Cerebrovasodilatory Contribution of Endogenous Carbon Monoxide During Seizures in Newborn Pigs

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ABSTRACT

Carbon monoxide (CO) and the excitatory amino acid glutamate both dilate cerebral arterioles in newborn pigs. The key enzyme in CO synthesis is heme oxygenase, which is highly expressed in neurons with glutamatergic receptor activity as well as cerebral microvessels. During seizures the extracellular level of glutamate is increased, which results in excessive depolarization of neurons. We hypothesized that CO is a mediator of excitatory amino acid–induced dilation of the cerebral microvasculature during seizures. Three groups of piglets were examined: *1*) i.v. normal saline (sham control), *2*) topical chromium mesoporphyrin (Cr-MP, 15×10^{-6} M), and *3*) i.v. tin-protoporphyrin (Sn-PP, 4 mg/kg). Synthetic metalloporphyrins (Cr-MP and Sn-PP) are heme oxygenase inhibitors, thereby reducing CO synthesis. Implanted closed cranial windows were used to monitor changes in pial arteriolar diameters. Seizures were induced by administration of i.v. bicuculline. Changes in pial arteriolar diameters were monitored during 30 min of status epilepticus. The percent increase in pial arteriolar dilation in the saline group during seizures was $68 \pm 3\%$. In the metalloporphyrin groups, the pial arteriolar dilation was markedly reduced ($35 \pm 3\%$ and $13 \pm 1\%$, for Cr-MP and Sn-PP, respectively; $p < 0.05$, compared with the saline group). We conclude that metalloporphy-

rins by inhibition of heme oxygenase and prevention of CO synthesis attenuate pial arteriolar dilation during seizures. Therefore, CO appears to be involved in cerebral vasodilation caused by glutamatergic seizures. (*Pediatr Res* 51: 579–585, 2002)

Abbreviations
aCSF, artificial cerebrospinal fluid
BBB, blood–brain barrier
BP, blood pressure
CBF, cerebral blood flow
CSF, cerebrospinal fluid
CO, carbon monoxide
Cr-MP, chromium mesoporphyrin
GABA, gamma aminobutyric acid
HLL, heme L-lysinate
HO, heme oxygenase
ICP, intracranial pressure
NO, nitric oxide
NOS, nitric oxide synthase
PAD, pial arteriolar diameter
Sn-PP, tin-protoporphyrin

Seizures result when there is an excessive depolarization of neurons within the CNS. One of the mechanisms of excessive depolarization is a relative excess of excitatory *versus* inhibitory neurotransmitters. Under certain conditions, extracellular levels of glutamate, the principal excitatory neurotransmitter, increase because of high release or diminished reuptake by energy-dependent transport in presynaptic nerve endings and glia. The results are neuronal excitation and seizures (1).

During seizures CBF increases to match neuronal activity and to preserve energy supplies to the brain (2, 3). Several mediators could be involved in maintenance of cerebral circulation during seizures. NO, dilator prostanoids, and recently CO have all been suggested as contributing factors (3, 4). Also, systemic hypertension in the absence of BBB integrity and metabolic disturbances such as hypoxia, hypercapnia, and acidosis during seizures may result in cerebral hyperemia (2).

Application of glutamate to the cerebral cortex results in vasodilation of pial arterioles (5). The mechanism of vascular dilation does not appear to involve dilator prostanoids but appears to involve NO through *N*-methyl-D-aspartate receptors

(6). Also, recently we have shown that glutamate-induced pial arteriolar dilation was significantly reduced by Cr-MP, which is an HO inhibitor, suggesting a contributory role of CO in the pial arteriolar dilation caused by the excitatory amino acid (7).

CO is produced endogenously through heme catabolism by HO (8). The HO-2 system is strongly expressed in the brain (9). Inasmuch as heme does not cross the BBB, it is clear that the function of HO in the brain does not involve peripheral heme degradation (10). Heme is an essential molecule for life as the prosthetic group of heme-bearing proteins, such as Hb, myoglobin, mitochondrial and microsomal cytochromes, and catalase, peroxidase, and many other enzymes, such as soluble guanylyl cyclase, NOS, and cyclooxygenase. Functional evidence for the potential presence of HO-2 in neurons with glutamatergic expression is suggestive of a special relationship between glutamatergic activity and endogenously produced CO (11). Furthermore, cerebral microvessels have very high expression of HO-2 and produce CO (7).

We hypothesized that pial arteriolar dilation during seizures is in part mediated via the release of CO. To test this hypothesis, the effects of metalloporphyrins, which are HO inhibitors, on pial arteriolar responses to status epilepticus were investigated in newborn pigs.

METHODS

The animal protocol was reviewed and approved by the Animal Care and Use Committee of The University of Tennessee Health Science Center. Thirty-four newborn pigs 1–3 d old and 2.0 ± 0.1 kg weight were used for this study.

Catheter placement. Piglets were anesthetized with i.m. ketamine hydrochloride and acepromazine, 33 and 3.3 mg/kg, respectively. A catheter was inserted into a femoral vein for injection of α-chloralose at an initial dose of 30 mg/kg followed by 3 mg/kg every 3 h to maintain the desired level of anesthesia. This catheter was also used for maintenance infusion of 5% dextrose water (D_5W) at 4 mL/kg per h. Another catheter was inserted into the abdominal aorta via a femoral artery for recording BP and for sampling of blood for pH and gases. The trachea was intubated, and the lungs were ventilated with room air using a newborn positive-pressure respirator (BP-200). All piglets were paralyzed with pancuronium bromide during seizures. The systemic arterial BP, pH, and gases were maintained within the normal range during the experiments before seizure induction. Body temperature was maintained between 37 and 38°C using a heating pad with a servo-control system.

Electrocortical activity was monitored during seizures using a filtered EEG (cerebral function monitor, model 870; Critikon, Tampa, FL, U.S.A.) (12). Disk electrodes were placed on the scalp over the frontal and biparietal areas. ICP was also recorded using a pressure transducer that was connected to a port of the cranial window that had a direct connection to periarachnoid CSF.

Cranial window placement. Closed cranial windows were implanted over the left parietal cortices for direct measurements of PAD, topical application of different compounds, measurement of ICP, and collection of CSF. To implant the cranial window, the head was immobilized, and the scalp was cut and reflected from the skull. A hole approximately 2 cm in diameter was made in the skull. An incision was made through the dura and arachnoid membranes, and these membranes were reflected over the edge of the bone. A stainless-steel ring with a premounted glass coverslip was inserted in the hole. The window was cemented in place with dental acrylic. Three needles piercing the ring allowed injection of aCSF under the window, ICP monitoring, and sampling of CSF. The space under the window was filled with aCSF that was equilibrated with 6% CO_2 and 6% O_2, which produced gases and pH and chemical composition within the normal range for CSF [pH = 7.33–7.40, P_{CO_2} = 42–46 mm Hg (5.6–6.13 kPa), P_{O_2} = 43–50 mm Hg (5.73–6.7 kPa), Na^+ 150 mEq/L, K^+ 3 mEq/L, Ca^{2+} 2.5 mEq/L, Mg^{2+} 1.2 mEq/L, Cl^- 132 mEq/L, glucose 3.7 mM, urea 6 mM, HCO_3^- 25 mEq/L]. The volume directly below the window was approximately 500 μL and was contiguous with the periarachnoid space.

Pial arterioles were observed with a dissecting microscope. PADs were measured using a television camera mounted on the microscope, a video monitor, and a video micrometer (Model VPA-1000, FOR-A, Los Angeles, CA, U.S.A.). Pial arterioles of different sizes (<100 μm in diameters) were selected and monitored in each piglet. Briefly, the images of arterioles were displayed on the television monitor, and the sides of each vessel were bracketed by parallel lines projected by the micrometer. As the arteriolar diameter changed, the lines were moved manually to correspond with the vessel walls. Precalibration of the distance between the lines allowed determination of arteriolar diameters. After implantation of the window, a 30-min stabilization period was allowed before experimentation.

Materials. The HO substrate, HLL, was used to determine an appropriate i.v. dosage of Sn-PP. HLL was prepared using methods described by Tenhunen *et al.* (13). It was protected from light at all times until placement beneath the cranial window, and the cranial window was only illuminated during vessel diameter measurements. HLL was stored at −30°C. HLL was diluted in aCSF (10^{-7} M) for placement under the cranial window. The HO inhibitors, Sn-PP and Cr-MP, were purchased from Porphyrin Products Inc. (Logan, UT, U.S.A.). Metalloporphyrins (Sn-PP and Cr-MP) were similarly protected from light. Among the metalloporphyrins, Sn-PP when systemically injected will cross the BBB. Sn-PP has a 3.4-h half-life in plasma and a significant concentration in brain 1–4 h after its injection (14, 15). Sn-PP has a marked inhibitory effect on cerebral HO activity (16).

Pancuronium bromide was purchased from Astra Pharmaceutical Products Inc. (Westborough, MA, U.S.A.). Bicuculline, isoproterenol, and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Experimental procedures. Because an i.v. dose for Sn-PP has not been established in newborn pigs, first in a separate group of piglets ($n = 13$) we examined dosage for i.v. Sn-PP. A dose range of 2 to 5 mg/kg of Sn-PP was tried. Pial arteriolar responses to topical cerebral cortical application of HLL (10^{-7} M) were evaluated before and after i.v. administration of normal saline or Sn-PP. Pial arteriolar dilation is an expected

response to HLL; this response is caused by heme degradation, releasing CO (7). This vasodilation is expected to be prevented by HO inhibition with Sn-PP. Furthermore, with an optimal dose of Sn-PP, pial arteriolar responses to topical isoproterenol (10^{-6} M) should be preserved. As to Cr-MP, we have previously shown that topical application of Cr-MP at 15×10^{-6} M is an appropriate dose for inhibition of HO activity (7).

In the second part of the study, the contributory role of CO in cerebrovascular dilation during seizures was investigated ($n = 21$). Three groups of newborn pigs were studied: sham control group that received normal saline at 4 mL/kg, i.v. ($n = 7$), Sn-PP group (4 mg/kg, i.v.; $n = 9$), and Cr-MP group (topical cerebral application of 15×10^{-6} M; $n = 5$).

Before induction of seizures, to confirm that metalloporphyrins were not nonspecifically altering pial arteriolar responses, responses to topical application of isoproterenol (10^{-6} M) and sodium nitroprusside (10^{-6} M) were evaluated separately in a consecutive manner before and after the treatment with i.v. Sn-PP, topical application of Cr-MP, or i.v. normal saline (sham control). A 10^{-6} M concentration is considered a middle dose for vasodilatory responses to isoproterenol and sodium nitroprusside. At this concentration responses are reversible in a reasonable time period. Seizures were induced by i.v. injection of 3 mg/kg bicuculline. Bicuculline is a γ-aminobutyric acid inhibitor; γ-aminobutyric acid is an inhibitory neurotransmitter, inhibition of which causes glutamatergic seizures.

Cyclic nucleotide analysis. Cortical periarachnoid CSF (0.4 mL) collected from under the cranial window was mixed with EDTA (5 μL) and stored at $-60°$C until assayed. Multiple CSF samples were collected during different interventions. Cyclic AMP and cGMP were measured by RIA (17).

Statistical analysis. Values for each variable are presented as mean $\pm$ SEM. Comparisons within and between 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. A $p < 0.05$ was considered significant.

RESULTS

The selected baseline PADs ranged from 40 to 96 μm at the onset of the study.

In the first part of the study, Sn-PP at 2 mg/kg caused a partial attenuation of pial arteriolar dilation that was induced by HLL. However, Sn-PP at 3 to 4 mg/kg was deemed to be an appropriate dose as vasodilatory responses to HLL were significantly attenuated (Fig. 1) without any reduction in isoproterenol-induced arteriolar dilation. Cerebrovascular responses to isoproterenol, obtained after administration of 5 mg/kg Sn-PP, varied from no response, to minimal vasodilation, to vasoconstriction. Furthermore, 5 mg/kg Sn-PP usually caused cardiovascular instability. It is unclear whether Sn-PP at 5 mg/kg injures cerebral microvessels or reduces cerebral vascular responses by another effect, possibly by compromising cardiovascular control. It is clear that 5 mg/kg is too much.

In the second part of the study, before the induction of seizures, cerebrovascular reactivity to isoproterenol at 10^{-6} M and sodium nitroprusside at 10^{-6} M was evaluated before and
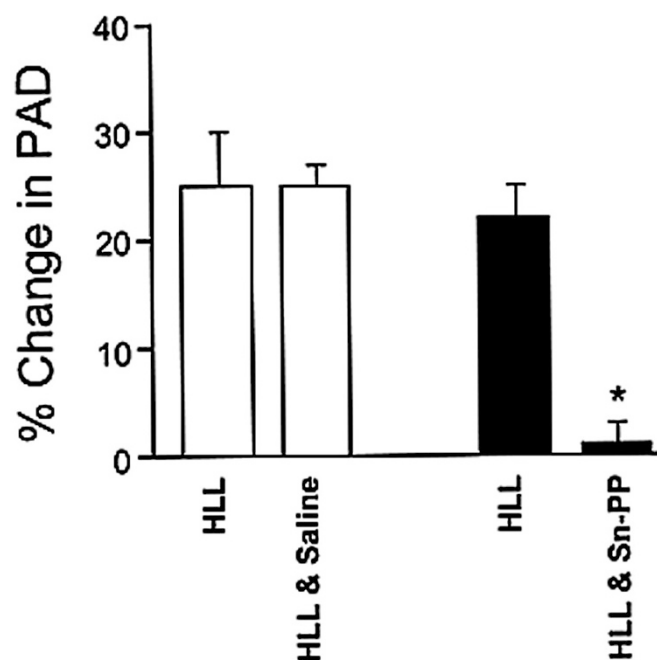

Figure 1. Effects of i.v. saline or Sn-PP (4 mg/kg) on PAD of newborn pigs that already received topical HLL (10^{-7} M) $n = 4$ newborn pigs. Values are mean $\pm$ SEM. *$p < 0.05$ HLL plus Sn-PP compared with HLL alone.

after the administration of Sn-PP, Cr-MP, and saline. Pial arteriolar dilations in response to these vasogenic stimuli were not significantly different within these three groups (Fig. 2).

The arterial pH and gases, BP, and temperature were kept within the normal range until the seizure induction. During seizures, BP, HR, and ICP increased significantly, which coincided with abnormal electrocortical activity. Also, Pa_{CO_2} increased and Pa_{O_2} decreased during status epilepticus. The percent change in arterial blood gases, vital signs, and ICP were similar in saline- and metalloporphyrin-treated groups during status epilepticus (Fig. 3).

Before induction of seizures, administration of metalloporphyrins caused a transient pial arteriolar dilation (10 $\pm$ 2%) that was followed by vasoconstriction (10 $\pm$ 1%). During status epilepticus, pial arteriolar dilation did occur in the saline- and metalloporphyrin-treated groups. However, this dilation was markedly attenuated in the metalloporphyrin-treated groups when compared with the saline-treated group (Fig. 4).

Periarachnoid CSF cyclic nucleotides (cGMP and cAMP) were measured during different interventions in the metalloporphyrin- and saline-treated groups. Before seizure induction, metalloporphyrins caused increased cortical CSF cGMP and cAMP ($p < 0.05$). During status epilepticus, cyclic nucleotides rose markedly in all three groups (Fig. 5). The rises in both cGMP and cAMP were more pronounced in the metalloporphyrin-treated groups compared with the saline-treated group (2- to 3-fold *versus* 4- to 6-fold).

DISCUSSION

In this study, pial arteriolar dilation secondary to seizures was significantly attenuated in the metalloporphyrin (HO in-
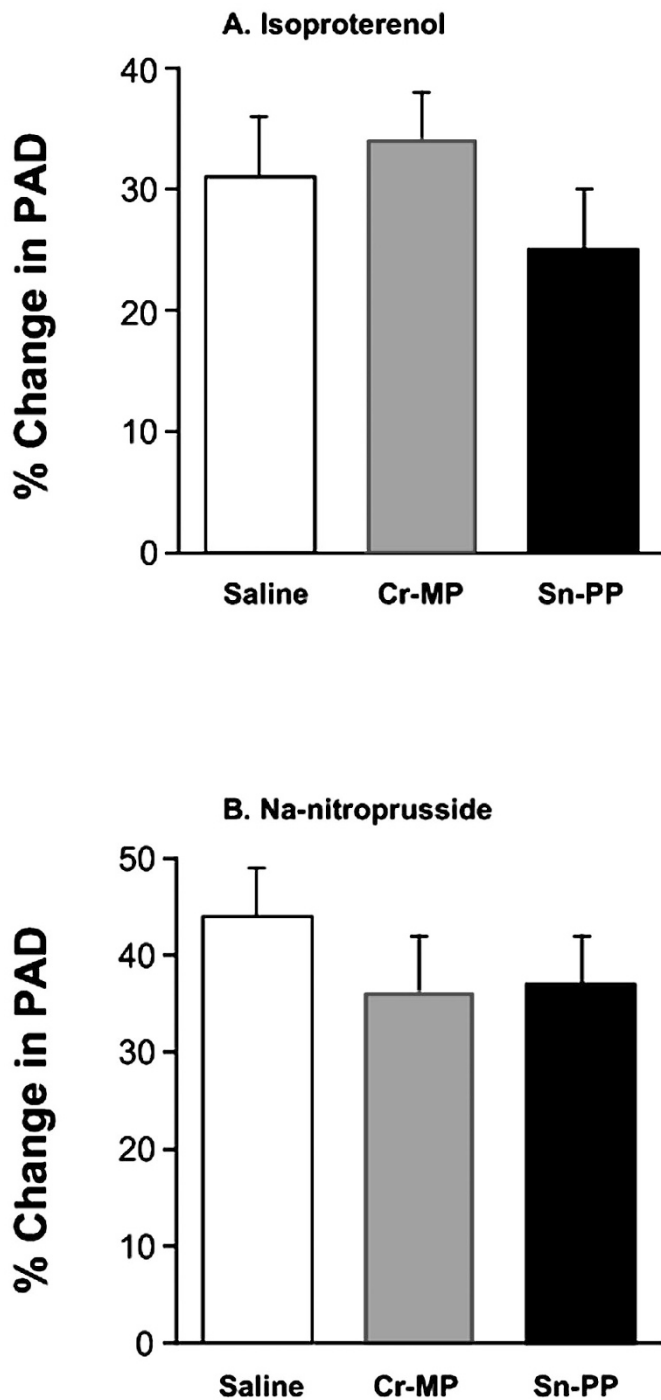
A. Isoproterenol

B. Na-nitroprusside

Figure 2. Effects of topically applied isoproterenol (10^{-6} M; *A*), and sodium nitroprusside (10^{-6} M; *B*), on PAD of newborn pigs after administration of saline, Cr-MP (15×10^{-6} M), or Sn-PP (4 mg/kg i.v.) $n = 13$ newborn pigs. Values are mean $\pm$ SEM.

Figure 3. Percent changes in blood gases ($Paco_2$ and Pao_2), BP, heart rate (HR), and ICP observed during status epilepticus in saline-treated, Cr-MP-treated (15×10^{-6} M), and Sn-PP-treated (4 mg/kg i.v.). groups ($n = 21$ newborn pigs). Values are mean $\pm$ SEM.

Figure 4. Percent changes in PAD that occurred during status epilepticus in saline-treated, Cr-MP-treated (15×10^{-6} M), and Sn-PP-treated (4 mg/kg i.v.) groups. ($n = 21$ newborn pigs). *$p < 0.05$ compared with saline; +$p < 0.05$ compared with Cr-MP. Values are mean $\pm$ SEM.

hibitor) -treated groups when compared with saline-treated newborn pigs. Both endogenously produced and exogenously administered CO are potent dilators of cerebral arterioles in newborn pigs (7). Our findings are in contrast to those obtained from adult rabbit and dog basilar and middle cerebral arteries *in vitro* (18). Therefore, CO may elicit less vasodilatory response in large cerebral arteries, in adults, or in other species (18). Because the previous studies were in isolated arteries, the
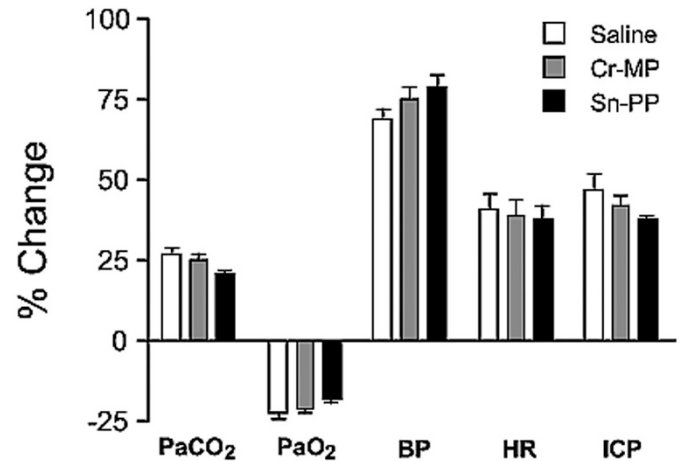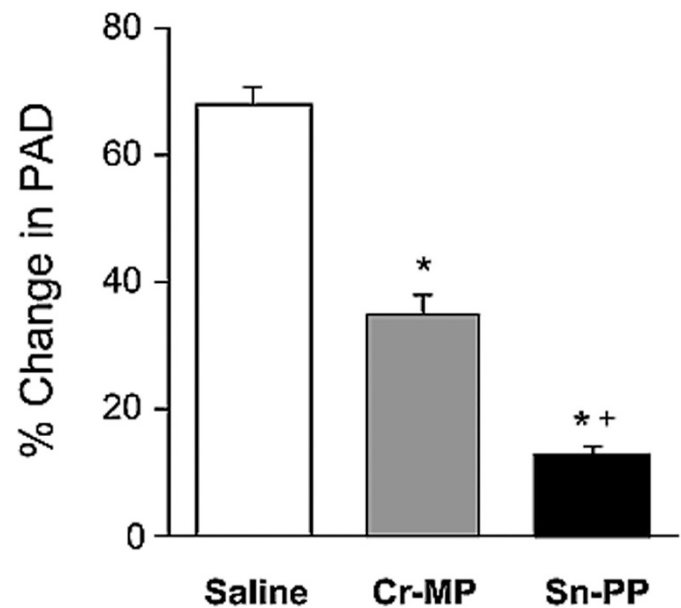
results are difficult to compare with the present ones on arterioles *in vivo*.

Endogenous CO is produced by heme destruction catalyzed by HO (19). In the brain, HO-1, the inducible form, and HO-2, a constitutive form, are both present in microvascular endothelial and smooth muscle cells (7, 20, 21). Under normal conditions, HO-1 is present in selected neurons; under stress it can be induced rapidly (22). Also, HO-2 in the brain is abundant in neurons of autonomic ganglia and also in the discrete neuronal population with glutamatergic expression (20, 23). Glutamate receptors are also expressed in cultured cerebrovascular endothelial cells (24). Seemingly, HO and glutamate receptors have similar localization in the brain. Glutamate has been reported
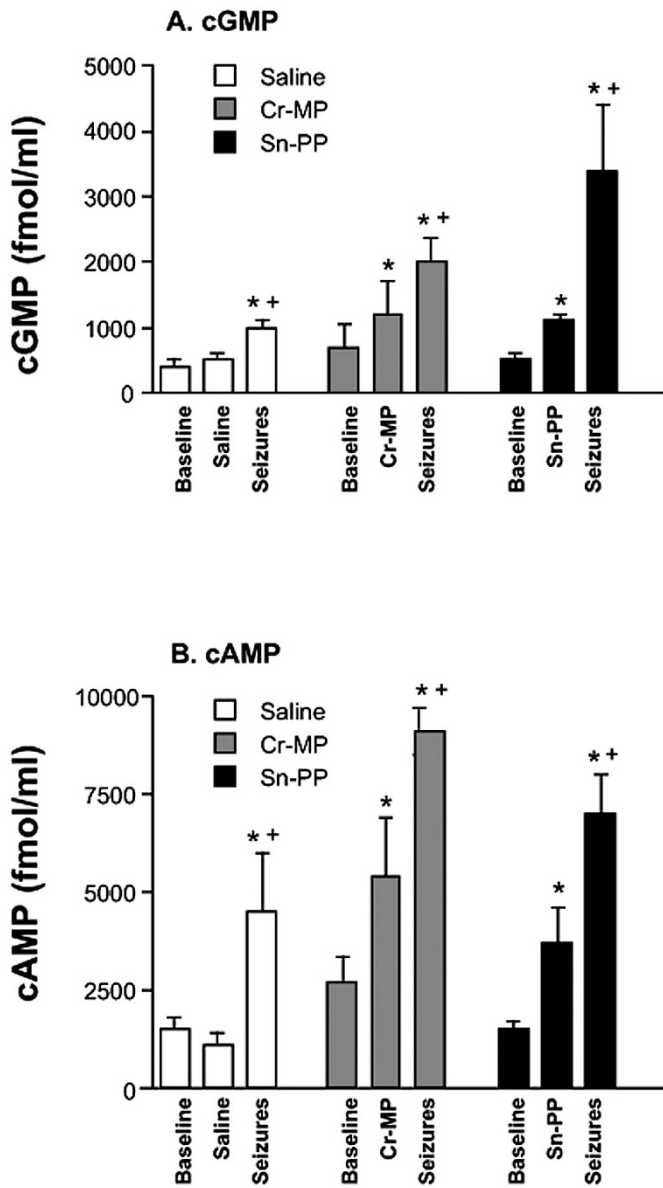
A. cGMP

B. cAMP

Fig. 5. Changes in CSF cGMP (*A*) and cAMP (*B*) levels during baseline; after treatment with saline, Cr-MP (15×10^{-6} M), or Sn-PP (4 mg/kg i.v.); and during seizures. ($n = 21$ newborn pigs). *$p < 0.05$ compared with baseline in each group; +$p < 0.05$ for seizures compared with treatment in each group. Values are mean ± SEM.

to regulate CO release through metabotropic receptors, possibly through an action on protein kinase C and stimulation of HO-2 synthesis (25).

Our findings that metalloporphyrins resulted in transient dilation of pial arterioles was surprising at first, as inhibition of endogenous CO production is expected to bring about vasoconstriction rather than vasodilation. This dilation appears to involve NO, because Cr-MP–induced dilation was abolished by pretreatment with N^{ω}-nitro-L-arginine which is a NOS inhibitor (7). Pertinent to this point, metalloporphyrins have previously been shown to be capable of NOS activation (26). Also, by inhibiting endogenous CO production, metalloporphyrins may remove tonic inhibition of NOS by CO binding to the NOS heme (27). 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.

Pial arteriolar dilation and increased CBF occur during seizures (2–4). In the present study both topical and systemically administered metalloporphyrins caused a significant attenuation of pial arteriolar dilation during status epilepticus, which we believe is caused by the inhibition of CO production. In rats, similar to piglets, the vasodilatory effects of heme on resistance vessels were promptly reversed by inhibitors of HO (7, 28). HO catabolizes heme to CO, free iron, and biliverdin. The vasodilatory manifestations in rats were not mimicked by biliverdin or attenuated by iron chelators but could be mimicked by exogenous CO (28). Therefore, seizure-induced vasodilation was most likely caused by the formation of CO, as HO inhibitors attenuated the vasodilation. HO-2– containing nerve fibers in the adventitial layer of pial vessels are one potential source of CO during seizures (20). Another potential source of CO during seizures is the glutamatergic neuronal hyperactivity, which results in stimulation of HO-2 synthesis (25). Also, much CO is produced in cerebral microvessels; namely, in endothelial and smooth muscle cells (7, 20).

During status epilepticus, pial arteriolar dilation was significantly attenuated in the metalloporphyrin groups, but it was not totally abolished. The partial pial arteriolar dilation during status epilepticus in the metalloporphyrin group can be explained by the influence of other mediators, such as NO or prostanoids, which may have provided a compensatory dilatory role. There is a similarity among cellular localization of HO, NOS, and prostaglandin cyclooxygenase with a potential for coordinated and complementary dilatory function (20). Montecot *et al.* (4) observed a significant reduction in cerebral hyperemia during seizures when rats were pretreated with Sn-PP; however, Sn-PP did not affect the cerebrovascular responses to hypercapnia. This finding can be explained by prostanoid-dependent mechanisms in hypercapnia-induced vasodilation, which is independent of CO production. Our previous study also indicated that metalloporphyrins had no effect on hypercapnia-induced vasodilation (7). These current findings suggest that CO participates in the regulation of CBF that is associated specifically with glutamate release.

CO can be an endothelially derived relaxing factor similar to prostanoids and NO (20). During seizures, the capacity of the cerebral tissue to generate CO is overwhelmingly higher than that of NO (8). Both NO and CO can exert their vasodilatory action by activation of soluble guanylyl cyclase, but CO can act predominantly by increasing the outward K^+ currents through Ca-activated K^+ channels in vascular smooth muscle cells (29). Furthermore, in our preliminary study, we have reported recently that in newborn pigs, Ca-activated K^+ channels in cerebral artery smooth muscle cells are sites of action for CO (30).

CO can affect BP through CNS-mediated influences on vascular tone in chronically hypertensive subjects (31). These influences are likely related to local interaction between HO and glutamatergic transmission in the nucleus tractus solitarius

in the medulla oblongata (32, 33). In our piglets, metalloporphyrins at the doses used had no influence on BP because the hypertension was not chronic but rather acutely induced by seizures.

CSF cyclic nucleotides were evaluated in our study. As one of the mechanisms for CO-induced vasodilation could be via activation of soluble guanylyl cyclase and production of cGMP, we did not discount the possibility of an attenuated level of cGMP after metalloporphyrin administration. However, we observed a significant rise in cGMP level independent of CO production. Other studies have shown that exogenous CO does not increase cerebral cGMP in piglets or rats (7, 34). Furthermore, we found that Cr-MP causes an acute dilation that involves NO (7, 26), which could account for the elevation of cGMP. As noted above (7), CO-induced dilation of cerebral microvessels does not appear to relate to CO activation of guanylyl cyclase but instead to Ca-activated K^+ channels.

cAMP also rose significantly in the metalloporphyrin groups. In our previous study, we found that cAMP production with exogenous CO administration was mildly elevated; the level was quantitatively insignificant when compared with cAMP produced by isoproterenol or iloprost (7).

cAMP and cGMP increased significantly during seizures regardless of whether the treatment was saline or metalloporphyrins. That brain cyclic nucleotides are increased during seizures has been reported, and appropriate treatment with anticonvulsants will modify the brain cyclic nucleotides (35). The greater rise in cyclic nucleotides in the metalloporphyrin-treated groups compared with the saline-treated group may be explained by a compensatory increase of NO and prostanoids (30), as CO production was inhibited by Sn-PP and Cr-MP in our experimental animals. cAMP and cGMP production are known to be stimulated by prostanoids and NO, respectively (17).

The heme-HO-CO system in the brain may be protective against neuronal cell death (36). In experiments using transgenic mice, HO-1–overexpressing neurons were found to be protected against chemical stimuli that were neurotoxic, such as glutamate (37). When HO activity was inhibited, protection against glutamate was lost (37). Heme degradation catalyzed by HO results not only in the production of CO but also of free iron and biliverdin. The protective role of the heme-HO-CO system could be related as well to release of other byproducts of heme degradation such as biliverdin or bilirubin. Bilirubin is a strong antioxidant. Considering the relative deficiency in glutathione and ascorbic acid of neuronal tissues, particularly in neonates, the antioxidant activity of bilirubin, which is released as a result of heme breakdown during seizures, may also be important for brain protection (38).

CONCLUSION

We conclude that in newborn pigs, HO inhibitors attenuate seizure-induced cerebral vasodilation. We propose that CO is involved in the cerebral vasodilation caused by seizures from excessive glutamatergic stimulation.

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