The nNOS/cGMP Mediation of the Depressor Response to NMDA Receptor Stimulation in the Caudal Ventrolateral Medulla

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Abstract

Using *in vivo* voltammetry to directly measure extracellular nitric oxide (NO) levels, our previous studies suggested that the neuronal NO synthase (nNOS) and cyclic guanosine monophosphate (cGMP) signal transducing systems are involved in the cardiovascular responses elicited by activation of *N*-methyl-D-aspartate (NMDA) receptors in the rostral ventrolateral medulla. In this study, we examined if the depressor responses elicited by activation of NMDA receptors in the caudal ventrolateral medulla (CVLM) also depend on the actions of nNOS and soluble guanylyl cyclase. In anesthetized cats, microinjection of NMDA into the CVLM produced hypotension and bradycardia associated with NO formation. These NMDA-induced responses were attenuated by prior injections of 2-amino-5-phosphonopentanoate (a NMDA receptor competitive antagonist), 7-nitroindazole (a nNOS inhibitor) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (an inhibitor of soluble guanylyl cyclase). These findings suggest that NO is also involved in the NMDA-induced depressor responses of the CVLM.

Key Words: nitric oxide; caudal ventrolateral medulla; 7-nitroindazole; 2-amino-5-phosphonopentanoate; N-methyl-D-aspartate

Introduction

Nitric oxide (NO) is a highly diffusible gas that produces a wide range of physiological and pharmacological actions in the cardiovascular system (2, 15). Many studies have demonstrated that the ventrolateral medulla (VLM) plays an important role in the maintenance of systemic arterial pressure (SAP) and vascular tone (3, 8, 16). In this system, the rostral VLM (RVLM) and caudal VLM (CVLM) play opposing roles in regulating cardiovascular functions (10, 19). In addition, NADPH diaphorase, a cofactor of NOS, has been localized in the VLM (13).

In the brain, neuronal NO synthase (nNOS) and N-methyl-D-aspartate (NMDA) receptors colocalize at presynaptic and postsynaptic sites (1). Upon activation of NMDA receptors, the increase of intracellular Ca^{2+} activates the Ca^{2+}/calmodulin-dependent NO system by stimulating nNOS, which further converts L-arginine to NO and L-citrulline. NO then activates soluble guanylyl cyclase (sGC), thereby raising intracellular cyclic guanosine monophosphate (cGMP) (4, 7, 18).

Our previous studies have suggested that nNOS and sGC were involved in the cardiovascular responses elicited by microinjection of NMDA in the RVLM (21). The aim of this study is to further elucidate if nNOS and sGC are also involved in the cardiovascular

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responses in the CVLM.

Materials and Methods

Adult cats of either sex (n=11), weighing 2-4 kg, were anesthetized intraperitoneally with urethane (400 mg/kg) and α-chloralose (40 mg/kg). General procedures and preparation of the working NO electrode have been described previously (21). Briefly, the systemic arterial pressures (SAP), mean SAP (MSAP), and heart rate (HR) were measured.

Voltammetric measurements of extracellular NO concentrations *in vivo* were performed using a microcomputer-controlled apparatus (IVEC-10; Medical Systems Co., Greenvale, NY, USA). A miniature Ag/AgCl reference electrode was inserted into the cortex. The working electrode, a double carbon-fiber filament (filament diameter 30 (m), was first coated with Nafion (5% solution) at 65°C. The coated electrode was electropolymerized with 2 mM Ni-meso-tetra (*N*-methyl-4-pyridyl) porphyrine-tetratosylate in 0.1 M NaOH and 5 mM o-phenylenediamine solution in 0.1 M phosphate buffered saline (PBS; pH 7.4) solution at +0.9 V for 25-50 min (21). Calibration of NO detection was carried out *in vitro* using 1.0-3.0 μM S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) in 0.1 mM PBS. One micromolar SNAP can generate 1 nM NO *in vitro* (5). Under *in vitro* conditions, the working electrode was sensitive to SNAP (2 μM), but not to norepinephrine (2 μM), dopamine (2 μM), serotonin (2 μM), tyrosine (2 μM), nitrite (2 μM), glutamate (2 μM), NMDA (20 μM), 7-nitroindazole (7-NI; 10 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM), 2-amino-5-phosphonopentanoate (AP-5; 20 μM) or ascorbate (2 μM).

The dorsal surface of the brain stem was exposed for brain stimulation. A double-barreled glass micropipette (outside tip diameter of 30–40 μm) was prepared for drug microinjections and a NO electrode was glued to the micropipette to form a single pipette/electrode. The distance between the tips of the micropipette and electrode was 150–250 μm. This pipette/electrode was inserted into the CVLM at the level of obex; 3.0 mm lateral to the midline and 3.2 mm ventral to the dorsal surface of the medulla oblongata. The following drugs were microinjected by means of a pressure pneumatic pump: NMDA (containing 0.1% Pontamine sky blue) and AP-5 (Tocris) dissolved in saline; ODQ (Tocris) first dissolved in dimethylsulfoxide (1% DMSO) and then diluted in saline; and 7-nitroindazole (7-NI; Tocris) dissolved in 15% methanol. At the end of the experiment, the animal was euthanased by an overdose of pentobarbital. The brain was removed and immersed in 10% formalin saline for 8 h. After fixation, frozen transverse sections (50 μm) of the brain were stained with Cresyl violet to identify the injection sites.

All data are presented as mean ± standard error of mean (SEM). Student's *t*- test and one-way ANOVA were used to test whether the changes were significantly different (P<0.05). NS indicates non-significant changes.

Results

To acquire a reproducible NMDA effect on extracellular NO levels, the selectivity and sensitivity of the NO electrode were first examined under *in vivo* conditions. Microinjection of ascorbate (100 μM, 50 nl; n=5), 1% DMSO (50 nl; n=5) and 15% methanol (50 nl; n=5) into the CVLM did not change the SAP or NO levels. Forty minutes after the first injection of NMDA into the CVLM, the peak NO levels and the levels of ΔMSAP induced by a second dose of the same agent in the same place were unchanged (92±6% and 87±5% of the controls; n=7).

Under control conditions, the resting MSAP and HR were 122.8±11.4 mmHg and 205.3±13.6 bpm, respectively. The drug-evoked NO release (ΔNO) was measured by comparing the peak NO values before and after each microinjection. Microinjection of NMDA (1.0±0.1 nmol in 50 nl; n=7) into the CVLM produced a depressor response (ΔMSAP -43.8±9.6 mmHg; P<0.05), bradycardia (ΔHR -34.4±8.5 bpm; P<0.05), and an increase in NO levels (ΔNO 1.0±0.2 nM; P<0.05). To evaluate the specificity of NMDA-evoked responses, we sought to determine if AP-5 could antagonize the responses induced by NMDA. AP-5 was microinjected into the CVLM 3 min before NMDA injection at the same site (n=7). Unilateral microinjection of AP-5 (1.0±0.1 nmol in 50 nl) alone did not significantly change the basal SAP (ΔMSAP 10.4±3.7 mmHg; NS), HR (ΔHR 9.3±4.7 bpm; NS), or NO level (ΔNO 0.2±0.1 nM; NS). However, AP-5 attenuated the NMDA-evoked depressor responses, bradycardia, and NO formation (Fig. 1). The inhibitor AP-5 (n=7) significantly reduced the following responses evoked by NMDA: 53.6% in ΔMSAP (from -43.8±9.6 to -20.3±5.4 mmHg; P<0.01), 45.6% in ΔHR (from -34.4±8.5 to -18.7± 6.5 bpm; P<0.05), and 70% in ΔNO level (from 1.0± 0.2 to 0.3±0.1 nM; P<0.01).

The effects of 7-NI, a nNOS inhibitor, on the responses induced by microinjection of NMDA into the CVLM were investigated (n=7). Microinjection of 7-NI (0.5±0.05 nmol in 50 nl) into the CVLM alone did not significantly change the basal SAP (ΔMSAP 9.6±4.5 mmHg; NS), HR (ΔHR 8.2±4.6 bpm; NS), and NO level (ΔNO 0.3±0.1 nM; NS). However, the NMDA-induced responses were significantly reduced

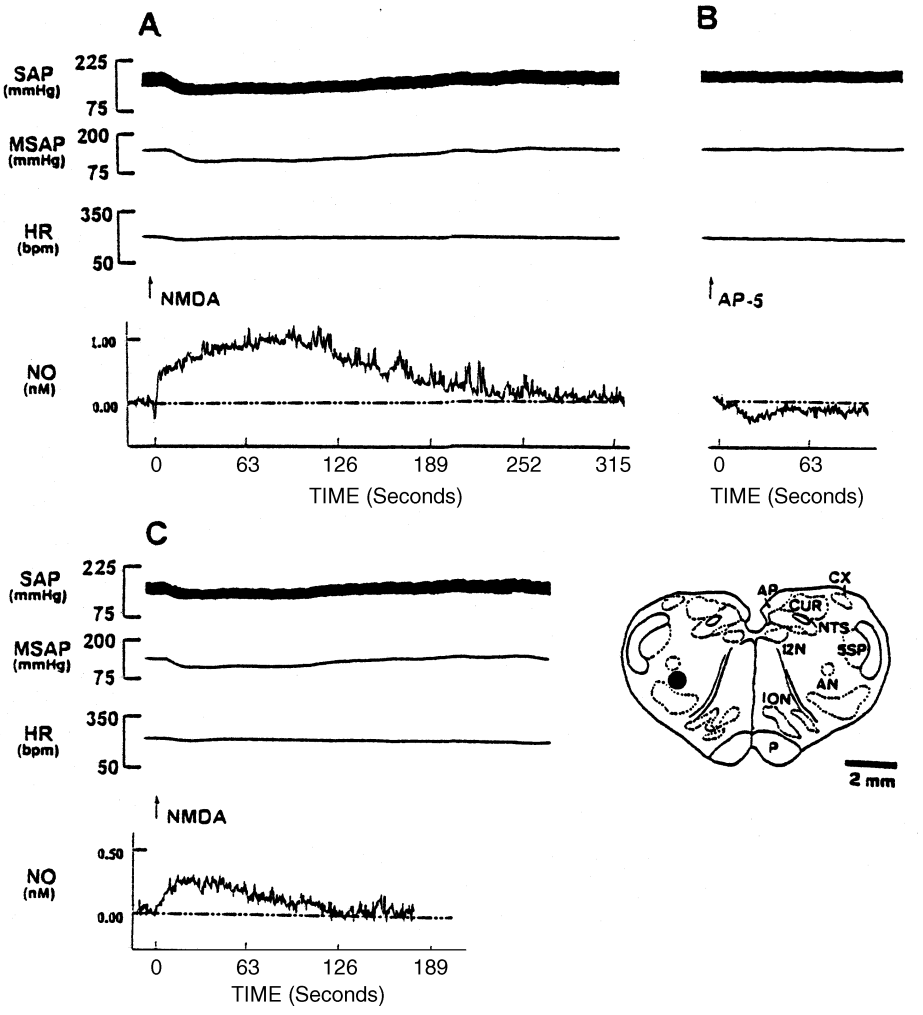

Fig. 1. AP-5 attenuated the NMDA-evoked cardiovascular responses and NO formation in CVLM. (A) Microinjection of NMDA (1.0 nmol) produced decreases in SAP (45.3 mmHg) and HR (40 bpm), and an increase in NO (1.2 nM). (B) Microinjection of AP-5 (1.0 nmol) produced no change in SAP but a small decrease in NO (0.3 nM). (C) Three minutes after AP-5, microinjection of NMDA again produced smaller decreases in SAP (25.2 mmHg) and HR (28 bpm), and a smaller increase in NO (0.3 nM). Tracings from top to bottom are: SAP, systemic arterial pressure; MSAP, mean SAP; HR, heart rate; NO, nitric oxide. Arrowheads (↑) under the tracings indicate the time of applying microinjection. A dot (•) in the brain drawing shows the location of stimulation. Abbreviations: AN, nucleus of ambiguous; AP, area postrema; CUR, cuneate nucleus; CX, external cuneat nucleus; ION, inferior olivary nucleus; NTS, nucleus tractus solitarius; P, pyramidal tract; 5sp, spinal trigeminal nucleus; 12N, hypoglossal nucleus.

by 7-NI as follows: 56.8% in ΔMSAP (from -45.2± 7.8 to -19.5±6.3 mmHg; $P<0.01$), 46.4% in ΔHR (from -32.3±8.7 to -17.3±5.5 bpm; $P<0.05$), and 70% in ΔNO level (from 1.0±0.3 to 0.3±0.1 nM; $P<0.01$; see Fig. 2).

We also studied if the NMDA-evoked responses depend on sGC activity. ODQ, a sGC inhibitor, was microinjected into the CVLM (n=8). ODQ (0.5±0.05 nmol in 50 nl) alone did not alter the basal SAP (ΔMSAP 9.2±3.7 mmHg; NS), HR (ΔHR 7.5±3.4 bpm; NS), or NO level (ΔNO 0.2±0.1 nM; NS). However, ODQ attenuated the NMDA-evoked effects as follows: 55.2% in ΔMSAP (from -41.5±8.2 to -18.6±6.5 mmHg; $P<0.01$), 41.9% in ΔHR (from -35.3±9.3 to -20.5±6.7 bpm; $P<0.05$), and 66.7% in ΔNO level (from 0.9±0.2 to 0.3±0.1 nM; $P<0.01$; see Fig. 3). The effects of AP-5, ODQ and 7-NI on the reduction of NMDA-evoked cardiovascular responses and NO formation gradually recovered within 60 min.

Discussion

The major findings of the present study were that microinjection of NMDA into the CVLM produced hypotension and bradycardia associated with NO formation. These effects were significantly reduced by prior injection of AP-5, 7-NI or ODQ. Thus, the cardiovascular responses elicited by activation of NMDA receptors in the CVLM depend on nNOS and sGC activities. Our findings of a NO

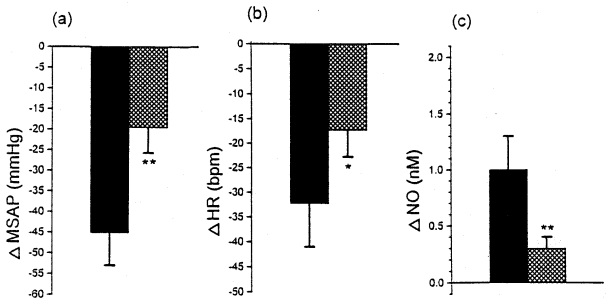

Fig. 2. Bar graphs show the cardiovascular responses and NO formation by NMDA before (▮) and after (▮) microinjection of 7-NI into the CVLM. NMDA (1 nmol; *n*=7) produced decreases in MSAP and HR associated with NO release. 7-NI (0.5 nmol) significantly attenuated the effects of MSAP (a), HR (b) and NO formation (c) induced by NMDA. Vertical bars represent means (SEM. **P*< 0.05, ***P*<0.01 compared with control.

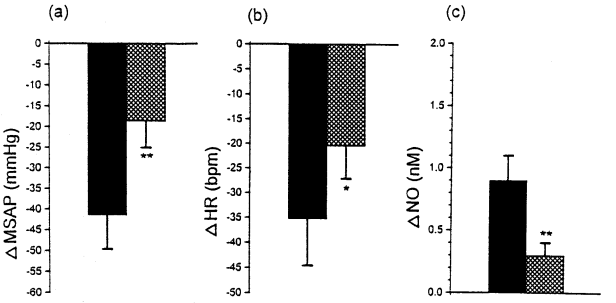

Fig. 3. Bar graphs show cardiovascular responses and NO formation by NMDA before (▮) and after (▮) microinjection of ODQ into the CVLM. NMDA (1 nmol; *n*=8) produced decreases in MSAP and HR associated with NO release. ODQ (0.5 nmol) significantly attenuated the effects of MSAP (a), HR (b) and NO formation (c) induced by NMDA. Vertical bars represent mean (SEM. **P*< 0.05, ***P*<0.01 compared with control.

signaling pathway that mediates the effects of activating NMDA receptors in the CVLM are similar to other results in the RVLM (11, 12, 21).

Exogenous application of L-arginine, a NO precursor, into the CVLM produced hypertension, whereas microinjection of N^G-monomethyl-L-arginine (L-NMMA), a NOS inhibitor, produced hypotension (6, 14). This observation seems to indicate that an elevation of NO level in the CVLM reduces the depressor actions of the CVLM, thereby eliciting a pressor response. In contrast, microinjection of L-arginine into the nucleus tractus solitarius (NTS), another depressor site in the brainstem, produced hypotension, while microinjection of N^G-nitro-L-arginine methyl ester and L-NMMA into the same site produced hypertension (17, 22). Microinjection of sodium nitroprusside or S-nitroso-glutathione, a NO donor, into the RVLM elicited increases in SAP and renal sympathetic activity (9). Based on these observations, it seems that exogenous manipulation of extracellular NO levels in the depressor sites does not necessarily result in a consistent effect on neuronal depressor activities. As NO is a highly diffusible gas, we speculate that a direct manipulation of extracellular NO levels by L-arginine or L-NMMA may affect all neurons in the vicinity and elicit a response that lacks spatial resolution. We observed that microinjection of NMDA into CVLM produced hypotension associated with endogenous NO formation. This endogenous NO synthesis elicited by exogenous application of NMDA may play a role that contrasts with the actions of NO acquired from exogenous application of L-arginine. As the NMDA-evoked depressor responses and NO formation in the CVLM are attenuated by either AP-5, 7-NI or ODQ, these observations strongly suggest that an attenuation of NO formation or suppression of NO-stimulated sGC activities may be responsible for the reduction of NMDA receptor-mediated depressor responses. Whether there is a spatial segregation of NO effects that characterizes the NMDA-mediated responses specifically in the CVLM requires further investigation.

It is interesting to note that, subsequent to NMDA receptor activation, NO generation is involved in both pressor and depressor responses. Previous studies have shown that NMDA produces cardiovascular responses and NO release in the RVLM and NTS (12, 20, 21). These are similar to our findings present that NMDA at CVLM produces depressor responses associated with NO release. Taken as a whole, these findings indicate that, on activation of NMDA receptors in either pressor or depressor sites of the brain, NO may serve as a subsidiary messenger mediating the NMDA-elicited signals. Because NO readily diffuses across the cell membrane to affect the adjacent neurons, this may serve as a common mechanism underlying the NMDA-receptor mediated effects.

In summary, NO formation elicited by microinjection of NMDA into the CVLM was concomitant with the depressor responses. Both NMDA-evoked depressor responses and NO formation were significantly attenuated by pre-treatment with AP-5, 7-NI and ODQ. We conclude that the nNOS/cGMP signal transducing system is involved in the cardiovascular responses induced by activation of NMDA receptors in the CVLM.

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