Reduced Functional Expression and Molecular Synthesis of Inducible Nitric Oxide Synthase in Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats

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Background—We demonstrated recently that the prevalence of neuronal (nNOS) over inducible (iNOS) nitric oxide synthase activity at the rostral ventrolateral medulla (RVLM), the medullary origin of sympathetic neurogenic vasomotor tone, and the associated dominance of sympathoexcitation over sympathoinhibition underlie the maintenance of sympathetic vasomotor outflow by the endogenous NO. Here, we evaluated the hypothesis that a significant downregulation of iNOS at the RVLM may play a crucial role in the genesis of augmented sympathetic vasomotor tone during hypertension.

Methods and Results—Spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats anesthetized with propofol were used. Compared with SHR, the hypotension, bradycardia, or depression in sympathetic vasomotor tone induced by bilateral microinjection of lipopolysaccharide (5 or 10 ng) into the RVLM of WKY rats exhibited significantly shorter-onset latency, appreciably steeper slope, and a greater incidence of mortality. All these effects of lipopolysaccharide (10 ng) were significantly blunted by coadministration of the selective iNOS inhibitor S-methylisothiourea (250 pmol). Reverse transcription–polymerase chain reaction and Western blot analyses further revealed significantly lower iNOS mRNA and protein levels at the ventrolateral medulla in SHR under basal conditions or on activation by lipopolysaccharide (10 ng). Conversely, nNOS mRNA and protein levels remained constant in the RVLM and were comparable in both strains of rats.

Conclusions—We conclude that a significant downregulation in both functional expression and molecular synthesis of iNOS at the RVLM may underlie the augmented sympathetic vasomotor tone during hypertension. (Circulation. 2001; 104:1676-1681.)

Key Words: blood pressure ▪ heart rate ▪ hypertension ▪ nervous system, sympathetic ▪ nitric oxide synthase

Apart from its well-known function as an endothelium-derived relaxing factor that promotes relaxation of peripheral vasculature,1 recent studies2 also assign a role to nitric oxide (NO) in central circulatory regulation. One of the potential sites in the central nervous system for NO to exert its modulatory action on cardiovascular functions is the rostral ventrolateral medulla (RVLM),3–8 which provides the sympathetic drive that maintains vascular vasomotor tone.9

Of the 3 isoforms of nitric oxide synthase (NOS), it is generally contended10–12 that whereas both the neuronal (nNOS) and endothelial (eNOS) isoforms are expressed constitutively, the activity of inducible NOS (iNOS) is induced principally by inflammatory stimuli. In a recent study,13 however, we demonstrated that both nNOS and iNOS in the RVLM are tonically active under physiological conditions at the levels of functional expression and molecular synthesis. Furthermore, the prevalence of nNOS over iNOS activity at the RVLM and the associated dominance of sympathoexcitation over sympathoinhibition may underlie the maintenance of sympathetic vasomotor outflow and stable arterial pressure by the endogenous NO.

A corollary to the above notion is that a significant downregulation of iNOS at the RVLM may play a crucial role in the genesis of augmented sympathetic vasomotor tone during hypertension. The present study validated this hypothesis. Our results indicate that spontaneously hypertensive rats (SHR) manifest a significant reduction in both functional expression and molecular synthesis of iNOS at the RVLM.

Methods

General Preparation
Experiments were carried out in 112 male, adult (8 to 10 weeks, 220 to 275 g) SHR or their normotensive Wistar-Kyoto (WKY) controls and were in accordance with the guidelines for animal experimentation endorsed by our institutional animal care committee. Under initial pentobarbital sodium anesthesia (50 mg/kg IP), the trachea was intubated and the right femoral artery and both femoral veins...
were cannulated. Thereafter, animals received IV infusion of propofol (Zeneca) at 30 mg · kg⁻¹ · h⁻¹. This scheme provided satisfactory maintenance of anesthesia while preserving the capacity of central cardiovascular regulation.¹⁴ The head of the animal was fixed to a stereotaxic headholder (Kopf 1430), and body temperature was maintained at 37°C with a thermoregulated heating pad. Animals were also paralyzed with pancuronium (2 mg · kg⁻¹ · h⁻¹ IV) and were mechanically ventilated (Harvard 683) to maintain end-tidal CO₂ to be within 4% to 5%, as monitored by a capnograph (Datex Normocap). Pulsatile pressure and mean systemic arterial pressure (MSAP), as well as heart rate (HR), were recorded on a polygraph (Gould ES3400).

**Microinjection of Lipopolysaccharide Into the Rostral Ventrolateral Medulla**

*Escherichia coli* lipopolysaccharide (LPS, serotype 0111:B4; Sigma), given alone or together with the selective iNOS inhibitor S-methylisothiourea,¹³,¹⁵ was microinjected bilaterally and sequentially, at a volume of 50 nL., into the RVLM. The coordinates used were 4.5 to 5 mm posterior to the lambda, 1.8 to 2.1 mm lateral to the midline, and 8.1 to 8.6 mm below the dorsal surface of the cerebellum.¹³,¹⁶ LPS or S-methylisothiourea was freshly prepared immediately before use with artificial cerebrospinal fluid (aCSF), which also served as the vehicle and volume control.

**Evaluation of Sympathetic Vasomotor Tone**

The SAP signals were simultaneously subjected to online power spectral analysis as detailed previously.¹³,¹⁴,¹⁶,¹⁷ We were particularly interested in the very-low-frequency (0 to 0.25 Hz) and low-frequency (0.25 to 0.8 Hz) components of SAP signals. Our laboratory demonstrated previously¹⁷ that these spectral components of SAP signals have their origin in the RVLM, and their power density reflects the prevailing sympathetic neurogenic vasomotor tone.

**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), and reverse transcription–polymerase chain reaction (RT-PCR) analysis of iNOS, nNOS, or β-actin mRNA were carried out as reported previously.¹³,¹⁶ The predominant cDNA amplification product predicted for iNOS, nNOS, or β-actin was 317, 345, or 440 bp, respectively, in length. 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 to β-actin mRNA product.

**Protein Extraction and Western Blot Analysis**

Western blot analysis of NOS protein at the ventrolateral medulla was performed with rabbit polyclonal antiserum against iNOS, nNOS, or β-tubulin (Santa Cruz Biotechnology) as the primary antiserum. This was followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG (Jackson). Specific antibody–antigen complex was detected with an enhanced chemiluminescence Western Blot detection system (NEN Life Science Products). The amount of iNOS, nNOS, or β-tubulin protein was quantified by Photo-Print Plus software (ETS Vilber-Lourmat).

**Histology**

At the end of each experiment, the animal was killed by an overdose of pentobarbital sodium, and the brain was removed and fixed in 10% formaldehyde in 30% sucrose solution for ≈72 hours. Histological verification of the microinjection site was carried out on 20-μm frozen sections stained with neutral red.

**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 Scheffé multiple-range test for post hoc assessment of individual means. Mortality rate was assessed by Fisher’s exact test. A value of *P*<0.05 was considered statistically significant.

**Results**

**Differential Cardiovascular Responses to Microinjection of LPS Into the RVLM of SHR or WKY Rats**

Our first series of experiments evaluated the relative iNOS activity in the RVLM of SHR and WKY rats by analyzing the cardiovascular responses to activation of this NOS isoform by LPS. Bilateral microinjection into the RVLM of LPS (5 or 10 ng) resulted in a significant and progressive hypotension (Figure 1) or bradycardia (Figure 2) in both strains of rats. Bilateral coadministration into the RVLM of an efficacious dose¹³ of the selective iNOS inhibitor S-methylisothiourea (250 pmol)¹⁵ significantly blunted the reduction in MSAP (Figure 1) or HR (Figure 2) induced by LPS (10 ng). We further noted that compared with SHR, the onset latency of significant LPS-promoted hypotension or bradycardia was discernibly shorter and the slope appreciably steeper in WKY rats.

Bilateral microinjection of LPS (5 or 10 ng) into the RVLM also elicited a significant and progressive reduction in the power density of the vasomotor components of the SAP spectrum, our experimental index for sympathetic vasomotor tone,¹⁷ in both SHR and WKY rats.
SHR and WKY rats (Figure 3). Again, the onset latency of significant LPS-induced sympathetic depression was appreciably shorter in WKY rats, along with a discernibly steeper slope. Coadministration of $S$-methylisothiourea (250 pmol) also significantly reversed the reduction in sympathetic vasomotor tone elicited by LPS (10 ng).

**Differential Survival Rate Induced by Microinjection of LPS Into the RVLM of SHR or WKY Rats**

Our second series of experiments further evaluated the relative iNOS activity in the RVLM of SHR and WKY rats by analyzing the survival rate after activation of this enzyme with LPS. Bilateral microinjection of LPS (5 or 10 ng) into the RVLM induced a dose-related decrease in survival time in WKY rats (Figure 4). Whereas both doses of LPS elicited comparable durations of survival in SHR, the survival time after they had received the higher dose was significantly longer than in WKY rats. In terms of mortality rate, 3 of 7 SHR and all 7 WKY rats died within 4 hours after local application of 5 and 10 ng of LPS into the RVLM. Conversely, none of the SHR (n = 7) succumbed within the same period after bilateral microinjection into the RVLM of the lower dose, and only 2 of 7 SHR died after receiving the higher dose of LPS. Of note was that coinjection into the RVLM of LPS (10 ng) and $S$-methylisothiourea (250 pmol) did not result in mortality within 4 hours in SHR and WKY rats (Figure 4).

**Relative iNOS mRNA or Protein Levels at the RVLM of SHR or WKY Rats**

Figure 5 depicts the results of our RT-PCR analysis of basal and evoked levels of iNOS mRNA in the RVLM of SHR and WKY rats. As in our recent observations, we detected the presence of a basal level of iNOS mRNA in the ventrolateral medulla in both strains of rats, although the level in SHR was significantly lower than that in WKY rats. Bilateral microinjection of LPS (10 ng) into the RVLM induced a gradual increase of iNOS mRNA in the ventrolateral medulla of both strains of rats that mirrored the progressive cardiovascular depression. Compared with WKY rats, the absolute magni-
amount of iNOS mRNA relative to β-actin mRNA detected from ventrolateral medulla 1.5 or 2.5 hours after animals received bilateral microinjection into RVLM of aCSF or LPS (10 ng). Lanes 1 and 4, extracts from ventrolateral medulla in animals that received aCSF. Lanes 2 and 5, samples obtained 1.5 hours after LPS administration. Lanes 3 and 6, samples obtained 2.5 hours after LPS administration. Values are mean ± SEM of quadruplicate analyses, n = 6 to 8 animals in each group. *P < 0.05 vs respective aCSF control group (C), #P < 0.05 vs corresponding WKY group in Scheffé multiple-range analysis.

Western blot analysis (Figure 6) revealed parallel results at the protein level. The basal level of iNOS protein in the ventrolateral medulla was again significantly lower in SHR. Again, whereas the magnitude of iNOS protein evoked at 1.5 or 2.5 hours by bilateral microinjection of LPS (10 ng) into the RVLM was discernibly less in the hypertensive rats, the relative increase in iNOS protein in SHR (1.3- and 1.7-fold) or WKY rats (1.5- and 1.9-fold) during these 2 time points was comparable.

It should be mentioned that concurrent RT-PCR or Western blot analysis (Table) revealed that the basal mRNA or protein levels of nNOS in the ventrolateral medulla were comparable in SHR and WKY rats. They also remained relatively constant 1.5 or 2.5 hours after bilateral microinjection of LPS (10 ng) into the RVLM.

Histological Verifications of Microinjection Sites
Histological verifications indicated that the tip of the micropipettes used to deliver LPS was located within the RVLM. Microinjection of LPS into areas outside the confines of the RVLM elicited minimal effect on SAP, HR, sympathetic vasomotor tone, survival rate, or expression of iNOS or nNOS at the mRNA or protein levels.

Discussion
Application of NO precursor or donor into the RVLM reportedly decreases sympathetic nerve activity and SAP. Other studies suggest a sympathoexcitatory and pressor role for NO in the RVLM. Our laboratory demonstrated recently that this controversy might be resolved by recognizing the differential roles of NOS isoforms at the RVLM in cardiovascular regulation. We found that under physiological conditions, both nNOS and iNOS in the RVLM are active at the levels of functional expression and molecular synthesis. Furthermore, the maintenance of SAP, HR, and sympathetic vasomotor outflow by endogenous NO at the RVLM may result from a prevalence of nNOS over iNOS activities, which are responsible for the sympathoexcitatory and sympathoinhibitory actions of NO, respectively. The present study expounded on these findings and provided the first demonstration of a crucial role for iNOS at the RVLM during hypertension.

An impairment of production and/or function of NO at the peripheral vasculature during hypertension has been suggested in animal and human studies. Experiments with iNOS-knockout mice also showed that they are resistant to LPS-induced mortality. In the area of central cardiovascular regulation, we recently demonstrated that iNOS at the RVLM is tonically active and is related to the reduction in sympathetic vasomotor tone. In addition, a shift in prevalence of iNOS over nNOS activity in the RVLM may be a crucial determinant for the reduction or loss in sympathetic neurogenic vasomotor tone and eventual death seen during endotoxemia. It is therefore intriguing to note that on the basis of the retarded efficacy of LPS to elicit cardiovascular depression or mortality, the present study demonstrated that iNOS at the RVLM is significantly reduced in SHR at the level of functional expression.

At the level of molecular synthesis, we further demonstrated a discernible reduction in the magnitude of basal and LPS-induced iNOS mRNA or protein expression in the RVLM of SHR. Closer observations revealed, however, that the relative increase in iNOS mRNA or protein expression evoked by LPS was comparable in both strains of rats. These results suggest that the reduction in iNOS activity at the
RVLM reflected an appreciably lower basal level of iNOS mRNA or protein in the ventrolateral medulla of SHR. Such a difference in genetic background between SHR and WKY rats may be polyallelic and affects the immune system as well as the cardiovascular system. SHR exhibit immune abnormalities of decreased delayed-type hypersensitivity, defective leukocyte–endothelial cell interactions, decreased neutrophil adhesion, and lowered production of proinflammatory cytokines to LPS treatment. These immune dysfunctions, which are closely associated with hypertension, are in part NO-dependent.

For the contention that a significant downregulation of iNOS at the RVLM may play a crucial role in the genesis of augmented sympathetic vasomotor tone during hypertension to be valid, it is imperative that both mRNA and protein levels of nNOS in the ventrolateral medulla either be comparable in SHR and WKY rats or be heightened in the hypertensive animals. Our results from RT-PCR and Western blot analyses supported the former notion. At the same time, we noted that an elevated expression of nNOS gene in the ventral medulla, including the RVLM, has been reported in SHR.

We recognize that to be a good experimental model for assessing the physiological role of iNOS in the regulation of sympathetic vasomotor tone, LPS must selectively activate iNOS at the RVLM. This notion was validated when microinjection into the RVLM of S-methylisothiourea significantly reversed the cardiovascular depression and mortality induced by LPS. Furthermore, microinjection of LPS into sites outside the confines of the RVLM did not elicit discernible circulatory changes. Because administration of LPS at 10 ng IV was also ineffective, it is unlikely that the differential hemodynamic response to application of LPS to RVLM was due to altered peripheral nervous or vascular mechanisms in SHR. We are aware that anesthesia may be a confounding factor in our study. In this regard, the anesthetic maintenance scheme that we used in this study has been demonstrated previously to induce minimal depressive action on the central cardiovascular machinery. It is also possible that the diminished cardiovascular suppression or mortality induced by microinjection of LPS into the RVLM may be attributed to the significantly higher SAP in SHR. This possibility, however, is deemed unlikely. Bernard et al reported that survival of SHR from LPS is not related to their hypertensive state and that WKY rats made hypertensive by clipping of one of the renal arteries showed fatality similar to that of normotensive WKY.

In conclusion, the present study revealed a significant downregulation in both functional expression and molecular synthesis of iNOS at the RVLM of SHR. Superimposed on maintained nNOS mRNA and protein, our findings support the notion that iNOS in the RVLM may underlie the genesis of augmented sympathetic vasomotor tone during hypertension. Several studies suggest that NO may be generated in the CNS by iNOS present in microglia or astrocytes. Speculatively, this stipulated downregulation of iNOS may originate from these glial cells in the RVLM, although the contribution from neurons cannot be ruled out.

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References

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