction. This scheme provided satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation, based on online observations of changes in the frequency spectrum of SAP signals.15 Animals were also paralyzed with an intravenous infusion of pancuronium (2 mg/kg per hour) and were mechanically ventilated (Harvard 683) to maintain end-tidal CO₂ to be within 4% to 5%, as monitored by a capnograph (Datex Normocap). Body temperature was maintained at 37°C with a heating pad.

Microinjection of Test Agents

Microinjection bilaterally of test agents by a glass micropipette, at a volume of 50 nL, was carried out stereotaxically and sequentially into functionally identified RVLM sites as detailed previously.11,14 The stereotaxic coordinates for adult or young animals were, respectively, 4.5 to 5 or 4.2 to 4.5 mm posterior to lambda, 1.8 to 2.1 or 1.5 to 1.7 mm lateral to the midline, and 8.1 to 8.4 or 7.5 to 7.8 mm below the dorsal surface of the cerebellum. Test agents used in this study included \textit{Escherichia coli} lipopolysaccharide (LPS; serotype 0111:B4, Sigma); the NO precursor L-arginine (L-Arg; RBI); a selective nNOS inhibitor,\textsuperscript{17} 7-nitroindazole (7-NI; RBI); a selective iNOS inhibitor,\textsuperscript{18} amino-guanidine (AG; RBI); and a non-nitrate NO donor,\textsuperscript{19} S-nitro-N-acetylpenicillamine (SNAP, RBI). The doses used were the same as in our recent studies\textsuperscript{11,12,20} when these test agents were used for the same purpose as in the present study. All chemicals were freshly prepared with artificial cerebrospinal fluid (aCSF) immediately before use. The only exception was 7-NI, which was dissolved in 3% methanol. Microinjection of the solvent served as the vehicle and volume control.

Evaluation of Sympathetic Vasomotor Tone

The SAP signals were simultaneously subjected to online power spectral analysis.\textsuperscript{11,12,20,21} We were particularly interested in the very low-frequency (0 to 0.25 Hz) and low-frequency (0.25 to 0.8 Hz) components in the SAP spectrum. Our laboratory demonstrated previously\textsuperscript{21} that these spectral components of SAP signals take their origin from the RVLM, and their power density reflects the prevailing sympathetic neurogenic vasomotor tone.\textsuperscript{11,12,20,21}

Histology

At the end of each physiological experiment, the animal was killed with an overdose of pentobarbital sodium, and the brain stem was removed and fixed in 10% formaldehyde in 30% sucrose solution for measurement once per week during the 4-week treatment period.
at least 72 hours. Histological verification of the microinjection site was carried out on 20-μm frozen sections stained with neutral red, according to the atlas of Paxinos and Watson.²²

**Isolation of Total RNA and Reverse Transcription–Polymerase Chain Reaction**

Isolation and extraction of total RNA from the ventrolateral part of the medulla oblongata, at the level of the RVLM (0.5 to 2.5 mm rostral to the obex for adult rats; 0.3 to 2.0 mm rostral to the obex for young rats) and reverse transcription–polymerase chain reaction (RT-PCR) analysis of iNOS, nNOS, or β-actin mRNA were carried out as reported previously.¹¹,¹²,²⁰ The predominant cDNA amplification product predicted for iNOS, nNOS, or β-actin was, respectively, 317, 345, or 440 bp long. The amount of mRNA products for iNOS or nNOS was analyzed by ImageMaster VDS analysis software (Amersham Pharmacia Biotech) and was expressed as the ratio (percentage) to β-tubulin mRNA.

**Protein Extraction and Western Blot Analysis**

Protein extraction from the ventrolateral part of the medulla oblongata and Western blot analysis of iNOS, nNOS, or β-tubulin were carried out as reported previously.¹² A rabbit polyclonal antiserum against iNOS, nNOS, or β-tubulin (Santa Cruz) was used as the primary antiserum, followed by horseradish peroxidase–conjugated goat anti-rabbit IgG (Jackson). Specific antibody-antigen complex was detected by an enhanced chemiluminescence western blot detection system (NEN Life Science Products). The amount of iNOS or nNOS protein was quantified by Photo-Print Plus software (ETS Vilber-Lourmat) and was expressed as the ratio (percentage) to β-tubulin protein.

**Statistical Analysis**

All values are expressed as mean±SEM. One-way or 2-way ANOVA with repeated measures was used to assess group means, as appropriate, followed by the Scheffe multiple-range test for post hoc assessment of individual means. *P<0.05 was considered statistically significant.

**Results**

**Lack of Effect of Antihypertensive Treatment on iNOS mRNA or Protein Levels in the RVLM of Adult SHR or WKY Rats**

Treatment with captopril for 4 weeks resulted in a significant decrease in MSAP in adult SHR (captopril, 97.6±3.5 mm Hg; water, 157.6±4.8 mm Hg; n=36 each). No comparable changes occurred in adult WKY rats (captopril, 101.4±4.3 mm Hg; water, 115.7±3.1 mm Hg; n=36 each).

The basal level of iNOS in the ventrolateral medulla or the progressive upregulation of iNOS expression 1.5 or 3 hours after microinjection bilaterally of LPS (10 ng) into the RVLM...
(Figure 1) was significantly lower in water-treated SHR than in WKY rats at both mRNA and protein levels. Intriguingly, this manifested reduction in molecular synthesis of iNOS at the RVLM persisted in SHR whose MSAP was normalized to the WKY level after captopril treatment. Whereas the absolute magnitude of such LPS-induced upregulation of iNOS mRNA or protein was significantly retarded in SHR, the relative increase in iNOS expression was similar in water- or captopril-treated SHR and WKY rats. On the other hand, concurrent RT-PCR or Western blot analysis (Table) revealed that the basal level of nNOS mRNA or protein in the ventrolateral medulla was comparable in water- or captopril-treated SHR and WKY rats and remained unaltered on microinjection of LPS into the RVLM.

Lack of Effect of Antihypertensive Treatment on Differential Cardiovascular Responses to Functional Activation of iNOS in the RVLM of Adult SHR or WKY Rats

Functional activation of iNOS in the RVLM by microinjection bilaterally of LPS (10 ng) elicited a significant and progressive hypotension, bradycardia, or decrease in the power density of the vasomotor components of the SAP spectrum in water-treated SHR (Figure 2) or WKY rats (Figure 3). However, the magnitude of these cardiovascular depressions was appreciably smaller, the onset latency longer, and the slope discernibly blunted in SHR. Intriguingly, superimposed on the reduced SAP or power density of the vasomotor components of the SAP spectrum, those differential cardiovascular responses to activation of iNOS at the RVLM were essentially duplicated after SHR were treated with captopril (Figures 2 and 3). We also found that 1 in 6 water-treated or 1 in 7 captopril-treated SHR and all 6 water- or captopril-treated WKY rats died within 4 hours after LPS treatment.

Reduced Molecular Synthesis and Functional Expression of iNOS in the RVLM of Young SHR

At 4 weeks, SHR and WKY rats exhibited comparable MSAP (113.4±5.6 mm Hg vs 98.9±4.8 mm Hg, n=45 each). Of note was that the basal level of iNOS in the ventrolateral medulla or the progressive upregulation of iNOS expression induced by bilateral microinjection of LPS (10 ng) into the RVLM (Figure 4) remained significantly lower in those young prehypertensive SHR. On the other hand, the level of nNOS mRNA or protein in the ventrolateral medulla was similar between young SHR and WKY rats under either basal conditions or on application of LPS into the RVLM (Figure 4). We also found that the basal iNOS or nNOS mRNA or protein in the ventrolateral medulla was not significantly different between young and adult SHR or WKY rats (cf Figure 1).
Functional activation of iNOS in the RVLM by bilateral microinjection of LPS (10 ng) elicited a significant and progressive hypotension, bradycardia, or decrease in the power density of the vasomotor components of the SAP spectrum in young SHR or WKY rats (Figure 5). Intriguingly, the magnitude of these cardiovascular depressions was again appreciably smaller and the slope discernibly blunted in young SHR.

**Differential Cardiovascular Responses to Production of Endogenous NO or Blockade of Basal nNOS or iNOS Activity in the RVLM of Adult SHR or WKY rats**

Increasing the production of NO in the RVLM by bilateral microinjection of the NO precursor L-Arg (40 nmol) resulted in a decrease in MSAP, heart rate, or the power density of the vasomotor components of the SAP spectrum in young SHR or WKY rats (Figure 6). The cardiovascular-depressive actions of L-Arg lasted for the entire 60-minute observation period. In contrast, the same treatment resulted in no significant changes in all 3 hemodynamic parameters in SHR (Figure 6).

Microinjection bilaterally of a selective nNOS inhibitor, 7-NI (1 or 2.5 pmol), into the RVLM resulted in progressive and significant hypotension, bradycardia, or decrease in power density of the vasomotor components of the SAP spectrum in both strains of rats (Figure 7). Intriguingly, the temporal profiles and maximal changes in these hemodynamic parameters were comparable between SHR and WKY rats. On the other hand, microinjection bilaterally of a selective iNOS inhibitor, AG (125 or 250 pmol), into the RVLM promoted a dose-related increase in MSAP, heart rate, or power density of the vasomotor components of the SAP spectrum in both SHR and WKY rats (Figure 7). However, AG was appreciably less efficacious and the duration of those cardiovascular responses discernibly shorter in SHR.

Coadministration of 7-NI (2.5 pmol) and L-Arg (40 nmol) into the RVLM of WKY rats elicited no discernible effect on the cardiovascular suppression induced by the NO precursor alone (Figure 6). On the other hand, comicroinjection bilaterally of AG (250 pmol) into the RVLM (Figure 6) of the normotensive rats antagonized significantly the bradycardia induced by L-Arg (40 nmol). The same treatment also reversed the hypotension or decrease in power density of the vasomotor components of the SAP signal induced by L-Arg to those that resembled qualitatively those promoted by the iNOS inhibitor alone (Figure 7). However, coadministration...
of 7-NI (2.5 pmol) or AG (250 pmol) into the RVLM of SHR elicited no appreciable action on the already minimal cardiovascular effects of L-Arg (Figure 6).

**Cardiovascular Responses to Exogenous NO in the RVLM of Adult SHR or WKY Rats**

Microinjection bilaterally into the RVLM of 2 low doses (0.25 or 0.5 nmol) of a NOS-independent NO donor, SNAP, promoted significant and comparable hypertension, tachycardia, or increase in sympathetic vasomotor tone in SHR and WKY rats (Figure 8). This gave way to no discernible cardiovascular changes when the NO donor was increased to 1 nmol. A significant reduction in MSAP, heart rate, or power density of the vasomotor components of the SAP spectrum was elicited when a high dose (5 nmol) of SNAP was administered into the RVLM.

**Discussion**

Reduction in iNOS expression was demonstrated in peripheral tissues during hypertension.\(^{23-25}\) Aerosol iNOS gene transfer increases pulmonary NO production and reduces hypoxic pulmonary hypertension.\(^{26}\) The augmented iNOS protein expression reported in the aorta and heart of SHR is, however, consequential to the hypertensive state.\(^{27,28}\) On the other hand, the present study provided novel genomic and phenotypic observations to reveal that the reduction in basal iNOS at the RVLM during hypertension is not a compensatory response to the elevated SAP. Instead, it is the innate abnormality in molecular synthesis and functional expression of iNOS at the RVLM that underlies the augmented sympathetic vasomotor tone seen during hypertension.

We demonstrated that the discernible reduction in basal and LPS-induced iNOS mRNA or protein expression in the ventrolateral medulla was already present in young, prehypertensive SHR, and that this trend persevered after normalizing the SAP of adult SHR to the WKY level. We also showed that the resistance to LPS-induced cardiovascular suppression demonstrated in young SHR persisted in captopril-treated adult SHR. Our assessment of functional expression further showed that the tonically active endogenous iNOS, which was unmasked by AG treatment to be responsible for sympathoinhibition,\(^{11}\) was reduced in the RVLM during hypertension. Thus, AG was discernibly less efficacious in promoting hypertension, tachycardia, and increase in sympathetic vasomotor tone in SHR, and the cardiovascular-depressive actions of L-Arg, which is iNOS dependent,\(^{11}\) was absent.
An increase\textsuperscript{25, 29} or decrease\textsuperscript{30} in nNOS mRNA expression in the RVLM was reported in hypertensive animals. Our results indicate that both nNOS mRNA and protein levels in the ventrolateral medulla remained unchanged under basal conditions or on microinjection of LPS into the RVLM in young normotensive SHR or captopril-treated adult SHR. Furthermore, the cardiovascular-depressive effects of 7-NI, which is indicative of a sympathoexcitatory role for nNOS,\textsuperscript{11} were comparable in both strains of rats. These findings provided crucial support for our contention that a significant, innate downregulation of iNOS at the RVLM may play a crucial role in the genesis of augmented sympathetic vasomotor tone during hypertension. They also complement our previous proposal\textsuperscript{12} that a shift in balance toward the already-prevalent nNOS\textsuperscript{11} in the RVLM and the associated dominance of sympathoexcitation occur during hypertension. We noted, however, that there is no apparent phenotypic deficiency in homoygous nNOS-mutant mice.\textsuperscript{31, 32} Thus, the significance of nNOS at the RVLM in the development of hypertension still awaits further investigation.

We found that whereas low doses of SNAP increased MSAP, heart rate, and sympathetic vasomotor outflow, high doses decreased the same cardiovascular parameters. This demonstration again substantiated the notion that, depending on the amount generated in the RVLM by nNOS (small) and iNOS (large),\textsuperscript{33} NO may elicit opposite cardiovascular responses.\textsuperscript{11, 34} More important, that SNAP induced comparable circulatory alterations in both strains of rats ruled out the possibility that the heightened sympathetic vasomotor outflow in the SHR arises from an abnormal response of RVLM neurons to NO. In this context, overexpression of NO by transfecting adenovirus vectors encoding endothelial NOS into the RVLM decreases SAP, heart rate, and sympathetic nerve activity in conscious rats.\textsuperscript{35} Furthermore, these elicited cardiovascular-depressive effects are exaggerated in stroke-prone SHR.\textsuperscript{36} We also noticed that despite a reduction in the magnitude of basal and LPS-induced expression of iNOS mRNA and protein in the RVLM of SHR, the relative increase in iNOS mRNA or protein expression evoked by LPS was comparable in both strains of rats. These observations are in agreement with our recent finding\textsuperscript{12} and suggest that the reduction in iNOS activity at the RVLM reflects a discernibly lower basal level of iNOS mRNA or protein.

In conclusion, based on results from antihypertensive treatment in adult SHR or young prehypertensive SHR and manipulation of endogenous iNOS or nNOS activity, the present study revealed that the reduced molecular synthesis and functional expression of iNOS in the RVLM is predisposed and may underlie the sympathetic vasomotor overactivity associated with hypertension.

**Perspectives**

A hallmark of hypertension is augmented central sympathetic outflow to the peripheral vasculature.\textsuperscript{1–5} At the same time, a key determinant of the prevailing sympathetic vasomotor drive is the balance between the physiologically active nNOS and iNOS at the RVLM, which are responsible, respectively, for the excitatory and inhibitory actions of endogenous NO on the premotor sympathetic neurons.\textsuperscript{11} It follows that additional shifts in balance toward nNOS by a significant reduction in both synthesis and activity of iNOS at the RVLM underlie the augmented sympathetic vasomotor tone during hypertension.\textsuperscript{12} As a prelude to hypertension, the present study further revealed that this downregulation of basal iNOS in the RVLM is innate. This newly identified role for iNOS should potentially provide a new direction for long-term control of hypertension through gene therapy.

**Acknowledgments**

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**References**


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