Influence of heme oxygenase metabolites and endocannabinoids on the cerebrocortical blood flow under physiological conditions and during hypoxia and hypercapnia

Ph.D. Thesis

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1. INTRODUCTION

The cerebral vasoregulation incorporates various local and global regulatory mechanisms, involving participation of neuronal and vascular elements. Cerebrovascular control pathways have been studied extensively for decades, but several important questions, like the exact mechanism of flow-metabolism coupling or hypercapnia- and hypoxia-induced vasodilation, are still not clarified completely. In our works we hypothesized that investigation of recently discovered vasoactive mediators may shed light on obscure details of the regulation of cerebral circulation.

Carbon monoxide (CO), which was previously considered only as a highly toxic and life-threatening pollutant, has been recognized recently as an important messenger in numerous physiological and pathophysiological processes. Endogenous CO is produced primarily by two heme oxygenase (HO) isoenzymes, HO-1 and HO-2. Analogous to nitric oxide synthase (NOS), the HO enzymes are widely distributed in the cardiovascular as well as in the central and peripheral nervous systems. CO and nitric oxide (NO) also display notable similarities with regards to their physiological roles, as both molecules appear to be neurotransmitters in the brain and peripheral autonomic nervous system. Furthermore, both CO and NO are capable of inducing vasodilation as well as inhibiting the proliferation of vascular smooth muscle cells. Although the expression of HO isoenzymes in the brain and cerebral vessels is well documented, the role of endogenous CO in the regulation of the cerebral circulation is far less understood than that of NO. In our studies we investigated the influence of the HO pathway on the cerebrocortical circulation in adult animals.

Endocannabinoids are endogenous bioactive lipid mediators exerting many of their effects in mammals through their specific G protein-coupled receptors. The main endocannabinoids are anandamide and 2-arachidonoyl glycerol (2-AG), the former favoring cannabinoid receptor 1 (CB1) and the latter, cannabinoid receptor 2 (CB2). These receptors are also involved in mediating the effect of several constituent compounds of the plant Cannabis sativa (marijuana). Endocannabinoids have been implicated in many physiological functions and also in pathophysiological processes, such as diseases and aging of the cardiovascular system, ischemia-reperfusion injury,

hypertension, diabetes and obesity. The role of the endocannabinoid system in the regulation of cerebral blood flow is, however, still largely unknown. It was recognized very early that Δ^9-Tetrahydrocannabinol is able to increase brain perfusion in dogs, and this observation has recently been verified with positron emission tomography in humans. Furthermore, administration of anandamide dilated cerebral arterioles of rabbits and isolated cerebral arteries of cats, but caused a decrease in cerebral blood flow in rats. In other in vivo observations in rats, however, both anandamide and the CB1-receptor agonist HU-210 elicited marked cerebral vasodilation, which was inhibited by a CB1-antagonist. To address these contradictory findings and to clarify the role of endocannabinoids and CB1-receptors in cerebral circulation, we carried out experiments in rats with the administration of a CB1 receptor antagonist / reverse agonist (AM-251) under resting physiological conditions and we also examined the role of CB1-receptors in hypoxia and hypercapnia.

2. SPECIFIC AIMS

The central nervous system is an independent functional HO unit which contains the substrate, enzymes and co-factors for the synthesis of CO. Furthermore, several cell types of the brain are able to produce endocannabinoids and express their receptors. Since both endogenous CO and endocannabinoids were previously shown to have vascular effects we hypothesized that they may be involved in the regulation of the cerebral circulation. Specifically, we aimed to describe the roles of both the HO pathway and the endocannabinoid system on cerebrocortical blood flow (CBF) under resting conditions as well as during combined hypoxia and hypercapnia (H/H).

1. First, we aimed to examine the effect of the HO inhibitor ZnDPBG on the CBF under resting conditions in anesthetized adult male rats, by measuring the CBF in the parietal cortex. We also addressed the potential interaction between the HO and NOS pathways in the regulation of the cerebrocortical blood flow under resting conditions.

2. Next, we observed the effect of the HO inhibitor ZnDPBG on the cerebrovascular effects of stepwise hypoxia/hypercapnia (H/H) by measuring the CBF in the parietal cortex of anesthetized adult male rats.

3. Following this, we aimed to study the effects of the cannabinoid CB1 receptor antagonist / reverse agonist AM251 on the cerebrocortical blood flow under resting conditions using similar methods.

4. Finally, we examined the effect of CB1-blockade by AM251 on the CBF rise to stepwise H/H.

3. Materials and Methods

3.1 Experimental animals, anaesthesia and surgical procedures

The experiments were carried out on adult male Wistar rats (300-400 g) anesthetized with urethane (1.3 g/kg ip.), and spontaneously breathing via the cannulated trachea. Depth of anesthesia was regularly controlled during the experiments by checking the corneal nociception reflexes, and when necessary, additional urethane was administered intravenously. Catheters were inserted into the right femoral artery for measuring systemic arterial blood pressure, into the left femoral artery for blood sampling, and into the left femoral vein for drug administration.

3.2 Routinely recorded physiological variables

Systemic arterial blood pressure was continuously recorded on a Grass polygraph (Model 7E, Natus Neurology Incorporated, Warwick, RI, USA). Arterial blood gas values ($aPCO_2$, aPO_2 and O_2-saturation) and acid-base parameters (apH and standard base excess) were measured by an ABL-300 Blood Gas Analyzer (Radiometer, Brønshøj, Denmark) in femoral arterial samples. Body temperature was kept constant between 36-38 ºC with a controlled heating pad.

3.3 Measurement of the cerebral blood flow

Cerebrocortical blood flow (CBF), more precisely red blood cell flux, was measured in the parietal cortex by laser-Doppler (LD) flowmetry. The skull of the rat was fixed in a stereotaxic head holder, 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. LD flow (LDF) was measured with a two-channel LDF monitor (Moor

Instruments, Devon, UK) and recorded continuously. 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 represented the characteristics of the parietal cortex. The two LD flow data sets gained from the parietal region of both hemispheres of the animal were evaluated and treated as independent measurements.

3.4 Experimental Protocols

3.4.1 The influence of HO pathway on cerebrocortical blood flow

3.4.1.1 First experimental protocol: the influence of HO pathway on CBF under normoxic/normocapnic conditions

In the first experimental protocol, the effect of HO blockade on the CBF was studied under physiological normoxic/normocapnic conditions. Animals of the **first experimental group** (n=9) served as a vehicle-treated control and CBF was determined before and after an intraperitoneal injection of 3 mL saline. In the **second experimental group** (n=9), 45 µmol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, Frontier Scientific, Logan, UT, USA) was applied in 3 mL saline intraperitoneally for the inhibition of the HO pathway. CBF was determined before as well as 15, 30, and 45 minutes after the administration of either saline 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. 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 order to investigate the interaction of the NOS and HO systems, more precisely, the effect of suppression of NO production on CO-induced CBF-changes, the synthesis of NO was inhibited. Therefore, animals in the **third** and **fourth experimental groups** were pretreated intravenously with the NO-synthase inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) in a dose of 50 mg/kg, 30 minutes prior to the administration of saline (n=5) or ZnDPBG (n=6), respectively. The CBF of the L-NAME pretreated rats was determined before as well as 15, 30, and 45 minutes after the administration of saline or ZnDPBG.

3.4.1.2 Second experimental protocol: the effect of HO blockade on the CBF rise to H/H

In the second experimental protocol, the effect of HO blockade on the CBF increase to H/H was observed. Stepwise H/H was induced by administration of different gas mixtures ($5\%O_2$-$20\%CO_2$-$75\%N_2$ for producing moderate H/H and $20\%CO_2$-$80\%N_2$ for producing severe H/H) with a constant flow rate of 3 L/min into a 5 mL chamber connected to the trachea. Since one side of the chamber was open, the pressure in the chamber was not different from the atmospheric pressure. CBF was recorded continuously. Peak CBF values were determined during the two steps of H/H, each with a duration of 10 minutes. After the first stepwise H/H round, the inhalation of the gas mixture was stopped, and following a thirty minute recovery period of normal air breathing, the animals were divided into two experimental groups, thus receiving intraperitoneally either 3 ml saline or 45 micromol/kg ZnDPBG dissolved in the same amount of saline. Thirty minutes following intraperitoneal drug treatment, the stepwise H/H was repeated in both experimental groups with continuous recording of the CBF, from which peak CBF values were determined again.

3.4.2 Influence of endocannabinoids on cerebral circulation

3.4.2.1 Third experimental protocol: the influence of CB1 receptor blockade on the CBF under normoxic/normocapnic conditions

In the third experimental protocol, the influence of CB1-receptors on the CBF under resting conditions was investigated. After a 15 min baseline period, the control (first) experimental group received vehicle-treatment (1 ml of saline, ethanol and emulfor iv. in a volume ratio of 1:1:8 of ethanol-emulphor-saline). The treated (second) experimental group was administered intravenously 10 mg/kg of CB1 receptor antagonist / reverse agonist AM251. AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) was obtained from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in 1 mL of vehicle containing ethanol/emulphor/saline (1:1:8; v:v:v). Blood samples were taken before as well as 15, 30, and 45 minutes after the administration of AM251 or its vehicle.

3.4.2.2 Fourth experimental protocol: the effect of CB1 receptor blockade on the CBF rise to H/H

With the fourth experimental protocol, the main goal of the investigation was to study the effect of the cannabinoid CB1 receptor antagonist AM251 on the cerebrocortical hyperemic response to controlled stepwise H/H. In this case, 3 steps of H/H were induced by inhalation of different gas mixtures with a constant flow rate of 3 L/min into a 5 ml chamber connected to the trachea as described above. Peak CBF values were determined during the three, 10-min long steps of H/H.

In the first step, during mild H/H, animals inhaled a gas mixture of $10\%O_2$-$10\%CO_2$-$80\%N_2$, which produced arterial pO_2 of 75-85 mmHg and pCO_2 of 50-60 mmHg. In the second step, during moderate H/H, animals inhaled a gas mixture of $5\%O_2$-$20\%CO_2$-$75\%N_2$, which produced arterial pO_2 of 55-65 mmHg and pCO_2 of 80-90 mmHg. In the third step, during severe H/H, animals inhaled gas mixtures of $0\%O_2$-$20\%CO_2$-$80\%N_2$, which produced arterial pO_2 of 45-50 mmHg and pCO_2 of 90-100 mmHg.

After the severe H/H step, the inhalation of the gas mixture was stopped, and following a thirty minute recovery period of normal air breathing, the animals received various drug treatments, depending on the experimental groups they were in. The animals were randomly divided into two experimental groups receiving either vehicle or AM251 (10 mg/kg i.v.). After another thirty minutes period following drug treatment, the stepwise H/H was repeated in all experimental groups with continuous recording of the CBF, and determination of peak CBF values.

3.5 Drugs and chemicals

ZnDPBG was purchased from Frontier Scientific, Logan, UT, USA, and AM251 from Cayman Chemicals, Ann Arbor, MI, USA. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.6 Statistical analysis

Statistical analysis was performed using Student's t-test for comparision of two values as well as ANOVA for repeated measurements or one way ANOVA followed by

Tukey's post-hoc test for three or more values. A p value of less than 0.05 was considered to be statistically significant.

4. RESULTS

4.1 Effects of heme-oxygenase blockade under resting normoxic/normocapnic conditions

Baseline arterial blood gas and acid-base parameters were within the physiological range and showed no significant changes after the administration of ZnDPBG or its vehicle (saline). Neither ZnDPBG nor its vehicle induced any significant changes in the mean arterial blood pressure (Figure 1). In contrast, administration of ZnDPBG but not its vehicle resulted in a marked increase of the CBF (Figure 2).
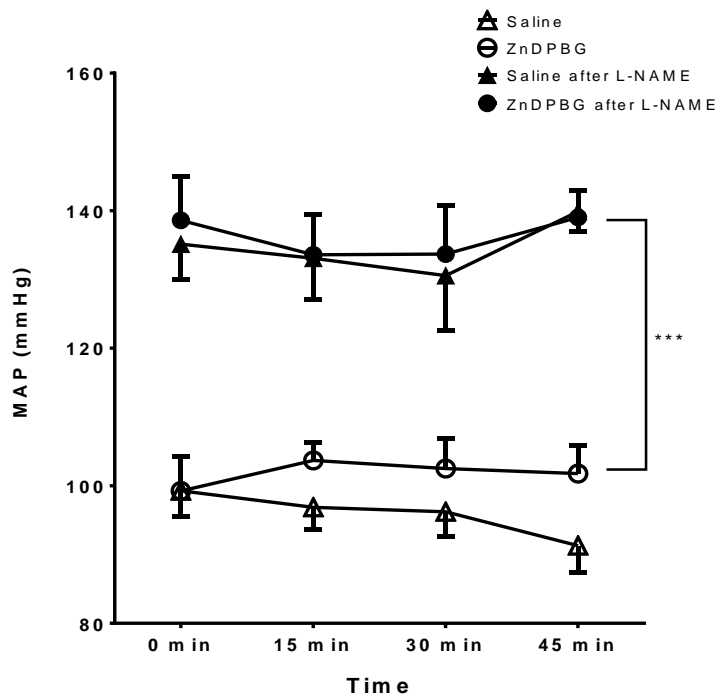
Figure 1. Mean arterial blood pressure (MAP) before (0 min) as well as 15, 30, 45 min after intraperitoneal injection of saline (open triangles, n=9) or 45 μmol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) (open circles, n=9) in control rats as well as in rats pretreated with N^G-nitro-L-arginine methyl ester (L-NAME) (filled symbols, n=5 for saline after L-NAME and n=6 for ZnDPBG after L-NAME). Values are presented as mean ± SEM. At all four timepoints, there was a significant (***$p<0.001$) increase of MAP in L-NAME pre-treated animals, compared to rats with intact NO synthesis.
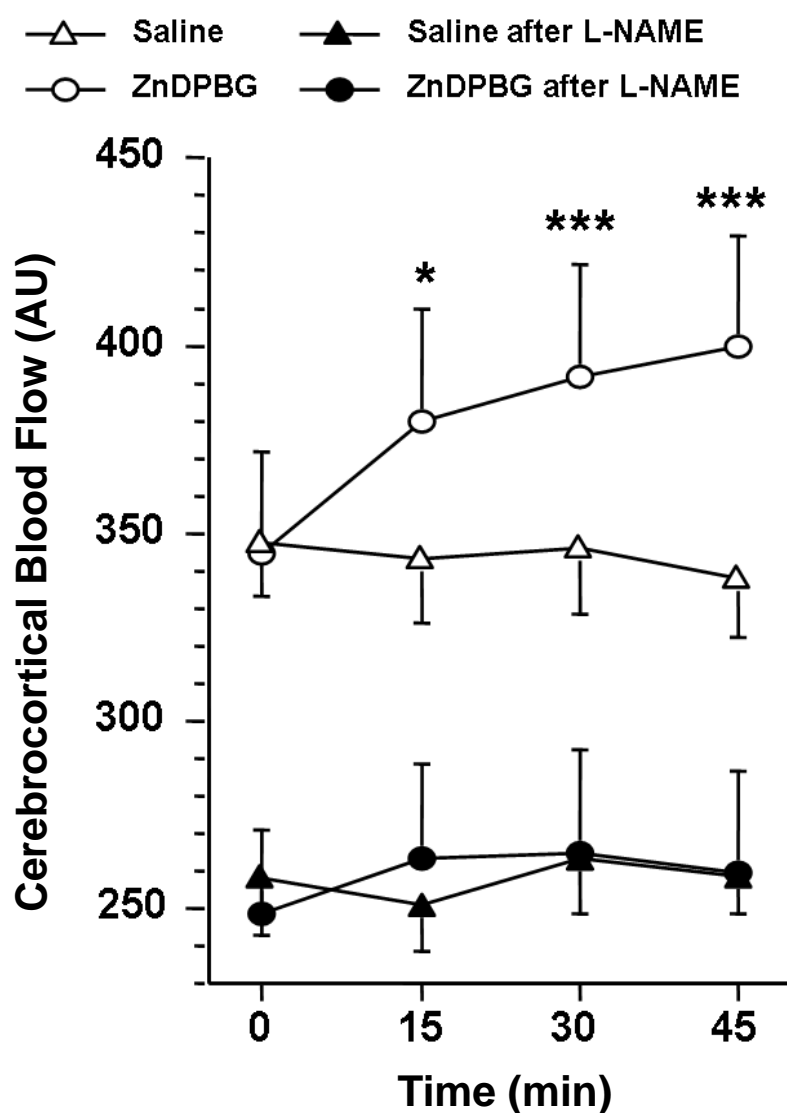
Figure 2. Cerebrocortical blood flow (CBF) before (0 min) as well as 15, 30, 45 min after intraperitoneal injection of saline (triangles) or 45 micromol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG; circles) in control (open symbols) or in N^G-nitro-L-arginine methyl ester (L-NAME) pretreated (filled symbols) rats. *p=0.016, ***p< 0.001 vs. '0min.' with repeated measures analysis of variance and Tukey's post hoc test, n= 10-16. AU, arbitrary unit.

In order to evaluate the role of NO in mediating the enhancement of the CBF after HO blockade, the experiments have been repeated in animals subjected to inhibition of the NO synthesis. Administration of L-NAME 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) without influencing arterial blood gas or acid-base parameters. In L-NAME pretreated

animals, neither ZnDPBG nor its vehicle induced any significant changes in the mean arterial blood pressure (Figure 1) or arterial blood gas and acid-base parameters. Most importantly, L-NAME prevented completely the enhancement of the CBF after inhibition of the endogenous CO production by administration of ZnDPBG (Figure 2).

4.2 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 later ZnDPBG treated or saline treated experimental groups. During moderate H/H, PaO_2 was reduced to 60-65 mmHg, $PaCO_2$ was increased to 80-85 mmHg (Figure 3) and pH decreased to 7.10-7.15 Despite no change in MAP (data not shown), CBF increased in both experimental groups by ~ 45% (Figure 4A). During severe H/H, PaO_2 was reduced to 45-50 mmHg, $PaCO_2$ was increased to 95-100 mmHg (Figure 3) and pH decreased to 7.05-7.10; CBF increased in both experimental groups by ~65% (Figure 4B), 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 (Figure 3), without affecting MAP. Most importantly, neither ZnDPBG nor saline treatment had any effect on CBF responses to moderate (Figure 4A) or severe (Figure 4B) H/H, when compared to the pretreatment values.
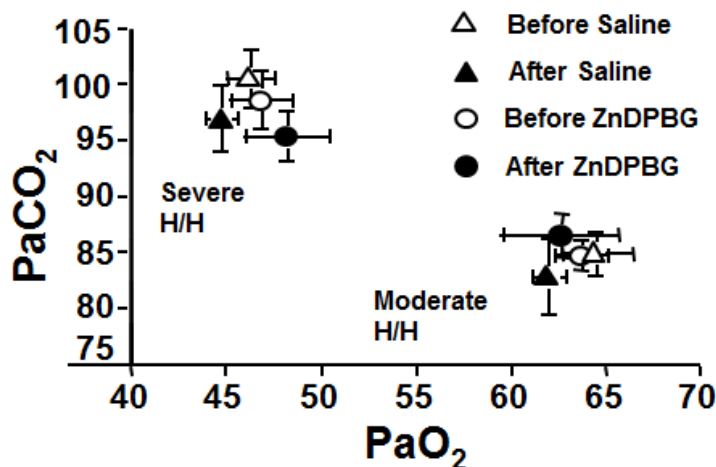

Figure 3. Arterial blood-gas tensions during mild and severe H/H before (open symbols) and after (filled symbols) intra-peritoneal injection of saline (triangles, n=8) or 45 μmol/kg zinc deutero-porphyrin 2,4-bis glycol (ZnDPBG; circles, n=8).
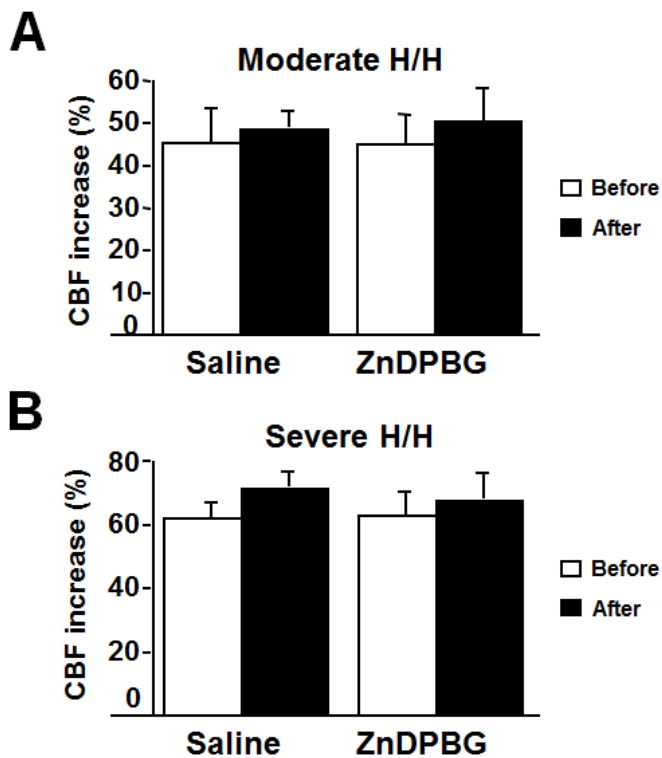
Figure 4. Changes of the cerebrocortical blood flow (CBF) during moderate (A) and severe (B) hypoxia/hypercapnia (H/H) before (open bars) and after (filled bars) intraperitoneal injection of saline (n=16) or 45 μmol /kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, n=16).

4.3 Influence of endocannabinoids on the cerebrocortical blood flow under resting conditions

The influence of constitutive CB1-receptor activity on the resting blood pressure and CBF was studied by i.v. administration of the selective CB1 antagonist / reverse agonist AM-251 in a dose of 10 mg/kg, which had been shown to be effective in blocking CB1 receptors in previous studies. Vehicle-treated animals served as controls. Neither AM-251 nor its vehicle induced any significant changes in the mean aterial blood pressure MAP (Figure 5A), heart rate HR (Figure 5B) or CBF (Figure 5C) up to 45 minutes after their administration. Furthermore, arterial blood gas tensions and acid-base parameters remained unchanged during the observation period. These findings indicate that constitutive CB1 activity has no significant influence on the systemic and cerebrocortical circulation under steady-state resting conditions in healthy normotensive rats.
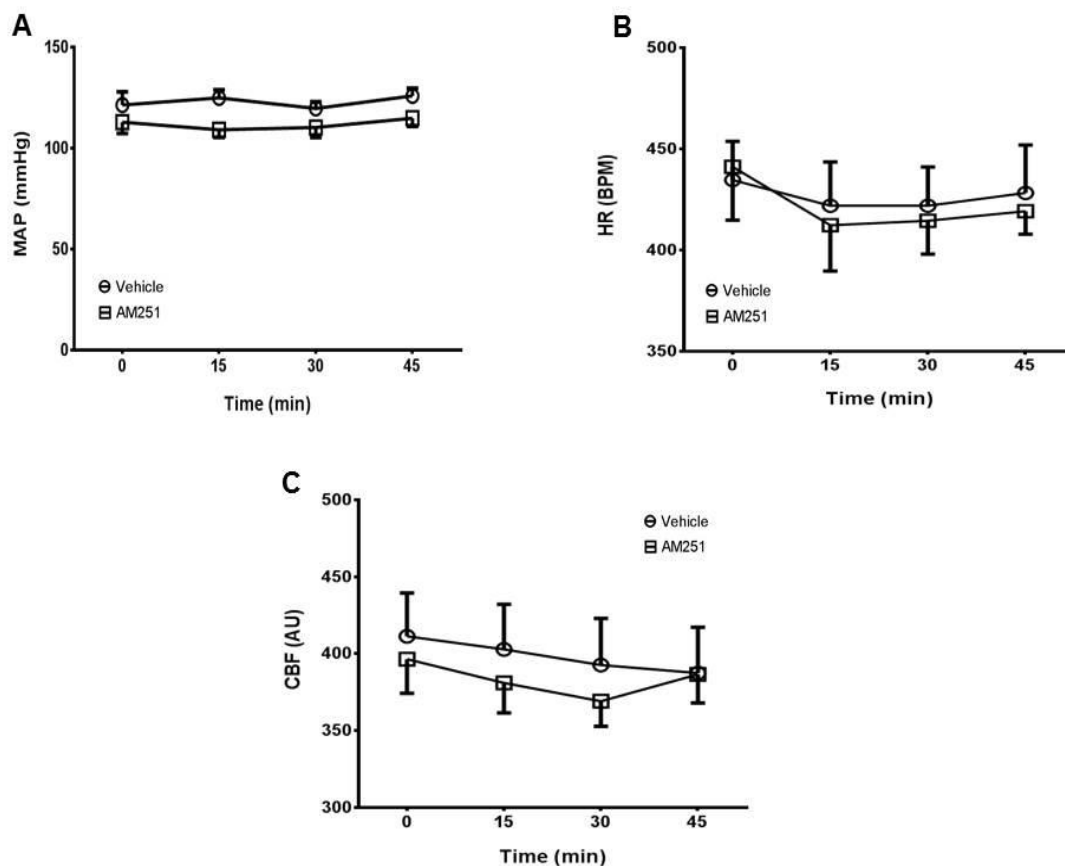
Figure 5:

Mean aterial blood pressure (MAP, panel A), heart rate (HR, panel B) and cerebrocortical blood flow (CBF, panel C) are shown before (0 minute) as well as 15, 30, 45 minutes after intravenous administration of 10 mg/kg of the CB 1 receptor antagonist AM251 (open squares, n=7) or its vehicle (open circles, n=5) in urethane-anaesthetized rats. Values are presented as mean ± SEM. No significant change of the measured variables was found at any given time points after the administration of vehicle or AM-251. AU, arbitrary unit.

4.4 Effects of endocannabinoid receptor blockade on the cerebrocortical blood flow during hypoxia and hypercapnia

To test the effects of endocannabinoid receptor blockade on the CBF elevation during hypoxia and hypercapnia, we produced stepwise H/H (as described in the Methods) before and after the administration of AM-251 or its vehicle, and determined the changes in CBF. Inhalation of three different gas mixtures containing decreased O_2- and increased CO_2-content (as compared to air) induced reproducible levels of hypoxia and hypercapnia before and after the intravenous administration of 10 mg/kg AM-251

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(Figure 6B) or its vehicle (Figure 6A), without significant changes in the BP. The H/H-induced enhancement of CBF was identical before and after the administration of the vehicle (Figure 7A). In contrast, AM-251 resulted in markedly increased CBF changes during mild and moderate H/H (by $28.1 \pm 8.8\%$ and $39.4 \pm 10.0\%$, respectively) without significantly influencing the peak CBF during severe H/H (Figure 7B).
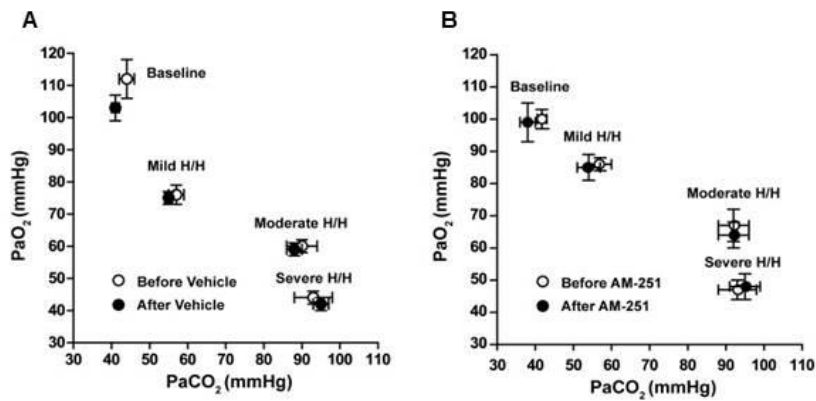

Figure 6.

Inhalation of three different gas mixtures containing decreased O_2- and increased CO_2-content (as compared to air) induced reproducible levels of hypoxia and hypercapnia before and after the administration of AM-251 (panel B) or its vehicle (panel A).
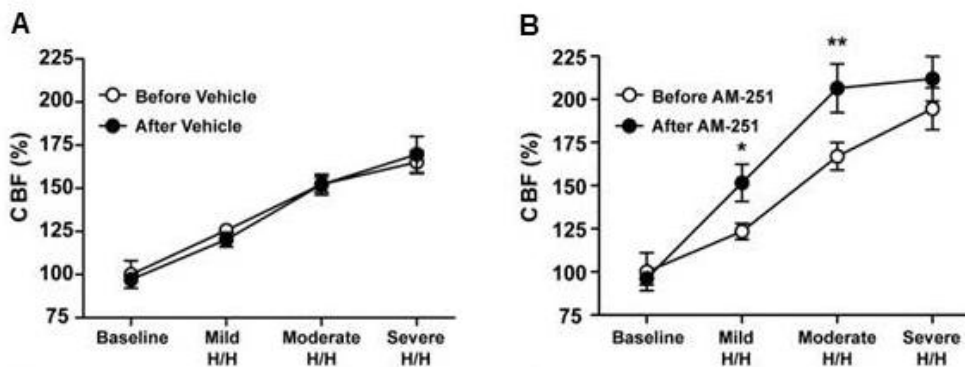

Figure 7.

The hypoxia/hypercapnia (H/H) induced enhancement of the cerebrocortical blood flow (CBF) was identical before and after the administration of the vehicle of AM-251 (panel A). In contrast, AM-251 resulted in markedly increased CBF changes during mild and moderate H/H (by $28.1 \pm 8.8\%$ and $39.4 \pm 10.0\%$, respectively) without significantly influencing the peak CBF during severe H/H (panel B).

5. CONCLUSIONS

1.   First, we examined the effect of the HO inhibitor ZnDPBG on the CBF under resting conditions in anesthetized adult male rats, by measuring the CBF in the parietal cortex. We also aimed to examine the interaction between the HO and NOS pathways in the regulation of the cerebrocortical blood flow under resting conditions. The results of our study indicate that endogenous CO influences tonically the resting cerebrocortical blood flow by interacting with the NO synthesis. Inhibition of the HO pathway leads to cerebrocortical hyperemia by increased neuronal and/or endothelial NO production.

2.   We observed the effect of the HO inhibitor ZnDPBG on the cerebrovascular effects of stepwise hypoxia/hypercapnia by measuring the CBF in the parietal cortex of anesthetized adult male rats under hypoxia/hypercapnia. We found that the HO – CO pathway does not appear to influence the CBF increase during hypoxia and hypercapnia in adult rats.

3.   Following this, we studied the effects of the cannabinoid CB1 receptor blockade by the reverse agonist / antagonist AM251 on the CBF under resting conditions using similar methods. Our study indicates that under resting physiological conditions CB1-receptor mediated mechanisms have limited tonic influence on the systemic and cerebral circulation.

4.   Finally, we examined the effect of AM251 on the CBF rise to stepwise H/H. Most importantly, our data suggest that the endocannabinoid system and CB1-receptors play an important role in the regulation of the cerebral circulation during hypoxia and hypercapnia and may potentially be involved in the autoregulation of CBF.

6. LIST OF PUBLICATIONS

Articles

1. **Leszl-Ishiguro M**, Horváth B, Johnson RA, Johnson FK, Lenzsér G, Hermán P, Horváth EM, Benyó Z: Influence of the heme oxygenase pathway on cerebrocortical blood flow. **NeuroReport**, 2007; 18: 1193-1197 **(IF: 2.163)**

2. *Iring A, *Ruisanchez É, ***Leszl-Ishiguro M**, Horváth B, Benkő R, Lacza Z, Járai Z, Sándor P, Di Marzo V, Pacher P, Benyó Z: Role of endocannabinoids and cannabinoid-1 receptors in cerebrocortical blood flow regulation. **PLoS One**, 2013; 8(1): e53390 **(IF: 3.730)**
   *These authors contributed equally to the publication.

Abstracts

1. Horváth B, **Ishiguro M**, Hrabák A, Káldi K, Lacza Z, Sándor P, Benyó Z: Interaction between the heme oxygenase and nitric oxide pathways in the regulation of the resting CBF. **Journal of Cerebral Blood Flow and Metabolism**, 2003; 23: S77 (XXIst International Symposium on Cerebral Blood Flow, Metabolism and Function, June-July 2003, Calgary, Canada)

2. **Ishiguro M**, Lacza Z, Horváth EM, Járai Z, Sándor P, Benyó Z: The cannabinoid receptor antagonist AM-251 enhances the cerebrocortical hyperemic response to hypoxia/hypercapnia. **The FASEB Journal**, 2004; 18: A267 (Experimental Biology, April 2004, Washington D.C., USA)

3. Horváth B, **Ishiguro M**, Lenzsér G, Hermán P, Hrabák A, Káldi K, Sándor P, Benyó Z: Interaction between the heme oxygenase (HO) and NO synthase pathways in the regulation of the resting CBF. **The FASEB Journal**, 2004; 18: A268 (Experimental Biology, April 2004, Washington D.C., USA)

4. Horváth B, **Ishiguro M**, Hortobágyi L, Lenzsér G, Hermán P, Hrabák A, Káldi K, Sándor P, Benyó Z: Role of the heme oxygenase pathway in the regulation of the resting CBF. **Acta Physiologica Hungarica**, 2004; 91: 302-303 (68th Annual Meeting of the Hungarian Physiological Society, June 2004, Debrecen, Hungary)

5. **Ishiguro M**, Lacza Z, Horváth EM, Járai Z, Sándor P, Benyó Z: Role of cannabinoid 1 receptor in the modulation of the cerebrocortical hyperemic response to hypoxia/hypercapnia. **Acta Physiologica Hungarica**, 2004; 91: 311-312 (68th Annual Meeting of the Hungarian Physiological Society, June 2004, Debrecen, Hungary)

6. **Ishiguro M**, Lacza Z, Horváth EM, Járai Z, Sándor P, Benyó Z: Inhibition of the cannabinoid-1 receptor enhances the cerebrocortical hyperemic response to hypoxia/hypercapnia. **Journal of Cerebral Blood Flow and Metabolism**, 2005; 25: S189 (XXIInd International Symposium on Cerebral Blood Flow, Metabolism, and Function, June 2005, Amsterdam, The Netherlands)

7. Ruisanchez É, Benkő R, **Leszl-Ishiguro M**, Lacza Z, Járai Z, Sándor P, Benyó Z: Cerebrovascular effects of endocannabinoids. **Diabetologia,** 2009; 52: S258, 2009 (45th Annual Meeting of the European Association for the Study of Diabetes, September-October 2009, Vienna, Austria)

8. Ruisanchez É, **Leszl IM**, Benkő R, Lacza Z, Járai Z, Sándor P and Benyó Z (2009). Role of endocannabinoids in the regulation of the cortical blood flow. Front. Syst. Neurosci. Conference Abstract: 12th Meeting of the Hungarian Neuroscience Society. Doi: 10.3389/conf.neuro.01.2009.04.172

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