Role of Arachidonic Acid Metabolites in the Regulation of Cerebral Blood Flow and Respiration

Ph.D. Thesis

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INTRODUCTION

The occurrence of cerebrovascular diseases, consisting of ischemic-, hemorrhagic stroke and subarachnoid hemorrhage, are the fourth leading cause of death in the modern world. On average, every 40 seconds someone experiences stroke, and every 4 minutes it causes death. During stroke, the cerebral blood flow is insufficient thus the consequent reduction in oxygen availability (hypoxia) impairs cellular respiration and energy production, resulting in the decreased availability of ATP; subsequently, ATP-dependent ion pumps, such as the Na^+/K^+ ATPase, are unable to maintain the physiological transmembrane ion gradients. The rapid accumulation of Ca^{2+}, due to influx from the extracellular space, results in the activation of lipases, for instance phospholipase A_2, which contributes to the formation of arachidonic acid metabolites. Furthermore, the increased intracellular Ca^{2+} concentration activates, via the Ca^{2+}-calmodulin complex, the neuronal and endothelial nitric oxide synthase (NOS); resulting in the enhanced production of the potent vasodilatory mediator, nitric oxide (NO). The initial burst of NO generation is followed by a gradual exhaustion in the enzymatic activity especially in cases associated with hypertension, traumatic brain injury and diabetes mellitus, resulting in the decreased bioavailability of nitric oxide.

It has been previously observed that under pathophysiological conditions coupled with limited

oxygen and NO availability, the vascular reactivity is enhanced; low frequency oscillations in the cerebral blood flow appears, described as vasomotion in isolated vessels, which phenomenon reportedly precedes the appearance of vasospasm.

Hence an improved understanding of the cerebrocortical blood flow regulation is essential to successfully treat cerebrovascular diseases and improve the chances of full recovery. In the current study, I have investigated the role of arachidonic acid metabolites, namely thromboxane A_2 (TXA_2) and endocannabinoids (EC) in the cerebrovascular blood flow regulation.

AIMS

Low frequency oscillations of cerebrovascular tone and cerebral blood flow have been linked to the decreased bioavailability of endothelium-derived nitric oxide. It has been shown that nitric oxide synthase inhibition provokes vulnerability to vasomotion that can be triggered by the administration of UTP or U-46619, the chemically stable analogue TXA_2, and is mediated by TP-receptors in isolated cerebral arteries. In the present study we hypothesized that in the absence of NO, hypersensitivity of TP-receptor mediated cerebrovascular signaling contributes to the development of vasomotion and CBF oscillations in the cerebral cortex of rats.

Additionally, we have investigated the role of a different arachidonic acid metabolite, endocannabinoids, in the regulation of cerebral blood flow (CBF). On one hand, it has been shown that systemic administration of phyto- and endocannabinoids increased CBF in dogs, cats and humans; on the other hand, contradictory results have been reported, where ECs reduced CBF in rats. To address these contradictory findings and clarify the role of endocannabinoids in the cerebral circulation, in the present study the endogenous cannabinoid levels were enhanced via the administration of an EC-reuptake inhibitor (AM-404). Furthermore, we have tested the effect of a CB1 receptor antagonist/inverse agonist (AM-251) in order to characterize the influence of constitutively active CB1 receptors in the cerebrovascular and cardiovascular regulation under resting conditions.

Numerous previous studies have observed that the marked cardiovascular depression elicited by endocannabinoids were dependent on the respiratory state of the animals; however, the exact mechanism underlying the influence of ECs on the respiratory regulation is still largely unknown. To unambiguously clarify the role of the endogenous cannabinoid system in respiratory control, respiratory parameters were recorded using pulse oximetry after enhancing EC levels by administration of an EC-reuptake inhibitor (AM-404). The experiments have been performed both in wild-type

control and CB1-deficient (CB1-KO) mice in order to analyze the involvement of CB1 receptors.

EXPERIMENTAL ANIMALS, MATERIALS AND METHODS

Animals

The experiments were performed in (**1**) adult male Wistar rats (300-400 g) according to the guidelines of the Hungarian Law of Animal Protection (243/1988) and all procedures were approved by the Semmelweis University Committee on the Ethical Use of Experimental Animals (590/99 Rh); and in (**2**) CB1-KO mice that were generated and kindly provided by Dr. Andreas Zimmer (Institute of Molecular Psychiatry, University of Bonn, Germany).

Materials

The CB1 receptor antagonist/inverse agonist AM-251 (1-(2, 4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) and the EC reuptake inhibitor AM-404 (N-(4-hydroxyphenyl)-5Z, 8Z, 11Z, 14Z-eicosatetrenamide) were obtained from Cayman Chemicals (Ann Arbor, MI, USA); both chemicals were dissolved in 1 ml of vehicle containing ethanol/emulphor/saline (1:1:8; v:v:v). Ketamine (Calypsol) and xylazine (CP-Xylazine) were purchased from Richter Gedeon Plc. (Budapest, Hungary) and CP-Pharma GmbH (Burgdorf, Germany), respectively. The

thromboxane A_2 receptor agonist U-46619 (9,11-dideoxy-9α,11α-methanoepoxy Prostaglandin F2α), the thromboxane A_2 inhibitor Ozagrel (sodium;(E)-3-[4-(imidazol-1-ylmethyl)phenyl]prop-2-enoate), inhibitor of nitric oxide synthase L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride), and the specific Rho-kinase inhibitor Y-27632 ((R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl) cyclohexanecarboxamide dihydrochloride), bradykinin, Uridine-5'-triphosphate (UTP), endothelin-1 (ET-1) and Urethane (Carbamic acid ethyl ester) were purchased from Sigma (St. Louis, MO, USA).

Methods

Wistar rats were anesthetized with urethane (1.5 g kg^{-1} intraperitoneally), the depth of anesthesia was regularly controlled during the experiments by checking the corneal or plantar nociception reflex and additional urethane was administered intravenously (i.v.) as necessary. The animals were spontaneously breathing through an intra-tracheal cannula. Catheters were inserted into both femoral arteries (for systemic arterial blood pressure measurement and for blood sampling) and into the left femoral vein (for drug administration). Body temperature was kept constant between 36.5–37.5 °C during the experiments using a heating pad controlled by a rectal probe. Systemic arterial pressure was recorded continuously on a polygraph (Model 7E, Grass, Quincy, MA, USA). Mice were anaesthetized by intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine

(10 mg kg^{-1}); the depth of anaesthesia was regularly checked and additional anaesthetics were administered intraperitoneally when necessary. The animals were spontaneously breathing. Body temperature was recorded by a rectal probe and kept constant between 36.5 – 37.5 °C during the experiments using a heating pad.

Cerebrocortical blood flow (CoBF) was measured by laser-Doppler (LD) flowmetry. The head of the rat 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 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 flux (LDF) was measured with a two-channel blood flow monitor (MBF3D, Moor Instruments, UK) and was recorded continuously. The LD monitor 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 blood flow in the parietal cortex. Blood pressure (BP) and CoBF were recorded continuously (BIOPAC Systems Inc, Goleta, CA, USA); the heart rate was calculated from the pulsating BP signal. Arterial blood gas and pH measurements were

performed throughout the *in vivo* rat experiments by a Radiometer (Bronshoj, Denmark) ABL-77 analyzer and by the use of a capnograph (Capstar-100, CWE Inc., Ardmore, PA, USA).

Hair on a randomly selected thigh of each mouse was removed using Veet gel (Unilever, UK). Oxygen saturation, heart rate, breath rate and breath distension were measured continuously using MouseOX pulse oximeter (Starr Life Sciences Corp., Oakmont, PA, USA) in accordance with the manufacturer's instructions, and recorded using the MP100 system and AcqKnowledge 3.72 software from Biopac Systems Inc. (Goleta, CA, USA). Arterial O_2 saturation values measured during the experiments were normalized to the O_2 saturation determined 5 min before the administration of AM-404.

Analysis of Data

The Discrete Fourier transform (spectrum) of the time series obtained *in vivo* (CoBF) was calculated by Fast Fourier Algorithm (FFT). The calculations were executed in the Matlab environment which uses an adaptive version of the FFT, called FFTW. Statistical analysis was performed using the GraphPad Prism software v.6.07 from GraphPad Software Inc. (La Jolla, CA, USA). Values are presented as mean ± SEM; *n* represents the number of experiments. Statistical analysis was performed using two-way ANOVA for repeated measurements followed by Bonferroni post-hoc test. A *p*

value of less than 0.05 was considered to be statistically significant.

RESULTS

Thromboxane receptor activation under physiological conditions have no effect on systemic physiological parameters or on cerebrocortical blood flow

In order to characterize the significance of TP-receptor activation in promoting CBF oscillations, we first investigated the effect of the TP-receptor agonist U-46619 in a dose of 1 $\mu g\ kg^{-1}$ during physiological conditions. Baseline physiological parameters before administration of 100 $mg\ kg^{-1}$ L-NAME or its vehicle, saline, were found to be within the normal range in all *in vivo* experimental groups; furthermore, neither i.v. administration of the solvent (saline) nor that of the agonist U-46619 induced any significant changes in acid-base, blood gas or systemic circulatory parameters in the control group. Additionally, neither the average CoBF nor its Fourier spectrum changed after the administration of saline or U-46619 in this experimental group.

NO synthase blockade increases mean arterial pressure, while decreases heart rate and cerebrocortical blood flow

NOS activity was pharmacologically blocked with 100 $mg\ kg^{-1}$ L-NAME. In this challenged state, we have observed no significant changes in acid base or blood gas

parameters but we have seen increased systemic blood pressure and decreased heart rate. These changes developed within 25 minutes after L-NAME administration and remained unaltered even after the intravenous administration of 1 μg kg^{-1} U-46619 (in Group IIa.), 1 ml kg^{-1} saline (in Group IIb.) or 10 mg kg^{-1} ozagrel (in Group IIc.).

Activation of thromboxane receptors aggravates while inhibition of TXA_2 synthesis attenuates vasomotion in the absence of NO

Low frequency CoBF oscillations, which were absent under resting conditions, developed after the administration of L-NAME with a dominant frequency of 148 ± 2 mHz and peak magnitude of 5.6 ± 0.5 AU. U-46619 significantly increased while ozagrel decreased the amplitude of these oscillations without changing the dominant frequency. In contrast, saline, the vehicle of U-46619 and ozagrel, failed to induce any changes in the magnitude or frequency of CoBF oscillations.

Inhibition of constitutive Cannabinoid-1 receptor activity has no effect on systemic blood pressure and cerebrocortical blood flow

To evaluate the potential influence of tonic EC release and constitutive CB1 receptor activity under resting conditions, blood pressure and CoBF were measured after i.v. administration of the selective CB1 antagonist/inverse agonist AM-251, and were compared

to vehicle treated animals. Neither 10 mg kg^{-1} AM-251 nor its vehicle induced any significant change in mean arterial blood pressure or CoBF up to 32 minutes after their application. Heart rate, arterial blood gas tensions, acid-base parameters and hematocrit remained unchanged during the observation period.

Tri-phasic effect of enhanced endocannabinoid release

First we tested the effect of enhanced endocannabinoid release on blood pressure and CoBF. Baseline physiological parameters were within normal range in the absence and presence of AM-251 before AM-404 treatment. After the i.v. administration of 10 mg kg^{-1} AM-404, a cannabinoid reuptake inhibitor, we observed three distinct phases of BP and CoBF. *Phase I* consisted of marked hypertension accompanied by a significant increase of CoBF with minor changes in arterial blood gas tensions and pH. The BP and CoBF elevations reached their maximum within 0.5 min and thereafter started to return towards their baseline levels until the onset of the second phase with a delay of 1–2 min. During *phase II* CoBF has increased most prominently with a peak at 3.5 min after administration of AM-404, accompanied by increased levels of expired CO_2 and BP. Blood gas analysis revealed marked hypoxia, hypercapnia and acidosis, and therefore, changes in the CoBF and BP were considered to be secondary to the depression of respiration. The *third phase* of changes was dominated by sustained hypotension, which reached its

maximum at 20 min. During this phase the arterial oxygen tension and saturation normalized, whereas the hypercapnia observed in *phase II* was reverted to a slight hypocapnia, and the acidic arterial pH returned towards the physiological level. Interestingly, CoBF showed a significant decrease during *phase III*, which was attributed to the reduction of the MAP and the arterial CO_2-tension. After testing the effect of enhanced endocannabinoid release on blood pressure and CoBF, we inhibited CB1 receptors to assess their involvement in the mediation of effects induced by inhibition of EC reuptake. Animals were pretreated with 10 mg kg^{-1} AM-251 or its vehicle, and the administration of AM-404 and subsequent measurements were repeated. During *phase I* the increase of MAP was comparable between the control and CB1-blocked group, indicating that CB1 receptors are not involved in the transient hypertension. However, the increase of the CoBF attenuated significantly in the presence of AM-251, implying an improved autoregulation of the cerebral circulation. Administration of AM-251 markedly attenuated hypoxia and hypercapnia as well as the acidosis seen during *phase II*, indicating that the increased EC availability after AM-404 suppresses respiration via CB1 receptor activation. The mild hypertension observed during *phase II*, and interestingly the increase of CoBF were both resistant to AM-251 treatment. Finally the sustained hypotension seen during *phase III* was attenuated after AM-251 treatment, indicating the involvement of CB1

receptors in mediating the effects of elevated EC levels. The accompanying changes in arterial O_2 tension and saturation as well as the pH were not affected by CB1-blockade, although the mild hypocapnia was attenuated.

Enhanced endocannabinoid levels induce transient respiratory depression in a CB1-dependent manner in mice

Respiratory parameters were within physiological range in both wild-type control and CB1-KO mice. Intravenous administration of 10 mg kg^{-1} AM-404 induced a transient depression in oxygen saturation and respiration rate and a concomitant increase in breath distension in control mice. Administration of the solvent alone had no effect. The rapid onset of respiratory depression started within 1 minute after treatment with AM-404, and reached its maximal level within 4 min, whereas its recovery started after 8 minutes; respiratory parameters gradually recovered to normoxic levels that was completed within 16 minutes. Additionally, there was a simultaneous increase in breath distension during respiratory depression in wild-type control animals, indicating that the lower respiratory rate was associated with larger fluctuations in the central venous pressure and cardiac output. In contrast to AM-404 evoked respiratory depression in wild-type control mice, CB1-KO mice showed no reduction in oxygen saturation and respiration rate; accompanying changes in breath distension were also absent.

CONCLUSIONS

The involvement of TP-receptor activation was explored in the appearance of cerebral blood flow oscillations under physiological conditions as well as after impaired NO synthesis. U-46619 elicited TP-receptor activation in control animals failed to evoke CBF oscillations; however, inhibition of NO-synthase by L-NAME resulted in increased blood pressure and reduction in CBF accompanied by oscillations in the blood flow that was further enhanced after TP-receptor agonist treatment. TXA_2 synthesis inhibition with ozagrel abrogated CBF oscillations *in vivo*. These results indicate that under pathophysiological conditions associated with NO-deficiency, such as subarachnoid hemorrhage, the hypersensitivity of TP-receptors augments the development of vasomotion, potentially leading to the propagation of vasospasm.

The involvement of CB1 receptors in the cardiovascular and cerebral blood flow regulation was investigated under physiological conditions and during increased endocannabinoid levels. Inhibition of CB1 receptors under resting conditions with AM-251 induced no changes in the systemic or cerebral circulation, indicating that CB1 receptor mediated mechanisms have limited influence on circulation under physiological conditions. During enhanced endocannabinoid activation that induced triphasic responses, the transient hypertension was CB1-independent; however, the sustained

hypotension observed during *phase III* was sensitive to CB1 receptor blockade. Furthermore, the marked respiratory depression observed during *phase II* proved to be CB1-sensitive.

Subsequently, the influence of increased levels of endogenously produced endocannabinoids on the respiratory regulation was explored. Treatment with the cannabinoid reuptake inhibitor, but not with the solvent, induced transient reduction in respiratory rate accompanied with depression in the arterial oxygen saturation and a concomitant increase in breath distension in wild-type control mice. However, CB1-deficient mice exhibited no alterations in the measured respiratory parameters after AM-404 administration; indicating that the endocannabinoid system has a pivotal role in the physiological control of respiration by regulating the respiratory rate and consequently influencing arterial oxygen saturation and this mechanism is entirely dependent on CB1 receptors.

LIST OF ORIGINAL ARTICLES

Original articles connected with the Ph.D. thesis:

Horvath B, Lenzser G, Benyo B, Nemeth T, Benko R, **Iring A**, Herman P, Komjati K, Lacza Z, Sandor P, Benyo Z. Hypersensitivity to thromboxane receptor mediated cerebral vasomotion and CBF oscillations during acute NO-deficiency in rats. *PLOS ONE 5:(12). (2010)* **(IF: 4.411)**

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Iring A, Hricisak L, Benyo Z. CB1 receptor-mediated respiratory depression by endocannabinoids. *RESPIRATORY PHYSIOLOGY & NEUROBIOLOGY 240: pp. 48-52 (2017)* **(IF: 1.773)**

Abstracts connected with the Ph.D. thesis:

Lenzsér G, Horváth B, **Iring A**, Hermán P, Komjáti K, Sándor P, Benyó Z. Thromboxane receptor-mediated vasomotion and cerebrocortical blood flow oscillations in NO-deficiency. *FRONTIERS IN NEUROSCIENCE 2010 (2010)*

Horváth B, **Iring A**, Benyó B, Hermán P, Lenzsér G, Lacza Zs, Sándor P, Benyó Z. Low frequency pial arterial vasomotion and cerebrocortical blood flow oscillations in rodents: role of nitric oxide and thromboxane A2. In: *SRBR Society for Research on Biological Rhythms 2012: 13th Biennial Meeting Society for Research on Biological Rhythms Conference Program. Florida, USA, 2012.05.19-2012.05.23.p. 266.*

Other publications

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Polycarpou A, Hricisak L, **Iring A**, Safar D, Ruisanchez E, Horvath B, Sandor P, Benyo Z. Adaptation of the Cerebrocortical Circulation to Carotid Artery Occlusion Involves Blood Flow Redistribution between Cortical Regions and is Independent of eNOS. *AMERICAN JOURNAL OF PHYSIOLOGY: HEART AND CIRCULATORY PHYSIOLOGY 311:(4) pp. H972-H980. (2016)* **(IF: 3.324)**

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Iring A, Horvath B, Benyo B, Tillson M, Bakken B, Benyo Z. Influence of unilateral carotid artery occlusion on the cerebrocortical microcirculation in mice: a laser-speckle study. *ACTA PHYSIOLOGICA HUNGARICA 97:(4) p. 447. (2010)*

Németh T, Ruisanchez É, Hricisák L, **Iring A**, Merkely B, Hunyady L, Smrcka AV, Offermanns S, Benyó Z. Signaling pathways of thromboxane receptor-mediated vasoconstriction: Major role of phospholipase C epsilon. *ACTA PHYSIOLOGICA 211:(Suppl. s697) Paper S4-B5. (2014). Joint meeting of the Federation of European Physiological Societies (FEPS) and the Hungarian Physiological Society. Budapest, Hungary: 2014.08.27 - 2014.08.30.*