The role of the heme – heme-oxygenase – carbon monoxide pathway in the regulation of the cerebral blood flow

Doctoral thesis

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I. Introduction

Endogenously formed carbon monoxide (CO) arises primarly from the degradation of monomeric free heme and is generated in a reaction catalyzed by the heme oxygenases (HO). During this reaction heme is degraded to release equimolar quantities of iron, biliverdin and carbon monoxide. The iron is primarily recycled into the formation of new heme while the biliverdin is rapidly converted to bilirubin by an abundance of biliverdin reductases. The carbon monoxide product eventually binds to circulating hemoglobin and is then transported as carboxyhemoglobin until it is excreted through ventilation.

In mammals three forms of HO have been identified to date. The regulation of HO isozymes markedly differs for each one; the HO-1 (also known as the stress protein HSP32) gene responds to all types of stimuli, which have in common only the ability to cause oxidative stress, and HO-2 is responsive only to adrenal glucocorticoids. New results suggested that there are no functional HO-3 genes in rat and that the HO-3a and HO-3b genes are processed pseudogenes derived from HO-2 transcripts.

Endogenous carbon monoxide thought to be simply a waste product, but now is known to serve as a messenger in numerous physiological and pathophysiological processes. The HO-CO pathway has been reported to evoke direct vascular effects and to influence other vasoregulatory mechanisms like the cyclooxygenase (COX) and nitric oxide synthase (NOS) systems. However, the participation of CO in the regulation of the cerebral circulation has received little attention.

II. Aims

We aimed to clarify the role of the heme – heme-oxygenase – carbon monoxide pathway in the regulation of the cerebral blood flow, therefore the aims of our studies were fivefold:

1., to investigate the involvement of endogenous carbon monoxide in the regulation of the resting hypothalamic circulation,

2., to clarify its possible interaction with the L-arginine – nitric oxide pathway, and

3., its possible interaction with the cyclooxygenase pathway.

4., Furthermore, we aimed to determine the influence of the HO pathway on the cerebrocortical blood flow in the presence and absence of a functional NOS system, and

5., to test the presence of these interactions in vitro in isolated middle cerebral artery (MCA) segments.

III. Methods

The experiments were carried out in adult (300-400 g bw.) male Wistar rats anesthetized with 1.3 g/kg ip. injected Urethane, and spontaneously breathing via a trachea cannula. Catheters were inserted into both femoral arteries (to measure blood pressure and for blood sampling) and into the left femoral vein (for drug administration). The skull was fixed in a stereotaxic head-holder. Body temperature was kept constant between 36-38 °C with a controlled heating lamp.

Systemic arterial pressure was continuously recorded on a Grass polygraph. Cerebral blood flow in the ventromedial hypothalamic area was determined by using Aukland´s H_2-gas clearance method. Briefly, a 100 μm in diameter teflon-coated Pt electrode with a 1 mm bare tip was introduced stereotactically into the ventromedial hypothalamic area. H_2-wash-out curves were produced by H_2-gas inhalation, and were recorded on the polygraph. HBF was calculated from the washout curves by using the initial slope technique, omitting the first 0.5 min.

For the measurement of cerebral blood flow in the parietal cortex the head of the animals was fixed in a stereotaxic head holder with the nose 5 mm down from the interaural line. The skull of the parietal region was exposed and the bone was thinned over the

parietal cortex on both sides with a microdrill, so that the lamina interna of the skull remained intact. Two laser-Doppler (LD) probes were placed above the thinned skull at a 12°-angle to the vertical to provide an optimal view of the cortex (4 mm caudal from bregma, 5 mm lateral from midline). LD flow was measured with a two-channel flow meter and was recorded continuously. The LD flowmeter was calibrated before each individual experiment with a constant movement latex emulsion. The laser light was in the infrared range (780 nm) and penetrated about 1 mm into the brain covering approximately 7 mm^2 of the parietal region, so that the data acquired mostly represented the characteristics of the parietal cortex .

The blood gas values ($PaCO_2$, PaO_2, O_2-saturation) and pH in femoral arterial samples were measured by a Radiometer Blood Gas Analyzer at the times of HBF determinations. Mean arterial blood pressure (MAP), heart rate (HR), ventilation rate (VR) were determined at the same time. After the last measurements CSF was obtained from the cisterna magna for cyclic GMP (cGMP) content determination. Thereafter the anesthetized animals were rapidly exsanguinated and hypothalamic tissue samples were excised, frozen rapidly and kept at –75 °C until measurement of NOS activity. The hypothalamic NOS activity was measured on the basis of labeled citrulline formation from labeled L-arginine.

In order to determine the effect of HO blockade on cerebral prostanoid production, two groups of animals were anesthetized as above and treated ip. with either the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, 45 μmol/kg ip.) or saline. Forty-five minutes later cerebrospinal fluid (CSF) was obtained from the cisterna magna and kept at –75 °C until further analysis. Concentrations of prostaglandin D_2, E_2, and $F_{2\alpha}$, as well as the stable prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ have been determined using gas chromatography/triple quadrupole mass spectrometry (GC/MS/MS). 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.

The in-vitro experiments were performed in the middle cerebral arteries (MCAs) supplying the parietal cortex, the site of our in-vivo CBF measurements. Adult male Wistar rats were exsanguinated rapidly under deep ether anesthesia. Ring segments of the middle cerebral arteries were prepared for measurement of isometric force. Special care was taken to preserve the endothelium during preparation. The segments were transferred to 8-ml organ baths filled with a modified Krebs solution of the following composition: 119 mM NaCl, 4,7 mM KCl, 2,5 mM $CaCl_2*2H_2O$, 20 mM $NaHCO_3$, 1,17 mM $MgCl_2*7\ H_2O$, 1,18 mM KH_2PO_4, 0,027 mM EDTA, 11 mM glucose and the bath solution was bubbled continuously with a humidified gas mixture (95% O_2 / 5% CO_2). The MCA segments were mounted on two L-shaped tungsten wires (50 µm diameter), one of which was fixed to the bath and the other to a force transducer. The vessels were allowed a 60-min equilibration period, during which the resting tension was adjusted to 1.5-2 mN and the bath solution was warmed to 37 °C with repeated washing every 25-30 min.

All chemicals have been obtained from Sigma except for ZnDPBG, which was purchased from Frontier Scientific Europe Ltd. Previous studies have verified that diclofenac, ZnDPBG and N^G-nitro-L-arginine methyl ester (L-NAME) in the doses used in our present study are effective in the brain for inhibition of COX, HO and NOS, respectively.

All values are presented as mean ± SEM; n represents the number of animals. Statistical analysis was performed using ANOVA for repeated measurements or one way ANOVA followed by Dunnett`s post-hoc test. For comparison of two groups, Student´s unpaired t-test was used. A P value of less than 0.05 was considered to be statistically significant.

IV. Results

IV./1. The role of the heme – heme-oxygenase – carbon monoxide pathway in the regulation of the resting hypothalamic blood flow

In the first part of the study, the effects of HO blockade on the systemic circulation and hypothalamic blood flow (HBF) were studied under physiological conditions. Animals in the first group (n=6) served as vehicle-treated controls and received an intraperitoneal injection of saline. In the second group (n= 8), zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, 45 μmol/kg), was applied intraperitoneally to inhibit the HO pathway. HBF was measured before and 15, 30, and 45 minutes after the administration of saline or ZnDPBG. The physiologic variables were within the normal range at the time of the first HBF determination before administration of saline or ZnDPBG. These cardiovascular, respiratory, and acid-base parameters did not change significantly during the whole experimental period. Furthermore, there were no differences with respect to the corresponding values between the two groups at any timepoint. HBF was similar in groups 1 and 2 during the first determination (103.1 ± 3.0 vs. 108.2 ± 8.8 mL/100 g/min in groups 1 and 2, respectively). Neither saline (group 1) nor ZnDPBG (group 2) induced any significant changes in HBF. Furthermore, there was no significant difference in the CSF cGMP concentration at the end of the experiments (450.7 ± 165.2 vs. 568.8 ± 148.0 pmol/L in groups 1 and 2, respectively). 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. ZnDPBG induced a reduction of cerebral HO activity from 4,6±0,9 to 2,5±0,4 μmol CO/kg tissue/per hour (P=0.025).

IV./2. Interaction of the heme – heme-oxygenase – carbon monoxide pathway with the nitric oxide synthase in the regulation of the hypothalamic blood flow

In order to study the possible interactions between the heme – heme-oxygenase – carbon monoxide pathway and the nitric oxide synthase, after the last hypothalamic blood flow measurements the anesthetized animals were rapidly exsanguinated and hypothalamic tissue samples were excised for the measurement of NOS activity. We observed a marked increase ($P<0.001$) in hypothalamic NOS activity in the ZnDPBG-treated animals (4.17 ± 0.87 pmol citrulline/mg protein/min) as compared to the vehicle-treated group (2.49 ± 0.38 pmol citrulline/mg protein/min).

Since HBF remained unchanged in ZnDPBG-treated animals despite an increase in NOS activity, we hypothesized that the HO blockade had a CBF-decreasing effect, which counteracted the action of NO. In order to study this possible direct effect of the HO blockade on the hypothalamic circulation in two additional experimental groups (groups 3 and 4), we repeated our measurements after NOS blockade induced by L-NAME.

Therefore, in these experimental groups (groups 3 and 4), NOS blockade was introduced by intravenous administration of 50 mg/kg NG-nitro-L-arginine methyl ester (L-NAME, Sigma). Animals in group 3 (n=9) served as vehicle-treated controls and received an intraperitoneal injection of saline 30 minutes after the onset of NOS blockade. In group 4 (n=8), ZnDPBG was administered at the same time. HBF was measured before and 15, 30, and 45 minutes after the administration of saline or ZnDPBG. The physiologic variables were within the normal range at the time of the first HBF determination before administration of saline or ZnDPBG. These respiratory, and acid-base parameters did not change significantly during the whole experimental period. Furthermore, there were no differences with respect to the corresponding values between the two groups at any timepoint.

Although the initial mean arterial pressure (MABP) was significantly higher in these groups (138 ± 6 Hgmm vs. 105 ± 7, $p<0{,}001$, Student`s unpaired t-test), it did not change further during the experiments. Moreover, despite an increased MABP, initial HBF

was significantly ($P<0.001$) lower in group 3 (65.7 ± 20.0 mL · 100 g−1 · min−1) and group 4 (65.9 ± 12.1 mL · 100g−1 · min−1) when compared to the groups without NOS blockade. The already diminished HBF was further reduced after administration of ZnDPBG in group 4, but did not change significantly after saline administration in group 3 (Fig. 1A). Moreover, there was a significant linear correlation between the HBF values after NOS blockade and the reduction of HBF in response to HO inhibition (Fig. 1B), indicating that HBF was better preserved in animals that expressed higher HO activity levels in the hypothalamus.

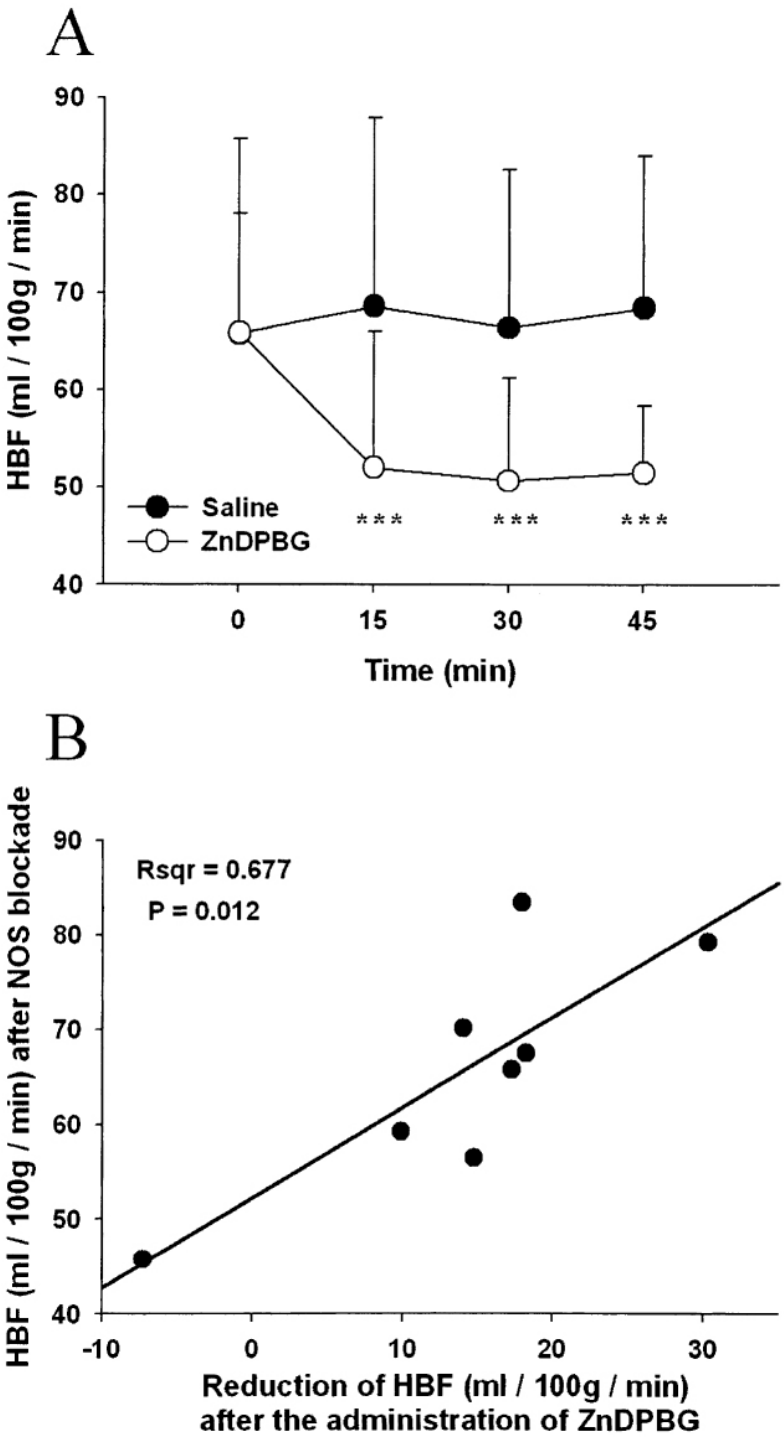
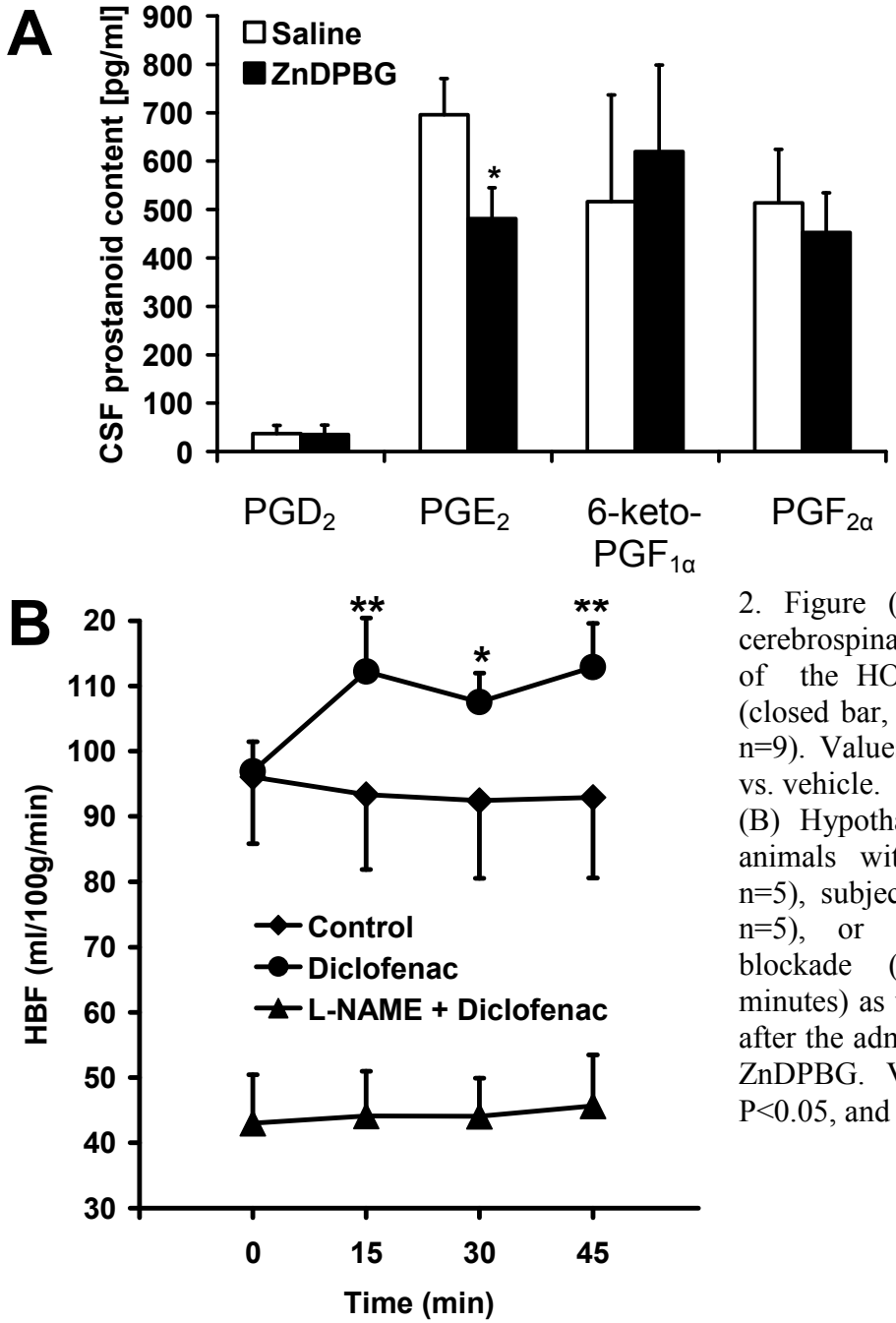


Figure 1. (A) Hypothalamic blood flow (HBF) in animals subjected to nitric oxide synthase (NOS) blockade before (0 minutes) and 15, 30, and 45 minutes after the administration of the HO synthase inhibitor ZnDPBG (open circles, n = 8) or saline (filled circles, n = 9). Values are mean ± SD. *** P <0.001 vs. 0 minutes. (B) The baseline HBF (0 minutes, A) after NOS blockade shows significant (P = 0.012) linear correlation with the reduction in HBF after ZnDPBG-induced inhibition of HO.

IV./3. Interaction of the heme – heme-oxygenase – carbon monoxide pathway with the cyclooxygenase in the regulation of the hypothalamic blood flow

Stimulation of cerebral PGE_2 production by constitutive HO activity has been reported in brain explants and primary cell cultures. To verify the presence of this interaction in vivo, PGE_2 concentration in the CSF of animals treated with ZnDPBG (45 μmol/kg ip.) was measured and compared to saline-treated controls. Interestingly, HO-blockade decreased CSF concentrations of PGE_2 without influencing the levels of other prostanoids such as PGD_2, the PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Figure 2A). These results together with the previous in vitro observations demonstrate that the HO pathway constitutively and selectively stimulates the production of PGE_2 in the brain.

Our second aim was to analyze the significance of this interaction in the regulation of the hypothalamic circulation. Therefore, the effect of HO on the hypothalamic blood flow (HBF) has been determined in two groups of animals with normal PGE_2-synthesizing capacity or after the inhibition of COX enzymes by diclofenac (10 mg/kg iv.). ZnDPBG had no effect in control animals but significantly increased HBF after diclofenac (Figure 2B) without influencing arterial blood gas or systemic circulatory parameters (data not shown). Interestingly, after diclofenac pretreatment the ZnDPBG-treated rats showed significantly ($P=0.007$) higher hypothalamic NO synthase activity (5.30±1.05 pmol citrulline/mg protein/min) as compared to saline-treated controls (2.45±0.32 pmol citrulline/mg protein/min). These results indicated that under physiological conditions HO-blockade may have two, equally potent influences on the hypothalamic vasculature: on the one hand a presumably NO-mediated vasodilation, and on the other hand vasoconstriction, due to the reduction of PGE_2-synthesis. After COX-blockade by diclofenac, however, only the NO-mediated pathway functions and results in the observed HBF-increase. To test the validity of this hypothesis we have determined the influence of HO-blockade on the HBF in animals subjected to simultaneous inhibition of COX and NOS by diclofenac and L-NAME (50 mg/kg iv.), respectively. ZnDPBG had no significant effect on the HBF in these animals (Figure 2B)



2. Figure (A) Prostanoid levels in the cerebrospinal fluid after the administration of the HO synthase inhibitor ZnDPBG (closed bar, n=8), or its vehicle (open bar, n=9). Values are mean ± SEM. * $P<0.05$ vs. vehicle.

(B) Hypothalamic blood flow (HBF) in animals without pretreatment (diamond, n=5), subjected to COX blockade (circle, n=5), or combined NOS and COX blockade (triangles, n=7) before (0 minutes) as well as 15, 30, and 45 minutes after the administration of the HO inhibitor ZnDPBG. Values are mean ± SEM. * $P<0.05$, and ** $P<0.01$ vs. 0 min.

IV./4. The role of the heme – heme-oxygenase – carbon monoxide pathway in the regulation of the cerebrocortical blood flow

The heme-oxygenase and nitric oxide synthase have a heterogenous distribution, therefore they can influence the cerebral blood flow in a regionally different manner. To explore these regional differences we investigated the effects of HO-blockade on the cerebrocortical blood flow (CBF).

The first experimental group served as a vehicle-treated control and CBF was determined before and after an intraperitoneal injection of saline. In the second group, ZnDPBG (zinc deuteroporphyrin 2,4-bis glycol, 45 μmol/kg) was applied intraperitoneally. Animals in the third and fourth experimental groups were pretreated with the NOS inhibitor L-NAME (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.

Arterial blood gas and acid–base parameters were within the physiological range during the experiments. L-NAME pretreatment increased mean arterial blood pressure (from 102±3 to 146±4 mmHg, $P<0.001$), 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 (Figure 3A). Inhibition of NO formation by L-NAME pretreatment completely blocked ZnDPBG-induced elevations of CBF (Figure 3A). Under in-vitro conditions, 10 μM ZnDPBG had no effect on the tension of isolated MCA segments (Figure 3B); which cannot be attributed to endothelial damage as functionally intact endothelium was confirmed by relaxation to 10 μM bradykinin (Figure 3B).

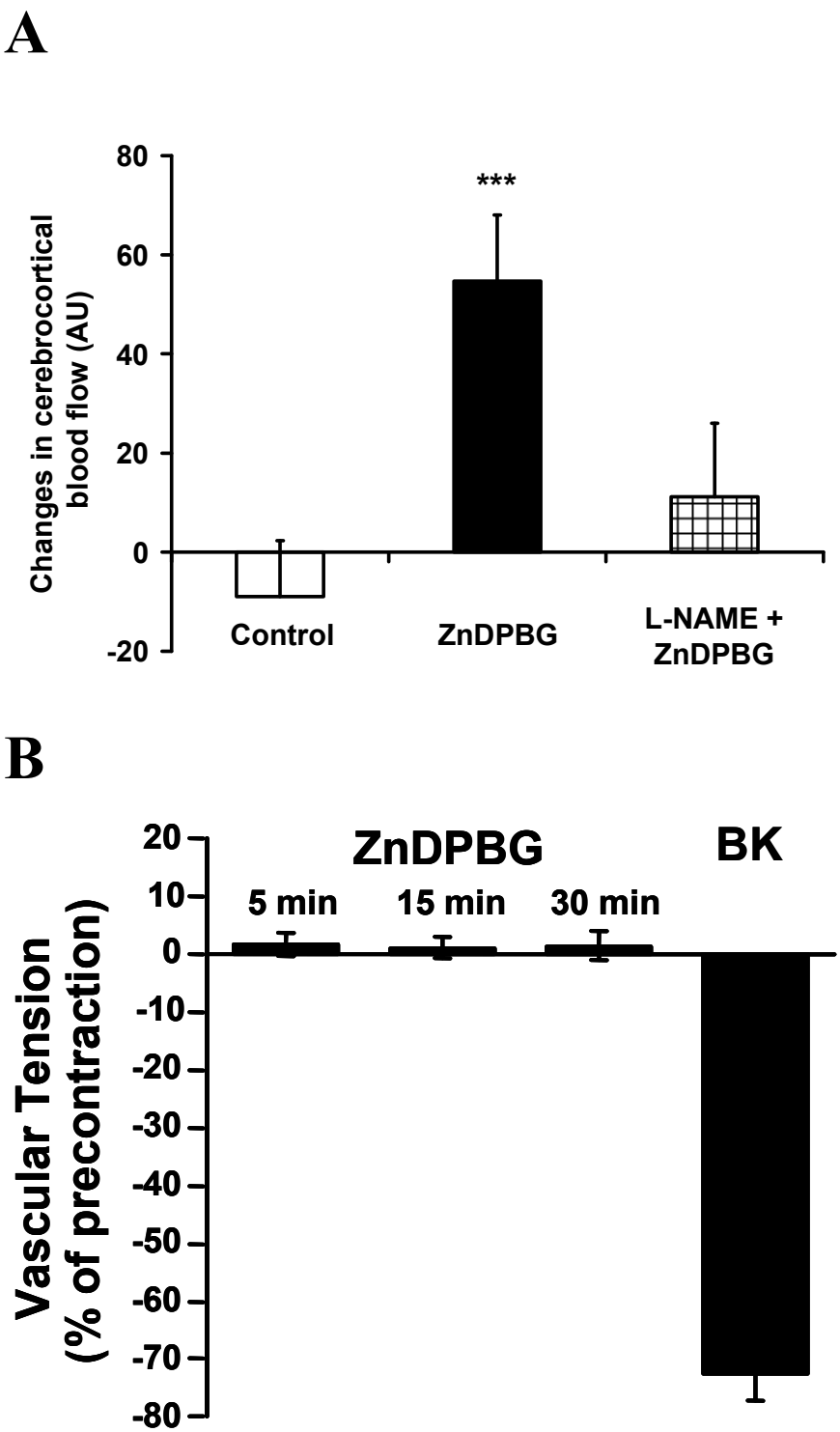


Figure 3. (A) Changes in the parietal cerebrocortical blood flow after the administration of vehicle (saline, white bar, n=6), and after the intraperitoneal administration of the HO-inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, 45 μmol/kg) in animals without pretreatment (black bar, n=8) and in animals with N^G-nitro-L-arginin metil ester pretreatment (L-NAME, 50 mg/kg, vertically and horizontally stripped bar, n=8). ***p<0,001 vs Control (one-way ANOVA and Dunett`s post-hoc test) (B) Changes in tension of middle cerebral artery (MCA) segments 5, 15, and 30 minutes after HO-blockade by 10 μM ZnDPBG and after the administration of 10 μM bradykinin.

V. Discussion

V./1. The role of the heme – heme-oxygenase – carbon monoxide pathway in the regulation of the hypothalamic cerebrocortical blood flow and its interaction with the nitric oxide synthase

We found that under physiologic conditions, HO blockade increased hypothalamic NOS activity without changing HBF. This finding indicates that endogenous CO has a dual effect on the hypothalamic circulation: a vasodilatory action that is compensated by the inhibition of NOS. After NOS blockade, inhibition of HO induced a further significant reduction of the HBF, indicating that in the absence of NO, CO becomes responsible for the maintenance of cerebral blood perfusion. Furthermore, a direct correlation between HBF in NOS-blocked animals and its reduction after HO inhibition was observed. In other words, the HO blockade induced most marked flow reduction in animals that showed relatively high blood flow in the absence of NO. This observation indicates that after NOS blockade the preservation of HBF is dependent on the vasodilatatory action of CO.

Although the HO pathway apparently has no significant influence on the resting HBF under physiologic conditions, one may not underestimate its potential importance in pathophysiologic states associated with diminished NO synthesis. It is noteworthy that in the second part of our study (i.e., in animals subjected to NOS blockade), the already compromised HBF further diminished after inhibition of endogenous CO production. Therefore, in the absence of NO, CO functions as a backup vasodilator. Although CO alone is unable to completely restore the blood flow in the hypothalamus, the partial improvement in perfusion may prevent the development of irreversible neurologic deficits. This role of the HO pathway is obviously important in circulatory shock or global cerebral ischemia, where the hypothalamus plays a central role in the coordination of defense mechanisms activated to prevent the collapse of the systemic circulation. Our observations provide a possible explanation for the relative preservation of the HBF during ischemia/reperfusion.

Our results indicates a dual role of endogenous CO in the hypothalamic circulation: a vasodilatory effect but at the same time suppression of NO release. Under resting conditions these two effects are equally potent and neutralize each other, at least in the hypothalamus. In the cerebral cortex, however, the NO-mediated pathway appears to be dominant and HO-blockade results in a NO-mediated pial vasodilation and in our experminents it resulted in cerebrocortical hyperemia. These observations support the view that the HO-pathway has a regionally heterogenous influence on the cerebral circulation. The fact that HO-blockade in vitro had no effect on isolated MCAs indicate that the carbon monoxide, which decreases cortical blood flow, originates by either nonvascular carbon monoxide sources, or mediates its effect by the inhibition of nonvascular NO release.

V./2. Interaction of the heme – heme-oxygenase – carbon monoxide pathway with the cyclooxygenase in the regulation of the hypothalamic blood flow

The most important finding of this part of the study is that the vasodilator influence of the HO-pathway on the hypothalamic vasculature is mediated by PGE_2. HO cleaves heme moieties liberating equimolar amounts of CO, free iron and biliverdin, which is converted to bilirubin by biliverdin reductase. From these metabolites CO and biliverdin are the putative regulators of PGE_2 synthesis, since they have been shown to stimulate the release of PGE_2 from primary cultures of rat hypothalamic cells in vitro. These observations and a recent report indicating that the HO pathway has no direct influence on the PGE_2 synthesis in cerebral microvessels clearly suggest that brain parenchymal rather than vascular PGE_2-release mediates the vasorelaxant effect of constitutive HO. This would also explain why exogenous CO has been shown to relax rat pial arteries only under in vivo conditions, but not in vitro.

Our experimental data are summarized on Figure 4A indicating that HO blockade by ZnDPBG has no influence on the HBF under resting conditions but results in either a decreased or an increased HBF after NOS- and COX-blockade, respectively. After simultaneous NOS- and COX-blockade, ZnDPBG fails to influence HBF. The proposed pathways mediating the effects of HO metabolites on the hypothalamic circulation are depicted on Figure 4B.

Although the HO pathway appears to have no direct cerebrovascular effect in adult rats, it can both increase or decrease HBF via stimulation of PGE_2-release or inhibition of NO-release, respectively. Under physiological conditions these pathways are equally potent and neutralize each other, at least in the hypothalamus. In pathophysiological states, however, one of these mechanisms may become dominant and evoke a significant effect on the HBF. For instance, in case of endothelial dysfunction and diminished NO production the PGE_2-pathway may dominate and contribute to the maintenance of the hypothalamic blood supply.

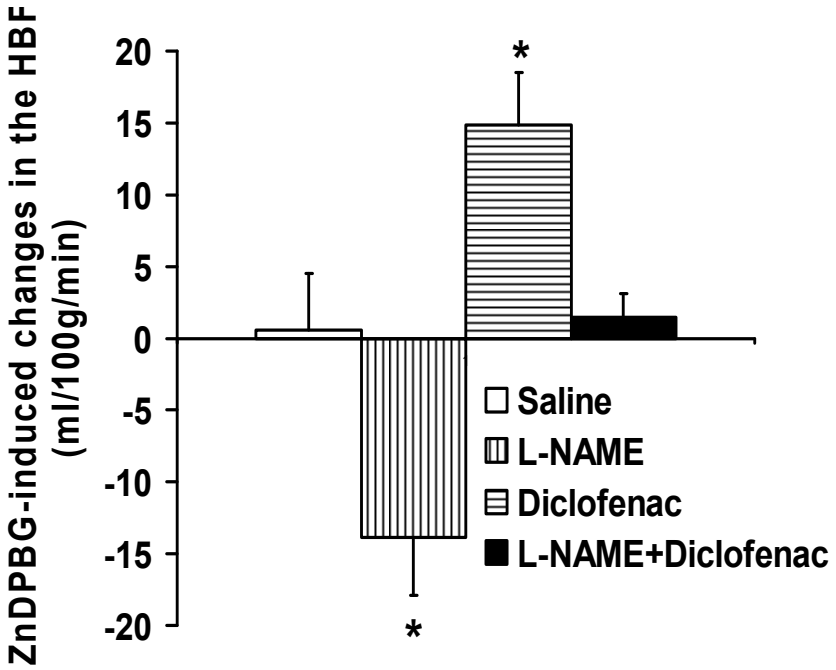


Figure 4. Hypothalamic blood flow (HBF) in animals without pretreatment (diamond, n=5), subjected to COX blockade (circle, n=5), or combined NOS and COX blockade (triangles, n=7) before (0 minutes) as well as 15, 30, and 45 minutes after the administration of the HO inhibitor ZnDPBG. Values are mean ± SEM. * $P<0.05$, and ** $P<0.01$ vs. 0 min.

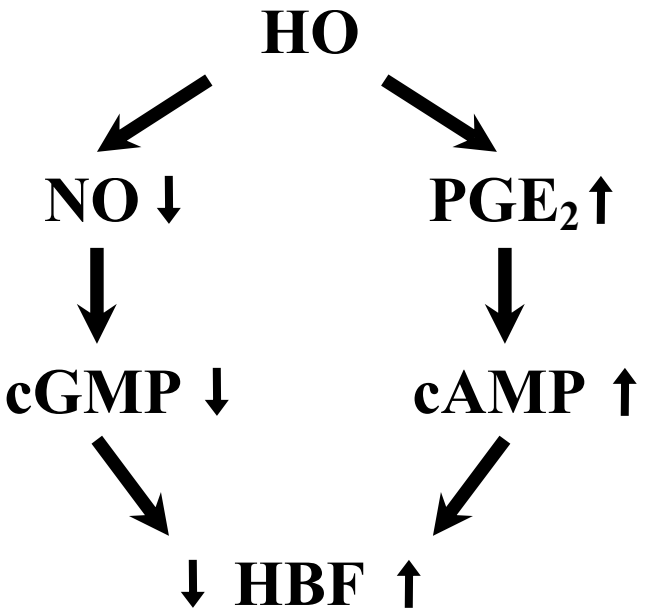


Figure 5. Proposed mechanism of the dual action of HO on the hypothalamic circulation. For abbreviations and for detailed explanation, see the text.

It is noteworthy that this PGE_2-mediated pathway seems to be independent from the morphological or functional integrity of the endothelium, since CO can be produced by vascular smooth muscle and brain parenchymal cells and the secondary PGE_2 release also appears to originate from non-vascular cells (as mentioned above). Therefore, the HO-CO-PGE_2-cAMP pathway is an ideal "backup" mediator in case of insufficient endothelial NO production.

Under pathophysiological conditions associated with markedly increased HO activity, however, the above described interaction between the HO and COX pathways can be changed substantially. Since COX enzymes are heme proteins and the heme prosthetic group is essential for their catalytic activity, heme degradation by HO may negatively influence COX activity. In accordance, HO-1 induction has been shown to reduce COX-expression and activity in endothelial cells and in the kidney. Furthermore, HO-1 induction in endothelial cells results in increased expression of prostaglandin transporter and in enhanced prostaglandin clearance. Our present study indicates that the constitutive HO-

activity in the brain fails to induce such effects since the PGE_2 levels decreased after HO-blockade while other prostanoids remained unaltered. In pathophysiological states associated with HO-1 induction in the brain (e.g. subarachnoid hemorrhage), however, the decreased production and enhanced clearance of PGE_2 and PGI_2 together with the CO-mediated inhibition of NOS activity may negatively influence the cerebral circulation.

VI. Conclusions

Endogenous CO production appears to increase the HBF via a PGE_2-dependent mechanism, but at the same time suppresses NOS activity which leads to the reduction of the HBF. These effects of CO may neutralize each other under physiological conditions. In pathophysiological states, however, which are associated with altered COX- or NOS activity, the HO pathway may significantly influence the resting HBF.

In the parietal cortex the primary effect of carbon monoxid is the inhibition of NOS activity, which effect seems to be mediated either by nonvascular carbon monoxide sources, or by the inhibition of nonvascular NO release. In conclusion, the HO-pathway appears to regulate the cerebral circulation of adults rats via interactions with the COX- and NOS-systems in a regionally heterogenous manner.

VII. The candidate's bibliography

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