Transmission of Arterial Baroreflex Signals Depends on Neuronal Nitric Oxide Synthase Synthase

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Abstract—Because inhibition of neuronal nitric oxide synthase in the nucleus tractus solitarii blocks cardiovascular responses to activation of local glutamate receptors, and because glutamate is a neurotransmitter of baroreceptor afferent nerves, we sought to test the hypothesis that neuronal nitric oxide synthase inhibition would block baroreflex transmission and cause hypertension. We determined reflex heart rate responses to intravenous phenylephrine and sodium nitroprusside in 5 anesthetized rats before and after bilateral microinjection (100 nL) of the neuronal nitric oxide synthase inhibitor AR-R 17477 (7.5 nmol) into the nucleus tractus solitarii. The inhibitor significantly increased mean arterial pressure without affecting heart rate, and it significantly reduced the gain of the baroreflex. After administration of the inhibitor, reflex responses of heart rate to changes in mean arterial pressure were always less than those responses to the same, or less, change in mean arterial pressure in the same animal without administration of the inhibitor. Microinjection of saline (100 nL) bilaterally into the nucleus tractus solitarii did not lead to hypertension or change baroreflex responses. These data support the hypothesis and suggest that neuronal nitric oxide synthase is critical to transmission of baroreflex signals through the nucleus tractus solitarii. (Hypertension. 2004;43:820-824.)

Key Words: baroreceptor reflex • nitric oxide • nitric oxide synthase • rat

As the primary site of termination of baroreceptor and other cardiovascular and visceral afferent nerve fibers,1–3 the nucleus tractus solitarii (NTS) plays a critical role in regulation of arterial pressure (AP) and peripheral blood flow. Stimulation of NTS leads to marked changes in AP and regional blood flow,4 and lesions lead to acute hypertension in humans5 and experimental animals.6 Changes in AP regulation may be persistent with chronic perturbations of chemoreceptor afferents.10 Changes in responsiveness to Glu injected into NTS in hypertensive rats11 suggest that alterations in Glu transmission may play a role in the genesis of some forms of hypertension. Thus, improved understanding of glutamatergic neurotransmission in the NTS in normotensive rats could shed new light not only on basic mechanisms of AP control but also on mechanisms that could contribute to hypertension.

Recent studies indicate that nitric oxide (NO•) may participate in generating responses elicited by Glu. For example, NO• may exert presynaptic and postsynaptic effects and may play a role in cardiovascular regulation at the level of NTS.12 Furthermore, responses to injection of NO• donors into NTS of anesthetized and awake animals mimic those elicited by injection of Glu agonists in anesthetized rats.13–15 However, it is not clear that NO• contributes to cardiovascular signal transduction in NTS16,17 or, if it does, to what extent its contribution is linked to actions of Glu in the nucleus. Garthwaite points to the need for studies to help elucidate the link between Glu and NO• in the brain in general.

We have recently shown that inhibition of neuronal nitric oxide synthase (nNOS) in NTS blocks cardiovascular responses to activation of glutamate receptors19 and that in NTS glutamatergic nerve processes lie closely apposed to neuronal elements that contain nNOS.20 Therefore, through the current study, we sought to test the hypothesis that baroreflex transmission in NTS depends on nNOS whose inhibition would lead to hypertension.

Methods

All methods were reviewed and approved by institutional animal care and use committees of the University of Iowa and the Iowa City VA Medical Center and complied with standards established in the Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). Adult male Sprague-Dawley rats were anesthetized with halothane (2%) delivered by inhalation with 100% oxygen, and they were instrumented with cannulae placed in a femoral artery and vein for recording AP and heart rate (HR) and for delivering drugs intravenously. The trachea was cannulated and the animals were artificially ventilated for the remainder of the experiments. As we have previously described,14 we then exposed the dorsal surface of the brain stem. After completion of this instrumentation, we discontinued halothane and maintained anesthesia with chloralose (20 mg/kg per hour after a loading dose of 40 mg/kg IV) for the remainder of the study. In 5 animals, we evaluated barore-
flexes before and after inhibition of nNOS. The baroreflex was tested before administration of the nNOS inhibitor and again 10 minutes after administration of the selective nNOS inhibitor AR-R 17477a (7.5 nmol; a gift from Astra Zeneca, Worcester, Mass) through glass micropipettes placed stereotaxically at predetermined14 coordinates in the nucleus. Baroreflex testing was begun while AP was still elevated from nNOS inhibition but after maximal elevation of AP. Testing was completed over the next 50 minutes. We confirmed that blockade by AR-R17477 of cardiovascular responses to injection of NMDA (3 pmol) into the NTS, as we have previously reported,22 persisted for 1 hour after injection of the inhibitor. We determined reflex decreases in HR in response to pressor effects of phenylephrine (0.05 to 2.5 μg in 0.1 to 5 μL of saline intravenous) and reflex tachycardic responses to depressor effects of nitroprusside (0.1 to 5 μg in 0.1 to 5 μL of saline intravenous). Each animal received multiple doses of each agent. Doses were delivered in random order and the 2 agents were randomly administered. Responses of mean AP (MAP) and HR to each agent were allowed to return to baseline before administration of the next dose. After quantifying changes in MAP and HR before and after inhibition of nNOS in NTS, we statistically analyzed only baroreflex responses before injections into NTS with those after injection of AR-R 17477 in the same 5 animals. In those animals, we analyzed baroreflex responses by subjecting all data from each animal to regression analysis (see later) to determine if nNOS inhibition significantly altered the gain of baroreflex responses. In 3 additional animals, we compared baroreflex responses before and after injection of saline bilaterally into NTS as a sham inhibitor of nNOS. Injection of saline into NTS did not affect baroreflex responses in those 3 animals.

Data, expressed as mean±SEM, were analyzed by paired t test. Random coefficient regression analysis was used to estimate the mean linear regression function for the relationship of change in HR with change in MAP for the control condition and the treated condition, and to compare the mean slope between control and treatment. A line was fit using data points derived for each animal, and the mean regression line was estimated from the individual lines. Changes were considered statistically significant at P<0.05.

Results
Bilateral injection of the nNOS inhibitor (Figure 1) increased MAP from 100.8±5.4 mm Hg to 145.2±5.3 mm Hg (n=5; P<0.001) without significantly changing HR (before blockade 341.4±9.6 bpm; after blockade 357.4±14.6 bpm; P=0.154). Microinjection of saline (100 nL) bilaterally into NTS of 3 animals did not lead to hypertension (data not shown).

We determined reflex bradycardic responses to pressor effects of intravenous phenylephrine and reflex tachycardic responses to depressor effects of intravenous sodium nitroprusside before and after bilateral microinjection (100 nL) of AR-R 17477 (Figure 2) into NTS in 5 animals or saline into NTS in 3 animals. Bilateral injections of saline into NTS did not change baroreflex responses when compared with responses in animals that had received no injection into NTS (see Methods; data not shown). In animals that had received bilateral injections of AR-R 17477, we began tests of baroreflex function before MAP had returned to basal values, but MAP gradually returned toward baseline in each animal before completion of testing. However, regardless of the level of MAP before administration of each dose of phenylephrine or nitroprusside, HR responses to comparable changes in MAP were always less after administration of AR-R17477 than they had been before administration of AR-R17477 into the NTS. Therefore, regression analysis (Figure 3) revealed a significant reduction in the gain of responses after nNOS inhibition (R=≈0.1; P<0.05) versus control (R=≈0.9).

Discussion
This study demonstrates that injection of an inhibitor of nNOS bilaterally into the NTS attenuates baroreflex responses to changes in blood pressure and leads to acute...
neurogenic hypertension without associated changes in HR, findings that have long been known to occur after acute interruption of the baroreflex by disturbances of NTS function.5,23 Thus, the data support the hypothesis. They suggest that nNOS is critical to normal transmission of baroreflex signals through NTS and participates in tonic regulation of blood pressure by NTS.6 The data support our earlier studies19 that demonstrated attenuation of responses to Glu agonists injected into the NTS. Because Glu is a neurotransmitter released in NTS on stimulation of the arterial baroreflex, attenuation of that reflex by nNOS inhibition would be consistent with a role for nNOS in mediating baroreflex responses through Glu receptors in the NTS.

Evidence for potential interactions between Glu and NO− is abundant. Activation of Glu receptors in brain leads to synthesis and release of NO−.24,25 Glu may act through these effects on NO− to activate sGC and increase cGMP, which may contribute to cellular responses to Glu itself.26,27 Some studies have shown that destruction of Glu receptors eliminated activation of sGC by Glu, even though the same neuronal pools of the enzyme could still be activated by an NO− donor acting “downstream” of the Glu receptor.26 Thus, some responses to Glu may depend on a link to NO− synthesis. Although influences of NMDA receptor activation on NO− production were described first, it is now clear that kainate, metabotropic (ACPD responsive), and α-amino-3-hydroxy-5-methylisoxazole-propionic acid receptor agonists have similar influences on production of NO−.26–31 However, antagonists of Glu receptors may themselves effect release of NO−;32 and in some systems NO−, which may act presynaptically and postsynaptically,33,34 may provide a feedback mechanism influencing release of Glu.35 Little is known of the physiological significance of joint actions of Glu and NO− in NTS, but one study suggests that NO− release also may be linked to Glu receptor activation in that nucleus.36

The current study supports others that have begun to show that NO−, like Glu, may participate in cardiovascular signal transduction in NTS. For example, NOS is transported bidirectionally from nodose ganglion neurons37 and is found in neurons and vagus nerve terminals in NTS.38–42 Staining for nNOS and nNOS mRNA is greatest in cardiovascular and gastrointestinal subnuclei of NTS,40 and NO− may be synthesized in vagus nerve terminals in NTS.40,41 Furthermore, we have found that nNOS immunoreactivity and Glu immunoreactivity, a marker of glutamatergic neurons, are colociliated in NTS dendrites and axons.43

Abundant evidence suggests that nitrooxidergic input to NTS may play a role in physiological control by that nucleus. Diminished neuronal activity as a result of nNOS inhibition suggests that NO− released from vagus nerve terminals may tonically influence activity of neurons in NTS.44 Injection of an nNOS inhibitor 7-nitroindazole (7-NI) into NTS has been shown45 to block Glu. Furthermore, microinjection into NTS of S-nitrosothiols, which may act as donors of NO−, elicits depressor and bradycardic responses like those produced by Glu.14 Actions of S-nitrosothiols, like those produced by NO− may, at least in part, be mediated through activation of sGC in that effects of the S-nitrosothiols are blocked by methylene blue,46 used to block activation of sGC by NO−.24 In addition, methylene blue inhibits actions of inotropic, but not metabotropic, Glu agonists.19 The biological relevance of this blockade has been supported by studies showing that methylene blue also blocks the Bezold Jarisch reflex.47 Other studies of a more selective blocker 1H-[1.2.4]oxadiazolo[4,3-c]-alquninoxalin-I-one (ODQ) of guanylate cyclase48,49 support earlier findings with methylene blue.

The similarity between physiological responses to Glu and NO− and in the distribution of Glu and NO− terminals in NTS suggests a possible link between the actions of the 2 compounds. One study even suggested that the 2 may act on the same neurons in NTS.12 We have found a high degree of homology in the immunostaining for nNOS and for Glu in the cardiovascular NTS,43 as well as for nNOS and NMDA receptors50 and for nNOS and α-amino-3-hydroxy-5-methylisoxazole-propionic acid receptors51 in that nucleus.

Previous studies have used other NOS inhibitors in their efforts to define the role of NOS in central cardiovascular transmission through the NTS. Nonselective inhibitors injected into NTS have not consistently attenuated the baroreflex.16,45 Such nonselective inhibitors would be expected to inhibit both eNOS and nNOS. In that eNOS may contribute to inhibition of baroreflex activity in NTS,52 the inconsistent findings could have been the result of action on nNOS and eNOS. Others have sought to selectively inhibit nNOS by injection of 7-NI into the NTS and have demonstrated attenuation of baroreflex responses to changes in blood pressure.53 However, some evidence suggests that 7-NI, a
highly hydrophobic compound, is not selective for nNOS under some conditions.\textsuperscript{54,55} Although there are other studies that support selectivity of 7-NI under a variety of conditions,\textsuperscript{56} we chose to use an agent, AR-R 17477, with acknowledged selectivity for nNOS and relatively little action on eNOS.\textsuperscript{21} The inhibitor was injected into NTS and baroreflex studies performed over approximately 1 hour, a period of time over which we have shown that responses to Glu agonists are blocked by AR-R 17477. Thus, although not directly studied here, blockade of the baroreflex with nNOS inhibition correlates temporally with blockade of responses to Glu agonists in NTS and supports a link between these 2 transmitter mechanisms in central baroreflex control within NTS.

**Perspectives**

This study has implications for understanding central cardiovascular reflex control in health and disease. The findings provide another example of chemical, not structural, changes in brain leading to hypertension, and they support the possibility that disturbed NO\textsuperscript{+} synthesis could contribute to hypertension and altered baroreflex function in humans. Identifying that nitroxidergic transmission may contribute to transduction of naturally occurring baroreflex signals through NTS provides a potentially important insight into integrative and transduction mechanisms not only in NTS but also in other central sites.

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**References**


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