Enhanced Depressor Response to Nitric Oxide in the Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats

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Abstract—Possible impairment of the L-arginine–nitric oxide (NO) pathway in the rostral ventrolateral medulla of adult spontaneously hypertensive rats (SHR) was investigated by microinjecting N^6-nitro-L-arginine methyl ester (L-NAME), NOC 18 (an NO donor), or L-arginine. Unilateral injection of L-NAME (10 nmol/50 nL) into the rostral ventrolateral medulla significantly increased mean arterial pressure (MAP) in both SHR and Wistar-Kyoto rats (WKY). The increases in MAP did not differ significantly between the two strains (15 ± 3 versus 10 ± 2 mm Hg, respectively; n = 8). In contrast, microinjection of L-arginine elicited significant (P < .05) dose-dependent decreases in MAP in both strains, and these depressor responses were significantly greater in SHR than in WKY (in 10 nmol of L-arginine: −29 ± 2 versus −15 ± 2 mm Hg, respectively; n = 8, P < .01). Similarly, microinjection of NOC 18 (10 nmol/50 nL) reduced MAP in both strains, and the depressor response was also significantly greater in SHR than in WKY (−38 ± 7 versus −22 ± 3 mm Hg, respectively; n = 8, P < .05). These results suggest that the L-arginine–NO pathway in the rostral ventrolateral medulla is impaired in SHR and that this impairment may contribute to the increase in arterial pressure in this animal model of genetic hypertension. (Hypertension. 1998;31:1030-1034.)

Key Words: L-arginine | NOC 18 | L-NAME | medulla oblongata | microinjection

The release of NO from endothelial cells reduces smooth muscle tone and regulates arterial pressure,1,2 whereas the intravenous injection of NOS inhibitors increases arterial pressure.3 This latter effect has been thought to result mainly from the vasoconstriction caused by deprivation of endothelial NO. However, NO is also produced from L-arginine in other cell types, including neurons in the brain.4 Although evidence suggests that NO within the central nervous system plays an important role in cardiovascular regulation,5,6 few studies have examined the role of central NO in genetically hypertensive rats. Cabrera et al7 reported that the central depressor action of endogenous NO was deficient in genetic hypertension. Because NOS is localized in discrete neuronal populations throughout the brain,5 several regions may be the candidates for the altered L-arginine–NO pathway within the brain of genetically hypertensive rats. In the RVLM, which is one of the important regions for cardiovascular regulation,5 NOS exists6 and the role of endogenous NO in the regulation of arterial pressure has been reported.11-14 Thus, we hypothesized that endogenous L-arginine–NO pathway might be altered in the RVLM of SHR. To address this issue, we microinjected an NOS inhibitor or L-arginine into the RVLM. We also microinjected an NO donor to assess the direct action of NO on the RVLM neurons.

Methods

Animal Preparation

All experiments were performed with adult male SHR/Izm (12 to 14 weeks old; body weight, 290 ± 5 g; n = 22) and age-matched WKY/Izm (body weight, 324 ± 6 g; n = 22) obtained from the Disease Model Cooperative Research Association (Kyoto, Japan).15 The experimental protocol was approved by the Committee on the Ethics of Animal Experimentation of the Faculty of Medicine, Kyushu University.

All rats were anesthetized with urethane (1.5 g/kg IP, supplemented with 100 mg/kg IV, if necessary). A polyethylene cannula (PE-50) was inserted into the right femoral vein for drug administration. MAP was measured directly with a cannula inserted in the right femoral artery and connected to a pressure transducer (P50; Gould Statham Instruments). HR was monitored with a cardiotachometer (model 1332; NEC San-ei), and body temperature was monitored with a rectal probe and was maintained at 37.0° ± 0.5°C with a heating pad.

Anesthetized rats were placed in the supine position with the head fixed in a stereotaxic frame (David Kopf Instruments). The trachea and the esophagus were transected in the lower neck and reflected rostrally. The distal trachea was cannulated to facilitate ventilation. After retraction of the bilateral longus capitis muscles, the inferior occipital bone was removed to provide a window (5 × 6 mm) to the surface of the ventral medulla oblongata. After incision and retraction of the dura, the ventral surface of the medulla was kept moist with either aCSF (in mmol/L: NaCl 133.3, KCl 3.4, CaCl2 1.3, MgCl2 1.2, NaH2PO4 0.6, NaHCO3 32.0, and glucose 3.4, pH 7.4) or endogenous cerebrospinal fluid. Paralysis was induced with d-tubocurarine (0.8 mg/kg IV); the tracheal cannula was then connected to a ventilator (model 681D; Harvard Apparatus) and the rats were artificially ventilated at a rate of 60 strokes per minute with a tidal volume of 3.0 mL. The adequacy of anesthesia was verified by the absence of withdrawal response to noxious stimulation of lower extremities before the injection of d-tubocurarine and by the stability of MAP and HR after paralysis had been induced.
**Microinjection Procedure**

Four-barreled micropipettes with tip diameters of 20 to 50 μm and made from calibrated microbore capillary glass tubing (Accu-Fill 90; Clay Adams) were used for microinjection of drugs. Drugs were injected in a volume of 50 nL over a 15-second period with a hand-held syringe. The injection volume was measured by observing the movement of the fluid meniscus along the reticle of a microscope.

The RVLM was identified by injection of L-glutamate (2 nmol/50 nL) on the following criteria: (1) the increase in MAP occurred ≤5 seconds after injection of L-glutamate, (2) the response plateau occurred within 20 seconds of microinjection, and (3) the change in MAP was ≥20 mm Hg. The RVLM injection sites were located 0.6 to 1.0 mm rostral to the most rostral rootlet of the hypoglossal nerve, 1.7 to 1.9 mm lateral to the midline, and 0.5 to 0.8 mm below the ventral surface.

The compounds microinjected into the RVLM were L-NAME (an NOS inhibitor), D-NAME (an inactive isomer of L-NAME), NOC 18 [(C2H5)2N[N(O)NO]Na, which spontaneously releases NO], L-arginine (a precursor of NO), D-arginine (an inactive isomer of L-arginine), and aCSF as a vehicle control. After verifying the RVLM by L-glutamate, only one agent was injected in the unilateral RVLM. After sufficient time for recovery, another injection was similarly performed in the contralateral RVLM. Thus, two different drugs were unilaterally injected in a randomized order in a rat. NOC 18 was obtained from Dojindo Laboratories, L-NAME and D-NAME from Wako, and L-glutamate, L-arginine, and D-arginine from Sigma Chemical Co. NOC 18 was dissolved in 0.1 mol/L NaOH and frozen at −20°C as a stock solution; after the pH of the stock solution was adjusted with 1 mol/L HCl to 7.4, the final solution was prepared by dilution with aCSF. Because NOC 18 releases NO in a linear manner for at least 1 hour, microinjections were performed within 1 hour of preparation of the final solution. All other drugs were dissolved directly in aCSF.

**Histological Analysis**

In some experiments, Alcian blue dye (50 nL) was injected from a separate barrel of the pipette. At the end of these experiments, the rats were deeply anesthetized with pentobarbital sodium (50 mg IV) and perfused transcardially with 150 mL of 0.9% NaCl followed by 150 mL of 10% phosphate-buffered formaldehyde solution. The brain stem was sectioned (50 μm) in the coronal plane and stained with neutral red. Microinjection sites were identified by the deposition of Alcian blue dye and referred to standard anatomic structures of the caudal brain stem according to the atlas of Paxinos and Watson.16

**Statistical Analysis**

Data are presented as mean±SEM and are expressed as the difference from baseline values. One-way ANOVA, followed by multiple comparisons by Dunnett’s multiple range test, was used in the analysis of the dose response to L-arginine in each strain. Differences in dose response between the strain were analyzed by two-way ANOVA. Other statistical analyses were performed with Student’s unpaired t test. A value of P<.05 was considered statistically significant.

**Results**

Baseline MAP was significantly higher in SHR than in WKY (113±2 versus 76±1 mm Hg, P<.01). Baseline HR was similar in both strains (364±5 and 381±6 bpm, respectively). Injection of L-glutamate into the RVLM increased MAP and HR in both SHR (33±1 mm Hg and 27±2 bpm, respectively) and WKY (26±1 mm Hg and 27±2 bpm, respectively). This depressor response was significantly (P<.01) greater in SHR than in WKY.

L-NAME (10 nmol) significantly increased MAP and HR in both SHR and WKY, whereas D-NAME (10 nmol) had no effect on MAP and HR (Fig 1). The pressor responses to L-NAME did not differ significantly between the two strains. Microinjection of L-arginine elicited significant dose-dependent reductions of MAP and HR in both SHR and WKY (Fig 2A and Fig 3). The depressor response to either a low dose (3 nmol) or high dose (10 nmol) of L-arginine was significantly greater in SHR than in WKY. D-Arginine (10 nmol) also reduced MAP and HR in both SHR (−11±3 mm Hg and −18±5 bpm, respectively; n=6) and WKY (−11±2 mm Hg and −16±3 bpm, respectively; n=6). The depressor responses to D-arginine were similar between the two strains. Similar to L-arginine, NOC 18 (10 nmol) significantly reduced MAP and HR in both SHR and WKY, and the depressor and bradycardic responses were significantly greater in SHR than in WKY (Fig 2B and Fig 4).

Histological examination revealed that the injection sites were located in the area that encompassed the dorsolateral aspect of the lateral paragigantocellular nucleus and the region dorsolateral to this nucleus (Fig 5A). This area is located at the caudal end of the facial nucleus. There was no difference in the distribution of injection sites between SHR and WKY (Fig 5B).
Discussion

We have shown that the microinjection of an NO precursor or an NO donor into the RVLM evokes depressor responses that are significantly greater in SHR than in WKY. NO acts as a neuromediator in the central nervous system and plays important roles in a wide variety of processes, including memory, learning and vision. Intravenous administration of NOS inhibitors increases arterial pressure. Because this pressor response is abolished by ganglion blockade or cervical spinal cord transection, NO in the central nervous system appears to play a critical role. However, it remains unclear whether the l-arginine–NO pathway in the central nervous system contributes to the pathogenesis of hypertension. The pressor response to intravenous administration of an NOS inhibitor is smaller in SHR than in WKY. Because the pressor responses to intravenous injection of NOS inhibitors are similar in SHR and WKY subjected to ganglion blockade or in pithed rats, the l-arginine–NO pathway may be impaired in some regions within the central nervous system of SHR. Cabrera et al showed that the depressor response to intracerebroventricular injection of an NO donor was greater, whereas the pressor response to intracerebroventricular injection of an NOS inhibitor was smaller, in stroke-prone SHR than in normotensive control rats. These researchers concluded that a reduced activity of the l-arginine–NO pathway might contribute to the increased arterial pressure of stroke-prone SHR.

The medulla oblongata is a possible site at which the action of NO might be altered in hypertension. NO in the nucleus tractus solitarii and the RVLM plays an important role in cardiovascular regulation. Microinjection of NOS inhibitors into the nucleus tractus solitarii, in which the vagal cardiopulmonary afferent fibers terminate, increased MAP and renal sympathetic nerve activity, whereas NO donors decreased MAP. Microinjection of NOS inhibitors in the RVLM increased MAP and renal sympathetic nerve activity, and these responses were attenuated by l-arginine or NO donors; conversely, l-arginine reduced MAP and renal sympathetic nerve activity, and these responses were counteracted by NOS inhibitors. Thus, NO exerts an inhibitory action on RVLM neurons. In the present study, we also observed an increase in MAP in response to L-NAME, and a decrease in MAP in response to l-arginine or NOC 18, in both SHR and WKY. Whereas L-NAME induced similar...
increases in MAP in SHR and WKY. L-arginine elicited a greater depressor response in SHR than in WKY. The decrease in MAP induced by NOC 18 was also greater in SHR. These observations support the hypothesis that the L-arginine–NO pathway is impaired in the RVLM of SHR. Such a defect in the L-arginine–NO pathway may be attributable to insufficient NOS activity or a deficiency of the substrate, L-arginine. The neuronal NOS gene expression in the RVLM and the NOS activity in the brain stem has been reported to be comparable between SHR and WKY. In contrast, evidence suggests that a deficiency of L-arginine contributes to hypertension. In the present study, the activation of glutamate receptors by microinjection of L-glutamate elicited greater increase in MAP in SHR than in WKY, consistent with our previous observations. The activation of glutamate receptors stimulates NO production by activation of NOS. Given that neuronal NO reduces pressor and sympathoexcitatory responses to L-glutamate, the augmented pressor response to L-glutamate in SHR might be related to the decreased activity of the L-arginine–NO pathway.

The precise cellular mechanism of NO in the RVLM remains unknown. In the paraventricular nucleus, microdialysis of NO-rich aCSF increases local concentrations of inhibitory neurotransmitters, including taurine and GABA, with significant reduction of arterial pressure. A recent report suggests that NO mediates GABAergic inhibition elicited by the activation of N-methyl-D-aspartate receptors and thus contributes to the regulation of neuronal excitability. The GABAergic system tonically inhibits the RVLM neurons and this inhibitory action of GABAergic input to the RVLM has been reported to be diminished in SHR. Thus, we speculate that the effect of NO in the RVLM may be mediated by the release of GABA, and the impaired L-arginine–NO pathway may lead to the diminished GABAergic input to the RVLM, resulting in the elevation of arterial pressure in SHR.

In the present study, not only L-arginine but also D-arginine reduced arterial pressure. D-Arginine is not a substrate for NO formation, but nonspecific actions of arginine, such as vasodilatory and hypotensive effects, have been demonstrated. D-Amino acid is detectable in mammalian brain tissues and the localization of D-serine correlates with N-methyl-D-aspartate receptors in the rat brain. Thus, we may speculate that D-arginine elicits cardiovascular actions as a neuromodulator in the same way as D-serine; in any case, the effect of D-arginine on MAP was similar between SHR and WKY.

In conclusion, the depressor responses to L-arginine or an NO donor injected into the RVLM were greater in SHR than WKY. Thus, a decreased activity of the L-arginine–NO pathway in this brain region may contribute to the increase in arterial pressure in this animal model of genetic hypertension.

References


