N-methyl-d-aspartate-induced vasodilation is mediated by endothelium-independent nitric oxide release in piglets

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Received 15 June 2001; accepted in final form 21 November 2001

Previous studies suggest that stimulation of N-methyl-d-aspartate (NMDA) receptors on cortical neurons results in dose-dependent pial arteriolar dilation via a mechanism involving neuronal nitric oxide (NO) synthase (nNOS) activation and subsequent NO release in newborn pigs (11, 29). Because cerebral resistance vessels and cortical astroglia lack NMDA receptors, cerebral vasodilation to NMDA must be initiated by substances released from activated neurons (30, 38). Similar involvement of NO in NMDA-induced pial arteriolar vasodilation or increased cerebral blood flow (CBF) have been observed in many other species (14, 32, 40). Glutamate receptor activation either by nervous or pharmacological stimulation results in increased blood flow in a variety of regions, such as the cerebral cortex (25, 33, 41), striatum (10), hippocampus (18), cerebellum (3), and medulla (16) in rats. In all these regions, NO seems to be involved in the mechanism of CBF increase, and the major glutamate receptor subtype appears to be the NMDA receptor, except in the cerebellum (3, 40). However, many of the details of this relationship between activation of NMDA receptors and cerebrovascular dilation are unclear. Whereas there is agreement that NO is essential for NMDA-induced arteriolar dilation, it is unclear whether parenchymal-derived NO can directly reach pial arterioles. Alternatively, pial arteriolar responses may follow the initial dilation of intracortical arterioles via retrograde flow-dependent vasodilation by endothelial factors (15).

The presence of alternative vasodilator agents is also suggested by the recovery of NMDA-induced hyperemia after chronic NOS inhibition (33). In addition, recent reports showed that inhibition of cytochrome P-450 epoxygenase (referred to as P-450 epoxygenase) blocks NMDA- or glutamate-induced increases in blood flow in the cerebral cortex or striatum in the rat (2, 10). Thus P-450 epoxygenase products such as epoxyeicosatrienoic acids (EETs) may be the final mediators of cerebrovascular dilation following NO production by NMDA-positive neurons. Indeed, cultured rat astrocytes produce and release EETs in response to glutamate, and in vivo astrocytes are activated by increased neuronal activity and glutamate release upon NMDA exposure (26). Nonetheless, the role of P-450 epoxygenase products in mediating NMDA-induced cerebral dilation has not been examined in piglets.

The present study addressed four aims concerning the mechanism of NMDA-induced pial arteriolar dilation in the piglet. First, we determined whether NMDA-induced arteriolar dilation is associated with...
increases in NO metabolites (NO\textsubscript{X}) in perivascular cerebrospinal fluid. Second, we examined whether NMDA-induced dilation is reduced in arteriolar segments separated from the parenchyma by underlying veins. Third, we determined whether endothelial dysfunction reduces arteriolar responses to NMDA. Finally, we determined whether pretreatment and coapplication of miconazole, a potent inhibitor of P-450 epoxygenase, would alter pial arteriolar responses to NMDA.

MATERIALS AND METHODS

Animals. Newborn piglets of either sex (1–7 days old, 1–2 kg body wt) were used. All procedures were approved by the Institution Animal Care and Use Committee. The animals were anesthetized with thiopental sodium (30–40 mg/kg ip) followed by intravenous injection of α-chloralose (75 mg/kg). The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. Supplemental doses of α-chloralose were given to maintain a stable level of anesthesia determined by continuous blood pressure monitoring and regular checking of the responsiveness to tactile stimuli. In groups 5 and 6, the left common carotid artery (CCA) was ligated and catheterized distally to provide access for intra-arterial phorbol 12,13-dibutyrate (PDB) injection. In piglets, ligation of one carotid artery has no detectable effect on CBF (19, 21). The piglets were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20 cycles/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. Body temperature was maintained at 37.5–38.5°C by a water-circulating heating pad. Body temperature, arterial pH, and blood gases were also in the normal ranges and did not vary significantly among different groups. For instance, in group 1, the values were the following: body temperature, 38.0 ± 0.2°C; pH, 7.44 ± 0.03; P\textsubscript{CO\textsubscript{2}}, 35.7 ± 1.8 mmHg; and P\textsubscript{O\textsubscript{2}}, 98.3 ± 8.9 mmHg.

The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19 mm in diameter) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate.

After surgery, the closed window was filled and gently perfused with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O\textsubscript{2} and 6.5% CO\textsubscript{2} in balance N\textsubscript{2} to give pH = 7.33, P\textsubscript{CO\textsubscript{2}} = 46 mmHg, and P\textsubscript{O\textsubscript{2}} = 43 mmHg. The aCSF consisted of the following (in mmol/l): 132 NaCl, 2.9 KCl, 1.2 CaCl\textsubscript{2}, 1.4 MgCl\textsubscript{2}, 24.6 NaHCO\textsubscript{3}, 6.7 urea, and 3.7 glucose. At the end of the experiments, the animals were killed with an intravenous bolus of KCl.

Study groups. Instrumented piglets were divided into eight study groups. In group 1 (n = 7), we determined aCSF levels of NO metabolites before and after NMDA application. In group 2 (n = 5), we examined reproducibility of arteriolar dilator responses to NMDA. Thus we determined whether two arterioles of the same size and arising from the same trunk vessel would show similar responses to NMDA. In group 3 (n = 4), and group 4 (n = 4), we determined whether adjacent sections of the same arteriole, one of which is on the cortical surface and one of which is over a large vein, would show the same response to NMDA or BK, respectively. In group 5 (n = 7) and group 6 (n = 7), we assessed the effect of impaired endothelial function by PDB on arteriolar responses to hypercapnia and NMDA or BK, respectively. In group 7 (n = 6) and group 8 (n = 5), we tested whether short or prolonged miconazole pretreatment reduces pial arteriolar responses to NMDA.

Experimental design. Diameters of pial arterioles were measured using a microscope (Wild M36) equipped with a videocamera (Panasonic) and a video microcalorimeter (IV-550, For-A-Co; Newton, MA). A pial arteriole with a baseline diameter of ~100 μm was chosen, and the cranial window was then gently perfused with aCSF until a stable baseline was obtained. Responses of pial arterioles to hypercapnia (group 5), NMDA (10, 50, and 100 μmol/l, groups 1, 2, 3, 5, 7, and 8), and BK (0.03, 0.3, 3, and 30 μmol/l, groups 4 and 6) were determined. Hypercapnia was elicited by ventilating the animals with a gas mixture containing 5% CO\textsubscript{2}-21% O\textsubscript{2}-balance N\textsubscript{2}. NMDA and BK were dissolved in aCSF and administered topically through the injectable ports of the cranial window onto the brain surface with single applications. Arteriolar diameters were monitored continuously for 4–7 min for each stimulus. The window was then flushed with aCSF, and the arteriolar diameters were returned to baseline values.

In group 1, aCSF samples (~500 μl) were collected by gently flushing the window 5 min after application of aCSF (baseline) and 100 μmol/l NMDA. NO released from the cortex was measured as its NO\textsubscript{X} metabolites using a chemiluminescence detector (model 280, Sievers NO analyzer) coupled to an Apple Macintosh (8100) computer as described previously (28). To quantify the concentration of NO\textsubscript{X} in aCSF samples, a standard curve for NaNO\textsubscript{2} concentration (0.1–10 μM) was generated. Unknown samples were compared with the standard curve using the software provided with the Sievers NO analyzer. This program takes into account both the peak response and the total area of the curve generated by standard and unknown samples. All measurements were performed in duplicate, and the background signal for aCSF was subtracted from each measured value.

In groups 4 and 5, 10 ml of PDB (10 μmol/l, dissolved in ethanol, diluted in saline, final concentration of ethanol = 1%) were injected in bolus (5 s) into the cerebral circulation through the left CCA catheter to attenuate endothelial function (4). In every case, we observed sudden whitening (blanching) in the observed vessels followed by gradual return of normal arterial blood flow. Vascular responses to hypercapnia, NMDA, or BK were reexamined after a stabilization period of 5–7 min. There was a variable effect of PDB injection on mean arterial blood pressure. In those cases where mean arterial blood pressure remained elevated for 5 min, venous blood was withdrawn to obtain similar blood pressure values for repeated measurements. The heparinized blood was reinjected after the measurements.

In group 7, piglets received a single topical application of miconazole (20 μmol/l, dissolved in dimethyl sulfoxide, diluted in aCSF, final concentration of dimethyl sulfoxide = 0.2%) for 20 min. In group 8, miconazole was applied four times every 20 min by repeated flushing of the cranial window; thus miconazole pretreatment lasted 80 min. After pretreatment, vascular dilation to NMDA coapplied with 20 μmol/l miconazole was reexamined in both groups.

Drugs. The drugs used in this study were NMDA, BK, miconazole, and PDB (Sigma).

Statistics. Data are expressed as means ± SE. Pial arteriolar diameter data were analyzed using repeated-measures analysis of variance, followed by pairwise comparisons using the Student-Newman-Keuls test where appropriate. Percent...
preservation of vasodilation data were analyzed with one-tailed t-test. Comparisons between branches of a larger arteriole or between adjacent branches of the same arterial were compared using paired t-tests. For paired arterioles in group 2, the Pearson Product Moment Correlation Coefficient was calculated to document the extent of similarity of dilator responses to NMDA of paired arterioles or of the relationship between baseline arteriolar diameter and degree of responsiveness to NMDA. P values of <0.05 were considered as statistically significant.

RESULTS

Mean arterial blood pressure was in the normal range and was not significantly different during repeated measurements of vascular responsiveness to hypercapnia, NMDA, or BK in any of the experimental groups. NMDA induced dose-dependent pial arteriolar vasodilation as previously reported. In addition to vasodilation, NMDA significantly increased NOx levels in the aCSF. Thus 100 μmol/l NMDA compared with baseline increased pial diameters by 47 ± 4%, and NOx concentrations simultaneously increased from 3.7 ± 1.0 to 5.3 ± 1.2 μmol/l (P < 0.05).

Paired arterioles reacted identically to NMDA (Fig. 1). Thus the correlation coefficient for responses to three doses of NMDA was 0.94 (P < 0.05; n = 15 comparisons). In contrast, NMDA elicited significantly smaller vasodilation in arteriolar segments overlaying veins compared with segments directly on the brain (Fig. 2). However, all doses of BK elicited remarkably similar responses in both arteriolar segments, indicating that there is no mechanical limitation of vasodilation in arterioles overlaying larger veins (Fig. 2).

Intra-arterial injection of PDB did not alter vascular responses to NMDA (Fig. 3). However, PDB attenuated vascular reactivity to hypercapnia (Fig. 3). Before PDB injection, inhalation of 5% CO2 significantly increased arterial Pco2 from 35.7 ± 1.8 to 52.9 ± 2.1 mmHg and reduced arterial pH from 7.44 ± 0.03 to 7.31 ± 0.03, resulting in arteriolar vasodilation from 93 ± 2 to 106 ± 5 μm. In contrast, after PDB injection 5% CO2 still increased Pco2 from 34.3 ± 1.8 to 51.9 ± 2.1 mmHg and reduced pH from 7.42 ± 0.05 to 7.25 ± 0.05, but resulted in only minimal changes in arteriolar diameters from 85 ± 5 to 89 ± 6 μm. PDB injection also attenuated vascular responsiveness to BK (Fig. 3). Remarkably, percent attenuation of vascular responsiveness to both endothelium-dependent stimuli (hypercapnia and BK) was very similar (>70%) in contrast to the unaltered NMDA-induced vasodilation.

Twenty minutes of pretreatment and coapplication of miconazole with NMDA did not affect NMDA-induced pial arteriolar responses (Fig. 4). Similarly, 80 min of miconazole pretreatment did not affect NMDA-induced dilation. Thus diameters for baseline and 50 and 100 μmol/l NMDA were 92 ± 8 versus 83 ± 4 μm, 122 ± 12 versus 113 ± 15 μm, and 130 ± 9 versus 131 ± 9 μm, respectively. Whereas there was a ten-
tivity for a decreased baseline diameter after repeated application of miconazole, NMDA reactivity was not changed (for example, 33 ± 13% vs. 34 ± 14% to 100 μmol/l NMDA).

DISCUSSION

There are four major new findings in the present study clarifying the mechanism of NMDA-induced pial arteriolar vasodilation. First, NMDA increases NO levels in perivascular CSF surrounding pial arterioles, implying a relationship between increased availability of NO and pial arteriolar dilation in the newborn pig. Second, the presence of underlying large veins decreases NMDA-induced dilation, suggesting the presence of a diffusible vasodilator reaching the arterial smooth muscle directly from the brain parenchyma. Third, endothelial stunning by PDB severely attenuates endothelial-dependent responses such as those to hypercapnia or BK, but leaves NMDA-induced vasodilation intact. Thus the mechanism of NMDA-induced pial arteriolar vasodilation appears to be independent of endothelial function. Finally, miconazole, a potent inhibitor of P-450 epoxygenase, failed to affect vascular dilation to NMDA, indicating that metabolites of cytochrome P-450 like EETs do not play a role in the mediation of pial arteriolar vasodilation in the piglet.

Whereas there is a strong association between arteriolar vasodilation and increase in blood flow with activation of neuronal glutamate receptors in the cerebral cortex, the precise mechanisms of coupling are unclear. The role of glutamate in metabolism/blood flow coupling has been demonstrated perhaps best in the cerebellum (3, 39). Considerable evidence suggests that NO of neuronal origin plays a major role in eliciting vascular responses to NMDA in newborn and adult animals. In the piglet, local treatment with NOS inhibitors virtually abolished pial responses to both NMDA and glutamate (29). Systemic pretreatment with nonspecific NOS inhibitors (Nω-nitro-l-arginine methyl ester) or specific (7-nitroindazole) inhibitors of nNOS also attenuated NMDA-induced vasodilation in proportion to their inhibitory effect on cortical NOS activity (6). In this study, we were able to show increased amounts of NOx accumulating in the aCSF simultaneously with the vasodilation during NMDA application, independently confirming previous studies in piglets. The actual increase in NO production may be actually higher than that detected in perivascular aCSF, because a high percentage of NO reaching the pial vessels from the neuropil is probably scavenged by hemoglobin and thus will never accumulate in the aCSF. This assumption is supported by our findings that arteriolar segments being “shielded” from the parenchyma by an underlying big vein show significantly smaller dilatory responses to NMDA, in accordance with the concept of a rapidly diffusing vasodilatory agent such as NO directly reaching the pial arterioles.

In the present study, administration of miconazole, a potent P-450 epoxygenase inhibitor, did not decrease NMDA-induced pial arteriolar dilation. We used the same concentration that others have shown to block NMDA-induced vasodilation in rats (10). In addition, a smaller dose was shown to inhibit hypoxic vasodilation in piglets (24). Furthermore, in our own laboratory at the same time that these experiments were done in piglets, this dose of miconazole was shown to be effective in blunting relaxation of rat mesenteric arteries to acetylcholine (data not shown). Therefore, we cannot confirm the involvement of P-450 epoxygenase metabolites in the mediation of NMDA-induced increases in the piglet.

In this study, we inhibited endothelium-dependent vascular responsiveness via intravascular administration of phorbol ester PDB to the cerebral circulation. We (present study) and others (4) have shown that this approach attenuates endothelium, but not smooth muscle-dependent, dilator responses in pial arterioles.

Fig. 4. Effect of miconazole pretreatment and coapplication with NMDA on pial arteriolar vasodilation. Baseline arteriolar diameters were not affected by 20-min pretreatment with miconazole; the values before versus after miconazole treatment were 97 ± 4 versus 94 ± 6 μm. Pial arteriolar responses to NMDA were unaffected by miconazole. Thus P-450 epoxygenase products do not appear to be involved in mediation of NMDA-induced vasodilation.
of various species. The mechanism of action is not precisely known, but phorbol esters induce reversible endothelial dysfunction in vivo probably secondary to actions of reactive oxygen species (ROS) from activated platelets and leucocytes on endothelium (4). Hypercapnia- and BK-activated vasodilations are believed to be mediated by NO or ROS (36) released from the endothelium, and thus dilator responses are eliminated by endothelium denudation or damage. In piglets, it has been shown that laser light dye induces endothelial injury eliminating arterial dilation to arterial hypercapnia (22, 23). ROS, which promote dilation to bradykinin, apparently arise from metabolism of arachidonic acid by cyclooxygenase (COX)-1 in mice (32). Whether this same mechanism occurs in piglets is unclear, because in this species COX-2 is the predominant isoform present under normal conditions both in brain and cerebral arteries (34). However, our finding that NMDA-induced dilation of pial arterioles was intact after PDB injection indicates that a contribution of endothelial NO or other endothelial-derived dilator substances to vasodilation is very unlikely.

In piglets, hypoxia, global ischemia, and asphyxia attenuate NMDA-induced dilation in a dose and time-dependent manner (7, 12, 13) via ROS produced by COX activity (5, 35). Endothelial cells are damaged by ischemia (20), but their role in the attenuation of NMDA-induced vasodilation is not supported by the present study. The site of injury appears to be neuronal. ROS produced during ischemia-reperfusion are likely to affect especially the NMDA receptors that are sensitive to ROS both in vivo (17, 27) and in vitro (1). In contrast, kainic acid elicits a similar but ischemia-resistant neuronal-vascular sequence (8). Our hypothesis that after ischemia the number of functional NMDA receptors determines the magnitude of the vascular response is supported by the results of Weiss et al. (37). They reported that chronic treatment with the NMDA receptor antagonist CGS-19755 for 7 days upregulated NMDA receptors and simultaneously enhanced NMDA-induced increases in CBF without affecting basal blood flow.

The least understood portion of NMDA-induced vasodilation remains to be the sequence of events from the activation of NMDA receptors to the activation of nNOS. In neuronal tissue cultures, NMDA treatment can directly result in NO release; however, in vivo this might not be the case. In the piglet, immunohistochemistry revealed a particularly distinct band of NMDA receptor (NMDAR 2A/B) immunopositive neurons interspersed with nNOS-positive neurons in cortical layer II/III. However, NMDAR 2A/B-nNOS double-immunopositive neurons were not found (9). In addition, tetrodotoxin was found to attenuate NMDA-induced vasodilation in the rat and the rabbit indicating that action potentials are necessary in evoking this response (14, 41).

In conclusion, the findings of this study extend and confirm previous findings on the mechanism of NMDA-induced vasodilation in piglets. Thus NMDA-induced pial arteriolar vasodilation in this species can be characterized as a neurally mediated multistep sequence, including 1) activation of NMDA receptor containing neurons, 2) subsequent activation of nNOS positive neurons, 3) release, 4) diffusion, and 5) actions of neuronal-derived NO, independent of endothelium, on vascular smooth muscle cells of pial arterioles. Preservation of this sequence by pharmacological or physiological interventions after anoxic stress must target neuronal elements of this response.

The authors gratefully acknowledge Dr. William G. Mayhan for invaluable help with the NOx measurements. This study was supported by National Heart, Lung, and Blood Institute Grants HL-30260, HL-46558, and HL-50587, American Heart Association Mid-Atlantic Affiliate Grant 9951272, and by OTKA Grant T-026295 from the Hungarian Science Foundation and FKFP Grant 0288/2000 from the Hungarian Ministry of Education.

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