NO Differentially Regulates Neurotransmission to Premotor Cardiac Vagal Neurons in the Nucleus Ambiguus

Harriet Kamendi, Olga Dergacheva, Xin Wang, Zheng-Gui Huang, Evgenia Bouairi, David Mendelowitz

Abstract—NO is involved in the neural control of heart rate, and NO synthase expressing neurons and terminals have been localized in the nucleus ambiguus where parasympathetic cardiac vagal preganglionic neurons are located; however, little is known about the mechanisms by which NO alters the activity of premotor cardiac vagal neurons. This study examines whether the NO donor sodium nitroprusside ([SNP] 100 μmol/L) and precursor, L-arginine (10 mmol/L), modulate excitatory and inhibitory synaptic neurotransmission to cardiac vagal preganglionic neurons. Glutamatergic, GABAergic, and glycineergic activity to cardiac vagal neurons was examined using whole-cell patch-clamp recordings in an in vitro brain slice preparation in rats. Both SNP, as well as L-arginine, increased the frequency of GABAergic neurotransmission to cardiac vagal preganglionic neurons but decreased the amplitude of GABAergic inhibitory postsynaptic currents. In contrast, both L-arginine and SNP inhibited the frequency of glutamatergic and glycineergic synaptic events in cardiac vagal preganglionic neurons. SNP and L-arginine also decreased glycineergic inhibitory postsynaptic current amplitude, and this response persisted in the presence of tetrodotoxin. Inclusion of the NO synthase inhibitor 7-nitroindazole (100 μmol/L) prevented the L-arginine–evoked responses. These results demonstrate that NO differentially regulates excitatory and inhibitory neurotransmission, facilitating GABAergic and diminishing glutamatergic and glycineergic neurotransmission to cardiac vagal neurons. (Hypertension. 2006;48:1137-1142.)

Key Words: nitric oxide ■ parasympathetic ■ vagal ■ ambiguous ■ cardiac ■ brain stem

NO, a gaseous neuromodulator, plays an important role in the central nervous system control of blood pressure and heart rate. In the brain stem, NO is localized to several medullary nuclei involved in cardiorespiratory and gastrointestinal function.1,2 Neurons that contain the NO synthesizing enzyme, NO synthase (NOS), have been observed in many critical sites of central cardiovascular regulation, including the dorsal motor nucleus of the vagus, rostral ventrolateral medulla, caudal ventrolateral medulla, nucleus of the tractus solitarius (NTS), and the nucleus ambiguus (NA).3 In the NA, NO is present in many of the terminal fields that surround cardiac vagal preganglionic neurons (CVPNs), including synaptic terminals from neurons that originate in the NTS.4 CVPNs are primarily located within the external formation of the NA.5-9 The CVPNs provide parasympathetic innervation to the heart and dominate control of the heart rate. CVPNs are intrinsically silent, and their activity is dominated by synaptic activation from other neurons that use neurotransmitters, such as acetylcholine, glutamate, GABA, and glycine, among others.5-8-12 NOS immunoreactivity is not directly colocalized with cholinergic neurons in the brain stem but rather is in small neurons, suggesting that NO is present in interneurons.13,14 This indicates that NO may exert its actions by modulating synaptic inputs to CVPNs. Microinjection of NO into the NA elicits decreases in heart rate, which are reversed by microinjections of Nω-nitro-L-arginine methyl ester, an NOS inhibitor.15 However, there is little known about the mechanisms by which NO alters parasympathetic cardioinhibitory activity in the brain stem. This study tests the hypotheses that NO modulates 3 critical synaptic inputs: glutamatergic, GABAergic, and glycineergic neurotransmission to CVPNs.

Methods

Slice Preparation

In an initial surgery, Sprague–Dawley rats (postnatal: days 2 to 6) were anesthetized with ketamine/xylazine and exposed to hypothermia during surgery (10 to 20 minutes) to slow the heart and aid in recovery. A right thoracotomy was used to expose the heart; rhodamine (XRITC, Molecular Probes, 2%, 20 to 40 μL) was injected into the pericardial sac to retrogradely label CVPNs. Specificity of the cardiac vagal labeling is confirmed by the absence of any labeled neurons in the brain stem when rhodamine is injected into the chest cavity while keeping the pericardial sac intact or when the injection into the pericardial sac is accompanied by section of the cardiac branch of the vagus nerve (n=4). In other control experiments (n=10), intravenous injection of ≤10 ng of rhodamine failed to label any neurons in the medulla except for rare labeling of neurons in the area postrema, an area with a deficient blood–brain barrier. Although a recent report16 suggests that there can be a lack

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of specificity using fluorescent tracers different from the one used in this study, Grkovic et al. injected tracers into the cardiac sac via an incision in the abdominal cavity and through the diaphragm, procedures that also greatly increase the probability of erroneous labeling.

On the day of the experiment (1 to 3 days after injection of the fluorescent tracer), the animals were anesthetized with halothane and euthanized by cervical dislocation. The brain was rapidly removed and placed in cold HEPES buffer (4°C) of the following composition (in mmol/L): NaCl (140), KCl (5), CaCl2 (1.5), MgCl2 (4), EGTA (2), Na-ATP (2), QX-314 (5), HEPES (10), oxygenated with 95% O2/5% CO2 gas mixture. Previous work has shown that the O2 tension within the tissue slice would be ~600 mm Hg at the surface and 200 mm Hg at the core of the tissue under these conditions. The osmolarity of both solutions was 285 to 290 mOsm.

Electrophysiological Recording

Individual cardiac vagal neurons were identified by the presence of the fluorescent tracer and imaged with differential contrast optics, infrared illumination, and infrared-sensitive video detection cameras to gain better spatial resolution and to visually guide and position the patch pipette onto the surface of the identified neuron. Pipettes were filled with a solution consisting of (in mmol/L): K gluconate (150), MgCl2 (4), EGTA (2), Na-ATP (2), QX-314 (5), HEPES (10), with pH 7.3 for glutamate excitatory postsynaptic currents (EPSCs). For GABA and glycine postsynaptic inhibitory events (IPSCs), a pipette solution containing (in mmol/L): KCl (150), MgCl2 (2), EGTA (2), Na-ATP (2), QX-314 (5), and HEPES (10) at pH 7.3 was used. Cardiac vagal neurons were studied using the whole-cell patch-clamp technique and were voltage clamped at a holding potential of ~80 mV.

To isolate glutamatergic currents, gabazine (25 μmol/L), a GABA<sub>A</sub> receptor antagonist, and strychnine (1 μmol/L), a glycine receptor antagonist, were added to the perfusate. Similarly, the non-NMDA and NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 50 μmol/L and D-2-Amino-5-phosphonovalerate (AP5) 50 μmol/L, respectively, as well as strychnine (1 μmol/L), were added to the perfusate to isolate GABA IPSCs. Gabazine (25 μmol/L), CNQX (50 μmol/L), and AP5 (50 μmol/L) were included in the perfusate to isolate glycine IPSCs. Tetrodotoxin (TTX) 10 μmol/L was included in the perfusate to isolate miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs).

After the application of sodium nitroprusside (SNP) or L-arginine, the neurons were allowed to recover, and at the end of each experiment, spontaneous EPSCs were blocked by CNQX (50 μmol/L) and AP5 (50 μmol/L), respectively, whereas GABAergic and glycine IPSCs were blocked by gabazine (25 μmol/L) and strychnine (1 μmol/L), respectively. Analysis of spontaneous events was performed using MiniAnalysis (Synaptosoft, version 4.3.1). The minimal acceptable amplitude of synaptic events was set by determining the lowest threshold that elicited no events in the presence of the appropriate antagonist at the end of each experiment (AP5 and CNQX for glutamatergic, gabazine for GABAergic, and strychnine for glycine IPSCs, respectively). Typical thresholds were 8 to 15 pA. Results are presented as mean±SEM, and data during control and drug applications were statistically compared with paired Student t test (for significant difference, P<0.05).

Results

Application of the NO donor SNP (100 μmol/L) and NO precursor L-arginine (10 mmol/L) differentially affected excitatory and inhibitory neurotransmission to CVPNs. Both SNP and L-arginine significantly inhibited the frequency of glutamatergic EPSCs (SNP reduced glutamatergic EPSC frequency from 14±1.3 Hz to 6.7±1.3 Hz; P<0.05; n=9) and L-arginine decreased EPSC frequency from 9.1±1.2 Hz to 6.3±1.1 Hz; P<0.05; n=11) but did not significantly alter EPSC amplitude. The results from a typical experiment and the summary data with application of SNP are shown in Figure 1 (left), whereas a typical experiment and the summary data with L-arginine are shown Figure 1 (right).

Differential effects of the NO donor and precursor were observed with GABAergic and glycine IPSCs in CVPNs. Both SNP and L-arginine significantly facilitated the frequency of GABA IPSCs in CVPNs. SNP (Figure 2, left) increased IPSC frequency from 3.7±1.2 Hz to 5.6±1.4 Hz (P<0.05; n=6), whereas L-arginine (Figure 2, right) increased frequency of GABAergic IPSCs from 4.0±0.7 Hz to 5.1±0.7 Hz (P<0.05; n=13). Interestingly, the amplitude of GABA IPSCs was significantly decreased by SNP (55±5 pA to 34±4 pA; P<0.05; n=6) and although L-arginine had similar effects (reduced GABAergic IPSC amplitude from...
33±5 pA to 27±3 pA; n=13), this reduction was not statistically significant.

In contrast, both SNP and L-arginine significantly inhibited glycinergic IPSC frequency. SNP decreased glycine IPSC frequency from 22±6 Hz to 10±4 Hz (P<0.05; n=8; Figure 3, left), whereas L-arginine decreased glycine IPSC frequency from 47±7 Hz to 18±3 Hz (P<0.05; n=8; Figure 3, right). In addition both SNP and L-arginine reduced glycinergic IPSC amplitude (SNP decreased amplitude from 54±8 pA to 44±5 pA; P<0.05; n=8; L-arginine significantly decreased amplitude from 115±18 pA to 72±13 pA; P<0.05; n=8).

To examine the specificity of L-arginine–evoked changes, L-arginine was applied in additional experiments in the presence of 7-nitroindazole ([7-NID] 100 μmol/L), a selective neuronal NOS inhibitor. 7-NID had no significant effect on EPSC or IPSC frequency, amplitude, or holding current. In the presence of 7-NID, L-arginine had no significant effect on glutamatergic (left), GABAergic (middle), and glycinergic (right) neurotransmission to CVPNs (see Figure 4).

To determine whether these changes in neurotransmission were action potential–dependent or could occur by altering presynaptic or postsynaptic function mEPSCs, GABAergic and glycinergic mIPSCs were isolated by inclusion of TTX (10 μmol/L) in the perfusate. SNP significantly inhibited glutamatergic mEPSC frequency, whereas the amplitude of mEPSCs was not significantly affected (SNP decreased mEPSCs from 9.9±1.7 Hz to 6.5±1.6 Hz; P<0.05; n=9; see Figure 5, left). In contrast, GABAergic mIPSCs were not significantly affected by application of SNP (Figure 5 middle). SNP did significantly inhibit both glycinergic mIPSC frequency (from 24±3 Hz to 3±1 Hz; P<0.05; n=9) and amplitude (from 31±3 pA to 24±2 pA; P<0.05; n=9; see Figure 5, right).

**Discussion**

Despite an extensive amount of literature on the role of NO in cardiac function, little is known about its role in central parasympathetic control of the heart. Although there is...
considerable information demonstrating that NO has peripheral effects that alter cardiac function,20–22 other work in the literature indicate that NO also modulates the activity of central CVPNs in the brain stem.15,23,24 Microinjections of NO into the NA decrease heart rate, a process that is reversed by \( \text{N}^\text{G}-\text{nitro-L-arginine methyl ester} \), a selective neuronal NOS (nNOS) inhibitor.15 Furthermore, lack of nNOS activity decreases baseline parasympathetic tone and responses to vagal stimulation, because nNOS knockout mice have a higher heart rate and diminished cardiac reflex responses.25,26 This study provides a potential cellular basis for the NO-mediated alterations of parasympathetic cardiac activity. The NO donor SNP and the NO precursor L-arginine significantly decreased the frequency of glutamatergic neurotransmission to CVPNs but had no effect on EPSC amplitude. SNP also significantly decreased the frequency of glutamatergic mEPSCs but not mEPSC amplitude, suggesting that the effect of NO occurs at presynaptic glutamatergic synaptic terminals and not at postsynaptic sites. Both SNP and L-arginine increased the frequency of GABAergic neurotransmission to CVPNs but decreased the amplitude of GABAergic IPSCs. SNP did not alter the frequency or amplitude of GABAergic mIPSCs, indicating that the effects of NO on GABAergic neurotransmission to CVPNs are action potential dependent and likely occur by changing the firing characteristics of the GABAergic neurons that innervate CVPNs. Surprisingly, both L-arginine and SNP decreased the frequency and amplitude of glycinergic IPSCs. The decrease in frequency and amplitude persisted in glycinergic mIPSCs, indicating that the NO-mediated depression of glycinergic neurotransmission occurs at both the presynaptic glycinergic terminals, as well as possibly postsynaptic glycinergic receptors.

Consistent with the present data, in other brain regions, NO modulates release of neurotransmitters, including glutamate, GABA, and glycine.27 NO has been shown to both increase and decrease glutamate release.28–30 In brain stem nuclei, such as the NTS, NO increases presynaptic release of glutamate in a positive feedback loop resulting from activation of
NO Modulation of Synaptic Inputs to Brainstem Cardiac Vagal Neurons

Figure 6. NO directly decreases both glutamatergic and glyciner
ergic neurotransmission to CVPNs, and this is likely mediated
terminals, as illustrated in Figure 6, because the modulation
of neurotransmission persists in the presence of TTX. In
contrast, NO facilitates GABAergic activity in CVPNs,
which is likely because of excitation of GABAergic neurons
at their cell bodies, because this increased activity is action
potential dependent.

Perspectives
The findings of the present study describe mechanisms
by which NO directly modulates synaptic inputs to CVPNs.
In particular, NO directly decreases glutamatergic and glycine-
ergic neurotransmission while enhancing GABAergic
transmission. The inhibition of glutamatergic and glycine-
ergic neurotransmission to CVPNs likely occurs at presynaptic

disclosures
None.

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