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# Nitric Oxide Mediates Depressor Responses by Activation of N-Methyl-D-Aspartate Receptors in the Nucleus Tractus Solitarius of Cat

**Wun-Chin Wu[1], Ching-Yue Yang[2] and Chok-Yung Chai[1]**

[1]*Institute of Biomedical Sciences*
*Academia Sinica*
*Taipei 115, Taiwan, ROC*
*and*
[2]*Department of Anaesthesiology*
*Chang-Gung Memorial Hospital*
*Taoyuan 333, Taiwan, ROC*

## Abstract

**Nitric oxide (NO) is involved in cardiovascular regulation and sympathetic nerve activity of the central nervous system (CNS). The nucleus tractus solitarius (NTS) is important to cardiovascular regulation. However, the physiological role of NO in cardiovascular regulation effecting through the NTS remains unclear. The purpose of this study is to investigate the effect of NO measured by *in vivo* voltammetry on the cardiovascular responses in NTS induced by N-methyl-D-aspartate (NMDA) in anesthetized cats. Extracellular NO concentration was monitored through a Nafion- and porphyrine-coated carbon fiber electrode, which has previously been demonstrated sensitive and selective to NO responses. Microinjection of NMDA into NTS elicited a dose-dependent decrease in cardiovascular responses associated with NO release. Following the dose-response curve, a dose of 3 nmol of NMDA was selected. Microinjection of NMDA into NTS produced depressor responses and NO release. These responses in NTS to NMDA were attenuated by pretreatment with a competitive antagonist, 2-amino-5-phosphonopentanoat (AP-5, 1 nmol), and methylene blue (MB, 1 nmol), an inhibitor of guanylate cyclase. These results suggest that NO is formed from NMDA activation in NTS and that NO diffuses out of neurons into the nearby target neurons to produce depressor response and NO release through cyclic guanosine monophosphate (cGMP) formation. In conclusion, NO mediates depressor response consequent to activation of NMDA receptors in neurons of NTS.**

**Key Words: nitric oxide, depressor, nucleus tractus solitarius, N- methyl-D-aspartate**

## Introduction

Nitric oxide (NO) is synthesized from L-arginine (L-Arg) by NO synthase (NOS) (22). It serves as a neuromodulator and neurotransmitter in the central and peripheral nervous system (11, 26). It has been demonstrated that NO is involved in cardiovascular regulation and sympathetic nerve activity (3, 29). The nucleus tractus solitarius (NTS) is known to be the major site of primary afferent fibers from peripheral baroreceptors. It plays an important role in cardiovascular regulation (4, 5). NOS is distributed in the central nervous system (CNS) including the NTS (21).

Excitatory amino acids are most abundant among the excitatory neurotransmitters in CNS. They appear to be the major stimulus for NO formation in neurons. For example, glutamate (Glu) and the glutamate analogue N-methyl-D-aspartate (NMDA) produce increases in neuronal activity and marked increases in cyclic guanosine monophosphate (cGMP) in the brain tissue that are mediated by NO, and this increase in cGMP can be prevented by NO synthase (NOS) inhibitors (2, 8). Microinjections of Glu and NMDA into NTS decreased the arterial blood pressure (SAP) (15, 28). It has been postulated that NO acts as a link

Corresponding author: Dr. C.Y. Chai, Institute of Biomedical Science, Academia Sinica, Taipei 115, Taiwan, ROC. Tel: 886-2-2789-9105, Fax: 886-2-2782-9224
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between NMDA activation and cell to cell signaling in the brain (10, 20, 30).

NO is highly diffusible and releases extracellularly by neurons during activation (16). We hypothesized that the neuronally derived NO may have an important influence on cardiovascular regulation. In this study we used *in vivo* voltammetry to measure changes of NO during microinjection of NMDA into NTS. We investigated whether the depressor response produced by NMDA depended on the extracellular formation of NO.

## Materials and Methods

*General Procedures*

Experiments were performed on 13 cats of either sex, weighing 2.3 - 3.5 kg, anesthetized intraperitoneally with urethane (400 mg/kg) and α-chloralose (40 mg/kg) and immobilized with gallamine triethiodide (2 mg/kg/30 min). The experimental procedures have been described previously (32). These included cannulation of the right femoral artery for monitoring SAP, mean SAP (MSAP) and heart rate (HR), cannulation of the right femoral vein for drug injections, tracheal intubation for artificial ventilation to maintain the end-tide $CO_2$ concentration at 4%, and maintaining the rectal temperature at 37.5°C with a thermostatically controlled heating pad. All recordings were made on a Gould ES-1000 recorder (Gould Co., Perkins Avenue, Cleveland, Ohio, USA).

*Brain Stimulation*

The head of each cat was fixed in a David-Kopf stereotaxic apparatus. The dorsal surface of the brain stem was exposed and the obex was used as the reference point. A three barrel-glass micropipette was pulled and broken to an outside tip diameter of 40 - 50 μm and mounted on a carrier apparatus. The pipette was inclined at 34° from the stereotaxic frame and lowed to NTS (2 mm anterior to the obex, 1.0 - 2.0 mm lateral to the midline and 0.5 - 1.7 mm ventral to the dorsal surface of the medulla). The three barrels containing different chemicals were connected to three separate pneumatic pressure systems (PPS-2, PPM-2, Medical Systems Corp., Great Neck, NY, USA) for chemical microinjection. These included the following chemicals: NMDA (3 nmol, Sigma), 2-amino-5-phosphonopentanoat (AP-5, 1 nmol, Sigma), and methylene blue (MB, 1 nmol, Sigma), all dissolved in artificial cerebrospinal fluid (aCSF, pH 7.4) containing 0.2% pontamine skyblue. The injection volume (30 nl) was measured directly by monitoring the movement of the fluid meniscus in the micropipette through a 40 × microscope (Wild M650) with an ocular reticle that allowed a resolution of 1 nl.

*NO Measurement*

Chronoamperometric measurements of extracellular NO level in NTS *in vivo* were performed with microcomputer-controlled apparatus (IVEC-10, Medical systems Co., Greenvale, NY, USA) as described previously (31). A miniature Ag/AgCl reference electrode was inserted into the cortex and cemented in place with dental acrylic. The working electrode was made of one carbon fiber filaments (30 μm in diameter; Textron, Lowell, MA, USA). The sensor was first coated with Nafion (5% solution; Aldrich Chemical Co., Milwaukee, WI, USA) at 65°C to decrease any interference from exracellular ascorbic acid (AA) (12). The electrode was then electropolymerized with 2 mM Ni meso-tetra (N-methyl-4-pyridyl) porphyrine tetratosylate (Ni-TMPP) in 0.1 M NaOH at +0.9 V for 25 - 50 min. Each electrode was tested for selectivity and sensitivity to NO *in vitro*. Calibration of NO (2.5 to 7.5 nmol/L) was made using 2.5 to 7.5 μmol S-nitroso-N-acetyl-DL-penicillamine (SNAP) in 0.1 mM phosphate buffer (PBS, pH 7.4) (Fig. 1A). One μmol/L of SNAP is equivalent to 1 nmol/L NO (7, 31). Only electrodes showing selectivity for NO, compared with AA greater than 100,000 : 1 *in vitro*, were used in the *in vivo* recordings. The NO current generated by application of an oxidation potential of +0.9V, relative to a Ag/AgCl reference electrode, was recorded *in vitro* continuously at a rate of 1 Hz. All *in vitro* signals were expressed as nanomolar changes in NO using the *in vitro* calibration factors. Because microinjection of AA (200 μM, 100 nl, n=5) into NTS did not induce detectable oxidation current, we believe that these sensors are selective to NO (Fig. 1B). The NO microsensors were also tested for simulation with various chemical solutions, i.e. dopamine, norepinephrine, glycine, glutamate, L-Arg, L-NAME, and AA *in vitro* in 0.1 mM PBS. We found that the NO microsensors were insensitive to these substances (data not shown). Control injection with the same volume (30 nl) of vehicle in the depressor points produced no response.

*Histology*

At the end of each experiment, the animal was killed with an overdose of pentobarbital. The brain was removed and immersed in 10% formaline saline for 8 h. After fixation, the frozen transverse sections (50 μm) were stained with cresyl violet to identify the stimulated sites.

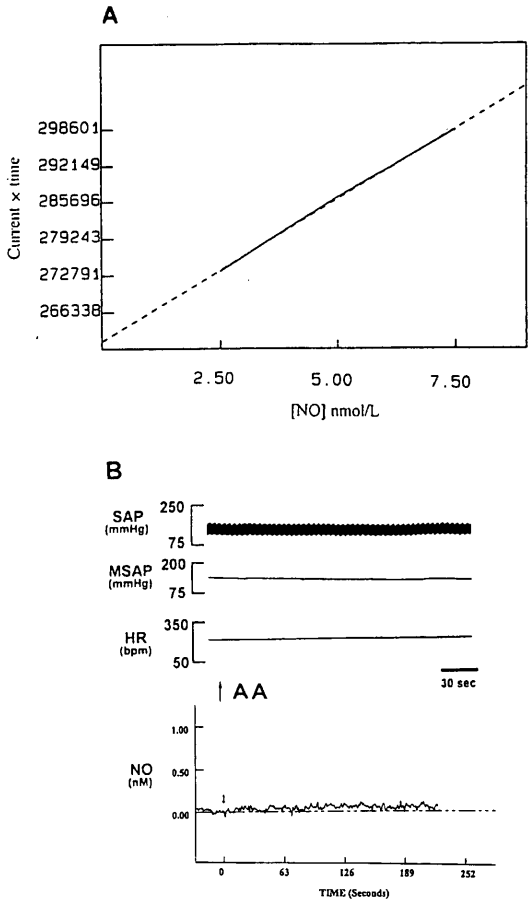


Fig. 1. Calibration for the sensitivity of NO electrode.
A, The NO electrode is calibrated *in vitro* using S-nitroso-N-acetyl-DL-penicillamine (SNAP, 2.5 to 7.5 μmol/L, pH 7.4) before the *in vivo* experiment. The *y*-axis represents the function of oxidation current (μA) × time (ms). The electrode shows a linear correlation between oxidation current and changes in [NO] (r = 0.991). B, Administration of ascorbic acid (AA, 200 μmol/L × 100 nl) to the NTS does not change the NO level. The electrode is insensitive to AA.
In this and the following figures, tracing from top to bottom: SAP = systemic arterial pressure; MSAP = mean SAP; HR = heart rate. NO = nitric oxide. Arrowhead (↑) indicates the microinjection of chemicals.

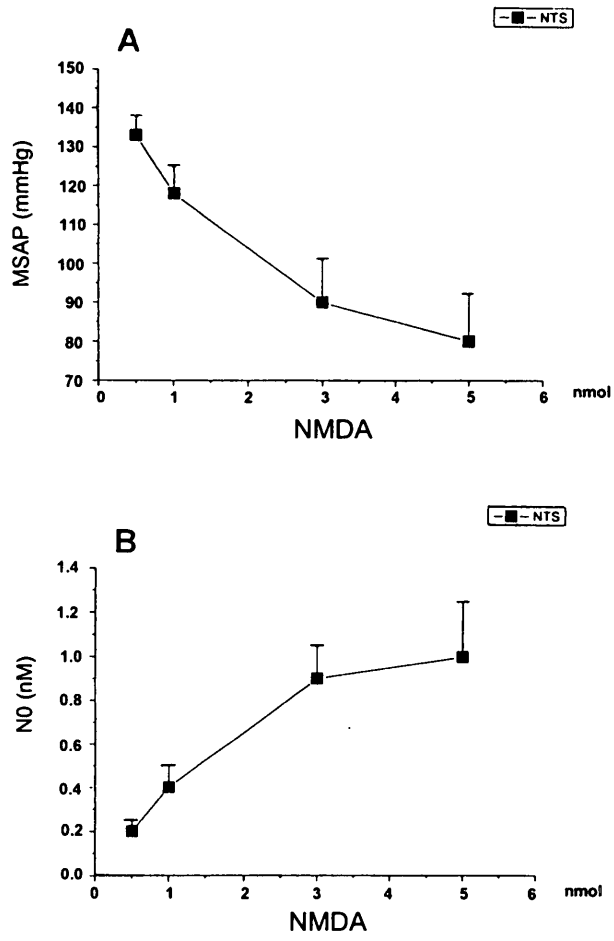


Fig. 2. Dose-response curves showing the changes of MSAP and NO level elicited by NMDA (0.5, 1, 3, 5 nmol) in NTS.
(A) The response of MSAP produced by NMDA. (B) The response of NO produced by NMDA. In each group, 5 points were studied. Vertical lines are SEM.

*Data Analysis*

Data were analyzed and compared by paired *t* test and unpaired *t* test methods. Differences of $p < .05$ were considered to be significant. All values were presented as mean±SEM.

## Results

*NO Level in NTS*

In 18 points of 13 experiments, the averaged extracellular NO level in NTS was 0.8 ± 0.5 nM (0.5 to 1.7 nM). For convenience of statistics and qualitative analysis, the NO level at these points receiving NMDA injection latter (arrowhead ↑) was set at zero.

*Determination of an Optimal Dose of NMDA for Microinjection*

Microinjections of NMDA (0.5, 1, 3, 5 nmol) to NTS produced a dose-dependent decrease in SAP response and increase in NO level (Fig. 2). Following the dose response curve, we selected a dose of 3 nmol for microinjection.

*Responses of NTS*

Microinjection of NMDA into NTS produced decreases of SAP and HR associated with NO release. On average, in 16 points of 13 cats, microinjection of NMDA (3 nmol) produced 37.5% decreases in SAP (from 138.5 ± 12.3 to 86.5 ± 9.8 mmHg; $p < .05$) and 21.2% decrease in HR (from 193.6 ± 13.7 to 152.4 ±

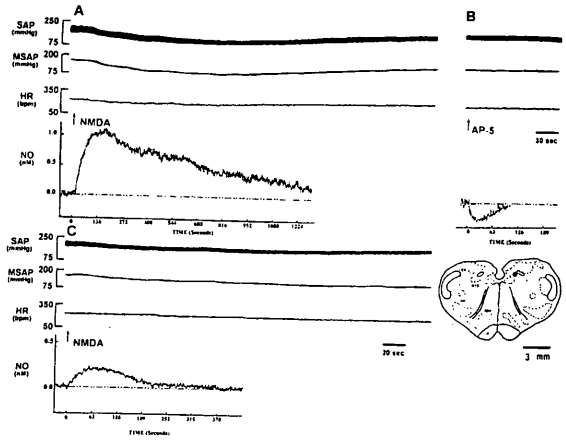


Fig. 3. AP-5 (1 nmol) blocked the cardiovascular responses and NO formation to NMDA (3 nmol) in NTS.
(A) Microinjection of NMDA produced decreases in SAP (75 mmHg) and HR (40 bpm), and increase in NO (1.2 nM). (B) Microinjection of AP-5 produced no change in SAP and small decrease in NO (0.3 nM). (C) Three min after AP-5, microinjection of NMDA again produced decrease in SAP (25 mmHg) and HR (25 bpm), and increase in NO (0.3 nM).
Dot (●) in the brain drawing shows the point receiving stimulation. Bar (–) indicates the time scale of SAP, MSAP and HR tracings. Abbreviations: AN = nucleus of ambiguus; CX = external cuneat nucleus; ION = inferior olivary nucleus; NTS = nucleus tractus solitarius; P = pyramidal tract; VIN = inferior vestibular nucleus; 5sp = spinal trigeminal nucleus; 5st = spinal trigeminal tract; 12N = hypoglossal nucleus; 12n = hypoglossal nerve.

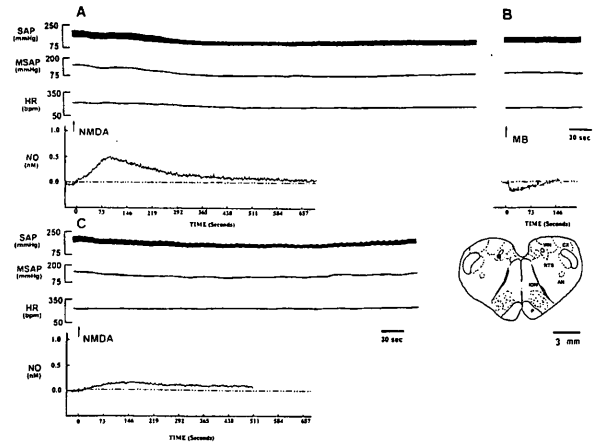


Fig. 4. Methylene blue (MB, 1 nmol) blocked the cardiovascular responses and NO formation to NMDA (3 nmol) in NTS.
(A) Microinjection of NMDA produced decrease in SAP (70 mmHg) and HR (40 bpm), and increase in NO (0.5 nM). (B) Microinjection of MB produced no change in SAP but small decrease in NO (0.2 nM). (C) Three min after MB, microinjection of NMDA again produced decrease in SAP (35 mmHg) and HR (10 bpm), and increase in NO (0.2 nM).

15.3 bpm; $p < .05$) associated with 0.7 ± 0.2 nM increase in NO. AP-5 and MB attenuated the responses of SAP, HR and NO to NMDA. Microinjection of AP-5 alone did not affect the resting SAP, but decreased NO level (0.5 ± 0.3 nM, n=8). On average, in 8 points of 7 cats, pre-treatment (3 min) of AP-5 (1 nmol) decreased the responses evoked by NMDA; 70.2% in SAP (from -58.3 ± 12.3 to -17.4 ± 8.3 mmHg; $p < .05$), 67.7% in HR (from -42.7 ± 8.6 to -13.8 ± 8.2 bpm; $p < .05$), and 63.7% in NO (from 1.1 ± 0.3 to 0.4 ± 0.2 nM; $p < .05$) (Fig. 3). On the other hand, microinjection of MB alone did not affect the resting SAP, but decrease NO level (0.4 ± 0.2 nM, n=5). On average, in 7 points of 6 cats, pre-treatment (3 min) of MB (1 nmol) decreased the responses evoked by NMDA; 58% in SAP (from -55.3 ± 9.7 to -23.2 ± 9.2 mmHg; $p < .05$), 67% in HR (from -38.5 ± 7.5 to -12.7 ± 5.6 bpm; $p < .05$), and 62.5% in NO (from 0.8 ± 0.3 to 0.3 ± 0.2 nM; $p < .05$) (Fig. 4).

## Discussion

The main finding of this study was that NO might be a mediator of depressor responses during the increase of neuronal activity in response to NMDA. Glu is a major neurotransmitters of the mammalian CNS that stimulates metabotropic and ionotropic receptors. The latter are NMDA or non-NMDA (quisqualate, AMPA and kainic acid) receptors. Stimulation of these ionotropic receptors, and mainly the $Ca^{2+}$-permeable NMDA receptor subtype, results in activation of a neuronal $Ca^{2+}$-dependent, constitutive NO synthase (6).

### *NMDA Induces Depressor and NO Release*

NO is produced enzymatically in various brain regions in response to activation of NMDA receptors (9). In this study, microinjection of NMDA into NTS produced depressor effect and NO release. The newly formed NO, acting both as a second messenger and neurotransmitter, readily diffuses across the cell membrane and then into presynaptic terminals, or excited adjacent sympathoinhibitory neurons in NTS to activate guanylate cyclase. It might cause the accumulation of cGMP resulting depressor responses. In the nervous system cGMP may directly act on the $Ca^{2+}$ and $Na^{+}$ channels to increase their firing rate, or acts on specific protein kinase (23) and phosphodiesterase (25). The increase in $Ca^{2+}$ influx in postsynaptic neuron of NTS may activate the NMDA receptor and causes decrease in SAP and NO release.

### *AP-5 Blocks Depressor Responses and NO Release Evoked by NMDA*

Antagonism of the ion channel linked to the NMDA receptor prevents neuronal $Ca^{2+}$ influx and may therefore prevent depressor responses. In the present study we found that prior microinjection of

AP-5, a competitive antagonist of NMDA, blocked depressor responses and NO release in NTS evoked by NMDA. It is suggested that NO is involved in the response of NTS neurons from activation of NMDA receptors. This is in agreement with a study showing that L-Arg is able to increase the firing of the neurons in NTS (27).

*Methylene Blue Blocks Depressor Responses and NO Release Evoked by NMDA*

NO may participate in local transcellular communication by facilitating cGMP formation in adjacent cells through the activation of soluble guanylate cyclase in cerebellum and NTS (14, 27). To determine whether the effects of NMDA in NTS target neurons were mediated through cGMP, we examined whether MB, a blocker of guanylate cyclase (18) inhibited the depressor effect and NO formation evoked by NMDA, as MB can inactivate NO extracellularly by formation of oxygen radicals (17). We have shown that the depressor responses and NO release evoked by NMDA in NTS were blocked by prior microinjection of MB. This result suggests that activation of guanylate cyclase, and thus cGMP synthesis, is involved in the excitatory effects of NMDA in NTS neurons. In other word, NMDA induces depressor effects through the pathways of activating soluble guanylate cyclase in NTS. Although microinjection of MB alone produced decrease in NO, the SAP and HR were not altered. The absence of cardiovascular changes may be attributed to the fact that MB blocked the cGMP in NTS neurons.

Two modes of NO functions have been postulated. First, NO is generated presynaptically following action potential-dependent influx of $Ca^{2+}$ ions. Second, NO is generated postsynaptically as a consequence of an increase in cytosolic $Ca^{2+}$ concentration resulting activation of excitatory amino acid, i.e., NMDA, receptors. NO then diffuses out and acts on glial cells or presynaptic terminals. In both cases, NO then activates, via guanylate cyclase, a cGMP-dependent mechanism that increases the release of Glu from presynaptic nerve terminals (10). In this study, we can not determine whether the depressor and sympathoinhibitory actions of NO in NTS are results of the postsynaptic or presynaptic release of NO. Because NO is a highly diffusible membrane-permeable molecule (disregard the precise site of generation), it may possibly act on neuronal structures some distance far from its source (24).

In the present study, although we used *in vivo* voltammetry to monitor the change of NO by local microinjection in NTS, however, there are various factors involved in the regulation of SAP and NO formation, i.e., different anesthetics and animal species (13), injection site (1), injection dose (29), aging (19), and the density of microvessel in brain.

In conclusion, in this study we confirm that NO is formed from NMDA activation in NTS and that NO diffuses out of neurons into nearby target neurons to produce cardiovascular responses and changes of NO formation through cGMP formation. The NO mediates depressor responses through activation of NMDA receptors in NTS.

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