Nitric Oxide Influences Neuronal Activity in the Nucleus Tractus Solitarius of Rat Brainstem Slices

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Abstract Nitric oxide (NO) is shown to be synthesized in the central nervous system as well as in vascular endothelial cells. However, the physiological role of NO in cardiovascular regulation in the central nervous system remains unclear. The present study examines whether NO plays a role in the regulation of neuronal activity in the nucleus tractus solitarius (NTS). Single-unit extracellular recordings were obtained from NTS neurons in slices (400 μm) of the rat brainstem, which had spontaneous discharges at a frequency of 0.5 to 3 spikes per second. Eighty-one neurons were tested for sensitivity to L-arginine, which is the physiological precursor of NO. L-Arginine (10⁻⁷ to 10⁻⁴ mol/L) increased neuronal activity dose dependently in 33 (40.7%) of 81 neurons tested, but D-arginine (10⁻³ mol/L) did not. The neurons that responded to L-arginine responded to glutamate as well. N⁵-Monomethyl-L-arginine (10⁻⁵ to 3 × 10⁻⁵ mol/L), an inhibitor of the formation of NO, dose-dependently blocked increases in the neuronal activity evoked with L-arginine (10⁻⁵ mol/L). Hemoglobin (1.5 mg/L), a trap of NO, and methylene blue (10⁻⁵ mol/L), an inhibitor of guanylate cyclase, also blocked increases in the neuronal activity evoked with L-arginine (10⁻⁵ mol/L). Sodium nitroprusside (SNP, 10⁻⁵ to 10⁻⁴ mol/L), which spontaneously produces NO, increased the neuronal activity in the neurons that responded to L-arginine. SNP did not alter the neuronal activity of the neurons that did not respond to L-arginine. Methylene blue (10⁻⁵ mol/L) blocked increases in the neuronal activity evoked with SNP (10⁻⁵ mol/L), but hemoglobin did not. These results suggest that NO is formed from L-arginine in the NTS neurons and that NO increases the neuronal activity of adjacent neurons in the NTS through an increase in cGMP. (Circ Res. 1994;75:70-76)

Key Words: nitric oxide • N⁵-monomethyl-L-arginine • L-arginine • nucleus tractus solitarius • brain slice

Previous studies have shown that nitric oxide (NO), which largely accounts for the biological effects of endothelium-derived relaxing factors,¹ is synthesized from L-arginine in the central nervous system²³ as well as in other tissues, including vascular endothelial cells,⁴⁻⁶ macrophages,⁶ and neutrophils.⁷ It is also suggested that in the cerebellum, NO may play a physiological role in local transcellular communication by facilitating cGMP formation in adjacent cells through the activation of soluble guanylate cyclase.⁸

The nucleus tractus solitarius (NTS) is the site where afferent fibers arising from arterial baroreceptors, chemoreceptors, cardiopulmonary receptors, and other visceral receptors⁹¹⁰ make the first synapses; thus, these fibers play an important role in the integration of autonomic control of the cardiovascular systems.¹¹

Togashi et al.²² have shown that in various tissues including the central nervous system, ⁵⁴-monomethyl-L-arginine (L-NMMA), an analogue of L-arginine, administered intravenously in sinoaortic denervated and vagotomized rats, increased arterial pressure and renal sympathetic nerve activity (RSNA) and have shown that an intracisternal injection of L-NMMA increased arterial pressure and RSNA. These results suggest that NO is involved in the regulation of RSNA as well as blood pressure in the central nervous system, particularly in the brainstem. Paola et al.¹⁴ have demonstrated in rats that L-NMMA microinjected into the NTS attenuated the depressor effect evoked with glutamate microinjected into the NTS. This result suggests the possibility that NO is involved in neural transmission in the NTS. In rabbits, we have observed that a microinjection of L-NMMA into the NTS in vivo elicited increases in RSNA, arterial pressure, and heart rate. These effects of L-NMMA were blocked by pretreatment of L-arginine microinjected into the NTS.¹¹ These findings suggest that NO is involved in the mechanism at the NTS to mediate the tonic inhibition of RSNA. Recent studies have demonstrated that in the cerebellum, glutamate evokes NO formation and that NO activates guanylate cyclase and increases cGMP.¹⁵⁻¹⁸ However, whether NO is formed in the NTS and the mechanisms responsible for the effects of NO in the NTS remain unclear.

In the present study, by using single-unit extracellular recordings of NTS neurons in rat brainstem slices, we examined whether L-arginine, which is the precursor of NO, and sodium nitroprusside (SNP), which produces NO spontaneously,¹⁹⁻²¹ influenced the neuronal activity of NTS neurons. We also examined whether L-NMMA, hemoglobin (a trap of NO),²⁰⁻²³ or methylene blue (a blocker of guanylate cyclase)¹⁹⁻²¹ inhibited the effects of L-arginine and whether methylene blue and hemoglobin inhibited the effects of SNP.
Materials and Methods

Slice Preparation

The present study was performed on 81 male Wistar-Kyoto rats (5 to 7 weeks old, 100 to 150 g). The animals were stunned by a blow to the back, and the whole brain from the forebrain to the upper cervical spinal cord was quickly removed after craniectomy and laminectomy of the upper cervical vertebrae. A small block including the medulla oblongata was removed from the brain and cut transversely, and 400-μm-thick slices at the area postrema level were obtained from the block with a vibratome (DTK-1000 D.S.K.). They were preincubated in oxygenated Krebs’ solution at room temperature. The standard medium had the following composition (mM/L): NaCl 124, KCl 5.0, KH2PO4 1.24, MgSO4 1.3, NaHCO3 26, glucose 10, and CaCl2 2.4 (pH 7.4). After 60 minutes of preincubation, the tissue slices were transferred to a 2.0-mL recording chamber in a solution flowing continuously at a rate of 1.5 mL/min and oxygenated with 95% O2/5% CO2 (36°C).24,25

Extracellular single-unit recordings were obtained from the NTS of the brainstem slices.26 The NTS was easily visualized as a translucent area in the slices under a light microscope (SV-6, Zeiss). Micropipettes were filled with 3 mol/L NaCl and had a tip DC resistance of 5 to 25 MΩ. Bioelectrical signals were amplified (MEZ-7200, Nihon-Kohden), displayed on an oscilloscope (DS-8605, Iwatani), and fed into a pulse counter (Model 110, Nihon-Kohden). Neuronal spikes and integrated spikes were simultaneously recorded on a four-channel optical hard-copy recorder (RTA-1200, Nihon-Kohden) at a paper speed of 25 mm/s. After confirming that stable neuronal activity was obtained, we waited at least 15 minutes. Then control neuronal activity was recorded for 4 minutes. Neuronal activity was expressed as spikes per second.

Protocol

Experiment 1

In this experiment, we examined whether L-arginine influenced the neuronal activity of the NTS neurons of rat brainstem slices. First, control recordings were obtained with standard Krebs’ solution. Thereafter, each slice was perfused with solution containing L-arginine at graded doses (10-7 to 10-5 mol/L) for 6 minutes at each dose and then perfused with standard solution for 15 minutes while neuronal activity was recorded continuously. Since L-arginine progressively increased neuronal activity, the maximal response to L-arginine at each dose occurred at the end of the application of L-arginine and lasted for several minutes after changing the perfusate to the standard solution. We used the maximal response for later analysis. Responses to L-arginine at 10-5 mol/L were obtained first. After neuronal activity had returned to the baseline level, we waited at least 15 minutes. Thereafter, application of L-arginine at each successive dose and recordings of neuronal activity were performed in a similar manner. Since the responses to L-arginine were almost maximal at 10-5 mol/L L-arginine, only 10-5 mol/L L-arginine was used in the subsequent protocols (experiments 2 through 9).

Experiment 2

To determine whether neurons that had responded to L-arginine responded to glutamate, we applied glutamate (10-5 mol/L). After experiment 1, we waited at least 15 minutes. Then the slice was perfused with Krebs’ solution containing glutamate (10-5 mol/L). The methods of perfusion and recording were similar to those in experiment 1.

Experiment 3

To determine whether the effects of L-arginine were related to the formation of NO, we examined whether d-arginine influenced the neuronal activity of the neurons that responded to L-arginine. d-Arginine is an isomer of L-arginine, which is not transformed to NO. After experiment 1, we waited at least 15 minutes. Then the slice was perfused with Krebs’ solution containing d-arginine. The methods of perfusion and recording were similar to those in experiment 1. In this experiment, we used d-arginine at a concentration of 10-5 mol/L.

Experiment 4

To determine whether the effects of L-arginine were due to NO formation from L-arginine, we examined whether L-NMMA, an inhibitor of the formation of NO from L-arginine, prevented the effects of L-arginine. After experiment 1, we waited at least 15 minutes. Then the slice was perfused with solution containing L-arginine at 10-5 mol/L plus L-NMMA at 10-5 or 3 × 10-5 mol/L. The methods of perfusion and recording were similar to those in experiment 1. Responses to L-arginine at 10-5 mol/L and L-NMMA at 10-5 mol/L were examined first, and then responses to L-arginine at 10-5 mol/L and L-NMMA at 3 × 10-5 mol/L were examined. We waited at least 15 minutes before beginning experiments with a higher dose of L-NMMA.

Experiment 5

To determine whether the effects of L-arginine were due to transcellular communication of NO formed from L-arginine, we used hemoglobin, which is a trapper of NO and cannot penetrate the neurons through the cell membrane. Thus, we examined whether hemoglobin prevented the effects of L-arginine. After experiment 1, we waited at least 15 minutes. Then the slice was perfused with Krebs’ solution containing L-arginine plus hemoglobin. The methods of perfusion and recording were similar to those in experiment 1. In this experiment, we used L-arginine at 10-5 mol/L and hemoglobin at 1.5 mg/L.

Experiment 6

To determine whether L-arginine activated guanylate cyclase in the NTS neurons, we examined whether methylene blue blocked the effects of L-arginine. Methylene blue is a blocker of guanylate cyclase and can penetrate the neurons. After experiment 1, the slice was perfused with Krebs’ solution containing L-arginine plus methylene blue. The methods of perfusion and recording were similar to those in experiment 1. In this experiment, we used L-arginine at a concentration of 10-3 mol/L and methylene blue at 10-3 mol/L.

Experiment 7

We examined whether the neurons that had responded to L-arginine responded to SNP, which produces NO spontaneously. After experiment 1, we waited at least 15 minutes. Then the slice was perfused with Krebs’ solution containing SNP at 10-3 or 10-4 mol/L. The methods of perfusion and recording were similar to those in experiment 1. Responses to 10-3 mol/L SNP were examined first, and then responses to 10-4 mol/L SNP were examined.

Experiment 8

To determine whether released NO was destroyed extracellularly, we examined whether hemoglobin and methylene blue blocked the effects of SNP. After experiment 1, the slice was perfused with Krebs’ solution containing SNP. Then we waited at least 15 minutes and perfused the slice with Krebs’ solution containing SNP plus hemoglobin or methylene blue. The methods of perfusion and recording were similar to those in experiment 1. In this experiment, we used SNP at a concentration of 10-3 mol/L, hemoglobin at 1.5 mg/L, and methylene blue at 10-3 mol/L.

Experiment 9

We examined the effects of L-NMMA, hemoglobin, and methylene blue on basal neuronal activity. The methods of perfusion and recording were similar to those in experiment 1.
In this experiment, we used L-NMMA at a concentration of 3×10⁻⁵ mol/L, hemoglobin at 1.5 mg/mL, and methylene blue at 10⁻⁵ mol/L, respectively.

In all slices, experiment 1 was performed first. Then one of experiments 2 through 9, but not experiment 7, was performed as the second protocol with neurons that responded to L-arginine in experiment 1. Experiment 7 was performed with neurons that responded to L-arginine as well as neurons that did not respond to L-arginine in experiment 1.

**Chemicals**

L-Arginine hydrochloride, D-arginine hydrochloride, L-NMMA, hemoglobin, and methylene blue were obtained from Sigma Chemical Co; SNP and glutamate were obtained from Wako Chemical Co. These drugs were dissolved in standard Krebs’ solution and diluted to the desirable concentrations immediately before use.

**Statistical Analysis**

Responses of neuronal activity to drugs were expressed as percent changes from the baseline value with Krebs’ solution. In all protocols, one-way ANOVA was used to test the treatment effect. If statistically significant, data were compared by Student’s t test. All values are expressed as mean±SEM. Values are considered to be statistically different at P<.05.

**Results**

Single-unit extracellular recordings were obtained from neurons located in the NTS, which fired spontaneously at frequencies of 0.5 to 3 spikes per second.

**Experiment 1**

Eighty-one neurons were tested for responses to L-arginine. L-Arginine increased neuronal activity in 33 (40.7%) of 81 neurons tested and decreased neuronal activity in 9 (11.1%) of 81 neurons tested. In 39 (48.1%) of 81 neurons tested, neuronal activity was not altered by L-arginine. In neurons that responded to L-arginine, L-arginine increased neuronal activity in a concentration-dependent manner (P<.01) (Fig 1). Fig 2 demonstrates the time course of neuronal activity of the neurons that responded to L-arginine. Neuronal activity increased progressively after the application of L-arginine, plateaued off at the end of the application, and gradually decreased to the baseline level.

**Experiment 2**

The neurons that responded to L-arginine also responded to glutamate (n=8) (P<.01). The magnitudes of the response were similar between L-arginine and glutamate at the same concentration (Fig 3).

**Experiment 3**

D-Arginine did not increase the neuronal activity of neurons that responded to L-arginine (n=6) (Fig 4).

**Experiment 4**

L-NMMA dose-dependently attenuated increases in the neuronal activity evoked with L-arginine (n=6) (P<.01) (Fig 5).

**Experiment 5**

Hemoglobin blocked increases in the neuronal activity evoked with L-arginine (n=10) (P<.05) (Fig 6).
Experiment 6

Methylene blue also blocked increases in the neuronal activity evoked with l-arginine (n=5) (P<.01) (Fig 7).

Experiment 7

SNP dose-dependently increased the neuronal activity of neurons that responded to l-arginine (n=12) (P<.01) (Fig 8). On the other hand, SNP did not alter the neuronal activity of neurons that did not respond to l-arginine (n=9). Thus, SNP increased the neuronal activity only of neurons that responded to l-arginine in the NTS.

Experiment 8

Methylene blue blocked increases in the neuronal activity evoked with SNP (P<.01), but hemoglobin did not (n=6) (Fig 9).

Discussion

It has been shown in the cerebellum by several physiological and biochemical methods that the excitatory effects of l-arginine on neuronal activity are mediated through NO formation and cGMP production.16,17,27 However, it is not known whether l-arginine alters neuronal activity by producing NO and cGMP in the NTS, where the baroreceptor afferents make the first synapse. Furthermore, it is not known whether NO acts as an autocrine or a paracrine modulator of neuronal activity in the NTS. Accordingly, we examined the effects of l-arginine on the neuronal activity of NTS neurons by recording extracellular single-unit activity and examined the underlying mechanisms of its action.
L-Arginine dose-dependently increased neuronal activity in about 40% of the NTS neurons in slices of the rat brainstem. We first examined whether the excitatory effect of L-arginine in the NTS involved the formation of NO. The following results suggest that this was indeed the case. First, D-arginine (an isomer of L-arginine), which is not transformed to NO, did not increase neuronal activity at the concentration (10^{-5} mol/L) at which L-arginine almost maximally increased neuronal activity. Second, L-NMMA, an inhibitor of the formation of NO from L-arginine, and hemoglobin, a trapper of NO, blocked increases in the neuronal activity evoked with L-arginine. These results strongly suggest that the excitatory effects of L-arginine on the NTS neurons were mediated by NO, which was locally formed from L-arginine. L-NMMA has anticholinergic properties. If L-NMMA affected neuronal activity through anticholinergic properties, basal neuronal activity would have been influenced by L-NMMA. However, L-NMMA did not alter basal neuronal activity in the NTS and only blocked increases in the neuronal activity evoked with L-arginine. Thus, we consider that the inhibitory effects of L-NMMA are mainly due to the inhibition of NO formation. To further elucidate the role of NO in the regulation of neuronal activity, we determined the effect of SNP, a substance that produces NO spontaneously, on the neuronal activity of NTS neurons. SNP (10^{-4} to 10^{-5} mol/L) increased the neuronal activity of neurons that responded to L-arginine but did not increase the neuronal activity of neurons that did not respond to L-arginine. Thus, these results suggest that some neurons in the NTS are sensitive to NO but others are not.

It has been shown in the cerebellum that NO diffuses out of the original neurons in which it is formed, penetrates the adjacent neurons, and increases the neuronal activity of the latter neurons. Thus, NO is considered to play a role in transcellular communication in the cerebellum. In the present study, the findings that hemoglobin blocked increases in the neuronal activity evoked with L-arginine in the NTS suggest that, as in the cerebellum, NO diffused out into the extraneuronal space in the NTS and excited the adjacent neurons, from which we recorded neuronal activity, since hemoglobin does not penetrate neurons. Thus, it appears that the neurons in which NO was formed from L-arginine were not the target neurons of NO. Furthermore, our results indicate that not all of the neurons in the NTS responded to NO. The neurons that did not respond to L-arginine did not respond to SNP, whereas those that responded to L-arginine responded to SNP as well. It is possible that the difference in responsiveness to NO might be related to the presence or absence of guanylate cyclase.

It is well known that the effects of NO are mediated through cGMP, and it has been shown in the cerebellum that L-arginine produced cGMP and thus excited the neurons. To determine whether the effects of L-arginine in the NTS target neurons were mediated through cGMP, we examined whether methylene blue, a blocker of guanylate cyclase, inhibited increases in the neuronal activity evoked with L-arginine. Methylene blue (10^{-5} mol/L) inhibited increases in the neuronal activity evoked with L-arginine. Although the inhibition by methylene blue was not complete, it was possible that a higher concentration of methylene blue might have inhibited increases in the neuronal activity completely. Since methylene blue can inactivate NO extracellularly by formation of oxygen radicals, we also examined whether methylene blue inhibited the neuronal activity evoked with SNP, which activates guanylate cyclase. Methylene blue inhibited increases in the neuronal activity evoked with SNP. We further demonstrated that hemoglobin, a trapper of NO outside the neuron, did not alter the neuronal activity evoked with SNP. Although we did not measure cGMP, these results suggest that activation of guanylate cyclase and thus cGMP synthesis are involved in the excitatory effects of L-arginine and SNP in the NTS neurons.

Previous studies in vivo have suggested that NO may play an important role in the regulation of sympathetic nerve activity in the brainstem, including the ventrolateral medulla and NTS. At the NTS, we have
observed that microinjection of L-NMMA into the NTS in vivo elicited increases in RSNA, arterial pressure, and heart rate and that these effects of L-NMMA were blocked by pretreatment of L-arginine microinjected into the NTS.\textsuperscript{9,10} Paola et al\textsuperscript{13} have reported that L-NMMA microinjected into the NTS in rats attenuated the depressor effect evoked with glutamate microinjected into the NTS. It is also shown that microinjection of S-nitrosoycteine, an S-nitrosothiol with the biological properties of NO, into the NTS decreased arterial pressure\textsuperscript{29} and that microinjection of methylene blue into the NTS inhibited the cardiopulmonary reflex activation induced by the intravenous infusion of serotonin.\textsuperscript{30} These results may suggest that NO plays a role in the regulation of sympathetic nerve activity in the NTS. The results of the present study were primarily to demonstrate directly that L-arginine increased the neuronal activity in some neurons of the NTS and that the effects of L-arginine were mediated through NO.

There are some differences in the results of the in vivo studies and those of the present study. A previous in vivo study\textsuperscript{29} demonstrated that microinjection of glutamate and S-nitrosoycteine into the NTS had different effects on arterial pressure, whereas L-arginine, SNP, and glutamate all increased neuronal activity in the present study. We believe that the results of these two studies are not necessarily contradictory, because the actions of glutamate may be twofold: through direct activation not mediated by NO and through the activation of NO synthesis. In our previous study, we observed that L-NMMA microinjected into the NTS elicited increases in RSNA, arterial pressure, and heart rate.\textsuperscript{14} However, in the present study, L-NMMA, hemoglobin, and methylene blue did not alter the basal neuronal activity in the slices. The differences in our previous in vivo study and the present study might have resulted from the absence of afferent inputs into the NTS in the present in vitro study.

It has been shown that supplementation of L-arginine does not increase NO formation or cGMP production in other tissues of normal animals.\textsuperscript{32-34} Our results suggest that supplementation of L-arginine increased NO formation and cGMP production. It has been shown in the cerebellum that supplementation of L-arginine increased cGMP production of neurons.\textsuperscript{16} Thus, intracellular availability of L-arginine may differ between neurons and other tissues. However, we cannot exclude the possibility that L-arginine may have been depleted in the neurons used in the present study, since the brain slices were incubated for some time before applying L-arginine. Thus, our results do not necessarily indicate that the administration of L-arginine into the NTS activates neuronal activity in vivo.

We have obtained slices of the NTS at the level of the area postrema, where the primary afferents from baroreceptors make the first synapse.\textsuperscript{9,10} Thus, we assume that the neurons that responded to L-arginine were those involved in cardiovascular regulation, although no inputs of afferent nerves were present. However, there are many kinds of neurons in the NTS; therefore, we do not know what kind of neurons responded to L-arginine. In the present study, the neurons that responded to L-arginine responded to glutamate as well. However, this does not imply that all responses to glutamate are mediated through NO.

In summary, the present study has demonstrated that L-arginine increased the neuronal activity in 40% of the neurons in the NTS and that L-NMMA, hemoglobin, and methylene blue blocked increases in the neuronal activity evoked with L-arginine. These results suggest that NO is formed from L-arginine in NTS neurons and that NO diffuses out of neurons to nearby target neurons and increases the neuronal activity through cGMP formation in the NTS. Together with the results of other studies in vivo,\textsuperscript{12-14} our results suggest that NO plays an important role in the autonomic control of the cardiovascular system in NTS neurons.

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