Influence of the heme-oxygenase pathway on cerebrocortical blood flow

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Heme-oxygenase (HO)-derived carbon monoxide (CO) is generated in the cardiovascular and in the central nervous systems. Endogenous CO exerts direct vascular effects and has been shown to inhibit nitric oxide synthase (NOS). In the current study, the heme-oxygenase blockade [zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), 45 \(\mu\)mol/kg intraperitoneally] decreased cerebral CO production and increased cerebrocortical blood flow (CBF) in anesthetized rats. This latter effect was abrogated by the NOS blockade (50 mg/kg L-NAME intravenously). Furthermore, inhibition of CO production had no effect on stepwise hypoxia/hypercapnia-stimulated increases in CBF. Our results indicate that endogenous CO reduces the resting CBF via inhibition of NOS but fails to influence the CBF response to hypoxia and hypercapnia in adult rats. NeuroReport 18:1193–1197 © 2007 Lippincott Williams & Wilkins.

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Introduction

Heme oxygenase (HO) catalyzes the degradation of monomeric free heme to generate carbon monoxide (CO) [1–4]. Previously thought to be simply a waste product, endogenously formed CO is now known to serve as a messenger in numerous physiological and pathophysiological processes [1–4]. In the cardiovascular system, CO is an activator of soluble guanylate cyclase and can promote the relaxation of vascular smooth muscle, activate large-conductance calcium-activated \(K^+\) channels and inhibit vascular smooth muscle cell proliferation [4]. In the central nervous system, CO plays an important role in synaptic plasticity, learning and memory processes, as well as in the regulation of hypothalamic neuropeptide release [1].

Expression of HO isoenzymes in neural and vascular tissues is well documented [5,6]. It is also known that freshly isolated cerebral microvessels, as well as microvascular endothelial cells, produce CO. The generation of CO is increased by stimulation of the ionotropic glutamate receptors [6,7]. The role of endogenous CO, however, in the regulation of the adult cerebral circulation is not well understood [3]. In newborn pigs, both exogenous CO and the HO substrate, heme, induce pial arterial dilatation; and the latter can be inhibited by the HO blocker chromium mesoporphyrin (CrMP) [5]. In addition, hypoxia-induced vasodilation decreases, whereas hypocapnia-induced vasoconstriction increases after topical application of CrMP [5,8]. Interestingly, pial arterial dilation in response to hypercapnia remains unaltered after topical CrMP treatment [5], but gets reduced after systemic administration of another HO inhibitor: tin protoporphyrin [9]. Cerebrospinal fluid (CSF) levels of endogenous CO increase during seizures [9,10], and seizure-induced pial arteriolar dilatation and the cerebral blood-flow increase are attenuated by HO inhibitors in piglets and adult male rats, respectively [10–12]. Glutamate receptor-mediated dilation of newborn pig pial arterioles also involves the HO pathway [7,13]. It appears that endogenous CO dilates pial cerebral arterioles by augmenting the coupling of \(Ca^{2+}\) sparks to \(K_{Ca}\) channels in smooth muscle cells [14].

Although the majority of the cerebrovascular studies have been carried out in newborn models, cerebral vasoregulatory mechanisms are known to change substantially during maturation. Furthermore, recent studies confirm that CO-synthesizing and nitric oxide-synthesizing enzymes are colocализed in cerebral vessels and perivascular neuronal cells [6,15]. As CO has been shown to inhibit nitric oxide synthase (NOS) activity [16], it raises the possibility of interactions between these pathways in cerebral circulation. We, therefore, wanted to determine the influence of the HO pathway on the cerebrocortical blood flow (CBF) in adult animals in the presence and absence of a functional NOS system, under resting and combined hypoxic/hypercapnic conditions.

Materials and methods

Experiments were performed using adult male Wistar rats (body weight 300–400 g), under the guidelines of the
Hungarian Law of Animal Protection (243/1988), and with approval from Semmelweis University Committee on Ethical Use of Experimental Animals (590/99 Rh). During the in-vivo experiments, the animals were anesthetized with urethane (1.3 g/kg intraperitoneally, Sigma), while spontaneously breathing via a fitted trachea cannula. Indwelling catheters were inserted into the right femoral artery for blood pressure measurements and sampling and into the left femoral vein for drug administration. Femoral arterial blood gases (PaCO₂, PaO₂, O₂ saturation) and acid–base parameters (pH, standard base excess) were measured with an ABL-300 Radiometer Blood Gas Analyzer (Brønshøj, Denmark). CBF was measured with a two-channel laser-Doppler (LD) monitor (MBF 3D, Moor Instruments, Devon, UK) as described previously [17], with infrared laser light (780 nm) penetrating 1 mm into the brain, spanning 7 mm² of parietal cortex.

In the first part of the study, the effect of HO blockade on the CBF was studied under physiological (normoxic/normocapnic) conditions. The first experimental group served as a vehicle-treated control and CBF was determined before and after an intraperitoneal injection of 3 ml saline. In the second group, ZnDPBG [zinc deuteroporphyrin 2,4-bis glycol, Frontier Scientific (Logan, Utah, USA), 45 μmol/kg intraperitoneally] was applied. This had previously been shown to effectively inhibit HO activity in the rat brain [18]. Animals in the third and fourth experimental groups were pretreated with the NOS inhibitor L-NAME (N⁵-G-nitro-L-arginine methyl ester, Sigma, 50 mg/kg intravenously). Thirty minutes later the third and fourth groups received saline or ZnDPBG, respectively. CBF was determined in all experimental groups before as well as 15, 30, and 45 min after the administration of vehicle or ZnDPBG.

To verify the inhibitory effect of ZnDPBG on brain HO activity in vivo, matched series of animals were treated with either ZnDPBG or vehicle as described above. Thirty minutes later, innate cerebral CO generation was determined using solid-phase gas chromatography (custom built Peak Performer 1 RCP, Peak Laboratories LLC, Mountain View, California, USA) as described in detail elsewhere [8,19]. Briefly, isolated midbrain tissues from each animal were individually sonicated in Krebs’ buffer and divided into eight aliquots. For each animal midbrain sample, head space CO was measured in quadruplicate vials after being maintained at 2°C or the remaining matched aliquots after being incubated at 37°C for 60 min. The differences between the incubated and cold vials were expressed as μmol CO generated/kg of wet tissue per hour. ZnDPBG induced a reduction of cerebral HO activity from 4.58 ± 0.87 to 2.47 ± 0.36 μmol/kg per hour (P = 0.025).

In the second part of the study, the effect of HO blockade on the cerebrocortical hyperemic response to hypoxia/hypercapnia (H/H) was examined. H/H was induced in a stepwise manner by the administration of different gas mixtures (5%O₂–20%CO₂–75%N₂ for producing moderate H/H and 20%CO₂–80%N₂ for producing severe H/H, respectively) with a constant flow of 31/min through a 5 ml chamber connected to the trachea, at atmospheric pressure. CBF was recorded continuously and peak CBF values were determined during the two 10-min long steps of H/H. After the first moderate and severe H/H challenge, the animals were divided into two experimental groups receiving either saline or ZnDPBG intraperitoneally. Thirty minutes later the moderate and severe H/H was repeated in both groups and peak CBF values were determined from the continuous recording of CBF.

The in-vitro experiments were performed in the middle cerebral arteries (MCAs) supplying the parietal cortex, the site of our in-vivo CBF measurements. MCA segments were prepared from adult male Wistar rats as described previously [20] and studied in a conventional myograph system (Danish Myo Technology A/S, Aarhus, Denmark). The effects of 10 μM ZnDPBG or 10 μM bradykinin (as positive control) were tested after precontraction of vessels by 10 μM prostaglandin F₂α.

**Results**

Effects of heme-oxygenase blockade under normoxic/normocapnic conditions

Arterial blood gas and acid–base parameters were within the physiological range (PaO₂: 90–105 mmHg, O₂ saturation: 95–97%, PaCO₂: 40–45 mmHg, pH: 7.32–7.36, standard base excess: −3–0 mM) during the experiments. L-NAME pretreatment increased mean arterial blood pressure (from 102.2 ± 3.0 to 146.3 ± 4.1 mmHg, *P < 0.001), decreased heart rate (from 424 ± 12 to 373 ± 12 beats/min, *P = 0.012) and reduced CBF (from 359 ± 18 to 258 ± 13 AU, *P < 0.001).

Administration of ZnDPBG, which inhibits CO formation, increased CBF, but saline vehicle alone had no effect (Fig. 1). Inhibition of NO formation by L-NAME pretreatment

![Fig. 1](https://example.com/fig1.png)

*Cerebrocortical blood flow (CBF) before (0 min) as well as 15, 30, and 45 min after intraperitoneal injection of saline (triangles) or 45 μmol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG; circles) in naive (open symbols) or in N⁵-G-nitro-L-arginine methyl ester (L-NAME) pretreated (filled symbols) rats. *P = 0.016, ***P < 0.001 vs. ‘0 min’, with repeated measures analysis of variance and Tukey’s post hoc test, n = 12–16. AU, arbitrary unit.*
completely blocked ZnDPBG-induced elevations of CBF (Fig. 1). Under in-vitro conditions, 10 μM ZnDPBG had no effect on the tension of isolated MCA segments; this effect cannot be attributed to endothelial damage as functionally intact endothelium was confirmed by relaxation to 10 μM bradykinin (79 ± 5% of the precontraction induced by prostaglandin F₂α).

Effects of heme oxygenase blockade on the hypoxia and hypercapnia induced increase of cerebrocortical blood flow
Before the initial H/H challenge, baseline cardiovascular, arterial blood gas and acid–base parameters were within physiological ranges in both (later ZnDPBG-treated or saline-treated) experimental groups. During moderate H/H, PaO₂ was reduced to 60–65 mmHg, PaCO₂ was increased to 80–85 mmHg and pH decreased to 7.10–7.15 (Fig. 2a). Despite no change in MAP, CBF increased in both experimental groups by ~45% (Fig. 2b). During severe H/H, PaO₂ was reduced to 45–50 mmHg, PaCO₂ was increased to 95–100 mmHg and pH decreased to 7.05–7.10 (Fig. 2a); CBF increased in both experimental groups by ~65% (Fig. 2c), whereas MAP remained unchanged.

The second stepwise H/H challenge, after ZnDPBG or saline, induced similar changes in the blood-gas tensions and pH as the initial challenge had done (Fig. 2a), without affecting MAP (data not shown). Most important, neither ZnDPBG nor saline treatment had any effect on CBF responses to moderate (Fig. 2b) or severe (Fig. 2c) H/H, when compared with the pretreatment values.

Discussion
Our current study provides evidence for the interaction between the HO and NOS pathways in the regulation of the CBF that had been described in the other vascular beds [19,21,22]. The CBF increase after administration of ZnDPBG and its inhibition with L-NAME pretreatment indicate that constitutive CO release tonically suppresses the NO production and consequently reduces blood flow in the cerebral cortex.

The interactions between CO and NO are well documented. Endogenous CO is known to relax smooth muscle, but in the presence of a functional endothelium CO-induced inhibition of NO can promote vasconstriction [4,21]. Systemic inhibition of CO formation, with CrMP, has no effect on hindlimb vascular resistance but promotes a marked and sustained vasconstriction after L-NAME pretreatment [19]. Delta-aminolevulinic acid, a heme precursor that promotes CO formation, causes vasocostriction in intact isolated rat gracilis muscle arterioles, but is converted to vasodilation by removal of the endothelium and by pretreatment with L-NAME to inhibit NO formation [22]. These earlier studies provide evidence that CO-induced constriction arises from the suppression of endothelial NO production. Furthermore, L-NAME increased the reduction of renal blood flow and augmented the contraction of isolated renal interlobar arteries in response to the HO inhibitor stannous mesoporphyrin II [23].

It has been suggested that HO-derived CO can be a primary regulator of NO production [16], and a growing body of evidence continues to support that contention. Ishikawa et al. [15] have demonstrated that HO-2 is colocalized with endothelial NOS (eNOS) in the cerebrovascular endothelium as well as with neuronal NOS (nNOS) in neurons and arachnoid trabecular cells, suggesting that the colocalization of the CO-generating and NO-generating pathways are sufficient for interaction. Tricarbonyldichlororuthenium (II) dimer, a CO-releasing molecule, was shown to reduce NO release from cultured endothelial cells. In addition, HO blockade by zinc protoporphyrin IX (ZnPP) induced CO-reversible and L-NAME-reversible increase of the cerebrovascular and perivascular NO production by 70–80%. These results are comparable with the ZnDPBG-induced 67% increase of the NOS activity in the rat hypothalamus observed in our previous study [24]. Most important, ZnPP induced a dose-dependent increase of the pial arteriolar diameter, which could be prevented by coadministration of either CO or L-NAME, indicating the
involvement of NO in the mediation of the vasodilation. Our current results confirm and complement these observations by providing direct evidence for the significance of the CO–NOS interaction at the level of cerebrocortical blood perfusion. In our study, however, ZnPDPBG failed to relax the isolated middle cerebral artery, whereas Ishikawa et al. [15] reported marked pial arteriolar dilatation in response to ZnP in rats. As, in the latter study, pial arteriolar responses were determined in vivo, the most plausible explanation of the discrepancy between the two findings is that reduction of nonvascular CO release or augmentation of nonvascular NO release plays an important role in CBF increase after inhibition of HO.

As HO degrades heme to form CO, iron and biliverdin, alternative interactions might explain the influence of HO activity on cerebrovascular NO functions [25]. It has been described that CO binds directly to NOS and inhibits NO production. Iron released in the course of heme degradation by HO can inhibit de-novo NOS synthesis by inhibiting its nuclear transcription, but our currently reported effect is rapid and is, therefore, unlikely to arise from changes in protein synthesis. Alternatively, HO activity might influence NOS synthesis by changing heme availability and the active site of NOS requires two heme molecules. Finally, the NOS and HO pathways might interact by competing for NADPH as a cofactor required for their enzymatic activity. It has, however, been shown that exogenous CO promotes vasodilation and mimics the effects of increased HO activity, suggesting a nicotine-mediated adenine dinucleotide phosphate-independent mechanism [22].

Our current study also shows that the HO–CO pathway does not play a discernible role in hypoxia/hypercapnia-induced increased CBF in adult rats. This observation is different from that of previous studies in newborn models in which HO blockade inhibited the pial arteriolar dilation in response to hypoxia or hypercapnia [5,9]. As the regulatory processes of the CBF during hypoxia or hypercapnia show marked alterations during maturation, we can only speculate that our contrasting findings arise from age-related differences in the models. Furthermore, we cannot exclude the possibility that endogenous CO can have dual cerebrovascular effects during H/H: (i) it directly facilitates the H/H induced CBF increase, but (ii) its simultaneous inhibitory influence on NO synthesis neutralizes this effect. Further studies can clarify this hypothesis and investigate the role of endogenous CO in the adaptation of the cerebral circulation to chronic hypoxia when the expression of HO-1 is increased in the brain [2].

Conclusions
Our results indicate that endogenous CO exerts a tonic influence on resting CBF by inhibiting NO synthesis. Furthermore, inhibition of the HO–CO pathway increases CBF most likely by increasing neuronal NO production. Finally, the HO–CO pathway does not influence hypoxia and hypercapnia-induced CBF increase in adult rats.

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