Carbon monoxide and cerebral microvascular tone in newborn pigs

CHARLES W. LEFFLER, ALBERTO NASJLETTI, CHANGHUA YU, ROBERT A. JOHNSON, ALEXANDER L. FEDINEC, and NICOLE WALKER

Leffler, Charles W., Alberto Nasjletti, Changhua Yu, Robert A. Johnson, Alexander L. Fedinec, and Nicole Walker. Carbon monoxide and cerebral microvascular tone in newborn pigs. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1641–H1646, 1999.—The present study addresses the hypothesis that CO produced from endogenous heme oxygenase (HO) can dilate newborn cerebral arterioles. HO-2 protein was highly expressed in large and small blood vessels, as well as parenchyma, of newborn pig cerebral. Topically applied CO dose-dependently dilated piglet pial arterioles in vivo over the range 10^{-11}–10^{-9} M (maximal response). CO-induced cerebrovascular dilation was abolished by treatment with the Ca^{2+}-activated K^+ channel inhibitors tetraethylammonium chloride and iberiotoxin. The HO substrate heme-L-lysinate also produced tetraethylammonium-inhibitable, dose-dependent dilation from 5 \times 10^{-8} to 5 \times 10^{-7} M (maximal). The HO inhibitor chromium mesoporphyrin blocked dilation of pial arterioles in response to heme-L-lysinate. In addition to inhibiting dilation to heme-L-lysinate, chromium mesoporphyrin also blocked pial arteriolar dilations in response to hypoxia but did not alter responses to hypercapnia or isoproprenol. We conclude that CO dilates pial arterioles via activation of Ca^{2+}-activated K^+ channels and that endogenous HO-2 potentially can produce sufficient CO to produce the dilation.

CO has been suggested to mediate ACh-induced vasorelaxation (29). CO can cause increases in cGMP in autocrine and paracrine fashions (6, 9, 19, 26) and can hyperpolarize vascular smooth muscle via modification of a histidine residue on the external membrane side of the large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel (26–28). It has been suggested that the similarity of cellular locations of HO and nitric oxide (NO) synthase (NOS), to which we add prostaglandin cyclooxygenase, may imply coordinated and potentially complementary roles of the paracrine mediators that are the currently identified endothelium-derived relaxing factors (29).

HO and CO appear to be important in prenatal and postnatal development. HO levels in cerebral are developmentally regulated, with maximal HO expression in the mature fetus compared with the immature fetus or adult in guinea pigs (8). CO appears to be important in control of the fetal vasculature, with a potential contribution of endogenously produced CO to ductus arteriosus patency (7). Expressions of HO-1 and HO-2 are 15 times higher in the pregnant than in the nonpregnant myometrium (1). Sex steroids can induce expression of both HO proteins. These findings further suggest that HO may be elevated in perinatal tissues.

Therefore, the present experiments were designed to address the hypothesis that CO produced from endogenous HO can dilate newborn cerebral arterioles in vivo.

METHODS

All procedures that involve animals were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee, Memphis. Newborn pigs (1–3 days old, 1–2.5 kg) were anesthetized with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im) and maintained on α-chloralose (50 mg/kg iv). The animals were intubated and ventilated with air. Catheters were inserted into the femoral vein for maintenance of anesthesia and drug injections and into the femoral artery to record blood pressure and draw samples for blood gases and pH analysis. Blood gases and pH were maintained within normal ranges. Body temperature was maintained at 37–38°C. The scalp was retracted, and a 2-cm-diameter hole was made in the skull over the parietal cortex. The dura was cut without touching the brain, and all cut edges were retracted over the bone so that the periarchnoid space was not exposed to bone or damaged membranes. 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) that was equilibrated with 6% CO_2 and 6% O_2, which produced gases and pH within the normal range for CSF (pH 7.33–7.40, P_{CO_2} = 42–46 mmHg, and P_{O_2} = 43–50 mmHg). aCSF could be injected and
samples collected from needle ports on the sides of the window. The volume of the fluid directly beneath the window was 500 μl and was contiguous with the periarachnoid space. Pial vessels were observed with a dissecting microscope. Diameters were measured with a video micrometer that was coupled to a microscope, television camera, and video monitor. Two pial arterioles of different sizes were measured in each piglet.

Materials. CO was purchased as compressed gas (99.5%). A saturated solution was assumed to be produced in ethanol at 37°C (7 × 10^-3 M) after ≥2 h of turbulent aeration with CO through a glass gas diffuser under pure CO atmosphere. Ethanol stocks were diluted in aCSF for injection under the cranial window at 10^-12-10^-5 M. Several ethanol dilutions were utilized, so that ethanol concentrations applied did not progressively increase with CO; ethanol concentration at maximal dilation (10^-9 M CO) was 0.1%. One percent ethanol does not affect pial arteriolar diameter (data not shown). The HO substrate heme-L-lysinate (36 mM) was prepared using methods described by Tenhunen et al. (25). It was protected from light at all times until placement beneath the cranial window, and the cranial window was illuminated only during vessel diameter measurements. Heme-L-lysinate was stored at -30°C. Lysinate vehicle (L-lysine, H2O, propylene glycol, ethanol) was used as zero heme-L-lysinate at a dilution equal to that with heme-L-lysinate at 2 × 10^-6 M. Lysinate vehicle did not affect pial arteriolar diameters. Heme-L-lysinate was diluted in aCSF (10^-8-2 × 10^-6 M) for placement under the cranial window. The HO inhibitor chromium mesoporphyrin was purchased from Porpyrin Products (Logan, UT). Polyclonal antibodies to HO-1 and HO-2 were purchased from Stress Gen (Victoria, BC, Canada). Secondary antibodies (goat anti-rabbit) conjugated to horseradish peroxidase were obtained from Bio-Rad.

Experiments. CO and heme-L-lysinate were applied directly to pial arterioles, and the maximal diameter attained over a 10-min period was recorded as the response to each dose. Repeat ascending dose-response curves to CO or heme-L-lysinate were produced before and after no treatment or tetraethylammonium (10^-6 M), iberiotoxin (10^-6 M), or tetraethylamonium chloride (TEA, 10^-3 M). N^+ -nitro-L-arginine (L-NNA, 10^-3 M) was topically applied 15 min before and during chromium mesoporphyrin treatment in one experimental group. CSF (300 μl of 500 μl total) was collected from beneath the cranial window at the end of each 10-min period for later measurements of cAMP and cGMP.

Responses to topical application of isoproterenol (10^-6 M), hypercapnia (10% CO2 ventilation), hypoxia (10% O2 ventilation), or topical application of sodium nitroprusside (10^-7 M) for 5 min were measured before and after treatments.

cAMP and cGMP were measured in the CSF by RIA, as described previously (20).

Assessment of HO protein. Analysis of HO-1 and HO-2 by immunoblotting was performed in samples of small cerebral vessels, large cerebral vessels, and cerebral parenchyma that were prepared by progressive passage through 300- and 60-μm mesh screens. Tissues were snap frozen in liquid nitrogen and stored at −70°C until used. The samples were homogenized in ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 25 µg/ml aprotinin, 25 µg/ml leupeptin, and 10 mM mercaptoethanol. Homogenates were centrifuged at 100,000 g for 60 min, and the resulting pellet was assayed for protein and saved for Western blot analysis. To this end, pellet proteins (50 μg) were subjected to electrophoresis on SDS-12% polyacrylamide gels and then transferred to nitrocellulose membranes. Each membrane was blocked at 4°C overnight with 5% BSA and 3% powdered milk in Tris-saline buffer (20 mM Tris·HCl and 150 mM NaCl, pH 7.5). Subsequently, the membrane was washed with Tris-saline buffer and then incubated with HO-1 antibody (SPA-895, 1:1,000 dilution) or HO-2 antibody (SPA-897, 1:100 dilution) for 1 h at room temperature. The membrane was washed again with Tris-saline buffer before incubation at room temperature for 60 min with secondary antibody (goat anti-rabbit IgG) conjugated to horseradish peroxidase. After the final washes, the immunocomplexed bands were visualized with the chemiluminescent ECL system (Amersham, Arlington Heights, IL).

Statistical analysis. Values are means ± SE. Comparisons among populations within each experimental group used ANOVA with repeated measures. Fisher's protected least significant difference test was used to determine differences between populations within each group. P < 0.05 was considered significant.

RESULTS

HO-2 is expressed in cerebral blood vessels of newborn pigs (Fig. 1). HO-2 was readily detectable in large vessels (>300 μm) and microvessels (60–300 μm) of piglet cerebrum. HO-2 was also highly expressed in the tissue of the cerebrum. Although conditions ~100-fold more sensitive were used to attempt to detect HO-1, none was detected in the piglet cerebrum or its vessels.

CO produced dose-dependent dilation of pial arterioles (Fig. 2). Although significance was reached at 10^-11 M in small arterioles (<60 μm) and at 10^-10 M in larger arterioles (>60 μm), no qualitative differences in the dose-response curves were apparent, and maximal dilation was reached by 10^-9 M CO in vessels of both sizes. The percent dilation was slightly greater in smaller (~30%) than in larger (~20%) arterioles. Repeat dose-response curves were virtually superimposable (data not shown, n = 5). Dilation to sodium

Fig. 1. Western blot analysis of heme oxygenase-1 (HO-1, A) and heme oxygenase-2 (HO-2, B) in 100,000-g pellet of homogenates of small cerebral vessels (SCV, 50 μg protein), large cerebral vessels (LCV, 50 μg protein), and cerebral parenchyma (CP, 50 μg protein). STD, HO-1 (2 ng) and HO-2 (200 ng) commercial standards.
nitroprusside (10^{-7} M) was not altered by the interposition of two CO dose-response curves (dilation to sodium nitroprusside: 76 ± 6 to 85 ± 6 µm before and 79 ± 5 to 90 ± 7 µm after the two CO dose-response curves, n = 5).

K<sub>Ca</sub> channel inhibitors TEA and iberiotoxin abolished CO-induced cerebrovascular dilation (Figs. 3 and 4). Neither TEA nor iberiotoxin significantly altered vasodilation in response to isoproterenol.

Heme-L-lysinate also caused dose-dependent dilation of pial arterioles. Repeat dose-response curves were superimposable, with minimal threshold at 10^{-8} M heme-L-lysinate and maximal dilation of ~25% at 5 × 10^{-7} M (Fig. 5). Insomuch as no qualitative differences related to vessel size were seen in the results in any experiments, data from ~60-µm-diameter vessels will be reported in the rest of this article. As was the case with CO, dilation to heme-L-lysinate was abolished by the K<sub>Ca</sub> channel inhibitor TEA. Dilation to 10^{-7} M heme-L-lysinate before TEA was 68 ± 4 to 85 ± 9 µm. During treatment with TEA, heme-L-lysinate did not cause dilation (73 ± 7 to 73 ± 6 µm, n = 6). Interposition of two heme-L-lysinate dose-response curves had minimal effects on dilations to hypercapnia and isoproterenol. Thus pial arterioles dilated in response to hypercapnia from 63 ± 7 to 81 ± 9 µm before and from 68 ± 8 to 88 ± 10 µm after two heme-L-lysinate dose-response curves. Similarly, dilations to isoproterenol (10^{-6} M) were from 61 ± 8 to 83 ± 11 µm before and from 67 ± 8 to 88 ± 12 µm after the heme-L-lysinate dose-response curve.

Dilation to heme-L-lysinate was blocked by chromium mesoporphyrin (Fig. 6). In the presence of chromium mesoporphyrin, heme-L-lysinate actually produced constriction. In contrast, chromium mesoporphyrin did not affect dilation to CO (dilations to CO at 10^{-11}, 10^{-9}, and 10^{-7} M were 4 ± 0.4, 16 ± 3, and 20 ± 3% before and 9 ± 5, 16 ± 5, and 19 ± 5% in the presence of chromium mesoporphyrin, respectively, n =
Chromium mesoporphyrin alone dilated pial arterioles. The pial arteriolar dilation in response to chromium mesoporphyrin was completely blocked in the presence of the NOS inhibitor L-NNA (Fig. 7). Chromium mesoporphyrin blocked pial arteriolar dilation in response to hypoxia (Fig. 8). The inhibition was reversible on removal of chromium mesoporphyrin. Dilation in response to hypoxia also did not occur when L-NNA and chromium mesoporphyrin were applied simultaneously. In contrast, chromium mesoporphyrin did not change dilation to hypercapnia (69 ± 4 to 98 ± 6 µm before and 72 ± 2 to 95 ± 8 µm in the presence of chromium mesoporphyrin) or 10⁻⁶ M isoproterenol (66 ± 5 to 86 ± 6 µm before and 72 ± 3 to 97 ± 10 µm in the presence of chromium mesoporphyrin). Furthermore, dilation to isoproterenol was unaffected by administration of L-NNA with chromium mesoporphyrin (26 ± 3% before and 26 ± 5% in the presence of L-NNA and chromium mesoporphyrin).

CO significantly increased cAMP in the aCSF, although the increase was not impressive and the dose-response relationship was poor. Thus cAMP concentrations were 1,112 ± 149, 1,307 ± 280, 1,981 ± 423, and 1,895 ± 342 pM at CO concentrations of 0, 10⁻¹¹, 10⁻⁹, and 10⁻⁷ M, respectively (n = 12). Similarly, heme-L-lysinate effects on cortical cAMP production were unimpressive: 1,505 ± 189, 1,839 ± 420, 1,513 ± 178, 1,670 ± 249, and 1,920 ± 468 pM cAMP at heme-L-lysinate concentrations of 0, 5 x 10⁻², 10⁻⁷, 10⁻⁶, and 2 x 10⁻⁵ M, respectively (n = 7).

CO did not affect cGMP concentration in aCSF: 683 ± 227, 675 ± 231, 799 ± 249, and 762 ± 288 pM at 0, 10⁻¹¹, 10⁻⁹, and 10⁻⁷ M CO, respectively (n = 7). Nor did heme-L-lysinate affect cGMP: 570 ± 138 pM cGMP for control and 804 ± 299 pM at 2 x 10⁻⁵ M heme-L-lysinate (n = 4).

**DISCUSSION**

New findings of the present study include the following: 1) HO-2 is highly expressed in newborn cerebral blood vessels, 2) CO is a potent vasodilator of the newborn cerebral microcirculation, 3) CO-induced cerebrovascular dilation appears to involve KCa channels, 4) endogenous HO can produce CO and dilation of the newborn cerebral microcirculation, and 5) HO may be involved in cerebrovascular dilation to hypoxia.

Our findings that CO dilates pial arterioles of newborn pigs in vivo contrast with those from adult rabbit and dog cerebral arteries in vitro (4). Brian et al. (4) were unable to dilate rabbit or dog basilar and middle cerebral arteries with CO even at concentrations 3,000 times higher than the highest concentration used in the present study. In our study the pial arterioles of newborn pigs were exquisitely sensitive to CO, dilating 27 ± 6% at 10⁻⁹ M. Although these two data sets seem incongruous, there are many differences between the studies. Four differences are particularly likely to contribute to the divergent findings. First, Brian et al.
examined responses of major cerebral arteries, basilar and middle cerebral, whereas the arterioles in the present studies ranged from 91 to 38 µm, among the smallest precapillary vessels on the brain surface. Second, the present study was conducted on intact brain in vivo, whereas the former used isolated, precontracted artery rings suspended in Krebs buffer with 95% O₂. It is conceivable that the mechanism involved in CO dilation (see below) is less functional under such conditions. Also it is possible that neurons underlying pial arterioles contribute to the response to CO. Third, the present study involves newborn animals, and, as noted in the introduction, the HO-CO system is developmentally regulated. Fourth, species differences could contribute, inasmuch as Brian et al. studied dog and rabbit cerebral arteries and the present experiments used pigs.

A most important aspect of our study is that heme-L-lysinate, like CO, increased the diameter of pial arterioles in newborn pigs. Heme-L-lysinate is an HO substrate that has been shown to produce dilation of gracilis muscle arterioles in rats (14). In experimental preparations in which the pial arterioles were bathed in aCSF containing the HO inhibitor chromium mesoporphyrin, the application of heme-L-lysinate elicited a constrictor rather than a dilatory response. The conclusion emerging from these findings is that the dilatory effect of heme-L-lysinate in pial arterioles is mediated by a product of HO activity. Because chromium mesoporphyrin did not affect dilatory responses to CO, it is reasonable to propose that HO-derived CO is the mediator of the dilatory effect of heme-L-lysinate.

The mechanism by which CO dilates pial arterioles appears to involve hyperpolarization via a K⁺ channel. Both of the structurally very different inhibitors of K⁺ channels that we used, TEA and iberiotoxin, totally abolished vasodilation in response to CO. Similarly, TEA blocked dilation to the HO substrate heme-L-lysinate, suggesting that dilations to heme-L-lysinate and CO occur via the same mechanism. That the observed inhibition of dilation in response to the K⁺ channel inhibitors is not due to a generalized inhibition of vascular reactivity is demonstrated by the complete absence of effects of these inhibitors on the dilator responses to isoproterenol.

CO is a potential paracrine mediator with multiple similarities to NO. Constitutively expressed enzymes responsible for the generation of both gases are found in endothelium, vascular smooth muscle, and perivascular neurons (present study; 3, 17, 29). Both can produce vasodilation and inhibit platelet activation, via activation of soluble guanylyl cyclase (6, 17, 26). However, considerable evidence suggests that CO can produce dilation independently of cGMP (10, 24), and exogenously administered CO did not increase cGMP in rat cerebral cortex (15) or newborn pig cortex (present study). Dilations to CO and NO can involve vascular smooth muscle hyperpolarization via K⁺ channel activity (22, 23, 26–28), which, as noted above, appears to be mechanistically involved in CO-induced dilation of piglet pial arterioles. CO and NO appear to have special roles in the brain, where they can function as cotransmitters or as modulators of neuropeptides. The localizations of HO-2 and bNOS in the brain suggest special roles for CO and NO in cerebral function, including regulation of cerebrovascular circulation.

Although NO and CO are capable of activating soluble guanylyl cyclase and thus potentially summatating in producing cGMP-dependent dilation, we could detect no increase in cerebral cGMP production coincident with CO or heme-L-lysinate-induced dilation, in contrast to NO-induced (sodium nitroprusside) dilation (2).

cAMP could be involved in the dilator responses to CO, inasmuch as CO and heme-L-lysinate increased cerebral cAMP production, although the changes were minuscule in comparison to the dose-dependent increases produced by iloprost or isoproterenol (21). Therefore, it appears that cyclic nucleotides are not the primary secondary messengers in CO-induced dilation in this system.

Our finding that addition of chromium mesoporphyrin to aCSF resulted in dilation of pial arterioles was surprising at first, since inhibition of endogenous CO production is expected to bring about vasoconstriction rather than vasodilation. This dilation appears to involve NO, because it is abolished by pretreatment with L-NNA. Pertinent to this point, metal protoporphyrins have previously been shown to be capable of NOS activation (5). Also, by inhibiting endogenous CO production, chromium mesoporphyrin may remove tonic inhibition of NOS by CO binding to the NOS heme (18). Chromium mesoporphyrin may also remove tonic inhibition by CO of NO-mediated cGMP production (11). The possibility that the heme-HO-CO system of cerebral arterial vessels is bifunctional, with endogenous CO being capable of subserving dilatory mechanisms, via its action on vascular smooth muscle, and vasoconstrictor mechanisms, via inhibitory actions on the NOS-NO system of the endothelium, must also be considered.

CO may be involved in vasodilation in response to hypoxia in the piglet cerebral microvasculature. Chromium mesoporphyrin, which blocked heme-L-lysinate dilation, abolished pial arteriolar responses to hypoxia. This response appeared specific for hypoxia, because dilator responses to hypercapnia and isoproterenol were unaltered. Because endothelial injury abolishes dilation to hypercapnia in this model (16), the effect of chromium mesoporphyrin does not appear to involve endothelial injury. We previously reported that dilation to hypoxia had an endothelial component but did not appear to be dependent on NO, ATP-activated K⁺ channels, KCa channels, or adenosine (16). We suggested that the dilation may involve a cytochrome P-450 epoxygenase metabolite of arachidonic acid. The ability of KCa channel inhibitors to block CO-induced dilation, but not hypoxia, would suggest that the contribution of NO to hypoxia-induced dilation is indirect. Maines (17) has proposed that HO-2 may be a cellular O₂ sensor.
In conclusion, CO is a potentially important dilator influence in the newborn cerebral circulation under physiological and pathophysiological conditions. The dilator actions of CO involve activation of KCa channels by CO. Cerebrovascular dilation to hypoxia, but not to hypercapnia, appears to involve HO-CO. Much further research in this area is warranted.

We thank Danny Morse and Laura Malinick for preparing the final figures and Barbara Rawls for secretarial assistance.

Address for reprint requests and other correspondence: C. W. Leffler, Dept. of Physiology, 894 Union Ave., Memphis, TN 38163.

Received 9 October 1998; accepted in final form 21 January 1999.

REFERENCES


