Endothelial NO Synthase Activity in Nucleus Tractus Solitarii Contributes to Hypertension in Spontaneously Hypertensive Rats

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Abstract—NO is implicated as a major modulator of central nervous circuits regulating cardiovascular activity. Based on previous data, we hypothesized that overactivity of endothelial NO synthase (eNOS) within the nucleus tractus solitarii (NTS) could contribute to the hypertension in the spontaneously hypertensive rat (SHR). Using real-time PCR, we found that endogenous eNOS mRNA was greater in the NTS of mature, but not juvenile prehypertensive SHRs compared with aged-matched Wistar Kyoto (WKY) rats. To test the functional significance of this, we chronically blocked eNOS activity in the NTS in the adult SHR by in vivo adenoviral-mediated gene transfer of a dominant-negative form of eNOS; data were compared with WKY rats. This resulted in a fall in arterial pressure in the SHR but not WKY rats. In both rat strains, cardiac baroreceptor reflex gain and the high-frequency spectral component of heart rate variability increased. Thus, endogenous eNOS activity in the NTS plays a major role in determining the set point of arterial pressure in the SHR and contributes to maintaining high arterial blood pressure in this animal model of human hypertension. (Hypertension. 2006;48:644-650.)

Key Words: nitric oxide synthase ■ nitric oxide ■ endothelium ■ blood pressure ■ baroreflex ■ sympathetic nervous system ■ neurogenic hypertension

As described by Doba and Reis,1 destruction of the nucleus tractus solitarii (NTS) leads to fulminating hypertension. This is highly suggestive that the NTS is a central brain stem structure that plays a vital role in maintaining the set point of arterial pressure. Equally, because baroreceptor afferents terminate in this nucleus, it is also one of the most effective central sites for modulating the gain of the baroreceptor reflex, a process that is critically important for blood pressure homeostasis. A potent neuromodulator of central cardiovascular autonomic activity is angiotensin II (Ang II). NTS microinjections of Ang II evoke concentration-dependent effects composed of either a depressor response2,3 or a pressor/tachycardic response.4 In addition, an Ang II antagonist increased cardiac baroreceptor reflex gain in anesthetized mature rats.5 Because of the work of Casto and Philips,6 it has been known that Ang II acting on Ang II type 1 receptors depresses cardiac baroreceptor reflex function in NTS. We showed that the depressant effect of Ang II type 1 receptor stimulation on cardiac baroreceptor reflex function in the NTS was mediated by NO.7 We confirmed recently that Ang II can release NO in the NTS using electron paramagnetic resonance.8 We also found that the effect of Ang II occurs via a Gq-PLC-IP3 pathway that releases intracellular calcium,9 which we assume is required for activating endothelial NO synthase (eNOS). Released NO enhances inhibitory GABAergic neurotransmission in the NTS,10 which may underlie the NO-induced cardiac baroreceptor reflex depression.7

In conscious normotensive Wistar rats, chronic blockade of eNOS activity in NTS elevated the cardiac baroreceptor reflex gain, supporting a depressant action of NO on baroreceptor reflex function.11,12 Based on this evidence, we hypothesized that NO in the NTS could contribute to the depressed baroreceptor reflex gain and hypertension that are both prevalent in the spontaneously hypertensive rat (SHR). We used an adenovirus to chronically express a dominant-negative protein to block eNOS activity in the NTS13; this protein is called truncated eNOS (TeNOS).14 Our findings indicate that endogenous eNOS in the NTS of the SHR is important for its hypertensive phenotype.

Methods

Procedures were carried out according to the United Kingdom Home Office Guidelines on Animals (Scientific Procedures) Act 1986. The animals were housed individually, allowed normal rat chow and drinking water ad libitum, and kept on a 12-hour light/12-hour dark cycle.
Measurement of Endogenous eNOS mRNA in the NTS

Extraction of RNA From the NTS

Three- and 15-week-old male rats were humanely killed by cervical dislocation. The NTS was rapidly microdissected out from each animal and homogenized in 400 μL of TRIzol reagent (Invitrogen). To avoid contamination with genomic DNA, the RNA samples were treated with RNase-free DNase I (Roche Diagnostics GmbH). RNA purity was verified by performing PCR on samples not treated with reverse transcriptase (RT).

Quantitative RT-PCR

The following primer sequences were used: β-actin (NM_031144), forward (CGTGCAATCCGTAAAGACC), reverse (GTAATCCTGCTTGATCCAC); and eNOS (AF085195), forward (AAGTGGGCAGCATCACCTAC), reverse (CTGGGAAACCCTCTTITGA). The sizes of the PCR products amplified with these primer pairs is comparable (data not shown). Real-time RT-PCR reactions were carried out using a DNA Engine Opticon 2 system (MJ Research). The QuantiTect SYBR Green RT-PCR kit (Qiagen) was used according to the manufacturer’s protocol. Two microliters of diluted RNA as a template were mixed with 1 μmol/L of each primer, 10 μL of 2× QuantiTect SYBR Green RT-PCR Master Mix, and 0.2 μL of QuantiTect RT mix (RT) and brought to 20 μL of total reaction volume using RNase-free water. The reactions were incubated at 50°C for 30 minutes for reverse transcription and then for 15 minutes at 95°C to inactivate RT and activate the HotStarTaq DNA Polymerase, followed by 50 cycles with 15-s denaturation at 94°C, 30-s annealing at 56°C, and 30-s extension at 72°C. After each extension step, the fluorescence signal was measured. After 50 cycles, a melting curve for each product was obtained for quality control.

In Vivo Gene Transfer Into NTS

Ad-CMV-TeNOS (1.5×10⁹ pfu/mL) is a replication-deficient recombinant adenoviral vector driving the expression of TeNOS under the constitutive control of the human cytomegalovirus (hCMV) promoter enhancer. TeNOS lacks catalytic activity yet retains the NH₂-terminal sequences required for cotranslational NH₂-terminal glycine myristoylation and membrane localization and acts as a dominant-negative inhibitor of wild-type eNOS activity through heterodimerization with the native protein to form nonfunctional dimers. As a control, Ad-CMV-eGFP (7.8×10⁹ plaque-forming units (pfu)/mL) expressing enhanced green fluorescent protein (eGFP) was used. Seven days after the implantation, animals were reanesthetized (as described above). Five 100-nL bilateral microinjections of viral suspension (either Ad-CMV-TeNOS or Ad-CMV-eGFP; injection speed, 1 minute per spot) were made into the NTS at separate sites spanning ±500 μm rostral/caudal to the calamus scriptorius and 350 to 700 μm from midline and 500- to 600-μm below the dorsal surface of the medulla. To avoid postsurgical cardiovascular effects, animals were allowed 7 days to recover before blood pressure data were sampled.

On these days, arterial pressure was measured for 30 minutes continuously between 9:30 and 10:00 AM (light phase) when animals were generally quiescent in their home cages. Heart rate (HR) was derived from the interpulse interval. Averaged systolic blood pressure (SBP), HR, spontaneous baroreceptor reflex gain (sBRG), and the variability of both HR and SBP were calculated.

Evaluation of sBRG and Variability of HR and SBP

sBRG

A computer-based data acquisition system (Maclab/8s, AD Instruments and PowerBook 3400c, Apple Computer Inc) was used to acquire, display, store, and analyze the telemetered data. The cardiac baroreceptor reflex gain was determined from spontaneous changes in SBP and pulse interval as described previously.

HR and Blood Pressure Variability

These were analyzed using the maximum-entropy method with high resolution (MemCalc, Suwa Trust). According to Murasato et al, the magnitude of power was integrated in both the low-frequency (LF) band between 0.27 and 0.75 Hz and the high-frequency (HF) band (0.75 to 3.3 Hz). We assumed that the HF component of power spectral density in HR variability is mediated by cardiac parasympathetic tone, whereas the ratio of LF/HF of HR variability is an index of cardiac sympathetic tone. Similarly, the LF power in the spectral density of arterial pressure variability reflects vasomotor sympathetic tone.

Immunohistochemistry to Visualize Adenoviral-Mediated Expression of TeNOS

To visualize expression of TeNOS after adenoviral-mediated gene transfer within NTS, immunohistochemical detection of eNOS was performed as described previously. We confirmed previously that eNOS antibody, sc-8311 (Santa Cruz Biotechnology), efficiently detects TeNOS expression compared with endogenous eNOS. This is based on the evidence that the Sc-8311 binding epitope is preserved in TeNOS (ie, NH₂-terminal side). The immunoreactivity in TeNOS-transfected rats compared with eGFP-transfected controls.

Data Analysis

Group data were expressed as mean±SEM. To evaluate time-dependent changes of cardiovascular variables by expressing TeNOS within NTS, we used repeated-measures ANOVA and the Bonferroni test for multiple comparisons of cardiovascular variables across time and between different groups. To evaluate the differences of eNOS mRNA level between rat strains, an unpaired t test was used. Differences were considered significant if P<0.05.

Results

Comparison of Levels of eNOS mRNA in the NTS of WKY Rats (n=6) and SHRs (n=6)

According to the real-time RT-PCR, the level of eNOS gene expression in the NTS of the adult SHR was significantly higher than that of adult WKY rats (1.01±0.22 versus 2.35±0.26; n=6 for each rat strain; P<0.01; Figure 1). In contrast, there was no difference in the level of eNOS gene expression between hypertensive SHRs (1.33±0.18; n=6) and age-matched WKY rats (1.05±0.14; n=6; P>0.1).

Effects of Chronic Inhibition of eNOS Activity in the NTS on Arterial Pressure, HR, and sBRG in WKY Rats (n=6) and SHRs (n=6)

Before viral injection, baseline levels of SBP, HR, and sBRG in the TeNOS-SHR group were 154±3 mm Hg, 326±11
Effects of Chronic Inhibition of eNOS Activity in the NTS on HR and SBP Variability in WKY Rats (n=6) and SHRs (n=6)

Before NTS viral injection, HF HR, LF/HF HR, and LF SBP in the TeNOS-SHR group were 11.1±1.3 bpm, 0.26±0.04, and 1.78±0.24 mm Hg, respectively. These values were not different from the eGFP-SHR group (HF HR: 11.4±1.4 bpm; LF/HF HR: 0.26±0.04; LF SBP: 1.90±0.25 mm Hg). LF SBP in both SHR groups was significantly higher than the WKY group (1.05±0.13 mm Hg; Figure 3; P<0.05), whereas there were no significant differences in HR variability between SHR and WKY groups (HF HR: 10.8±1.4 bpm; LF/HF HR: 0.39±0.03; Figure 3). Seven days after viral transfection, in both TeNOS-SHR and WKY groups, HF HR was significantly increased (SHR: 27.3±4.7 bpm; P<0.01; WKY: 20.7±3.0 bpm; P<0.01; Figure 3), whereas no changes were found in LF/HF HR (SHR: 0.33±0.06; WKY: 0.43±0.06) compared with baseline values (Figure 3). For LF SBP, only the TeNOS-SHR group showed a significant decrease after viral transfection (from 1.78±0.24 mm Hg to 0.75±0.10 mm Hg; P<0.05; Figure 3). These changes returned to pretransfection levels 14 days after viral transfection. No significant changes were observed in the eGFP-SHR group over the entire period (Figure 3).

Immunocytochemical Verification and Longevity of Adenoviral-Mediated Expression of TeNOS in the NTS in WKY Rats (n=6) and SHRs (n=6)

In the TeNOS-transduced rats (WKY and SHR), numerous NTS cells, including glia and blood vessels, were immunopositive for eNOS at day 7 after transduction (Figure 4). By contrast, much less eNOS immunoreactivity was found in eGFP-transduced rats, but there was significant expression of eGFP in the NTS (data not shown). In all of the cases, we found no obvious differences in immunoreactivity or eGFP expression between rat strains. In a separate group of rats, we found that by day 14 after transduction with AdV-CMV-TeNOS, there was little eNOS immunoreactivity (Figure 4), which correlated well with the return of the measured cardiovascular variables to control levels.

Location of NTS Sites Transduced With Ad-CMV-TeNOS and Ad-CMV-eGFP
Based on the localization of eNOS immunoreactivity and eGFP expression, highly comparable regions were transduced with both viruses (Ad-CMV-TeNOS and Ad-CMV-eGFP) and consistent between WKY and SHR. These regions spanned −13.8 to −14.6 mm relative to Bregma (ie, 400 μm rostral to Bregma) and consistent among rats of both strains.
and caudal to calamus scriptorius), 350 to 700 μm from midline and 500 to 800 μm below the dorsal surface of the medulla (Figure 4).

Discussion
The present study has revealed a number of novel findings. First, we found that eNOS mRNA is upregulated in the NTS of the adult SHR compared with age-matched WKY rats. Second, eNOS within the NTS of both WKY and SHR is constitutively active and modulates central cardiovascular autonomic activity. Third, in both rat strains, chronic inhibition of eNOS increases both the sBRG and HF component of HR variability and lowers HR. The WKY rat data are consistent with our earlier findings in the Wistar rat.11 Finally, in the SHR, there was a substantial reduction in arterial pressure (16 mm Hg), which was not evident in the WKY rat. All of the responses returned to control levels 2 weeks after viral transduction, which is comparable with the transgene expression profile mediated by adenovirus in the NTS as reported by others.25

Figure 3. Comparison of effects of disabling eNOS activity in NTS on the cardiovascular system in SHRs and WKY rats. In SHRs, SBP and LF of BP variability decreased. In both SHRs and WKY rats, the sBRG and HF component of HR variability increased, whereas HR decreased. The ratio of LF/HF of HR variability (which could reflect preferentially cardiac sympathetic tone) was unaltered. *P<0.05, **P<0.01, and ***P<0.001 vs before and after gene transfer. +P<0.05, ++P<0.001 vs WKY.

Figure 4. Transverse sections through the medulla indicating the extent of transgene expression within the NTS of a representative SHR. The photomicrograph shows the enhanced eNOS immunoreactivity in an SHR that received an NTS injection of Ad-CMV-TeNOS (viral titer: 1.5×109 pfu/mL) compared with a control animal (bottom right). AP indicates area postrema; DVN, dorsal vagal motor nucleus; CC, central canal.
Possible Mechanisms Underpinning the Changes in Cardiovascular Variables After eNOS Blockade in the NTS of WKY Rats and SHRs

There were similarities and differences in the response evoked by NTS eNOS blockade in the WKY and SHR. In both rat strains, a bradycardia and an elevation of the HF of HR variability suggested an enhanced vagal tone.22 This was paralleled by an increase in sBRG. Because the baroreceptor reflex provides a major excitatory drive to cardiac vagal motoneurones,26 the enhanced sBRG may contribute to the bradycardia observed. Based on previous data from our laboratory, we propose that the increase in sBRG after eNOS blockade may relate to a reduction in GABA<sub>A</sub> receptor-mediated synaptic inhibition. This is based on the following findings: (1) microinjection of either l-arginine or an NO donor into the NTS depressed the cardiac component of the baroreceptor flex,27 and this can be blocked by bicusculine, a GABA<sub>A</sub> receptor blocker<sup>28</sup>; (2) NO enhanced inhibitory neuronal activity of NTS neurones,28 which was abolished by bicuculline.29 The NO-induced release of GABA has been demonstrated in the ventrolateral medulla<sup>30</sup> and hypothalamus.31,32

By comparison, eNOS blockade in the NTS of the SHR reduced arterial pressure but was without effect on this variable in the WKY rat. The evoked depressor response in the SHR was associated with a reduction in the LF of SBP, suggesting a reduction in sympathetic vasoconstrictor tone.23,24 We confirmed that lability of blood pressure (ie, mean values of SD in SBP) was also decreased after eNOS blockade in the SHR (before: 5.62 ± 0.85 versus 7 days after: 5.64 ± 0.59; P < 0.05), whereas no changes were found in SHRs.25,56 Although no data were reported on the activity of either nNOS and/or iNOS, but this remains to be determined.

How Is the NTS of the SHR Different From the WKY Rat?

We found that eNOS mRNA is upregulated in the SHR NTS compared with WKY rat but in juvenile SHRs, eNOS mRNA was not different from age-matched WKY rats. Thus, the heightened eNOS in the adult SHR NTS may be secondary to the hypertension. Although we concede that differences in eNOS mRNA might not translate necessarily into either increased protein levels or eNOS bioactivity, our in vivo data unequivocally demonstrate a functional difference in eNOS activity between the 2 rat strains, and eNOS activity is important for maintaining arterial pressure at a high level in SHRs. It is unclear whether the elevated eNOS activity in the NTS of the SHR is only because of its higher concentration, or there is also a change in the mechanisms of eNOS activation leading to a higher rate of NO release. We propose that in the NTS of the SHR, NO production is enhanced and that different actions occur because of a concentration dependency of its effects and increased effective spread stimulating additional targets. By reaction with superoxide, high NO concentrations can lead to production of peroxynitrite, which can affect neurotransmission independently of NO.37

Diversity of Effects of Nitrergic Mechanisms in the NTS for Cardiovascular Control

Previous acute studies in normotensive animals have blocked NO, used antisense against neuronal NOS (nNOS), or trapped NO in the NTS and shown either no change38,39 or an increase in arterial pressure.40,41 Data are also inconsistent when either NO donors or l-arginine are microinjected into the NTS (no change<sup>40,42</sup> or decreased<sup>43–49</sup>). Previous studies describing the effects of NOS inhibitors in the NTS on baroreceptor reflex gain are also inconsistent (no change,40,49 increased,<sup>38</sup> or decreased<sup>38,50,51</sup>). It should be noted that both nNOS and eNOS isoforms are found in the NTS, and both neurones and fibers, some of which are vagal afferents, contain nNOS.<sup>52–54</sup> Thus, microinjection or viral gene transfer approaches to release NO<sup>55,56</sup> will not discriminate between these different cellular compartments. Further, these methods cannot be regarded as the opposite of blocking NO, because the resultant NO concentration, its degree of spread, and additional targets stimulated will not reflect native conditions. Specific NOS isoform blockade, either pharmacologically or by viral gene transfer, may allow a more meaningful interpretation of data than applying l-arginine, NO donors, or overexpressing an NO isoform.37 Blocking an isoform-specific NOS will reduce endogenous NO production, thereby respecting the subtleties that may permit NO to achieve specific actions.38 One caveat is that blocking endogenous eNOS might cause compensatory alteration in the activity of either nNOS and/or iNOS, but this remains to be determined.

eNOS has been overexpressed chronically in the NTS using an adenovirus in conscious telemeterized WKY rats and SHRs.25,55,56 Although no data were reported on the baroreceptor reflex gain, a bradycardia and a depressor response were observed, with the largest response seen in the SHR. In addition, Tai et al<sup>38</sup> reported a rebound pressor response occurring ≈ 3 weeks after a depressor effect after adenovirus-mediated overexpression of eNOS in the NTS. The reason for this was not explained but is unlikely to be attributable to the presence of the transgene. The depressor effect in the SHR after eNOS overexpression is in stark contrast to the present study. The reasons for these differences are unclear but probably relate to the differences between NOs overexpression versus selective antagonism of an NO isoform described above.

In some NTS neurones, nNOS colocalizes with the vesicular glutamate transporter 2.70 Moreover, exogenous NO in the NTS enhances both glutamate release in vivo<sup>60</sup> and evoked excitatory postsynaptic potentials in vitro,<sup>61</sup> and both of the latter may explain the bradycardia/depressor responses (for references see above) and depressed baroreceptor reflex gain after nNOS blockade.<sup>38,51</sup> In addition, Dias et al<sup>38</sup> have
described that Nω-nitro-L-arginine methyl ester blockade of NOS in the NTS reduces the depressor response evoked by N-methyl-D-aspartate. Considering a positive interaction between NO and glutamatergic transmission in the NTS in the regulation of arterial pressure, the question arises as to why selective eNOS antagonism seems to produce cardiovascular effects (bradycardia, depressor, and increased sBRG in SHR) that are opposite to those produced using eNOS overexpression or pharmacological antagonism of eNOS/nNOS or nNOS. Perhaps endogenous eNOS affects GABAergic transmission preferentially, whereas endogenous nNOS modulates glutamatergic mechanisms as suggested by Kano et al.62 We believe that the effects of Ad-CMV-TeNOS in this study result from the blockade of eNOS residing in the endothelium. First, we have described recently that adenoviruses with hCMV promoter drive extremely low levels of gene expression in most types of NTS neurones.14 Second, these vectors clearly can transduce at least some of the local vasculature (Figure I, available online).7,18 Third, our data support that the bulk of eNOS in the NTS is localized to the endothelium (Figure I), whereas eNOS expression in glia has never been demonstrated in any recent study.

In conclusion, using a highly selective genetic tool, we have shown that endogenous eNOS activity in the NTS of the SHR is raised relative to the WKY rat, and plays a major role in the maintenance of the hypertension and reduced cardiac baroreceptor reflex gain that are characteristic of this animal model.

Perspectives
We described recently a novel form of communication within brain stem circuits controlling arterial pressure, this being vascular-neuronal signaling.38 We hypothesized that NO released from the endothelium of the vessel wall can diffuse to modulate synaptic transmission; in the NTS, we have shown that NO enhances GABAergic transmission via soluble guanylyl cyclase.8 Enhanced GABAergic drive in the NTS has multiple actions: it depresses baroreflex and enhances arterial pressure. We suggest that this NO mechanism is part of the homeostatic processes regulating arterial pressure in normotensive rats. It is likely that NO release and its concentration within NTS are dynamic and, for example, dependent on blood flow through this nucleus. However, in the SHR, we have described a pathological role for eNOS-generated NO. Normally, NO production from the endothelium is not thought of as pathological; rather, it provides a protective role. We raise the issue that too much eNOS-generated NO might be deleterious in the brain, especially in an environment of oxidative and nitrative stress causing peroxynitrite to form, the latter being neurotoxic. We now need to address the question of downstream molecules mediating the effects of NO from eNOS in the SHR, which are responsible for its pathological actions on baroreflex and arterial pressure. The issue of why eNOS is elevated in the SHR raises an intriguing question as to what else is different in the SHR brain stem that contributes to its hypertensive state. Also, can these changes be found in human NTS from deceased subjects with essential hypertension?

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Disclosures
None.

References
20. Oosting J, Struijker-Boudier HAJ, Janssen BJA. Validation of a continuous baroreceptor reflex sensitivity index calculated from spontaneous
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