Nitric oxide modulates the cardiovascular effects elicited by acetylcholine in the NTS of awake rats

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Nitric oxide modulates the cardiovascular effects elicited by acetylcholine in the NTS of awake rats. Am J Physiol Regul Integr Comp Physiol 295: R1774–R1781, 2008. First published September 24, 2008; doi:10.1152/ajpregu.00559.2007.—Microinjection of acetylcholine chloride (ACh) in the nucleus of the solitary tract (NTS) of awake rats caused a transient and dose-dependent hypotension and bradycardia. Because it is known that cardiovascular reflexes are affected by nitric oxide (NO) produced in the NTS, we investigated whether these ACh-induced responses depend on NO in the NTS. Responses to ACh (500 pmol in 100 nl) were strongly reduced by ipsilateral microinjection of the NOS inhibitor Nω-nitro-L-arginine methyl ester (l-NAME; 10 nmol in 100 nl) in the NTS: mean arterial pressure (MAP) fell by 50 ± 5 mmHg before l-NAME to 9 ± 4 mmHg, 10 min after l-NAME, and HR fell by 100 ± 26 bpm before l-NAME to 20 ± 10 bpm, 10 min after l-NAME (both P < 0.05). Microinjection of the selective inhibitor of neuronal nitric oxide synthase (nNOS), 1-(2-trifluoromethylphenyl) imidazole (TRIM; 13.3 nmol in 100 nl), in the NTS also reduced responses to ACh: MAP fell from 42 ± 3 mmHg before TRIM to 27 ± 6 mmHg, 10 min after TRIM (P < 0.05). TRIM also tended to reduce ACh-induced bradycardia, but this effect was not statistically significant. ACh-induced hypotension and bradycardia returned to control levels 30–45 min after NOS inhibition. Control injections with l-NAME and saline did not affect resting values or the response to ACh. In conclusion, injection of ACh into the NTS of conscious rats induces hypotension and bradycardia, and these effects may be mediated at least partly by NO produced in NTS neurons.

Cholinergic transmission; nitric oxide synthase inhibition; nucleus of the solitary tract; cardiovascular control

The nucleus of the solitary tract (NTS) is the primary site of cardiovascular afferent termination in the central nervous system (CNS) (1). The main neurotransmitter released in the NTS by terminals of cardiovascular afferents appears to be glutamate (39, 40, 42).

We have previously shown that the effects of glutamate in the NTS depend partly on locally produced neuronal nitric oxide (NO). NO increases the number of discharges evoked by excitatory amino acids in NTS neurons that receive vagal afferent inputs, and action potentials induced by iontophoretic application of AMPA in the NTS are reduced by Nω-nitro-L-arginine methyl ester (l-NAME) (10). In addition, the renal sympathoinhibition induced by activation of baroreceptors and cardiopulmonary receptors is attenuated by microinjection of l-NAME in the NTS (11). Therefore, we proposed that the release of glutamate induced by the activation of cardiovascular afferents triggers the activation of nNOS in the postsynaptic neuron. The NO produced may diffuse to adjacent cells and increase the release of glutamate and other neurotransmitters. The specific function or mechanism of this interaction remains to be elucidated. NO plays a second-messenger role, mediating cyclic guanosine monophosphate (cGMP) synthesis in the brain. The soluble guanylyl cyclase (sGC) is the target for NO in the CNS (3, 13, 19, 31, 37). NO is a diffusible compound, and it may act in the same and/or adjacent cells, changing the release of other neurotransmitters, such as ACh.

In the present study, we tested whether the cardiovascular effects of acetylcholine chloride (ACh) in the NTS similarly depend on NO. Several researchers suggested that ACh in the NTS may contribute to central autonomic regulation (21, 29, 30). The cholinergic system was identified within neurons and terminals of the NTS by the presence of acetylcholinesterase (20, 35), choline acetyltransferase (17, 35), ACh (17), and muscarinic receptors (20, 35, 47). Nicotinic receptors were identified by intracellular and extracellular studies on subpopulations of neurons in the NTS (32, 34, 45). However, there have been few functional in vivo studies, and the role of ACh in the NTS in cardiovascular regulation is still unclear. ACh microinjected into the NTS of anesthetized rats causes hypotension and bradycardia (6, 41). The hypotensive response to ACh was blocked by microinjection of atropine, but not hexamethonium in the NTS (6), suggesting the involvement of muscarinic receptors. Possibly, the M2 receptors mediate this effect, because their activation reduced arterial blood pressure and heart rate (HR) (38). It appears that these receptors are not tonically stimulated because injection of methylatropine into the NTS does not increase blood pressure (44). In addition, stimulation of nicotine receptors in the NTS causes hypotension and bradycardia (28, 43).

It has been observed that ACh can stimulate the local release of NO in several brain areas (7), including the hippocampus (36). Double immunostaining for cGMP and the vesicular ACh transporter in the rat hippocampus and nucleus ambiguous suggested that activation of muscarinic receptors (probably M2 receptors) on NO-containing neurons results in retrograde NO signaling in some subpopulations of cholinergic fibers (8), since only a small percentage of NO-containing neurons contain muscarinic receptors (25). Activation of NO through glutamatergic receptors would result in anterograde signaling to the remaining cholinergic fibers (8). Cells from the striatum of nNOS-knockout mice released ACh in the presence of the NO donor (SIN-1) but not in the presence of the glutamatergic

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agonist (NMDA). This study is evidence that ACh release in the CNS can be affected by the formation and release of NO induced by activation of glutamatergic receptors (5).

The cardiovascular effects of microinjection of ACh in the NTS have not yet been investigated in awake animals. In the present study, we characterized the cardiovascular effects elicited by microinjection of ACh in the NTS in conscious rats and investigated whether the inhibition of central NO production in the NTS affects these responses.

MATERIALS AND METHODS

Animals. Experiments were performed in Male Wistar rats weighing between 300 and 350 g. Rats were maintained on 12:12-h light-dark cycle in temperature-controlled rooms, and food and water were available ad libitum, except during the experiments. During the experiments, animals were awake and moving freely in the cage. All experimental protocols were approved by the Institutional Ethics Committee.

Surgical procedures. Five days before the experiments, rats were anesthetized with ketamine (50 mg/kg ip, Cristália, Itapira, Brazil) and xylazine 2% (25 mg/kg ip; Virbac, Jurubatuba São Paulo Brazil) and were placed in a stereotaxic frame (model 1940; David Kopf Instruments, Tujunga, CA) with the incisor bar 3.5 mm below the interaural line. Bilateral guide cannulas (15 mm long) were implanted above the intermediate NTS, 14.5 mm caudal to the bregma, 0.5 mm lateral to the midline, and 5.5 mm below the skull surface at the level of bregma (27). Guide cannulas were fixed with methacylate to the skull and to watch screws inserted in the skull. A stainless steel wire closed the cannula until the beginning of the experiments. Animals were allowed to recover for 3–5 days.

One day before the experiment, rats were anesthetized with a mixture of halothane (2%) in oxygen (100%), and a polyethylene catheter (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ) was introduced in the femoral artery. The arterial catheter was tunneled subcutaneously and exteriorized at the back of the neck. All procedures were performed with sterile tools and solutions. Animals that appeared to show distress or pain after surgery were euthanized.

Measurement of blood pressure. To measure pulsatile arterial blood pressure, mean arterial pressure (MAP), and HR, the arterial catheter of the conscious rat was connected to a Statham P23Db transducer (Statham Instruments, Hato Rey, Puerto Rico). Signals were amplified with an ETH-200 Bridge amplifier (CB Sciences, Dover, NH), digitized with a Power Lab 4SP (AD Instruments, Colorado Springs, CO), and stored and analyzed on a Macintosh G3 computer (Apple) with Chart 4.1.1 software.

Drugs and drug administration. Injected drugs include the nonselective cholinergic agonist ACh (Sigma, St. Louis, MO), the NOS inhibitor L-NAME (Sigma), the inactive negative enantiomer of L-NAME, N\textsuperscript{G}-nitro-D-arginine methyl ester (D-NAME; Sigma), and the selective inhibitor of neuronal NOS 1-(2-Trifluoromethylphenyl) Imidazole (TRIM; Sigma). All drugs were dissolved in saline (0.9% NaCl), and the pH was adjusted to 7.4 with NaOH.

Drugs were microinjected unilaterally into the intermediate NTS with a 1-μl syringe (Hamilton, Reno, NV) that was connected with PE-10 tubing to a stainless-steel injection needle with an outer diameter of 0.2 mm. The injection needle protruded 1.5 mm beyond the tip of the guide cannula. The volume of all injections was 100 nl.

Protocols. Microinjections started after baseline MAP and HR were established. Cardiovascular effects elicited by unilateral microinjection of ACh (500 pmol/100 nl) in the NTS were determined before, and 10, 20, and 30 to 45 min after ipsilateral microinjection of L-NAME (10 nmol in 100 nl) or D-NAME (10 nmol in 100 nl), TRIM (13.3 nmol in 100 nl), or 100 nl 0.9% saline in the NTS. A maximum of five microinjections were performed in each animal.

The dose of ACh (500 pmol/100 nl) used in this study was based in previous studies of the literature (6) and in the dose-response curve in awake rats presented in this manuscript. This dose caused cardiovascular changes similar to baroreflex activation. The dose of L-NAME and D-NAME (10 nmol/100 nl) used was based on our previous studies in awake (9) and anesthetized rats. We have shown before that microinjection of 10 nmol/100 nl of L-NAME into the NTS does not change resting blood pressure, heart rate, regional blood flow, and renal sympathetic nerve activity (11), or resting neuronal discharge of vagus-nerve evoked NTS neurons (10).

The dose of TRIM (13.3 nmol/100 nl) was chosen on the basis of Scislo et al. (33) and previous studies from our laboratory. This dose reduced ACh-induced responses without changing baseline blood pressure and heart rate, and this inhibition was transient. In some of the experiments, we tried higher concentrations of TRIM, and it increased the resting blood pressure and caused total blockade of the ACh response without a recovery, probably because of neuronal damage.

Histology. After the experiments, methylene blue (100 nl of a 2% solution) was microinjected in the same NTS site. Animals were anesthetized with urethane (400 mg/ml ip) and perfused transcardially with 0.9% saline, followed by 10% formalin solution. The brain was removed and stored in buffered formalin for at least 2 days before 40-μm coronal sections were made with a microtome. Slices were stained by the Nissl method (Neutral red) (16). Only rats whose microinjection site was located in the intermediate NTS were used for data analysis. A typical example of the injection site is shown in Fig. 1.

Data analysis. Peak changes in MAP and HR induced by microinjection of ACh were measured. All data were expressed as means ± SE and were analyzed with SigmaStat statistical software (ver. 2.03; SPSS, Chicago, IL). To compare differences between time points, we used one-way ANOVA with repeated measures, followed by Newman-Keuls post hoc test. Significance was accepted at P < 0.05.

RESULTS

Changes in MAP and HR elicited by microinjection of ACh in the NTS. Unilateral microinjections of ACh into the NTS of awake rats elicited hypotension and bradycardia in a dose-dependent manner. Injections of 0.1, 1.0, and 10 pmol ACh in 100 nl did not significantly reduce MAP and HR (n = 9, Fig. 2A). However, in a second group of rats, the 100 pmol in a 100-nl dose elicited hypotension (−28 ± 7 mmHg) and bradycardia (−61 ± 14 bpm; P < 0.05, n = 7). Higher doses...
of ACh were more effective (500 pmol reduced MAP by 34 ± 3 mmHg and HR by 93 ± 11 bpm, and 1,000 pmol reduced MAP by 55 ± 6 mmHg and HR by 104 ± 15 bpm; \( P < 0.05, n = 7 \), Figs. 2B and 3). The injections did not cause obvious behavioral or respiratory activation.

To test whether ACh-induced hypotension was secondary to bradycardia, we assessed cardiovascular responses to microinjection of ACh (500 pmol/100 nl) before and 20 min after intravenous injection of the muscarinic receptor antagonist methylatropine (1 mg/kg). The bradycardia induced by ACh fell from 144 ± 18 bpm before methylatropine to 6 ± 2 bpm after methylatropine (\( P < 0.05, n = 5 \)), but methylatropine did not reduce the ACh-induced hypotension (41 ± 1 mmHg before methylatropine, 39 ± 2 mmHg after methylatropine).

Effects of ACh were only observed when microinjections reached the intermediate NTS; injections in other areas of the NTS or adjacent sites failed to elicit any response. The sizable heart rate responses are probably due to the condition of the experiment performed in awake, freely moving rats.

**Effects of NOS inhibition with l-NAME on changes in MAP and HR caused by microinjection of ACh in the NTS.** Repeated unilateral microinjections with ACh (500 pmol in 100 nl) were made before and after microinjection of l-NAME (10 nmol in 100 nl) into the ipsilateral NTS of conscious rats. l-NAME did not change baseline MAP (113 ± 4 mmHg before l-NAME; 112 ± 3 mmHg after l-NAME, \( n = 8 \)) or baseline HR (331 ± 8 bpm before l-NAME; 325 ± 10 bpm after l-NAME, Fig. 4, A and B). However, l-NAME almost completely blocked ACh-induced hypotension (50 ± 5 mmHg before l-NAME, 9 ± 4 mmHg, 10 min after l-NAME, \( P < 0.05 \)) and bradycardia (100 ± 26 bpm before l-NAME, 20 ± 10 bpm, 10 min after l-NAME, \( P < 0.05 \)). Responses to ACh were still depressed 20 min after injection of l-NAME and returned to control values between 30 and 45 min after injection of l-NAME (Fig. 4B, left).

In a separate group of rats, we injected d-NAME instead of l-NAME to control for nonspecific effects of l-NAME. Microinjection of 10 nmol d-NAME into the NTS did not affect
the NTS did not affect baseline MAP (112 ± 2 mmHg before saline, 113 ± 2 mmHg after saline) and HR (324 ± 18 bpm before saline, 324 ± 19 bpm after saline). Saline also did not alter the hypotension and bradycardia induced by ipsilateral microinjection of 500 pmol ACh (Fig. 5B).

**DISCUSSION**

In the present study, we determined the cardiovascular effects elicited by microinjection of ACh in the NTS of awake rats and whether NO released centrally can modulate these responses. Although several studies analyzed cholinergic transmission in the NTS and other brain stem areas related to cardiovascular control, there are no reports in awake rats. We found that unilateral microinjection of ACh into the NTS of nonanesthetized rats elicited hypotension and bradycardia. These responses were reduced after the inhibition of the production of NO in the NTS.

Our results are in line with previous findings that showed that microinjection of ACh and carbachol in the intermediate NTS produce hypotension and bradycardia in anesthetized rats (6). The minimal effective dose in rats anesthetized with halothane (4.4 pmol) is similar to that observed in the awake rats in our study (10 pmol). The optimal dose was around 500 pmol, both in anesthetized (6) and in awake rats.

The hypotension induced by ACh was not secondary to reduced heart rate, because blockade of ACh-induced bradycardia with methylatropine did not reduce the hypotensive response. The mechanism that mediates ACh-induced hypotension is not yet clear, but it seems likely that activation of the cholinergic system in the NTS reduces sympathetic activity. Despite the fact that all microinjection sites that were histologically located outside the NTS did not elicit any cardiovascular response, we must not exclude the possibility of some spread of the injection to the dorsal motor nucleus of the vagus. Since the response induced by ACh (bradycardia and hypotension) is similar to that induced by baroreceptor activation, it may be that ACh is part of a mechanism that facilitates baroreceptor transmission. This idea is also supported by the finding that NTS neurons containing ACh and choline acetyltransferase (ChAT) and activity of ChAT is influenced by peripheral sinoaortic denervation (26). This mechanism appears inactive in basal conditions, because the baroreflex is not affected by blockade of the cholinergic receptors in the NTS (6, 44). The conditions that stimulate the release of ACh in the NTS are not yet known.

NO in the NTS has been shown to affect cardiovascular responses (9, 10, 11). The location of neuronal NOS in the NTS, juxtaposed to baroreceptor synapses, suggests a role for NO in the modulation of baroreflex (23). NO is produced by high-intensity stimulation of glutamate receptors (15). After its production, it may act in the same or in adjacent cells to stimulate the release of glutamate through presynaptic receptors (9), and it may also change the release of other neurotransmitters such as GABA and substance P.

NO can also affect cholinergic transmission. The histochemical staining NADPH-diaphorase was reported in neurons in a number of cholinergic nuclei (12, 46), even before NO was identified (18). Our results showed that l-NAMe could be blocking ACh responses better than the neuronal NOS inhibitor (TRIM) compound, because it blocks eNOS and additionally
blocks muscarinic receptors (4). The effect of TRIM suggests that nNOS contributes to ACh-induced cardiovascular responses. Similar effects of l-NAME and TRIM on glutamatergic transmission were previously observed in modulation of NTS neurons discharge (10, 24). A study by Scislo et al. (33) reported that l-NAME and TRIM are equipotent in inhibiting cardiovascular responses elicited by the stimulation of adenosine A2a receptors in the NTS. However, the dose used in these experiments was twice the dose used by us. When we tested this higher dose in our experiments, it increased baseline MAP. We found this high dose of TRIM highly efficient in reducing ACh-induced hypotension, but this effect was not reversible.

It is difficult to discriminate effects from each NO source because nNOS and eNOS are present in adjacent structures in the NTS (22), and NO diffuses through cell membranes (14). A recent study from Wang et al. (48) suggested that endothelial production of NO in the NTS would take place under some conditions to raise the local concentration of NO to a level that activates GABAergic inhibition to generate a excitatory-inhibitory balance in reflex transmission. Then, the quantity of NO released from nNOS would have a limited role, mainly in intracellular pathways of glutamatergic transmission.

On the basis of our results and previous finds in the literature, we could speculate that the cholinergic transmission in the NTS (34, 38) involves the activation of a nitroxeidergic pathway that partially produces NO from nNOS and facilitates the sympathoinhibitory response elicited by ACh microinjection (Fig. 6). The exact afferent inputs (cardiovascular or others) that lead to the release of ACh in the NTS are still unknown (17, 44). Maybe, the cardiovascular inputs to the NTS that activate glutamatergic transmission, in situations of high-frequency discharge (cardiovascular reflexes activation), also sig-

Fig. 4. Cardiovascular responses to unilateral microinjection of ACh (500 pmol/100 nl) into the NTS of awake rats before and after microinjection of the NOS inhibitor l-NAME (10 nmol/100 nl) in the same NTS site. A: typical recordings. ACh-induced responses were measured before injection of l-NAME (ACh control), and 10 and 30–45 min after l-NAME (ACh 10 min and recovery, respectively). PAP, pulsatile arterial pressure; MAP, mean arterial pressure; HR, heart rate. B: group data showing effects of l-NAME (n = 8) and the inactive enantiomer d-NAME (10 nmol/100 nl, n = 7) on ACh-induced responses. *P < 0.05 with control.
nal the activation of a cholinergic modulatory pathway. This signalization could be triggered by NO.

Garthwaite (13) showed that activation of NOS in cholinergic cells can be triggered by activation of NMDA receptors. Postsynaptic activation of glutamatergic receptors produces NO that diffuses out of the cell to neighboring cells to activate sGC in a subpopulation of cholinergic fibers containing muscarinic receptors (8).

The methods used by us (microinjections) may affect both presynaptic and postsynaptic receptors for ACh and NO in the NTS. cGMP was detected both on the presynaptic and postsynaptic side of the nerve terminal and colocalized with the ACh vesicular transporter, suggesting that NO may affect both presynaptic and postsynaptic mechanisms in subsets of cholinergic neurons (7). Neuronal NOS was found in presynaptic and postsynaptic terminals in the NTS; however, the presynaptic structures were not of vagal origin, suggesting that second-order neurons in the NTS or neurons from other regions of the CNS that use other neurotransmitter systems account for presynaptic nNOS immunoreactivity (2).

The cholinergic receptor subtype that mediates the effect of ACh in the NTS has not yet been identified. The identification of the cholinergic receptor subtype that mediates the cardiovascular changes induced by microinjection of ACh in the NTS and the localization of this receptor are crucial in understanding the modulatory role of ACh in blood pressure signaling. The consequences of increase in cGMP synthesis by NO in cholinergic fibers of different areas of the CNS remain to be
elucidated. The role of ACh and NOS-cGMP activation in the brain stem nuclei involved in cardiovascular control has received little attention so far, but our results are the first evidence of a functional role for centrally released NO in cholinergic transmission in the NTS of conscious rats. We suggest that one of the pathways by which NO can modulate glutamatergic transmission in the NTS involves the release of ACh in this nucleus.

**Perspectives and Significance**

This study characterized the cardiovascular effects elicited by ACh microinjection within the NTS of awake rats and showed that NO released in this nucleus from nNOS may modulate these responses. The findings in the current study are the first step to physiological investigation of the cholinergic transmission in the NTS since there are few functional studies in vivo. Also, NO mechanisms and its integration with glutamatergic system in the NTS seem to be complex. Further investigation is necessary to conclude how ACh produces its cardiovascular effects and whether this involves the release of other neurotransmitters. It is a perspective of our study to understand the relationship of glutamate-NO-ACh systems in modulation of the cardiovascular reflexes. The subtype of cholinergic receptor (muscarinic or nicotinic) mediating the cardiovascular responses induced by microinjection of ACh is still not clear. The search for differences between subpopulations of NTS neurons with muscarinic and nicotinic receptors and the specific function of NO during their activation could add significant information about baroreflex modulation in the NTS.

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