Cyclooxygenase Products Stimulate Carbon Monoxide Production by Piglet Cerebral Microvessels

ALIE KANU, DAVID GILPIN, ALEXANDER L. FEDINEC, AND CHARLES W. LEFFLER[1]

Laboratory for Research in Neonatal Physiology, Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Products of arachidonic acid (AA) metabolism by cyclooxygenase (Cox) are important in regulation of neonatal cerebral circulation. The brain and cerebral microvessels also express heme oxygenase (HO) that metabolizes heme to carbon monoxide (CO), biliverdin, and iron. The purpose of this study in newborn pig cerebral microvessels was to address the hypothesis that Cox products affect HO activity and HO products affect Cox activity. AA (2.0–20 μM) increased prostaglandin E_2 (PGE_2) measured by radioimmunoassay (RIA) and also CO measured by gas chromatography/mass spectrometry (GC/MS). Further, 10^{-4} M indomethacin, which inhibited Cox, reduced both AA and heme-induced CO production. Conversely, neither exogenous 2×10^{-6} M heme, which markedly increased CO production, nor the inhibitor of HO, chromium mesoporphyrin, altered PGE_2 synthesis. Because AA metabolism by Cox generates both prostanoids and superoxides, we determined the effects of the predominant prostanoid and superoxide on CO production. Although PGE_2 caused a small increase in CO production, xanthine oxidase plus hypoxanthine, which produces superoxide, strongly stimulated the production of CO by cerebral microvessels. This increase was mildly attenuated by catalase. These data suggest that Cox-catalyzed AA metabolites, most likely superoxide and/or a subsequent reactive oxygen species, increase cerebrovascular CO production. This increase seems to be caused, at least in part, by the elevation of HO-2 catalytic activity. Conversely, Cox activity is not affected by HO-catalyzed heme metabolites. These data suggest that some cerebrovascular functions attributable to Cox activity could be mediated by CO. Exp Biol Med 231:181–185, 2006

Key words: cerebrovascular circulation; heme oxygenase; reactive oxygen species

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[1] To whom correspondence should be addressed at Department of Physiology, 894 Union Avenue, Memphis, TN 38163. E-mail: cleffler@physio1.utmem.edu

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Introduction

The gaseous molecule, carbon monoxide (CO), is a neurotransmitter in the brain and peripheral nervous system and is an important regulator of vascular tone (1). In the cerebral microvasculature, CO causes vasodilation *via* activation of large conductance calcium-activated potassium channels (2). In addition, in some conditions, CO can modulate intracellular cGMP levels in both autocrine and paracrine fashions (3).

CO is endogenously produced in the brain *via* enzymatic degradation of heme *via* heme oxygenase (HO) to CO, biliverdin IXa, and free iron (1). All heme degradation products are potentially toxic, but may also provide strong cytoprotection, depending on the amounts generated and the microenvironment. Besides its function as the prosthetic moiety in heme proteins, heme affects a wide spectrum of biochemical processes, including gene expression, by regulating transcription, mRNA stability, protein synthesis, splicing, and post-translational modification (4).

HO is expressed as three known isoforms, which are products of different genes and differ markedly in their tissue distribution as well as their molecular properties. Expression of HO-1 (heat-shock protein-32) is easily induced by numerous stimuli (1), whereas HO-2 is constitutively expressed and is known to be upregulated only by steroids (1). HO-3, a third isoform, has a much lower heme-degrading activity (1). HO generates CO from cellular heme, which is produced in cells from glycine and succinyl CoA (5). Of the vascular tissues examined, the cerebral microvessels are among the greatest producers of CO (6). The cellular mechanisms of regulation of CO production by the constitutive HO-2 enzyme include control of catalytic activity and substrate delivery (7).

The prostanoids are also important autocrine/paracrine dilators in the control of cerebral circulation in the newborn pig. The cells that produce CO and can respond to CO also produce and respond to prostanoids (8). Cyclooxygenase (Cox) products are synthesized by the catalytic conversion of arachidonic acid (AA) into prostaglandin H_2, which is subsequently processed by different enzymes into various

prostanoids (9). In addition, Cox metabolism of AA produces superoxide anion (10, 11). The similarity of the cellular locations of HO and Cox suggests that these paracrine mediators could be part of a coordinated system (12). Cox-1 and Cox-2 are the two predominant isoforms of Cox (13). The pattern of Cox-1 and Cox-2 expression is cell and tissue specific. Cox-1 is constitutively expressed in a variety of cell types (14). Cox-2 is inducible and rapidly and transiently upregulated after stimulation of cells with serum, growth factors, inflammatory mediators, and tumor promoters (15). However, in some tissues, Cox-2 is also expressed under nonstimulated conditions (16).

This study, that uses freshly isolated piglet cerebral microvessels, was designed to address the hypothesis that Cox products affect HO activity and CO production and that HO products affect Cox activity and prostanoid production.

Materials and Methods

The animal protocols were performed in adherence to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and 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- to 3-days old; 1–2.5 kg) were used for these experiments. Animals were anesthetized with intramuscular administration of 33 mg/kg ketamine hydrochloride and 3.3 mg/kg acepromazine.

Isolation of Cerebral Microvessels. The brain was removed, placed in ice-cold Krebs solution: 120 m*M* NaCl; 5 m*M* KCl; 0.62 m*M* $MgSO_4$ in seven volumes of H_2O; 1.8 m*M* $CaCl_2$; 10 m*M* HEPES; and 10 m*M* glucose (pH 7.4 with NaOH). The dura mater and attached vessels were removed. The brain cortex tissue was minced and gently homogenized in a Dounce homogenizer with a loose pestle. The homogenate was passed through a 300-μm nylon mesh screen, and the passage was refiltered through a 60-μm nylon mesh screen. The microvessels collected are those that pass through the 300-μm mesh screen but are stopped by the 60-μm mesh screen. The definition of "microvessel" remains nebulous. Although some investigators contend that only capillaries are microvessels, we use the more common definition that includes arterioles, capillaries, and venules. Although our cut-off may include small arteries and veins, 200- to 300-μm in diameter, observation of the actual sizes collected shows the predominant vessels to be less than 100 μm in diameter. Although the mesh is 300 μm, intact vessels of that diameter are unlikely to pass because the segment lengths cause the vessel to drape over the mesh. Experiments on freshly isolated cerebral microvessels began immediately after vessel collection, with resuspension of the microvessels in Krebs solution.

Experimental Treatments. Treatments were started by replacement of the Krebs solution in the vials with fresh Krebs solution that contained the experimental treatment. Heme, prepared as heme-L-lysinate (HLL), indomethacin, and chromium mesoporphyrin (CrMP) were dissolved in Krebs solution. AA and prostaglandin E_2 (PGE_2) were dissolved in ethanol and diluted a minimum of 100-fold in Krebs solution. The light-sensitive HLL and CrMP were protected from light exposure. The catalytic activity of HO in intact cerebral microvessels was determined by providing exogenous heme (HLL), so that endogenous substrate delivery would not affect CO production. To investigate Cox products that may increase CO production, endogenous Cox was blocked with indomethacin and PGE_2, or a superoxide generator was exogenously added and CO production was measured. PGE_2 was used as the prostanoid because it is produced in greatest quantity from exogenous AA. Two concentrations of PGE_2 were used that were equimolar to the exogenous AA. To investigate the effect of superoxide anion and subsequent reactive oxygen species (ROS) on CO production in piglet cerebral microvessels, the activated oxygen-generating system, 1 U/ml xanthine oxidase and 0.2 m*M* hypoxanthine, was administered to the microvessels for incubation (17). These concentrations maximally produced 13 μ*M* $\bullet O_2^-$ (17). To evaluate the potential role of H_2O_2 and ROS produced from H_2O_2, H_2O_2 was removed with 50,000 U/ml catalase, beginning 10 mins before the addition of xanthine oxidase and hypoxanthine.

Measurements of CO Production. Freshly isolated microvessels were placed inside 2.0-ml amber vials containing Krebs solution. For the experiments in which 2×10^{-5} *M* CrMP was used, the vessels were pretreated with CrMP for 30 mins before the experiment was started, and the inhibitor was maintained throughout. The internal standard (see next paragraph) was injected into the bottom of the vial and the vial was immediately sealed with a rubberized Teflon-lined cap. Cerebral microvessels were incubated for 30 mins at 37°C. Incubations were terminated by placing the samples in hot water (75°C) and CO production was determined immediately.

A saturated solution of the isotopically labeled CO ($^{13}C^{16}O$; isotopic purity > 99%) was used as an internal standard for quantitative measurements by gas chromatography/mass spectrometry (GC/MS; Ref. 6).

GC/MS analysis of the headspace gas was performed on 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 mole sieve capillary column (30 m; 0.32-mm inner diameter) with a linear temperature gradient from 35°C to 65°C at 5 °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 *via* a gas-tight syringe into the splitless injector with a temperature of 120°C. Mass-to-charge ratios (m/z) 28 and 29 corresponding to $^{12}C^{16}O$ and $^{13}C^{16}O$, respectively, were recorded *via* selective ion monitoring. The amount of CO in samples was calculated from the ratio of peak areas of m/z 28 and 29. The results are expressed as picomoles of CO released into the

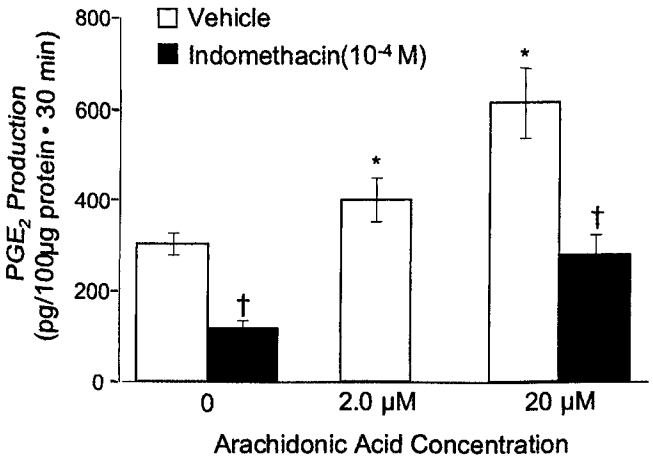

Figure 1. Effect of AA and 10^{-4} *M* indomethacin on PGE_2 production by piglet cerebral microvessels. Means ± SE of five experiments. *P < 0.05 compared with zero AA; †P < 0.05 compared with corresponding values without indomethacin.

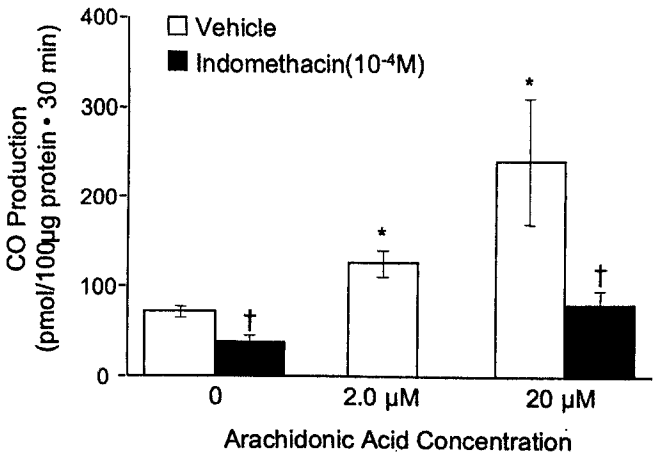

Figure 2. Effect of AA and 10^{-4} *M* indomethacin on CO production by cerebral microvessels of piglets. Means ± SE of five experiments. *P < 0.05 compared with zero AA; †P < 0.05 indomethacin compared with corresponding values without indomethacin.

headspace gas per 100-μg protein in 30 mins. Protein was measured by the Lowery method.

Measurements of PGE_2 Production. Concentrations of PGE_2 in the cell incubation medium were determined by radioimmunoassay (RIA; Ref. 18). Antibodies to PGE_2 were produced in rabbits immunized with PGE_2 coupled to thyroglobulin. Our antibodies cross-react minimally (<1%) with other biologically relevant prostanoids. Moreover, PGE_2 was not displaced from its antibodies by 20 μg/ml AA; 5-hydroxyeicosateraenoic acid (HETE), 1 μg/ml of 15-HETE; 5 μg/ml of leukotriene (LT)-B_4, LTC_4, LTD_4, or LTE_4; or 10 ng/ml of lipoxin A_4 or B_4. The free tracer fraction was separated from the fraction bound to antibodies using dextran-coated charcoal. Concentrations were calculated from the second-order regression of tracer bound to the antibody versus unlabeled prostanoid.

PGE_2 production and Cox activity, detected as PGE_2 production from exogenous AA (2.0–20 μ*M*), were determined. After 30 mins of incubation at 37°C, the medium was aspirated and stored at –20°C for PGE_2 determination.

Materials. CO was purchased as compressed gas (99.5%), and saturated solutions (10^{-3} *M*) were produced in Krebs. The HO substrate, HLL, was prepared using methods described by Tenhunen *et al.* (19). The HO inhibitor, CrMP, was purchased from Frontier Scientific (Logan, UT). AA and PGE_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI). Water-soluble indomethacin (indomethacin trihydrate) was a gift from Merck Sharp & Dohme Research Laboratories, Rahway, NJ. Xanthine oxidase, hypoxanthine, catalase, and all other chemicals were of analytical grade and purchased from Sigma Chemical (St. Louis, MO).

Statistical Analysis. Values are presented as means ± SE. Results were subjected to a one-way ANOVA for repeated measures with Tukey's post hoc test to isolate differences between groups. Determination of difference from zero was by assessed by Student's *t* test. A level of P < 0.05 was considered significant.

Results

Effects of AA on CO Production in Piglet Cerebral Microvessels. As expected, AA increased PGE_2 production and indomethacin inhibited basal PGE_2 production and PGE_2 production from exogenous AA (2 μm–20 μ*M*; Fig. 1). Treatment with AA also increased CO in a dose-dependent manner, and this increase was inhibited by indomethacin (Fig. 2). CO production from exogenous heme also was reduced by indomethacin (Fig. 3).

Effect of HO-2 Metabolites on PGE_2 Production. As shown in Figure 3, the HO substrate, HLL, increases CO production from freshly isolated microvessels that express HO-2, but not HO-1 (6, 20). However, neither HLL nor CrMP affected PGE_2 production (Fig. 4). The 2 ×

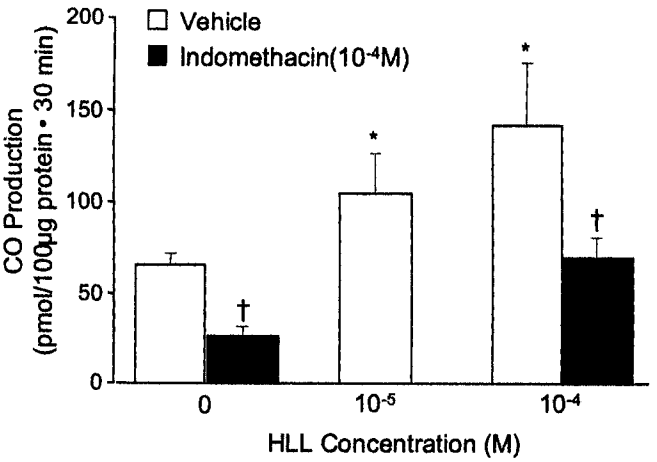

Figure 3. Effect of HLL and 10^{-4} *M* indomethacin on CO production in piglet cerebral microvessels. Means ± SE of five experiments. *P < 0.05 compared with no HLL; †P < 0.05 compared with no indomethacin.

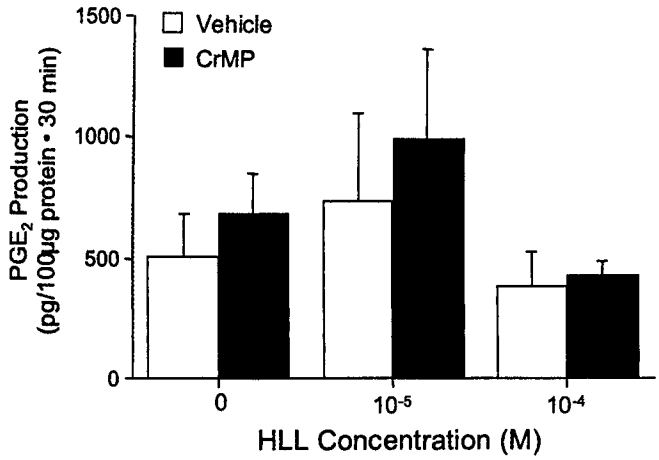

Figure 4. Effect of HLL and 2×10^{-5} *M* CrMP on PGE_2 production by piglet cerebral microvessels. Means ± SE of five experiments.

10^{-5} *M* dose of CrMP was selected because, *in vivo*, this concentration blocked dilation of pial arterioles caused by HLL but not dilation caused by CO (6). These data suggest that products of HO-2 metabolism of heme, CO, bilirubin, and iron, do not affect either free AA levels or Cox catalytic activity.

Effect of Exogenous PGE_2 in Indomethacin-Pretreated Cerebral Microvessels. In piglet cerebral microvessels pretreated with 10^{-4} *M* indomethacin, 2 µ*M* and 20 µ*M* PGE_2 caused small, dose-dependent increases in CO production (Fig. 5).

Effects of the Superoxide-Generating System of Xanthine Oxidase and Hypoxanthine. Xanthine oxidase with hypoxanthine, which produces superoxide, stimulated the production of CO approximately three-fold (Fig. 5), which is similar to the increase caused by 2 µ*M* and 20 µ*M* AA (Fig. 2) as compared with PGE_2. The increase in CO production was mildly attenuated by catalase.

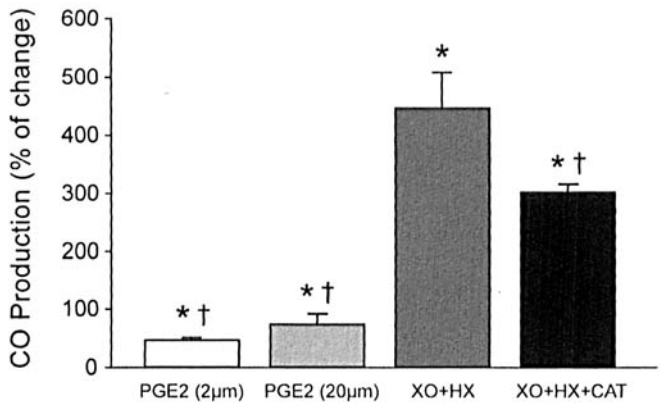

Figure 5. Effects of PGE_2 and the superoxide generating system of 1 U/ml xanthine oxidase (XO) plus 0.2 m*M* hypoxanthine (HX), with and without 50,000 U/ml catalase (CAT) on CO production by piglet cerebral microvessels treated with 10^{-4} *M* indomethacin ($n = 11$, 10, 10, and 7). Values are percentage change from vessels without either PGE_2 or XO/HX, expressed as means ± SE. $^*P < 0.05$ compared with zero (no change); $\dagger P < 0.05$ compared with no XO and HX.

Discussion

This study demonstrates that Cox products, apparently ROS, increase CO production by freshly isolated newborn pig cerebral microvessels. This conclusion is based on our new findings that: (i) AA, which increases Cox activity, increases CO production from both endogenous and exogenous heme; (ii) indomethacin, which decreases Cox activity, decreases CO production; (iii) xanthine oxidase with hypoxanthine, which produces superoxide, stimulates CO production in cerebral microvessels similarly to AA; and (iv) PGE_2 does not increase CO production nearly as much as AA or xanthine oxidase plus hypoxanthine. Conversely, because increasing HO activity with heme and inhibiting it with CrMP failed to affect PGE_2 production, it seems that neither CO nor other heme metabolites affect phospholipase or Cox activity.

Cox (PGG/H synthase) has both Cox and peroxidase activity. Cox oxidizes AA to the cyclic endoperoxide intermediate PGG_2, which is then converted to PGH_2, from which subsequent prostanoids are synthesized. AA metabolism can also be a source of ROS production (10, 11). In particular, Cox isoforms and 5-lipoxygenase contain heme iron and generate superoxide anion (10). PGE_2, even at an equimolar concentration to AA, did not increase CO production nearly as much as AA. Conversely, $\bullet O_2^-$ increased CO production similarly to AA. These results suggest that Cox metabolism of AA increases CO production *via* production of ROS.

The reaction of hypoxanthine plus xanthine oxidase generates $\bullet O_2^-$ and H_2O_2 (17, 21). To study the effect of $\bullet O_2^-$ on cerebral microvessels, superoxide was generated by xanthine oxidase acting on hypoxanthine. Because this reaction produces both $\bullet O_2^-$ and H_2O_2, 50,000 U/ml catalase was used to eliminate the effect of H_2O_2 produced *via* dismutation of $\bullet O_2^-$. When catalase was added to the xanthine oxidase plus hypoxanthine, CO production was reduced, but was still greater than 2.5 times the basal level. The maximal $\bullet O_2^-$ concentration produced, 13 µ*M* (17), is similar to the AA concentration used in these experiments.

HOs are the main producers of CO, although small amounts of CO can be derived from other sources, such as lipid peroxidation (22). CO production can be controlled either by regulation of substrate (heme) delivery or of HO-2 catalytic activity. HO-2 catalytic activity may be altered by cofactor availability, cellular localization, and/or post-translational modifications of the enzyme. Necessary cofactors for heme metabolism by HO are oxygen, NADPH, and NADPH–cytochrome-*c* reductase (23). Under the experimental conditions used in the present experiments, it is highly unlikely, although not impossible, that any of these cofactors would be low and, thus, limiting. Because indomethacin decreased and AA increased CO production from exogenous heme, a Cox product increases HO-2 catalytic activity. However, the possibility that Cox activity also can increase cellular heme cannot be excluded.

Recent studies suggested that low levels of ROS, such as $\bullet O_2^-$ and H_2O_2, modulate signal transduction pathways in mammalian cells (24). H_2O_2 stimulates protein phosphorylation (25), activates protein kinases (26), inhibits tyrosine phosphatases (27), alters intracellular Ca^{2+} (28), and stimulates phospholipases. In piglet cerebral microvessels, tyrosine phosphorylation seems critical (29). Conversely, in rat neurons, serine phosphorylation increases HO-2 activity (30).

In contrast to the present results on cerebral microvessels, elevation of HO increases Cox activity (31) in the rat hypothalamus. In the present study, increasing heme up to 10^{-4} *M* did not modify PGE_2 production. Furthermore, the HO inhibitor, CrMP, did not affect prostanoid production either. Because increasing HO activity with heme and inhibiting it with CrMP failed to affect PGE_2 production, it seems that neither CO nor the other heme metabolites change phospholipase or Cox activity in newborn pig cerebral microvessels.

In conclusion, these data suggest that one or more products of AA metabolism by Cox, apparently ROS, increase cerebrovascular CO production. This increase seems to include an increase in HO-2 catalytic activity, but a concomitant increase in free heme cannot be excluded. Conversely, neither CO nor other heme metabolites seem to markedly affect the activity of Cox.

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