Role of carbon monoxide in glutamate receptor-induced dilation of newborn pig pial arterioles

JAMES S. ROBINSON, ALEXANDER L. FEDINEC, AND CHARLES W. LEFFLER
Laboratory for Research in Neonatal Physiology, Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163

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CARBON MONOXIDE (CO) is a potent vasodilator of newborn pig cerebral arterioles in vivo (11). CO is produced from the degradation of heme to biliverdin, iron, and CO via the enzyme heme oxygenase (HO) (4). The HO-1 isoform is induced by numerous stimuli including cAMP (3) and hypoxia (7), whereas the HO-2 isoform is found in its highest concentration within the brain (13). Both of these isoforms are present in vascular smooth muscle cells and endothelium (2). Because of this, CO must be considered a potential physiological gaseous vasodilatory mediator of cerebral blood flow, analogous to nitric oxide (NO).

Glutamate is a vasodilator of newborn cerebral arterioles (1). We recently showed specific binding of glutamate to piglet cerebral microvascular endothelial cells in primary culture that stimulated CO production by these cells (16). Two HO inhibitors, zinc deuteroporphyrin bisglucuronate and chromium mesoporphyrin, have been shown to attenuate the pressor and bradycardic changes induced by L-glutamate microinjections into the nucleus tractus solitarius of rats (17). It was suggested that these cardiovascular effects of L-glutamate may be due to production of CO from HO (17).

Therefore, the following experiments were undertaken to test the hypothesis that endogenously produced CO contributes to the vasodilatory effects of L-glutamate on cerebral arterioles of newborn pigs.

METHODS

The animal protocols used were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Newborn piglets of either sex (1–3 days old) were used for these experiments. Animals were anesthetized with ketamine and acepromazine, and anesthesia was maintained with α-chloralose (30–40 mg/kg initially, supplemented with 7 mg·kg⁻¹·h⁻¹). Catheters were placed in the femoral artery and vein to permit sampling for blood gases and pH, monitoring arterial pressure, and drug and anesthesia administration. A tracheotomy was performed, an endotracheal tube was inserted, and the piglet was mechanically ventilated with room air supplemented with oxygen when needed. Core temperature was monitored with a rectal probe and maintained between 37 and 38°C.

Cranial window placement. After instrumentation, the scalp was surgically removed, and a 2-cm-diameter hole was cut in the skull over the parietal cortex. The dura was cut and reflected over the cut bone edge. Care was taken to avoid contact between the brain surface and the cut edges of the skull.

Address for reprint requests and other correspondence: C. W. Leffler, Dept. of Physiology, Univ. of Tennessee Health Science Center, 894 Union Ave., 426 Nash, Memphis, TN 38163 (E-mail: cleffler@physiol1.utmem.edu).

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dura. A stainless steel and glass cranial window was placed in the hole and cemented into place with dental acrylic. The space under the window was filled with artificial cerebrospinal fluid (aCSF; in mg/ml: 220 KCl, 1.132 MgCl₂, 221 CaCl₂, 7.710 NaCl, 401 urea, 665 dextrose, and 2.066 NaHCO₃). aCSF was warmed in a water bath to 37°C and bubbled with a mixture of N₂, O₂, and CO₂ (pH 7.33, Pco₂ 46 mmHg, Po₂ 43 mmHg). A pial arteriole ~60–80 μm in diameter was selected and monitored through the window with a dissecting microscope with a mounted video camera. Arteriolar diameter was measured with a video microscalar (model IV 550; For-A-Co, Los Angeles, CA).

Experimental design. After pH and blood gas values were established within normal ranges, the window was flushed with fresh aCSF and allowed to equilibrate for 10–15 min. A baseline pial arteriole measurement was recorded, and the mean arterial pressure was also recorded with each measurement. Initially, positive and negative controls previously shown to be inhibited and not inhibited by HO inhibitors, respectively (11), were run as a means to confirm that the HO dose was sufficient and selective. Hypoxia, 5-min ventilation with 10% O₂, and hypercapnia, 5-min ventilation with 10% CO₂, were used as positive and negative controls, respectively. Pial arterioles were allowed to return to baseline between hypoxic and hypercapnic challenges.

Response to glutamate. l-Glutamic acid (monosodium salt) was applied topically at a dose of 10⁻⁵ M. All compounds flushed under the window were dissolved in fresh aCSF. Pial arteriolar diameters were recorded for 10 min. Arterial blood samples were drawn at the 5-min mark for blood gases and pH to ensure physiological stability. Then the glutamate was removed with fresh aCSF.

HO inhibitors. Once all control responses were established, a HO inhibitor was applied. Initially, topical application of chromium mesoporphyrin (CrMP) (15 × 10⁻⁶ M) was applied. The CrMP was allowed to sit under the window for 30 min in darkness and flushed with fresh CrMP every 10–15 min. With the use of metal protoporphyrins, the lights were only turned on for measurements.

AMPA receptors. (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) was used as the AMPA glutamate receptor agonist at doses of 10⁻⁷, 10⁻⁶, and 10⁻⁵ M for 5-min dose-response experiments. Isoproterenol (10⁻⁷ M) was used as a negative control, and the HO substrate heme-1-lysinate, at 10⁻⁷ and 5 × 10⁻⁷ M, was used as a positive control for effectiveness of HO inhibition. Topical copper mesoporphyrin (CuMP), 15 × 10⁻⁶ M in aCSF, was used as an inactive inhibitor control (a metal porphyrin that does not inhibit HO). AMPA, isoproterenol, and heme-1-lysinate challenges were then run again with the inactive porphyrin CuMP. Topical tin protoporphyrin (SnPP), 15 × 10⁻⁶ M in aCSF, was next used as the active HO inhibitor, and the RS-AMPA, isoproterenol, and heme-1-lysinate exposures were repeated.

Kainate receptors. To test kainate receptors, (RS)-2-amino-3-(3-hydroxy-5-t-butylisoxazol-4-yl)propionic acid (ATPA) at doses of 10⁻⁷, 10⁻⁶, and 10⁻⁵ M was used as the agonist. Isoproterenol (10⁻⁶ M) was again used as a negative control. Topical application of tin mesoporphyrin (SnMP; 15 × 10⁻⁶ M in aCSF) was the HO inhibitor, and the ATPA and isoproterenol were repeated.

N-methyl-d-aspartate receptors. 1-Aminocyclopentaneboxylic (ACPD) at doses of 10⁻⁷ and 10⁻⁶ M was used as the agonist of N-methyl-d-aspartate (NMDA) receptors. Isoproterenol (10⁻⁷ M) was the negative control, and heme-1-lysin- nate (10⁻⁷ M) was the positive control. SnPP, 15 × 10⁻⁶ M in aCSF, was used as the HO inhibitor, and ACPD, isoproterenol, and heme-1-lysinate were repeated.

Measurements of CO production. To determine whether glutamate increases CO production by the pial vessels and whether metal porphyrins do, in fact, inhibit that production, we measured the production of CO by freshly isolated cerebral microvessels (60–300 μm) isolated from neonatal pig brains. Cerebral microvessels were isolated and collected as described previously (16) using gentle brain homogenation, serial passage through 300- and 60-μm screens, and collection of the microvessels trapped by the smaller screen. Freshly isolated microvessels were placed inside amber vials (2.0 ml) containing aCSF. All subsequent assay steps were carried out in the dark to prevent nonenzymatic photooxidative production of CO ex vivo due to the photodegradation of organic compounds. In the experiments using metal protoporphyrin inhibitors, both CrMP and zinc protoporphyrin (ZnPP) (15 × 10⁻⁶ M) were used, and both inhibited CO production equally. Because more data were collected using ZnPP, only those data are presented in the results. Vessels were pretreated with ZnPP for 30 min before beginning the experiment. aCSF in each vial was replaced with fresh aCSF, fresh aCSF containing glutamate, or fresh aCSF containing glutamate and ZnPP. The internal standard (see below) was injected into the bottom of the aCSF in the vial, and the vial was immediately sealed with a rubberized Teflon-lined cap. The vessels were incubated for 60 min at 37°C. Incubations were terminated by placing the samples in hot water (75°C).

A saturated solution of the isotopically labeled CO (¹³C¹⁶O, isotopic purity >99%) was used as internal standard for quantitative measurements by gas chromatography-mass spectrometry (GC-MS).

GC-MS analysis of the headspace gas was performed using a Hewlett-Packard 5970 mass-selective ion detector interfaced to a Hewlett Packard 5890A gas chromatograph. The separation of CO from other gases was carried out on a Varian-5A molesieve capillary column (30 m; 0.32 mm ID) with a linear temperature gradient from 35 to 65°C at 15°C/min. Helium was the carrier gas at a column head pressure of 4.0 psi. Aliquots (100 μl) of the headspace gas were injected using a gas-tight syringe into the splitless injector having a liner-lined cap. Ions at a mass-to-charge ratio (m/z) of 28 and 29 corresponding to ¹²C¹⁶O and ¹³C¹⁶O, respectively, were recorded via selective ion monitoring. The amount of C O in samples is calculated from the regression line of standard curves according to the ratio of peak areas of m/z 28 and m/z 29. The results are expressed as picomoles of CO released into the headspace gas per milligram protein.

Data analysis. Differences among groups were detected with ANOVA for repeated measures with Fischer’s protected least significant difference test to compare individual groups with each other. P < 0.05 was required for inference that populations were different.

RESULTS

Before HO inhibitor treatment, vasodilation occurred in response to hypercapnia, hypoxia, and glutamate (Fig. 1). CrMP inhibited dilation to hypoxia and glutamate but not to hypercapnia. The change in diameter from baseline in response to glutamate (10⁻⁵ M) was reduced from 79 ± 12 to 96 ± 15 μm before CrMP to 77 ± 12 to 84 ± 12 μm after addition of CrMP (n = 7). These data suggest CO could be involved in glutamate-induced cerebral vasodilation.

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GLUTAMATE-INDUCED DILATION

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CrMP solution that was exposed to light for 1 wk or more was used as an inactivated CrMP control. The inactivated CrMP resulted in no inhibition of dilations to hypercapnia, hypoxia, or glutamate (Fig. 2).

Additional experiments were conducted to determine which glutamate receptors can cause dilation that is attenuated via HO inhibition. Agonists for AMPA, NMDA, and kainate glutamate receptors were all administered topically in the absence and presence of a variety of HO inhibitors.

**AMPA receptors.** RS-AMPA caused dose-dependent dilation of pial arterioles from a baseline of 61 ± 6 μm to a maximal dilation of 76 ± 7 μm at an agonist dose of 10⁻⁵ M (Fig. 3). Heme-L-lysinate and isoproterenol dilated pial arterioles to 79 ± 7 and 77 ± 7 μm, respectively. The metal porphyrin that does not inhibit HO, CuMP, caused no inhibition of dilation to RS-AMPA (Fig. 3), heme-L-lysinate, or isoproterenol. Topical SnPP inhibited dilation to all doses of RS-AMPA (Fig. 3). SnPP also inhibited dilation to heme-L-lysinate (64 ± 6 μm) but not to isoproterenol (72 ± 6 μm).

**Kainate receptors.** ATPA caused a small but significant dilation of pial arterioles with a maximum dilation to 72 ± 6 μm from a baseline of 62 ± 5 μm (Fig. 4). Addition of SnMP blocked the dilation to ATPA (Fig. 4) but did not block dilation to isoproterenol (76 ± 10 μm).

**NMDA receptors.** Pial arterioles dilated dose-dependently to the NMDA receptor agonist ACPD similarly or more strongly than to AMPA (Fig. 5). SnPP blocked
the dilation to ACPD. The SnPP also blocked dilation to heme-L-lysinate but not to isoproterenol. The inactive metal porphyrin CuMP resulted in no inhibition to either dose of ACPD (Fig. 5).

To determine if cerebral microvessels are stimulated to produce CO by glutamate, freshly isolated piglet cerebral microvessels were exposed to glutamate and the CO production was measured (Fig. 6). Glutamate stimulated CO production by the microvessels dose-dependently. The metal protoporphyrin inhibitor of HO, ZnPP, blocked glutamate-stimulated CO production by the microvessels (Fig. 7).

DISCUSSION

The new findings of this study are as follows: 1) HO inhibitors block dilations of newborn pig cerebral arterioles in vivo to glutamate and to specific NMDA, AMPA, and kainate agonists; and 2) glutamate stimulates CO production by cerebral microvessels in vitro that is blocked by HO inhibitors. These data suggest vascular production of CO may be involved in glutamate-induced cerebral vasodilation. These findings support the results of other studies, suggesting that HO can be involved in the effects of glutamate (17). In these studies, the cardiovascular effects of microinjections of glutamate into the nucleus tractus solitarius of conscious rats were inhibited by the HO inhibitors CrMP and zinc deuteroporphyrinbis-gluconate (Zn-DPBG). Our data are also in concert with our recent report that glutamate increases CO production by cerebral microvascular endothelial cells in primary culture (16).

Glutamate is an excitatory neurotransmitter in the brain. The increased neuronal activity caused by glutamate requires increased cerebral blood flow, which appears to be supplied, at least in part, by glutamate stimulation of CO production.

The production of CO and its vasodilatory effects can be increased under specific conditions. In adult rabbits, a HO inhibitor was found to significantly reduce the cerebral blood flow during epileptic seizures but had no effect on cerebral blood flow in hypercapnic conditions, or in basal conditions (15). In newborn pigs, a HO inhibitor was found to reduce the increase of pial arteriolar diameter in response to hypoxia (11).

Initially, we used glutamate itself to activate all glutamate receptors. Once inhibition via HO inhibitors was established, responses to specific glutamate receptors were individually tested. The initial 10^{-5} M dose in the original experiment maximally dilated some of the vessels. For this reason, lower concentrations were used to test responses to specific glutamate receptor agonists. Agonists of the glutamate receptor types tested, NMDA and AMPA, and to a lesser extent kainate, dilated pial arterioles. These dilations were attenuated by metal porphyrin inhibitors of HO. These

Fig. 5. Effect of topically applied SnPP and CuMP on pial arteriolar responses of newborn pigs to ACPD (10^{-7} to 10^{-6} M). Topical inhibitors were applied for 30 min before and during application of ACPD (n = 10 arterioles in 5 piglets). *P < 0.05 compared with no ACPD.

Fig. 6. Effect of glutamate on carbon monoxide (CO) production by freshly isolated piglet cerebral microvessels (n = 5 separate microvessel preparations, each run as a glutamate dose-response). *P < 0.05 compared with no glutamate.

Fig. 7. CO production by untreated piglet cerebral microvessels and microvessels treated with glutamate in the absence and presence of zinc protoporphyrin (ZnPP) (15 × 10^{-6} M) (n = 4 separate microvessel preparations, each run as a set of the treatments). *P < 0.05 compared with no glutamate.
results suggest CO is involved in the mechanism by which all three agonists produce vasodilation. Whether or not metabotropic glutamate receptors produce vasodilation was not examined.

The metal porphyrin that does not inhibit HO, CuMP, and light-inactivated CrMP were both used as inactive controls for the HO inhibitors in our studies. Neither of these inhibited dilations to the HO substrate heme-L-lysinate, indicating that they were indeed inactive on HO. Furthermore, we used several different metal porphyrins to inhibit HO, and all had the same effect on glutamate receptor-induced dilation. In our earlier work (11), we showed that topical CrMP blocked arteriolar dilation to heme-L-lysinate. Other metal porphyrins can also inhibit dilation to heme-L-lysinate (8). Our current studies also showed inhibition to heme-L-lysinate with topical CrMP or SnPP, without affecting dilatory responses to isoproterenol or CO2. These data suggest that metal porphyrin actions are specific for HO, since responses to isoproterenol and CO2, which depend on a cAMP mechanism (9), were unaffected. Finally, we measured CO production by piglet cerebral microvessels and showed the metal porphyrin ZnPP inhibited glutamate-stimulated CO production by these vessels. These data, in total, strongly suggest that the metal porphyrins used are both effective and relatively specific inhibitors of HO.

HO-2 is widely and greatly expressed in the brain compared with other organs. HO-2 is in very high concentration in vessels and neurons (13). Certainly CO that could contribute to cerebral vasodilation could be produced by neurons, glia, smooth muscle, and/or endothelium. We previously showed that cultured endothelial cells produce CO, and this production is stimulated by glutamate (16). In the present study, we demonstrate that freshly isolated cerebral microvessels that had been removed from the other brain cell types dose dependently produce CO in response to glutamate. These data suggest strongly that glutamate-induced cerebral dilation depends, at least in part, on CO production from HO-2 in the vasculature, since freshly isolated cerebral microvessels do not express HO-1 (16).

The dilator actions of CO intimately involve the prostanoid and NO systems as permissive enablers (10). Of particular interest to the present subject is the involvement with NO because, in piglets, glutamate-mediated dilation appears to involve NO (14). Some dilations attributed to NO could relate to its permissive action for CO-induced dilation. Both CO and NO can activate soluble guanylyl cyclase, although guanylyl cyclase is much more sensitive to NO (5, 13), and we could detect no increase in cerebral cGMP production coincident with CO-induced dilation, in contrast to NO-induced dilation (11). Clearly, in piglet cerebral arterioles, dilation to CO is caused by Ca2+-activated K+ (KCa) channel activation (11). Treatment of piglets with N5-nitro-L-arginine (l-NNA) to block NO synthase abolishes cerebral vasodilation in response to CO. In piglets treated with l-NNA, the NO donor sodium nitroprusside, at a constant concentration that causes little dilation directly, completely restores dose-dependent cerebral vasodilation to CO (10). Thus NO can be a permissive factor for CO-induced cerebrovascular responses. NO acts upstream of KCa channels because NO cannot restore dilation to CO after KCa channel inhibition (10). We speculate that the action of NO involves cGMP because dilation to CO appears to be inhibited by the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoline-1-one (unpublished observations). Because the dilation to CO appears to be mediated by KCa channels, the permissive action of NO may be to alter the KCa channel response to CO.

In conclusion, the present experiments suggest that CO may be involved in glutamate and specific glutamate receptor-induced vasodilation of newborn pig cerebral arterioles. As such, CO could contribute to matching cerebral blood flow to neuronal activity.

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