Nitric Oxide Mediates Vasodilatation in Response to Activation of N-Methyl-d-Aspartate Receptors in Brain

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Neurons release nitric oxide (NO) in response to activation of receptors for the excitatory amino acid N-methyl-d-aspartate (NMDA). We examined the hypothesis that activation of receptors for NMDA produces dilatation of the cerebral microcirculation that is mediated by NO. Diameters of cerebral arterioles were measured using a closed cranial window in anesthetized rabbits. Under control conditions, topical NMDA produced concentration-related dilatation of pial arterioles. Dilatation in response to NMDA was inhibited selectively by MK-801 (an NMDA receptor antagonist) and tetrodotoxin, suggesting that responses to NMDA were receptor mediated and dependent on neuronal activation. Increases in arteriolar diameter in response to NMDA were not affected by L-arginine but were inhibited by NG-nitro-L-arginine, suggesting that the vasodilatation was mediated by NO. Dilatation of cerebral arterioles in response to NMDA was not inhibited by indomethacin, suggesting that cyclooxygenase products do not mediate the response. Using isolated cerebral arteries, we also examined whether NMDA elicited direct cerebral vascular effects. In intact arteries studied in vitro, NMDA had no effect on vascular tone, suggesting that cerebral arteries lack receptors for NMDA. These findings suggest that NO generated in response to activation of receptors for NMDA in vivo is neuronally derived and not due to a direct vascular effect. Thus, NO may mediate increases in local blood flow during increases in neuronal activity in response to excitatory amino acids. (Circulation Research 1993;72:476-480)

KEY WORDS: • NMDA • cerebral arterioles • nitroprusside • NG-nitro-L-arginine • glutamate • acetylcholine • L-arginine

A wide range of cell types, including endothelium and neurons, produce the potent vasodilator nitric oxide (NO) or a related nitrosothiol.\textsuperscript{1-4} NO synthase has been isolated from brain and is distributed throughout a number of brain regions.\textsuperscript{5-8} Excitatory amino acids appear to be a major stimulus for production of NO in neurons. For example, glutamate and the glutamate analogue N-methyl-d-aspartate (NMDA) produce increases in neuronal activity and marked increases in cyclic GMP (cGMP) in brain tissue that are mediated by NO.\textsuperscript{9-12} Isolated neurons release NO in response to activation of receptors for NMDA,\textsuperscript{2} and computer modeling suggests that neuronally derived NO may influence local vascular tone.\textsuperscript{13}

Because NO is highly diffusible and is released extracellularly by neurons during activation,\textsuperscript{2,14,15} we hypothesized that neuronally derived NO may have an important influence on the local cerebral microcirculation. NO synthase and receptors for NMDA have been localized in the outer layers of the cerebral cortex.\textsuperscript{5,8,16} Thus, we used an experimental approach of measuring changes in diameter of pial arterioles during topical application of NMDA to the cerebral cortex. We examined whether NMDA produced dilatation of cerebral arterioles in vivo and whether the response to NMDA was dependent on activation of neurons and formation of NO. The major implication of this study is that NO may be a mediator of local vasodilatation during increases in neuronal activity in response to excitatory amino acids.

Methods

Animal Preparation

Experiments were performed on New Zealand White rabbits (weight, 2.5-3.5 kg) that were anesthetized with pentobarbital sodium (30 mg·kg\textsuperscript{-1} i.v.). Pentobarbital was supplemented regularly at approximately 10 mg·kg\textsuperscript{-1}·hr\textsuperscript{-1}. The trachea was cannulated, and the animals were ventilated mechanically with air and supplemental oxygen. A catheter was placed into a femoral artery for measurement of systemic pressure and to sample arterial blood. A femoral vein was cannulated for infusion of drugs.

Rabbits were placed in a headholder, and a closed cranial window was placed over the parietal cortex as described in detail elsewhere.\textsuperscript{17} The cranial window was filled with artificial cerebrospinal fluid warmed to 37°C. Diameters of pial arterioles were measured by use of a
microscope equipped with a TV camera coupled to a video monitor. Images were recorded on videotape, and vessel diameters were measured later with an image analyzer.

**Experimental Protocol**

Seven groups of animals were studied. In group 1 (n=7), arteriolar diameter was measured under control conditions and 1–2 minutes after the window was filled with cerebrospinal fluid containing NMDA (100 and 300 μM), acetylcholine (1 and 10 μM), or sodium nitroprusside (1 and 10 μM). Doses of a given agonist were applied in a cumulative manner, but the order of application of agonists was altered. A 15–60-minute recovery period was included after each agonist to allow diameters of arterioles to return to baseline. flushing the window with fresh cerebrospinal fluid maintained at 37°C did not alter baseline diameter of arterioles. Application of the three agonists was then repeated after a 60-minute recovery period. This group of animals served as a time control to establish whether responses to NMDA, acetylcholine, and nitroprusside were reproducible.

In the remaining six groups of animals, responses of pial arterioles to agonists were examined in the absence and then in the presence of antagonists used to examine the mechanism that mediates vasodilation in response to NMDA. In general, responses to acetylcholine, nitroprusside, and NMDA were tested in all animals before and again during treatment with a specific inhibitor. In group 2 (n=6), the effect of an antagonist to NMDA, MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycloheptan-5,10-imine maleate] (10 μM), was examined. In group 3 (n=7), the effect of tetrodotoxin (0.1–1 μM), an inhibitor of sodium channels and action potentials, was examined. In group 4 (n=9), the effect of Nω-nitro-λ-arginine (L-NNA, 100 μM), an inhibitor of NO synthase, was examined. In group 5 (n=5), the effect of L-arginine (300 μM), the substrate for NO synthase, was examined. In group 6 (n=4), the effect of L-NNA (100 μM) in the presence of L-arginine (300 μM) was examined. The cranial window was treated for 15 minutes with antagonists before application of agonists. In group 7 (n=6), the effect of indomethacin on responses of pial arterioles to NMDA was examined. Changes in diameter of cerebral arterioles were measured before and 20 minutes after intravenous injection of indomethacin (5 mg · kg⁻¹).

**Isolated Cerebral Arteries**

Basilar and middle cerebral arteries were taken from anesthetized rabbits, cut into 3–4-mm rings, and suspended in organ baths by use of two stainless steel stirrups. A force transducer was used to record isometric tension in vessels continuously bathed with a modified Krebs solution with the following composition (mM): NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, EDTA calcium 0.026, glucose 11.1; pH 7.4. The buffer was maintained at 37°C and aerated with 95% O₂ – 5% CO₂. Vessels were gradually stretched to a resting tension of approximately 400 mg, which produced optimal constrictor to KCl, histamine, and serotonin. To examine relaxation responses, rings were precontracted submaximally with histamine or serotonin. After reaching a stable contraction plateau, vessels were exposed to either acetylcholine, NMDA, or nitroprusside.

**Statistics**

Statistical analysis was performed using a paired t test to compare various interventions with the preceding controls. Bonferroni correction was used for multiple comparisons. All values are expressed as mean±SEM. A value of p<0.05 was considered significant.

**Results**

**Responses of Pial Arterioles to NMDA**

Under control conditions, topical application of NMDA and nitroprusside produced concentration-related dilatation of pial arterioles. Responses to both agonists were reproducible (data not shown). Increases in diameter of cerebral arterioles during application of NMDA was inhibited completely in the presence of MK-801 (10 μM), indicating that responses were receptor-mediated. NMDA (300 μM) increased arteriolar diameter by 40±8% in the absence and −3±2% in the presence of MK-801. MK-801 (10 μM) alone had no significant effect on baseline diameter of cerebral arterioles and did not inhibit dilatation of pial arterioles in response to nitroprusside (data not shown).

Under control conditions, L-NNA (100 μM) decreased baseline diameter of pial arterioles by 11±2% (109±6 versus 97±6 μm, p<0.05). Dilatation of pial arterioles in response to NMDA was inhibited by L-NNA (Figure 1), indicating that the response was dependent on production of NO. In contrast, dilatation of cerebral arterioles in response to nitroprusside was not inhibited and tended to be enhanced by L-NNA (Figure 1).

L-Arginine (300 μM) had no effect on baseline diameter and did not alter dilatation of pial arterioles in response to NMDA (data not shown). L-arginine (300 μM) inhibited the effect of L-NNA (100 μM) on responses of pial arterioles to NMDA (data not shown).

Tetrodotoxin (0.1–1 μM) had no effect on baseline diameter of pial arterioles (112±10 versus 109±9 μm) but inhibited dilatation in response to NMDA (Figure 2). This finding indicates that responses to NMDA are dependent on neuronal activation. In contrast to NMDA, dilatation
of cerebral arterioles in response to nitroprusside was not inhibited by tetrodotoxin (Figure 2).

Dilatation of pial arterioles in response to NMDA was not inhibited and tended to be enhanced by indomethacin. NMDA (300 μM) increases arteriolar diameter by 28±9% in the absence and 41±13% in the presence of indomethacin. These findings suggest that products of cyclooxygenase do not mediate cerebral arteriolar dilatation in response to NMDA.

Acetylcholine produced reproducible, concentration-related dilatation of cerebral arterioles that was inhibited significantly by L-NNA. Acetylcholine (1 and 10 μM) increased vessel diameter by 14±3% and 40±5% in the absence and 7±1% and 26±4% in the presence of L-NNA, respectively. These results suggest that dilatation of pial arterioles in response to acetylcholine is dependent on production of NO or an NO-containing compound. In contrast to NMDA, however, dilatation of pial arterioles in response to acetylcholine was not altered by tetrodotoxin, indicating that responses were not dependent on neuronal activation. Acetylcholine (1 and 10 μM) increased vessel diameter by 16±6% and 41±8% in the absence and 13±6% and 41±9% in the presence of tetrodotoxin, respectively.

Responses of Isolated Cerebral Arteries

In intact rings of the middle cerebral artery precontracted submaximally with histamine, NMDA had no effect on vascular tone (Figure 3). These same arteries exhibited marked relaxation in response to nitroprusside and acetylcholine (Figure 3). A similar lack of effect of NMDA on vascular tone was observed in 15 experiments (five middle cerebral, 10 basilar arteries), including some experiments in which serotonin was used to precontract the vascular rings. These findings suggest that NMDA has no direct effect on cerebral blood vessels.

Discussion

There are several major new findings in the present study. First, activation of NMDA receptors produces dilatation of the cerebral microcirculation. Dilatation of cerebral arterioles in response to NMDA is inhibited by tetrodotoxin and L-NNA, suggesting that responses to NMDA are dependent on neuronal activation and production of NO. Indomethacin did not inhibit vasodilatation in response to NMDA, suggesting that cyclooxygenase products do not mediate responses to NMDA. Second, dilatation of pial arterioles in response to acetylcholine is inhibited by L-NNA but not tetrodotoxin. These findings support the concept that dilatation of cerebral vessels in response to acetylcholine is dependent on production of an endothelium-derived relaxing factor and not dependent on activation of neurons. Third, isolated middle cerebral and basilar arteries did not respond to NMDA, suggesting that cerebral blood vessels lack receptors for NMDA. Thus, these findings support the hypothesis that dilatation of cerebral arterioles in response to NMDA in vivo is mediated by NO produced by neurons in response to activation of receptors for NMDA.

Glutamate is the major excitatory neurotransmitter in brain and mediates its effects through activation of three distinct receptor subtypes (NMDA, quisqualate, and kainate). Isolated neurons release NO in response to activation of receptors for NMDA. Glutamate and NMDA produce large increases in cGMP levels both in brain slices and in vivo that are blocked by inhibitors of NO synthase. These findings suggest that neuroglial derived NO mediates increases in cGMP levels in brain in response to excitatory amino acids.

Topical application of the excitatory amino acids glutamate and NMDA produces dilatation of pial arterioles (present study). Dilatation of pial arterioles in response to NMDA was not inhibited by indomethacin in the present study and a previous study in newborn pigs, suggesting that activity of cyclooxygenase is not involved in mediating the response. In the present study, dilatation of the cerebral microcirculation in response to NMDA was inhibited by L-NNA and tetrodotoxin, suggesting that responses were mediated by NO and dependent on neuronal activation. Effects of L-NNA on responses to NMDA appear to be specific, because vasodilatation in response to nitroprusside was not inhibited by L-NNA. Tetrodotoxin is considered to be highly specific as an inhibitor of sodium channels in electrically excitable cells. In the present study, evidence that effects of tetrodotoxin were specific for responses to NMDA is provided by the finding that vasodilatation in response to acetylcholine and nitroprusside was not inhibited by the toxin.
We considered the possibility that NMDA could have direct effects on endothelium or smooth muscle. Cerebral arteries are innervated by nonadrenergic noncholinergic nerve fibers that can produce NO-mediated relaxation when activated chemically or electrically. In isolated cerebral arteries, however, NMDA had no effect on tone, suggesting that cellular elements in the vessel wall were not activated. These same vessels exhibited marked relaxation in response to acetylcholine and nitroprusside. Our findings in rabbit cerebral arteries confirm previous studies that suggested that receptors for NMDA are not present in cerebral blood vessels. Receptors for NMDA are absent in ovine cerebral microvessels, and isolated cerebral blood vessels from several species (basilar and middle cerebral arteries in cats, basilar artery and parenchymal arterioles in rats, and pial arteries in humans) are unresponsive to NMDA and glutamate in vitro.

In addition to lack of effect on isolated cerebral blood vessels, several lines of evidence strongly suggest that responses of the cerebral microcirculation to NMDA are mediated via an effect on cortical neurons. Neurons are generally considered to be the only cells with receptors for NMDA, and neurons produce NO in response to activation of NMDA receptors. Responses to NMDA in the present study and a previous study that examined increases in cGMP levels in brain slices in response to NMDA were inhibited by tetrodotoxin, indicating that responses were dependent on neuronal activation. In brain slices, excitatory amino acids enhance neuronal activity and the rate of oxygen consumption. Topical application of NMDA and glutamate to the cerebral cortex produces neuronal depolarization and a marked increase in local cerebral metabolism. Although astrocytes can also produce NO in response to some stimuli, it is unlikely that they contribute to observed responses, because astrocytes lack receptors for NMDA.

A recent study suggests that dilatation of cerebral arterioles in response to nitroglycerin and nitroprusside is mediated, in part, by activation of trigeminal fibers that innervate cerebral vessels and release calcitonin gene-related peptide. We cannot exclude the possibility that release of calcitonin gene-related peptide from trigeminal afferents in response to NO released by cortical neurons contributes to dilatation of pial arterioles in response to NMDA.

More than a century ago, Roy and Sherrington suggested that products of cerebral metabolism influence cerebral vascular tone. A number of compounds, including hydrogen and potassium ions and adenosine, have been suggested to participate in such a mechanism. Previous studies suggest that vascular muscle in brain is under the influence of NO or a NO-containing compound produced by endothelium. The present study suggests that NO produced by neurons in response to activation of receptors for NMDA also influences local vascular tone and may represent a major mechanism to couple local perfusion with neuronal activity.

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**References**


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