

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

Ph.D. Thesis

Mirjam Leszl-Ishiguro, M.D.

Semmelweis University
Doctoral School of Basic Medicine



Supervisor: Zoltán Benyó, M.D., D.Sc.

Reviewers: Ferenc Bari, M.D., D.Sc.
Violetta Kékesi, M.D., Ph.D.

Members of the examination board: Viktor Bérczi, M.D., D.Sc.
Rudolf Urbanics, M.D., Ph.D.
Ákos Zsembery, M.D., Ph.D.

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1. LIST OF ABBREVIATIONS

AA arachidonic acid

ABP arterial blood pressure

ACh acetylcholine

2-AG 2-arachydonyl-glycerol

ANA anandamide

AT anandamide transporter

AU arbitrary unit

Ba²⁺ barium

Ca²⁺ calcium

cAMP cyclic AMP

CBF cerebral blood flow

CB1 cannabinoid 1 receptors

CB2 cannabinoid 2 receptors

cGMP cyclic GMP

CGRP calcitonin gene-related peptide

CO carbon monoxide

CO₂ carbon dioxide

COX cyclooxygenase

CrMP chromium mesoporphyrin

DPCPX 8-cyclopentyl-1,3-dipropylxanthine

EC endocannabinoid

EDHF endothelium-derived hyperpolarizing factor

EDRF endothelium-derived relaxing factor

ET-1 endothelin-1

FAAH fatty-acid amide hydrolase, anandamide amidohydrolase

H/H hypoxia hypercapnia

HO heme oxygenase

ip. intraperitoneally

K⁺ potassium

K_{ATP} ATP-sensitive K⁺ channels

K_{Ca} Ca²⁺ sensitive K⁺ channels
K_{ir} inwardly-rectifying K⁺ channels
K_v voltage-gated K⁺ channels
L-NAME nitro-L-arginine methyl ester
L-NMMA N-mono-methyl-L-arginine
LPI Iysophosphatidylinositol
mACh muscarinic acetylcholine receptor
Mg²⁺ magnesium
NE norepinephrine
NMDA N-methyl-D-aspartate
NO nitric oxide
NOS NO synthase
PGE₂ prostaglandin E₂
PGH prostaglandin G/H synthase
PGI₂ prostacyclin
PLC phospholipase C
PLA phospholipase A
sGC soluble guanylyl cyclase
SMC smooth muscle cell
SnMPIX tin mesoporphyrin IX
SNP sodium nitroprusside
8-SPT 8-p-sulfohenyl-theophylline
SUR sulphonylurea receptor
TEA tetraethylammonium ion
TxA₂ thromboxane A₂
VR1 vanilloid 1 receptors
YC-1 3-(5'-hydroxymethyl-2'-furyl)-1-benylindazol
ZnDPBG zinc deuteroporphyrin 2,4-bis glycol
ZnPPIX zinc protoporphyrin IX

2. INTRODUCTION

2.1. Main regulatory mechanisms of the cerebral circulation

2.1.1. Local regulatory mechanisms

Cerebral autoregulation is the ability of cerebral blood flow (CBF) to remain constant in spite of changes in the systemic arterial blood pressure (ABP) within the range of 60-150 mmHg, thereby ensuring a steady, optimal level of blood supply to the brain. If ABP falls outside this range, either at its lower or upper limit, this autoregulation becomes inadequate (Lavi et al., 2003). Cerebral autoregulation involves the transient responses of the ABP-CBF relationship that can be observed during spontaneous fluctuations in ABP or sudden changes in ABP, such as following changes in posture (Panerai, 2007). Mechanisms that may be involved in the cerebral autoregulation are not completely understood. Most likely, local regulatory mechanisms of the cerebral blood flow include myogenic, metabolic, endothelium-mediated, and neuronal regulations.

2.1.1.1. Myogenic autoregulation

The myogenic hypothesis is based on the Bayliss effect. It was shown in rat cerebral arterioles that at elevated intravascular pressures, vasoconstriction occurred, while at decreased intravascular pressures, vasodilation occurred (Bohlen and Harper, 1984). Increased intraluminal arterial pressure results in an increased transmural pressure, which opens mechanosensitive cation channels (Wu and Davis, 2001). The Na⁺ influx into the vascular smooth muscle results in depolarization and as a consequence, voltage-sensitive Ca²⁺ channels open and the Ca²⁺ influx causes cerebral vasoconstriction. The activation of voltage-sensitive Ca²⁺ channels is essential for myogenic contraction in rat cerebral arteries (McCarron et al., 1997).

2.1.1.2. Flow-metabolism coupling

Roy and Sherrington hypothesized in the late 1800s that the brain possesses an intrinsic mechanism by which its vascular supply can be varied locally via vasodilatory properties of the chemical products of cerebral metabolism, released in correspondence to local variations of functional neuronal activity (Roy CS and Sherrington CS, 1890). The Roy-Sherrington principle suggests that CBF changes are a function of a tight

coupling between cellular energy requirements and the supplies of glucose and oxygen. Increased neuronal activity in the brain is accompanied by a rise in the local CBF, which serves to satisfy enhanced glucose and oxygen demand (Filosa et al., 2006). Moment-to-moment regulation of vascular smooth muscle tone involves various mechanisms that ultimately determine the concentration of free Ca^{2+} within the vascular SMCs (Brayden, 2002).

2.1.1.2.1 Cellular activity and metabolism

There is less knowledge of the role of local metabolites in the cerebral autoregulation, although metabolites released as a result of hypoxia, hypercapnia, pH alterations (such as adenosine and K^+), may have a role. The coupling of increased local CBF to neuronal activity, the functional hyperaemia, is facilitated by the proximity of sites of neuronal electrical activity and neurotransmitter release to the cerebral vasculature (Nguyen et al., 2000). These mechanisms include synaptic, metabolic and ionic signals that interact together.

Earlier researchers favored the idea of a negative feed-back system, in which decreased O_2 -tension or increased CO_2 -tension in the brain, as a consequence of insufficient CBF, would initiate the release of vasodilator compounds (adenosine, H^+ , lactate, K^+) in order to reset the balance between metabolic demand and energy supply. Recent advances in functional neuroimaging, called our attention to the pivotal role of neuronal mechanisms in the coupling of the CBF to the nutrient demand of the brain. Most notably it has been demonstrated that during neuronal activation the CBF changes precede the reduction of the O_2 -tension and the increase of the CO_2 -tension in the brain tissue, and a close interplay between neurons, astrocytes and microvessels (i.e. the “neurovascular unit”) is responsible for the effectiveness of this regulation (Hamel 2006, Schwedt and Dodick 2009, Figley and Stroman 2011). Within this concept, increased glutamate release during enhanced synaptic activity would activate NMDA and metabotropic glutamate receptors in postsynaptic neurons and neighboring astrocytes, and these cells would release arachidonic acid metabolites, NO and K^+ leading to relaxation of the cerebrovascular smooth muscle (Attwell et al. 2010, Koehler et al. 2009).

Metabolic regulators of CBF that participate in the flow-metabolism coupling, involve substances released in the abluminal space during periods of increased metabolic demand, as a result of immediate changes in tissue energy balance following neuronal activity and synaptic transmission (Nguyen et al., 2000). An example is the ATP-derived neurotransmitter and vasodilator adenosine, which may be released by neurons during metabolic deprivation induced by neuronal firing (Nguyen et al., 2000). Adenosine matches metabolic activity to CBF in conditions such as neuronal activity, hypoxia and hypercapnia (see later), with a major role attributed to the adenosine receptor A2A receptor (Phillis, 2004). The primary effect of receptor signaling is the activation of K_{ATP} channels with consequent smooth muscle relaxation and elevated CBF (Phillis, 2004). ATP, also an important metabolic regulator, takes part in glial cell control of cerebral arteriolar diameter (Koehler et al. 2006).

In addition to neurons, cortical astrocytes play a role in the neurovascular coupling. It is known that astrocytes send processes called ‘endfeet’ to synapses and to arterioles and are capable of sending signals to pial arterioles, and communicate with other astrocytes through gap junctions (Dunn and Nelson, 2010). Pial arteries, extrinsically innervated by sympathetic nerves, run on the surface of the brain, then penetrate into the cortical parenchyma and become parenchymal arterioles (see Diagram 1, Dunn and Nelson, 2010). In the cortical parenchyma beyond the Virchow-Robin space, extrinsic innervation is lost and the arteriole becomes surrounded by astrocytic endfeet (Dunn and Nelson, 2010). Information from active neurons is integrated by astrocytes, which generate increases in endfoot Ca^{2+} as a result of astrocytic metabotropic glutamate receptor activation and inositol 1,4,5-trisphosphate production (Filosa et al., 2006). This Ca^{2+} signal in the endfoot may activate neuronal NOS as well as Ca^{2+} -sensitive phospholipase A_2 (PLA₂), thereby releasing arachidonic acid (AA). AA dilates cerebral arterioles and microvessels through a COX-dependent mechanism, with prostaglandin E_2 (PGE₂), a COX metabolite, mediating a significant component of vasodilation (Filosa et al., 2006). Neuronal NOS-derived NO dilates cerebral vessels via activation of soluble guanylyl cyclase in the vascular smooth muscle.

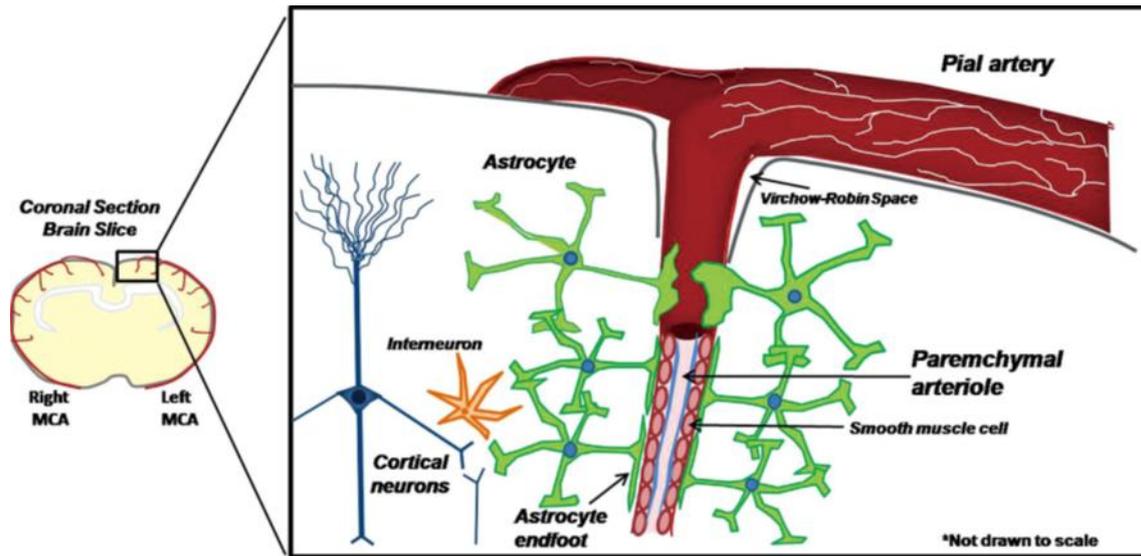


Figure 1: Cortical parenchymal arterioles and the surrounding astrocyte network (Dunn and Nelson, 2010)

2.1.1.2.2 The role of potassium channels in the cell signaling

Functional hyperaemia may also be directly linked to electric current flow through excitable membranes, which involves potassium ions (K^+) lost during action potential repolarization. K^+ released by activated neurons to regions of lower K^+ near the microvasculature act as an important regulator, coupling increases in cerebral metabolism and blood flow (Nguyen et al., 2000). The elevation of astrocytic endfoot Ca^{2+} accompanying neuronal activation activates astrocytic Ca^{2+} -activated K^+ channels (K_{Ca}), which also release K^+ into the restricted space between the endfoot and the SMC of the arteriole (Filosa et al., 2006).

Vascular K^+ channels are a diverse group of transmembrane proteins that regulate membrane potential, thereby modulating Ca^{2+} entry and cell signalling (Brayden 2002). The following K^+ channels are functionally important in the vasculature, located on both endothelium and vascular SMCs: Ca^{2+} -activated K^+ channels (K_{Ca}), voltage-dependent K^+ channels (K_V), ATP-sensitive K^+ channels (K_{ATP}) and inwardly rectifying K^+ channels (K_{ir}) (Dunn and Nelson, 2010).

In large cerebral arteries AA causes vasodilation through activation of Ca^{2+} -activated K^+ (K_{Ca}) channels, as AA-induced vasodilatation was markedly attenuated by inhibitors of

such channels, namely 1 mM tetraethylammonium ion (TEA) and 50 nM iberiotoxin (Faraci et al., 2001). The basilar artery produced relatively few prostanoids when treated with AA. COX does not play a major role in the AA-induced relaxation of the basilar artery (Faraci et al., 2001). It was shown, instead, that AA is metabolized in the basilar artery largely via the lipoxygenase enzyme. This pathway mediates vasodilatation by activation of K_{Ca} channels in the basilar artery (Faraci et al., 2001).

The K_{ir} channels have been extensively studied in the cerebral circulation, and were found to be important in coupling cerebral metabolism and CBF (Chrissobolis and Sobey, 2003). The extracellular concentration of K^+ in the brain increases from 3 mmol/L to 7 mmol/L during neuronal activity (Chrissobolis and Sobey, 2004). In this concentration range, K^+ activates SMC K_{ir} channels, elicits marked SMC membrane potential hyperpolarization, which closes voltage-dependent Ca^{2+} channels, decreasing intracellular Ca^{2+} , and thus leading to dilatations of cerebral arteries and arterioles. In pial arteries, the disruption of the gene for the K_{ir} channel, abolished K^+ induced vasodilations (Dunn and Nelson, 2010).

In isolated basilar arteries 30 μ mol barium (Ba^{2+}), which, at that particular concentration, is a selective inhibitor of K_{ir} channels, abolished the hyperpolarization caused by K^+ , suggesting that such channels exclusively mediate K^+ -induced vascular hyperpolarization (Chrissobolis et al., 2000). Findings from in vivo studies indicated that only about one-half of the vasodilator response to K^+ was inhibited by the same concentration of Ba^{2+} that abolished K^+ -induced hyperpolarization in vitro. In addition, this Ba^{2+} -resistant component of the response to K^+ in vivo is insensitive to inhibitors of K_{ATP} channels, K_{Ca} channels and $Na^+-K^+-ATPase$, therefore, it is probable that the entire response is indeed mediated by K_{ir} channels, however, Ba^{2+} cannot fully access and block K_{ir} channels when applied topically in vivo (Chrissobolis et al., 2004).

The K_{ATP} channels are present in neurons, cerebral vascular SMCs, and brain microvascular endothelial cells, and are known to play an important role in the regulation of vascular tone, depending on metabolic need and energy supply (Jansen-Olesen et al., 2005). K_{ATP} channels exist as an octameric complex containing two distinct types of protein subunits. The channels consist of four inwardly rectifying potassium channel subunits (Kir6.1 or Kir6.2), with each K_{ir} subunit being associated

with a larger regulatory sulphonylurea receptor (SUR) (Brayden 2002). Intracellular ATP at micromolar concentrations binds to the K_{ir} subunit and inhibits, while following dissociation of ATP, the ADP, a nucleotide diphosphate, in the presence of magnesium (Mg^{2+}) interacts with the SUR subunit and activates K_{ATP} channels (Brayden 2002). This activation of K_{ATP} channels causes hyperpolarization of SMCs, which prevents the opening of depolarization-activated Ca^{2+} channels, thus blocking Ca^{2+} entry to the cell, resulting in vasodilation in cerebral arteries (Jansen-Olesen et al., 2005). Hyperpolarization of the endothelial cells during impaired energy supply elevates the concentration of intracellular Ca^{2+} via the transmembrane Ca^{2+} influx due to the membrane potential gradient (Luckhoff and Busse, 1990) and thereby promotes the Ca^{2+} -dependent formation of the vasorelaxant NO (Jansen-Olesen et al., 2005).

Via myo-endothelial communication, hyperpolarization may be transmitted electrically from SMCs to endothelium through gap junctions, which have been described in rat basilar arteries (Ploug et al., 2006). The significance of SMC hyperpolarization spreading to the endothelial cells may be that the production of NO by the endothelial cells may increase the effect of vasodilation.

There is some regional heterogeneity with respect to the apparent distribution of K_{ATP} channels in different sized cerebrovasculatures. Ploug et al. showed that compared to that measured in the aorta, there is a higher expression level of Kir6.1 and SUR2B proteins in the basilar and middle cerebral arteries. The differential expression seen at the protein level suggests a more significant role for the Kir6.1/SUR2B K_{ATP} channel complex in the basilar and middle cerebral arteries, and thus in the regulation of CBF (Ploug et al., 2006).

2.1.1.2.3 The role of protein kinase C in the cell signaling

The enzyme protein kinase C (PKC) exerts numerous cellular effects, including mediation of responses to vasoconstrictor agonists in cerebral arteries (Chrissobolis and Sobey 2002). The K_{ir} channels and the K_{ATP} channels are structurally related, and in the basilar artery, vasodilator responses mediated by activation of both K_{ir} and K_{ATP} channels are inhibited by the activity of the PKC. Furthermore, it has been reported that PKC exerts inhibitory actions on K_v and K_{Ca} channels as well, and so PKC-induced inhibition of K^+ channels may normally modulate basal cerebral artery tone and may contribute to pathophysiological vascular conditions such as cerebral vasospasm

(Chrissobolis and Sobey 2002). PKC phosphorylates ryanodine receptors located on the sarcoplasmic reticulum, which modulates calcium influx into the cell cytoplasm, termed calcium sparks (Liu QH et al., 2009). Ca^{2+} sparks can generate hyperpolarizing spontaneous transient outward currents in cerebral arterial SMCs, which results in the inhibition of voltage-dependent Ca^{2+} channels, prevention of Ca^{2+} influx, and thus cause cerebral vasorelaxation (Nelson et al., 1995). As PKC reduces the frequency of Ca^{2+} sparks in cerebral arterial SMCs by phosphorylating ryanodine receptors, it acts in the direction of cerebral vasoconstriction (Bonev et al., 1997).

2.1.1.3 Neural regulation

CBF may increase out of proportion to metabolic demands, may increase without significant change in local metabolism, and may increase much faster than the accumulation of the metabolic end products. Therefore, the 120 year old metabolic hypothesis of Roy and Sherrington cannot fully explain the increases of CBF during increased functional activity of the central neurons (Sandor, 1999). Neurogenic stimuli via perivascular nerve endings may act as rapid initiators, to induce a moment-to-moment dynamic adjustment of CBF to the metabolic demands, and further maintenance of these adjusted parameters is ensured by the metabolic and chemical factors (Sandor, 1999).

Nerve fibers that make up perivascular neuronal networks surround not only extracortical, pial vessels, but also follow both intracortical arteries and veins into the cerebral parenchyma (Sandor, 1999). These nerve fibers contain sympathetic adrenergic nerves of superior cervical ganglionic origin, parasympathetic cholinergic, trigeminovascular and sensory nerves. The number of nerve fibers in the walls of the vessels becomes smaller, as the diameter of the vessels decreases (Lee, 2002). Parenchymal arterioles lack the extrinsic innervation of larger pial arteries (Dunn and Nelson, 2010). Sympathetic nerve stimulation releases norepinephrine (NE) from perivascular nerves, although synaptic concentration of NE upon maximum transmural nerve stimulation is too low to directly affect vascular smooth muscle tone (Lee, 2002). Furthermore, α -adrenoceptor antagonists were unable to alter pial arteriolar diameter in species such as cats, piglets and lambs, and transection of sympathetic nerves in resting conditions resulted in minimal effect on CBF in several different species (Sandor,

1999). Similarly, isolated human pial arteries were shown to have poor innervation and demonstrated weak responsiveness to adrenoceptor agonists (Lavi et al., 2003). These results suggest that NE is not the major transmitter for cerebral vasoregulation in large cerebral arteries. Instead, NE may act on presynaptic receptors of neighboring nerve terminals and modulate the release of transmitter substances from these nerves. For example, Lee and colleagues showed that in pig cerebral arteries NE acts on β_2 adrenoceptors located on nitrenergic nerve terminals, and releases NO, causing vasodilation (Lee et al., 2000).

Similar to NE, acetylcholine (ACh) is not the main dilatory transmitter at terminal synapses (Lee, 2002). It is co-released with NO, a potent nonadrenergic, noncholinergic vasodilator transmitter substance in cerebral blood vessels. Choline acetyltransferase, which synthesizes and releases ACh, coexists with NOS in the parasympathetic ganglia and perivascular nerves in cerebral blood vessels.

ACh-induced relaxation depends on the intact functioning of the endothelium (Dong et al., 2000). In guinea-pig mesenteric and cerebral arteries, ACh induced relaxation, neither mediated by NO nor mediated by prostanoids, was abolished completely with removal of the endothelium as well as when arterial segments were precontracted with high K^+ concentrations, which is known to cause membrane depolarization. Therefore, it seems that membrane hyperpolarization is essential in the vasorelaxation process involving the yet unknown endothelium derived hyperpolarizing factor (EDHF).

The cellular mechanism of EDHF action shows tissue and species variability, and in guinea pig basilar arteries EDHF-mediated relaxation possibly involves the activation of K_{ir} and K_v channels (Dong et al., 2000). The toxins apamin and charybdotoxin inhibit K_{Ca} and K_v channels, respectively, both of which inhibit EDHF-mediated relaxation. In addition, ciclazindol and glibenclamide, inhibitors of K_v and K_{ATP} channels, have been reported to suppress EDHF-mediated relaxation in guinea-pig basilar artery and ACh-induced smooth muscle hyperpolarization in rabbit middle cerebral artery (Petersson et al., 1997).

The source of endogenous ACh is presumably the cholinergic neurons that innervate the basilar artery, while its target M_2 mACh receptors are expressed in neurons, endothelial cells and vascular SMCs (Chrissobolis et al., 2004). Since endothelial denudation has no

effect on K^+ -induced cerebral vasorelaxation in rats, it seems unlikely that endothelial M_2 mACh receptors are involved in K^+ -induced responses.

Another type of nerve fibers, the sensory nerve fibers arrive either via somatosensory pathways or from the sensory organs, and the axon terminals form synapses in the thalamus or in the geniculate bodies (Sandor, 1999). The neurotransmitters involved in the transmissions of the signals may influence the tone of the cerebral resistance vessels in the relay nuclei along the sensory pathways and in the appropriate cerebrocortical regions. Perivascular C-type afferent sensory neurons release substance-P and calcitonin gene-related peptide (CGRP), both of which cause vasodilation in pial arterioles (Sandor, 1999). Substance-P acts via an endothelium-derived hyperpolarizing factor (EDHF) and NO pathway, while CGRP has endothelium-independent actions, by involving activation of smooth muscle adenylate cyclase, with a subsequent vasodilation (Edvinsson et al., 1992).

In the guinea-pig basilar artery, ANA induces vasorelaxation via activation of capsaicin-sensitive sensory nerves (Zygmunt et al., 1999). Moreover, the vasodilatory responses of isolated arteries exposed to ANA were shown to be mediated through the vanilloid TRPV1 receptor and involve the release of CGRP from perivascular sensory nerves (Golech et al., 2004).

2.1.2 Adaptation to hypoxia and hypercapnia: Local and global regulatory mechanisms

It is known that CBF is increased by hypoxia, hypercapnia and concomitant hypoxia and hypercapnia (Spicuzza et al. 2005). Hypoxic and hypercapnic stimuli can act on cerebral blood vessels by releasing various local metabolites. Both endothelial and neural processes take part in the CBF regulation in hypoxia and hypercapnia. The endothelial cells of the cerebral blood vessels are not passive barriers, but rather play an important role in the regulation of the cerebral circulation. Both the NO and the prostanoid pathways are considered to be important endothelium-derived relaxing factors (EDRFs), with the prostanoids being the dominating EDRF mediating cerebrovascular responses in the neonatal period, and NO being the dominating EDRF in the juvenile and adult life (Willis and Leffler, 2001). The mechanisms involved in the endothelial dependent dilator responses in newborns and in juveniles are not the same. In cultured endothelial vascular cells from adult pigs, Parfenova et al. found higher

endothelial NOS expression and activity, compared to cells from newborn pigs while finding no differences in the COX expression and activity between the adult and newborn pigs (Parfenova et al., 2000).

2.1.2.1 The role of nitric oxide

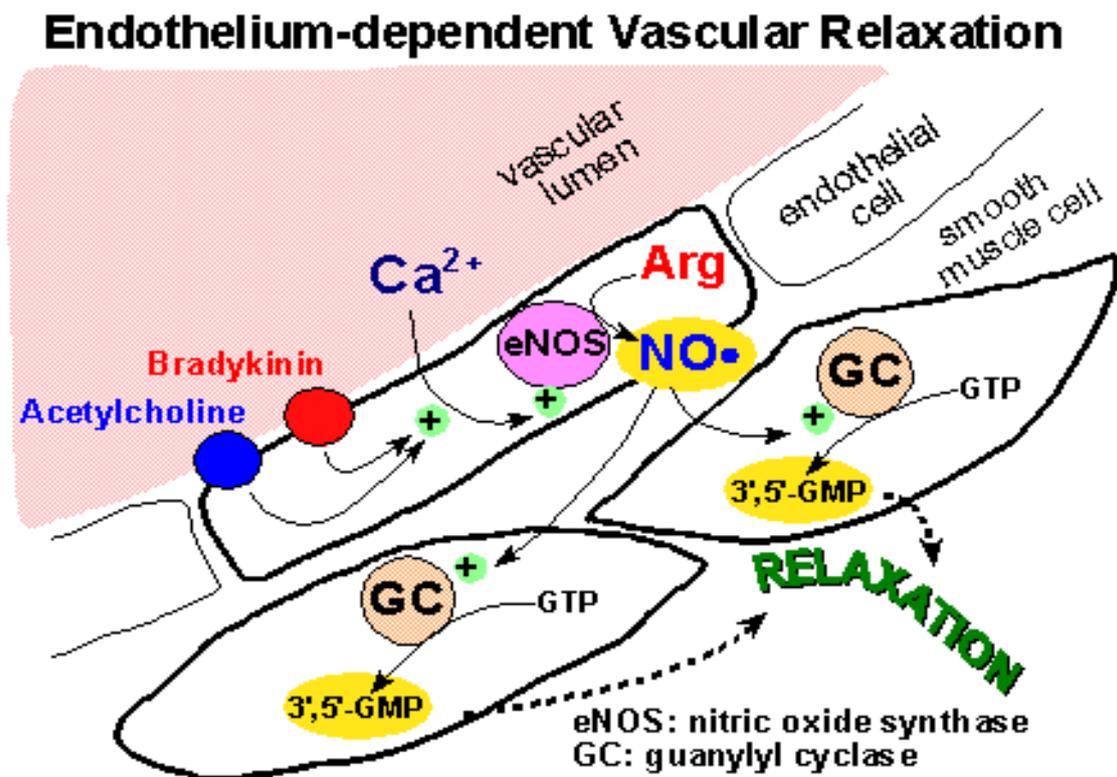


Figure 2: Synthesis of nitric oxide in the endothelium

(Source: <http://www.kumc.edu/research/medicine/biochemistry/bioc800/sig02-11.htm>)

Nitric oxide (NO) is a highly potent dilator of cerebral arteries and arterioles (Sandor, 1999). The endothelium synthesizes NO via the constitutive eNOS (see Diagram 2). The shear force acting on the endothelial membrane, such as in response to an increase in CBF velocity, as well as substances like histamine, bradykinin, epinephrine, NE, ACh, substance-P, ADP, serotonin and thrombin, stimulate phospholipase C (PLC), thereby increasing the intracellular calcium (Ca^{2+}) level, and with it the Ca^{2+} -calmodulin complex level, which activates NOS in the endothelium (Faraci et al., 1994). Neurons,

besides releasing glutamate, adenosine and other neurotransmitters that may act as vasoactive signals, also release NO. The neuronal type of the constitutive form of nitric oxide synthase (nNOS) is related to the endothelial eNOS. The increase in the Ca^{2+} levels in the neurons activates nNOS, and the NO produced acts not only as a cotransmitter in different neuronal functions, but also activates soluble guanylyl cyclase (sGC) in the vascular smooth muscle cells, increasing the cGMP level and causing vasodilation. This mechanism of vasodilation may play an important role in the flow-metabolism coupling (Edvinsson et al., 2001).

The variation of the CBF during metabolic perturbations partly involves a chemoregulatory mechanism, independent of ABP fluctuations. The CBF increases by 50% during hypercapnia and decreases by 35% during hyperventilation, without major changes in ABP (Lavi et al., 2003). Several studies indicate that NO is necessary to maintain carbon dioxide (CO_2)-mediated CBF. The studies of Sandor et al (1994) provided clear evidence that NO plays a major role in the mediation of regional CO_2 -responsiveness of the cerebral and spinal cord vessels of the cat: flow response of 11 brain and spinal cord regions to CO_2 was abolished by 95% after NOS blockade by NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME). This corresponds well to Lavi et al.'s investigation in which another NOS inhibitor, N-mono-methyl-L-arginine (L-NMMA), was shown to attenuate the vasodilatory CBF response to hypercapnia in rats (Lavi et al., 2003).

There are different results from studies carried out on different species regarding the importance of NO as a mediator in hypercapnic vasodilation. Goadsby et. al. showed in cats that NO is not the sole determinant of hypercapnic vasodilation in the cerebrovascular bed (Goadsby 1994). Lavi et al. showed in dogs that hypocapnia did not cause a significant change in CBF during infusion of the NO donor sodium nitroprusside (SNP) (Lavi et al. 2003).

The size of the blood vessel also seems to matter, concerning the contribution of NO pathway. In larger cerebral arteries as well as arterioles, NO plays a role in cerebral vasodilation in response to hypercapnia, while in the cerebral parenchymal arteriole and microvessels, NO derived from endothelial as well as neuronal enzymes does not seem to mediate vasodilation induced by hypercapnia (Nakahata et al. 2003).

Another way to view the varying results is that not the endothelium but perivascular NO-releasing nerves from extracerebral origin are the main source of basal NO in large cerebral arteries originating from the anterior portion of the circle of Willis (Iadecola et al., 1993 and Lindauer et al. 2001). Significantly lower perivascular NO concentrations may be present at arterioles which are not surrounded by NO-producing tissue and lack perivascular nerve endings from extracerebral origin. NOS inhibition nearly abolished the vasodilation response to extraluminal acidic buffer solution of rat MCA, while in cat pial arterioles, there seemed to be no permissive effect of NO donors under NOS inhibitor application during hypercapnia (Lindauer et al. 2003).

Several mechanisms might be involved in the molecular relation between CO₂ and NO pathway. NO effect may possibly be mediated by cGMP because cGMP application during NOS inhibition or during soluble guanylyl cyclase inhibition restored pH-reactivity (Lindauer et al. 2003). In the vascular smooth muscle cell, hypo- and hyperpolarizing K⁺ channel activity may possibly be modulated by NO or cGMP and by intracellular proton concentration (Lindauer et al. 2003). In the rat MCA, both K_{ATP} and K_{Ca} channels were shown to mediate vasodilation to acidosis, and the functions of both channels were dependent on basal perivascular NO concentration, since NOS inhibition reduced vasodilation to specific K⁺ channel openers (Lindauer et al. 2003).

Vasodilation of cerebral arteries during elevation of proton concentrations, producing pH values of 6.5, is highly dependent on the basal perivascular NO level, whereas at even lower extraluminal pH (6.0) vasodilation becomes independent of NO and is probably mediated by different mechanisms (Lindauer et al. 2003). Extracellular acidosis is associated with increased K⁺ conductance causing hyperpolarization that, in turn, leads to closure of voltage-gated Ca²⁺ channels and smooth muscle relaxation (Lindauer et al. 2003).

Other authors provided evidence against a permissive action of NO/cGMP on K⁺ channels involved in vasodilation to acidosis. For instance, it was shown that arginine analogues inhibit hypercapnic vasodilation by blocking K_{ATP} channels independently of NO or cGMP, and that K_{ATP} channels may have an arginine site that influences their function instead of the proposed specific role of NO or cGMP. In rat pial arterioles, NOS inhibition had no effect on vasodilation to specific openers of K_{ATP} channels

(Lindauer et al. 2003). The diminished functional role of basal perivascular NO in pial and larger penetrating arterioles compared with larger cerebral arteries of the anterior part of the circle of Willis may possibly explain the observation that in vessel segments adapted to lower perivascular NO concentrations, K_{ATP} channel function may occur less dependent of NO, whereas in vessels with higher perivascular NO concentration, at least part of the channel activity may be specifically influenced by NO (Lindauer et al. 2003).

Both hypoxia and hypercapnia may be accompanied by extracellular and/or intracellular acidosis and acidosis itself produces marked dilatation of the cerebral arteries (Santa et al. 2003). Also, the fast changes in pH that occur during hypercapnia are important modulators of NOS (Lavi et al. 2003). It has been proposed that the mechanisms by which acidosis produces dilatation of the cerebral arteries may be quite different between hypoxia and hypercapnia, and that extracellular pH rather than intracellular pH may be the major determinant of hypercapnia-induced, NO-dependent relaxation of the cerebral arteries in vitro (Santa et al. 2003).

It seems that in the case of the cerebral arteriole, the extracellular change of proton levels and not the CO_2 molecule levels is responsible for vasodilation in hypercapnia, which is supported by the observation that under the condition of normal pH, applied CO_2 produced no vasodilation of the parenchymal arterioles (Nakahata et al. 2003).

Results concerning contribution of K_{ATP} channels in the vasodilation of cerebral arteries to acidosis are inconclusive, although pharmacological manipulations of K_{ATP} channel activation were found to be possibly involved in the modulation of CO_2 -NO cerebral vasomotor reactivity (Faraci et al. 1994). A study of rabbits demonstrated that glibenclamide, a selective K_{ATP} channel antagonist, is partly capable of reducing dilation of pial arterioles induced by hypercapnia, suggesting that K_{ATP} channels may marginally contribute to cerebral vasodilation during hypercapnia (Faraci et al. 1994). In addition, study of the canine basilar artery demonstrated that extracellular acidosis that accompanies hypercapnia causes vasorelaxation partly via K_{ATP} channels. In humans it was shown that in larger cerebral arterioles, mild hypercapnia-induced vasodilation was only partly mediated by K_{ATP} channels. In cerebral parenchymal arterioles, mild hypercapnia-induced vasodilation was completely abolished by glibenclamide. These data suggest that K_{ATP} channels, expressed in microvessels of

cerebral cortex are very sensitive to the mild increase in levels of CO₂, and that these channels are important mediators of hypercapnia-induced vasodilation in the rat cerebral parenchymal microvessels (Nakahata et al. 2003).

As in the case of hypercapnia, NO may contribute to the increase of CBF evoked by hypoxia. Endothelial contribution to hypoxic vasodilatation increases throughout early postnatal life and becomes prominent in adult cerebral arteries (Pearce 2006). A local role for NO in the fetal cortical vasculature is strongly supported by the finding that the release of cGMP from the brain increased in response to hypoxia (Hunter et al. 2003). Hypoxic increases in cortical cGMP production were completely eliminated by NOS inhibition, strongly suggesting a direct role for NO in hypoxic cortical relaxation. NO produces vascular relaxation by interacting with the heme group of soluble guanylate cyclase (sGC), the enzyme that synthesizes the second messenger cGMP, which in turn promotes vasodilation in many vascular beds, including cerebral arteries. The tonic release of NO is governed by continuous activation of endothelial cells by stimuli such as pulsatile flow and sheer stress, and NO directly stimulates cerebrovascular cGC, which is highly abundant in cerebral arteries, particularly in the fetus, as the vasorelaxant capacity of the cGMP pathway is attenuated by maturation (Hunter et al. 2003). Pearce et al. have shown that fetal cerebral arteries are more sensitive to the vasodilator effects of cGMP than adult arteries, suggesting that hypoxia-induced release of NO from the endothelium and subsequent cGMP formation are an important and age-dependent component of the cerebral response to acute hypoxia (Pearce 2006).

In the adult rat brain, NOS synthesis seems to be important in the CBF responses to hypercapnia but not hypoxia (Hunter et al. 2003). In support of this, is that reduced tissue oxygen levels may drastically reduce NOS activity, as shown by the reduction of the NO generating capacity in rat brains as a result of reduction of oxygen supply (Bari et al. 1998). According to studies done on piglets and rats, activation of NMDA receptors is not an important mechanism involved in promoting arteriolar dilation during hypoxia; moreover, NMDA-induced cerebral arteriolar dilation was inhibited by hypoxia and by exogenous adenosine (Bari et al. 1998).

Hypoxia may also promote release of vasodilator opioids, such as methionine enkephaline, linked to production of NO and cGMP, suggesting important interactions

between opioid release and NO during acute hypoxia in the immature brain (Pearce 2006).

As seen in hypercapnia, acute hypoxia also has been reported to activate K_{Ca} channels in neonatal pial arteries through a NO-independent mechanism, while within the adventitia of the arterial wall, hypoxia may also promote the release of vasodilatory sensory neuropeptides, while inhibiting the release of NO from perivascular nerves in adult cerebral arteries (Pearce 2006). Thus one sees the complexity of the cerebrovascular regulation in hypoxia, which involves the endothelium, the smooth muscle and the adventitia of the cerebral blood vessel, as well as the cerebral parenchyma.

2.1.2.2 The role of prostanoids

Prostanoids, such as prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) are important vasodilator molecules. The endothelium synthesizes prostanoids via the cyclooxygenase enzyme 1 (COX-1) from arachidonic acid. Both PGI_2 and PGE_2 act on the same PGI_2 receptor, resulting in adenylate cyclase activation (Parfenova et al., 1995). The COX-1 isoform participates in the maintenance of resting CBF and in the vasodilation produced by hypercapnia or endothelium-dependent vasodilators, and the COX-2 isoform contributes exclusively to vascular responses initiated by neuronal activity, as COX-2 is constitutively expressed in glutamatergic neurons (Niwa et al., 2001).

Several reports suggested that prostanoids play an important role in the regulation of basal CBF of adults, newborns, and fetuses; although others suggested that there is no effect of COX inhibition in adults. In the case of hypoxic regulation of CBF, data regarding the role of prostanoids are even less clear. Although hypoxia can increase cerebral PGE_2 levels, it has no significant effect on PGI_2 or thromboxane A_2 (TxA_2) release, suggesting that prostanoids play a modest role in hypoxic cerebral vasodilatation in the immature brain (Pearce 2006).

Blockade of prostanoid synthesis by diclofenac attenuates the CBF rise in response to hypoxia, which is related to systemic effects rather than direct effects on the cerebral vasculature in fetal sheep, because arterial blood pressure was significantly lower due to diclofenac (Nishida et al. 2006). This indicates that in the brain during hypoxia, prostanoids do not act locally on resistance vessels directly and instead these mediators

and others such as carbon monoxide interact and compensate for one another in a complex and redundant system to mediate responses to hypoxia (Nishida et al. 2006).

2.1.2.3 The role of adenosine

The cerebral vascular response to hypercapnia seems to be also mediated in part by adenosine, an endogenously produced purine nucleoside. Adenosine mediates various physiological processes acting via at least four different adenosine receptor types named A1, A2A, A2B and A3. The receptors are all coupled to G-proteins and have been found in a wide range of species and types of tissues (Blood et al. 2003). A1 receptor expression is notably high in the brain, where activation of the A1 receptor results in neuronal membrane stabilization and decreased neuronal firing, while A2A and A2B receptors can be found in cerebral vascular smooth muscle, where both mediate vasodilation in response to hypoxia and neuronal stimulation (Blood et al. 2003). Adenosine was shown to depress fetal cerebral oxygen consumption through activation of neuronal A1 receptors and mediate vasodilation through activation of A2 receptors on cerebral arteries, suggesting that adenosine is a critically important mediator of cerebrovascular homeostasis during acute hypoxic insult in the fetus as well as in the adult (Pearce 2006). In preterm fetal sheep, adenosine A2 receptor was shown to play a greater role in cerebral vasodilatation than does the A1 receptor, as is supported by the observations that in adult rats, A2 agonists resulted in greater cerebral vasodilatation than A1 agonists, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective adenosine A1 receptor antagonist, had no effect on the hypoxic increases in CBF (Blood et al. 2003). Similarly, in rats selective antagonists of A2A adenosine receptors significantly attenuated the CO₂-evoked increase in cortical arteriole diameter, showing the involvement of the adenosine receptor during hypercapnia-induced vasodilation as well, although this antagonism is not complete, suggesting that other mechanisms are also involved (Phillis et al. 2004).

Adenosine is elevated during hypoxia in the brain of piglets and other animals (Bari et al. 1998). Plasma and intracerebral adenosine concentrations also increase during hypoxia in the fetal sheep, newborn lamb and adult rat (Blood et al. 2003). In piglets, it was shown that blocking adenosine receptors with theophylline, a non-selective adenosine receptor antagonist, blocked pial arteriolar dilation to hypoxia and N-methyl-

D-aspartate-induced dilation (NMDA) during hypoxia (Bari et al. 1998). Adenosine could reduce Ca^{2+} entry into nerve cells and activation of NOS by promoting hyperpolarization or by blocking neural N-type voltage dependent Ca^{2+} channels, and also might reduce presynaptic glutamate release, thus suppressing auto-amplification of glutamate and/or NMDA effects (Bari et al. 1998). It is important to note, however, that some studies indicate that activation of NMDA receptors is not an important mechanism involved in promoting arteriolar dilation during arterial hypoxia in piglets or rats (Bari et al. 1998). The brain parenchyma includes many different cell types, and each of these can release a different combination of vasoactive factors in response to hypoxia. Hypoxia initiates a regionally heterogeneous change in the interstitial milieu that may not only promote vasodilatation but may also attenuate vasodilation to some receptor agonists such as NMDA (Pearce 2006). The reasons for this complexity arise not only from the mixtures of cerebral cell types that vary from region to region but also from the arteries and arterioles whose reactivity are labile and varies with age, artery size, and region (Pearce 2006).

As in the case of piglets, in the case of fetal sheep, intravenous infusion of theophylline, resulted in a lack of CBF increase during hypoxia (Blood et al. 2003). Theophylline crosses cell membranes freely, resulting in blockade of adenosine receptors on either side of the blood brain barrier, while 8-p-sulfophenyl-theophylline (8-SPT), another non-selective adenosine receptor antagonist, is relatively impermeable to the plasma membrane and does not cross the blood brain barrier. 8-SPT infusion resulted in an inhibition of much of the characteristic increase of CBF provoked by hypoxia, suggesting that these adenosine receptors are located outside the blood brain barrier (Blood et al. 2003). In the adult rat, topical application of 8-SPT to the exposed cortex prevented hypoxic CBF increase, also suggesting that the receptors mediating cerebral vasodilatation are located outside the blood brain barrier (Blood et al. 2003).

2.1.2.4 The role of sympathetic control

Normocapnic hypoxia increases sympathetic control of the cardiovascular system more than normoxic hypercapnia, and it is likely that sympathetic stimulation can prevent excessive cerebral vasodilation in response to combined hypoxia and hypercapnia,

which may be important in reducing a possibly dangerous rise in intracranial pressure in extreme hypoxia and hypercapnia (Spicuzza et al. 2005).

The role of sympathetic activation must not be neglected in the regulation of CBF, within the autoregulatory range of blood pressure in hypoxia and hypercapnia. This is interesting also, because although parasympathetic nerves innervate the cerebral vessels, the parasympathetic fibers of the 7th cranial nerve, which contains the efferent limb of the peripheral chemo- and baroreceptor reflex arcs, were found to have no significant role in the cerebral vasodilation that occurs during hypoxia and hypercapnia. This was shown on baboon brain, which responded normally to hypoxia and hypercapnia both before and after unilateral and bilateral transection of the 7th cranial nerve (Hoff et al. 1977).

Golanov et al., have proposed that at least 50% of hypoxic cerebrovascular vasodilation is neurogenic, involving a rapid, patterned, sympathetic response originating in the lower brainstem, with excitation of oxygen detectors, namely sympathoexcitatory reticulospinal neurons of the rostral ventrolateral medullary nucleus (Golanov et al. 2001). These neurons are directly, selectively, rapidly and reversibly excited *in vitro* or *in vivo* by hypoxia (Golanov et al. 2001). Neurons of the rostral ventrolateral medullary nucleus do not innervate directly the cerebral cortex but rather synapse in the medullary vasodilator area, the bilateral lesion of which blocks the cerebrovascular responses to stimulation of neurons of the rostral ventrolateral medullary nucleus as well as hypoxia-induced cerebrovasodilation. The vasodilator effect of the excitation of rostral ventrolateral medullary nucleus is relayed by the subthalamic cerebrovasodilator area to other brain areas (Golanov et al. 2001).

Cortical cerebrovasodilation evoked by excitation of the above pathway is likely to depend upon the integrity of cortical neurons (Ilch and Golanov 2004). Selective excitotoxic lesion of cortical neurons, achieved by ibotenic acid microinjection of the parietal cortex, blocks CBF increase triggered by stimulation of subthalamic cerebrovasodilator area and also attenuates hypoxia-induced CBF elevation by 54% in the cortex (Ilch and Golanov 2004). This supports the view that hypoxic cerebrovasodilation is partially neurogenic and requires activity of local neurons with oxygen sensing properties.

Hypoxia-induced blood flow increase seen in the brainstem, which is the major site of cardiorespiratory controls, was shown to be stronger compared to that seen in the cortex. More precisely, in the ventral respiratory groups of the brainstem, the blood flow increase during hypoxia was higher than in the dorsal part of the brainstem, containing the nucleus tractus solitarius and pontine respiratory groups (Montandon et al. 2006). These results suggest that blood flow response to hypoxia favours oxygen delivery in brainstem regions involved in respiratory rhythm generation (Montandon et al. 2006).

2.2. Influence of the heme oxygenase pathway on the cerebral circulation

2.2.1 Heme oxygenase enzymes and the brain

Heme oxygenase (HO)-mediated heme degradation is the primary cellular mechanism for production of endogenous carbon monoxide (CO) (Baranano and Snyder, 2001). In this reaction, heme is degraded into equimolar quantities of iron, biliverdin, and CO (Tenhunen et al. 1969). Iron is mainly used up for production of new heme, while biliverdin turns into bilirubin rapidly, via enzymatic action of bilirubin-reductase, which is at excess (Kutty and Maines 1984). CO in the end, attaches itself to circulating haemoglobin in the bloodstream, and is transported as carboxyhemoglobin until it is expired from the lungs. The highest HO expression is in the brain and cerebral circulation, particularly in endothelium and perivascular astrocytes, and the HO activity of the brain tissue exceeds that of systemic organs (Maines MD 2000).

Analogous to NOS, the HO enzyme has different constitutive and inducible isoforms, namely HO-1, HO-2, and HO-3 (Baranano and Snyder, 2001) isoforms. The HO-1 isoform, also known as heat-shock protein 32, is extremely sensitive to heavy metals, and may be activated by agents that produce oxidative stress and pathological states, such as heat-shock, GSH-depletion, radiation, hypoxia, and hyperoxia (Maines 1984). HO-1 induction in vivo occurs in response to hemorrhage, hyperthermia, and ischemia (Leffler CW et al. 2011). However, HO-1 is not expressed in cerebral vessels or the brain under physiological conditions, and it does not contribute to acute vascular responses that involve rapid CO-mediated increases in blood flow. HO-1 inducers including hydrogen peroxide, arachidonic acid, NO donors, transition metals CoCl_2 ,

FeCl₂, FeCl₃, TNF- α and glutamate failed to acutely induce HO-1 in brain endothelial cells (Leffler CW et al. 2011).

The HO-2 isoform is abundantly expressed in the brain of various mammalian species, including mice, rats, pigs, and humans. In newborn and mature animals, HO-2 is detected in neurons, glial cells, and the cerebral vasculature (Vigne et al. 1995; Zakhary et al. 1996; Parfenova et al. 2001; Leffler CW et al. 2011). Physiological and pathophysiological stimulations, including glutamate, seizures, hypoxia, and hypotension, rapidly increase the in vivo activity of HO-2. A chemical inducer of HO-2 is the adrenal gland-derived glucocorticoid (McCoubrey Jr et al. 1997).

In cerebral vessels and endothelial cells, HO-2 activation by glutamate is mediated by protein tyrosine kinases and Ca²⁺/calmodulin-dependent mechanisms (Leffler CW et al. 2011 and Xi Q et al. 2010). Ca²⁺ and calmodulin are important regulators of HO-2 activity in freshly isolated cortical astrocytes and in cultured glial cells as well (Xi Q et al. 2011). In cerebral microvessels, NO may directly inhibit HO-2 catalytic activity but indirectly stimulate the activity via elevation of cGMP (Leffler CW et al. 2005).

Local heme availability is an important determinant of the rate of CO production. Factors that increase the heme substrate availability also increase CO production by cerebral vessels, indicating that HO-2 activity is substrate dependent (Leffler CW et al. 2003). At resting conditions, the concentration of free heme is approximately 0.5-1 μ mol/L, while at excesses of 50 times greater heme concentrations, the plasma bilirubin and CO production rise only two or three times more (Johnson et al. 1996). The difference is due to the physical properties of heme. In an aqueous medium, at physiological pH, free heme remains monomeric until 2 μ mol/L concentration, and above this concentration it forms dimers and polymer chains (Falk 1964). These forms represent a potential “heme-supply” and are not substrates of HO enzymes (Tenhunen et al. 1969). Therefore it may be seen that by addition of heme, the free monomeric heme concentration increases only by two-three times, and this substrate increase is parallel to the increase of production of bilirubin or CO (Johnson et al. 1996).

Endogenous CO is not only produced by the degradation of heme. The competitive inhibition of HO enzyme activity by metalloporphyrins leads to only 30-50% decrease in CO production (Vreman et al. 1991). While normally heme degradation is the

primary route for CO production, under HO inhibition, alternative metabolic pathways become important for CO production, such as lipid metabolism, although these associations are scarcely known yet (Vreman et al. 1991; and Wolff 1976). CO generation is associated with the process of lipid peroxidation in tissues with limited antioxidant reserves (Vreman et al. 1998).

Several HO inhibitors, such as the zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), are able to cross the blood brain barrier, since given intraperitoneally, they are able to inhibit the HO activity in the brain as well as the peripheral tissues (Vreman et al. 1991; Johnson et al. 1995), while heme does not cross the blood brain barrier (Linden et al. 1987). However, d-aminolevulinic acid synthase, the rate-determining enzyme for new heme synthesis, as well as other enzymes acting in the synthesis of heme, were shown in brain tissue (Moore et al. 1987). The discovery that the brain is not reached by the circulating heme molecules, but rather locally synthesizes its own heme supply via the metabolic pathways needed for the synthesis of heme, shows that the central nervous system is an independent functional HO unit.

2.2.2 The heme oxygenase pathway in the brain and cerebral vasculature

The mechanism of signalling pathways of the HO-CO system under physiological conditions displays various forms. CO activates the soluble guanylyl cyclase (sGC), its rate of activity increasing twofold (Makino et al. 1999 and Vogel et al. 1999). There is a possibility, that CO does not activate sGC directly, because the effect of CO is dramatically increased by the xenobiotic called 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazol (YC-1) (Friebe et al. 1996; Stone and Marletta 1998). YC-1 and CO together activate sGC as strongly as NO does. There may be a possible endogenous material, which increases the ability of CO to activate sGC.

CO may also bind to heme-containing proteins, and thereby alters their enzymatic activities (White and Marletta 1992; Schmidt 1992; Raff and Janowski 1994; Wada et al. 1985). It follows, that heme proteins are potential signalling messengers for CO.

Enzymes of the cytochrome P450 family are potential mediators of CO actions (White and Marletta 1992). The binding of CO to these enzymes increases during a rise in metabolic need and decreased oxygen tensions. The cytochrome P450 enzymes located on the mitochondrial membrane are involved in the electron transport chain and ATP

production, and may be involved in a complex effect of the action of kinases and other ATP-dependent processes.

Under resting conditions, the HO pathway has a dual influence on the hypothalamic circulation: a vasodilation mediated by PGE₂, and a simultaneous vasoconstriction due to the reduction of NO synthesis, the two effects being equally potent and neutralizing each other (Horvath et.al. 2008).

The addition of hemin, a HO inducer and substrate of the HO enzyme increased the production of PGE₂ in the hypothalamus, an action which was blocked by HO inhibitors, such as zinc protoporphyrin IX (ZnPPiX) and tin mesoporphyrin IX (SnMPiX) (Mancuso et al. 1997). CO may play a basic role in the hypothalamus PGE₂ production. Studies done on piglet cerebral microvessels showed that brain parenchymal rather than vascular PGE₂ release mediates the vasorelaxant effect of constitutive HO (Kanu et.al. 2006).

In piglets, both exogenous CO and the HO substrate heme-L-lysinate induced pial arterial dilatation and the latter could be inhibited by the HO blocker chromium mesoporphyrin (CrMP). Furthermore, hypoxia-induced vasodilation was inhibited by application of CrMP (Leffler et al. 1999; Leffler et al. 2001; Winestone et al. 2003). During seizures, the pial arteriolar dilation in piglets and the CBF-increase in adult rats were reported to be attenuated by HO inhibitors (Montecot et al. 1998; Pourcyrus et al. 2002).

As an in vitro paradigm for ischaemia, cultured astrocytes were exposed to hypoxia, which triggered marked increase in the expression of 33 kDa stress protein, identified as HO-1, whose induction was observed within 4 hours of hypoxia and peaked at 12 hours, accompanied by an accelerated transcription of HO-1 mRNA (Imuta et al. 2007). Consistent with the induction of HO-1, a platelet bioassay revealed the production of CO by reoxygenated astrocytes, and the presence of CO in the medium decelerated the hypoxia-mediated apoptotic type of cell death in cultured cerebral neurons via lowering the activity of caspase-3, a key enzyme regulating apoptotic cell death. This protection against apoptosis was likely mediated by CO-mediated increases in intracellular cGMP, because exposure of hypoxic neurons to CO increased intracellular cGMP levels and addition of cGMP analogue to hypoxic neuronal cultures suppressed caspase-3 activity

and promoted neuronal survival, suggesting that a potentially important paracellular pathway may exist through which astrocytes may rescue nearby neurons from ischaemic death (Imuta et al. 2007).

Recently it was demonstrated that endogenous CO dilates cerebral arterioles by augmenting the coupling of Ca^{2+} sparks to K_{Ca} channels in SMCs (Jaggard et al. 2002). CO elevates the coupling of Ca^{2+} sparks to the large-conductance Ca^{2+} -activated K^+ channels (K_{Ca}) in cerebral arterial SMCs and elevates K_{Ca} channel Ca^{2+} sensitivity, leading to more effective K_{Ca} channel activation by coupled sparks and K_{Ca} channel activation by previously uncoupled Ca^{2+} sparks (Leffler CW et al. 2011).

It was observed that an astrocytic signal, notably HO2-derived CO, is used by glutamate to stimulate arteriole myocyte K_{Ca} channels and dilate cerebral arterioles, as glutamate stimulated CO production by astrocytes with intact HO-2, but did not do that in genetically deficient HO-2 cells. Glutamate activated transient K_{Ca} currents and single K_{Ca} channels in cerebral arteriole myocytes that were in contact with astrocytes, but did not affect K_{Ca} activity in myocytes that were alone (Li et al. 2008).

In vivo, both glutamate and hypoxia dilate newborn pig cerebral arterioles, both dilations are blocked by inhibition of CO production, and both increase cerebrospinal fluid CO concentration (Kanu and Leffler 2007). However, although dilation of newborn pig pial arterioles to glutamate is mediated by activation of K_{Ca} channels, consistent with the intermediary signal being CO, neither K_{Ca} channel blockers nor the guanylyl cyclase inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) blocked dilation to hypoxia, suggesting that the contribution of the HO/CO system to hypoxia-induced dilation is not due to the stimulation of vascular smooth muscle K_{Ca} channels or guanylyl cyclase (Kanu and Leffler 2007). This needs to be investigated further.

2.2.3 The interaction of HO-NO pathways in the brain tissue and cerebral vasculature

The physiological roles of NO and CO display notable similarities, as both molecules appear to be neurotransmitters in the brain and the peripheral autonomic nervous system. Furthermore, NO and CO both are endothelial-derived relaxing factors for blood vessels (Baranano and Snyder, 2001). The effect of CO on vascular tone has been studied frequently in the past, and it was found that CO is able to relax smooth muscle-

preparations (Graser et al. 1990). At the same time, CO was shown to bind to NOS and to inhibit the production of NO (White and Marletta 1992; Matsuoka et al. 1994; McMillan and Masters 1995), thereby causing vasoconstriction.

This interaction may also be seen the other way around; that is, NO affects the CO production. For instance, endotoxins and inflammatory cytokines induce HO-1 not directly, but rather via NO release, since their activity was blocked by NOS inhibition (Billar et al. 1992). In further experiments, NO-donors were able to induce HO-1 mRNA expression in aorta endothelium (Mottlerlini et al. 1996), SMCs (Durante et al. 1997) and rat hepatocytes (Kim et al. 1995).

The participation of CO in the regulation of the cerebral circulation has been poorly studied yet (for review please see: Koehler and Traystman 2002). Freshly isolated cerebral microvessels produce CO from endogenous heme under basal conditions and increased CO production was reported after stimulation of ionotropic glutamate receptors (Parfenova et al. 2003; Leffler et al. 2003a and 2003b). It was observed that in freshly isolated cerebral microvessels from piglets, glutamate activates NOS, producing NO that leads to CO synthesis via a cGMP-dependent elevation of HO-2 catalytic activity, which is consistent with the *in vivo* findings that either HO or NOS inhibition blocks cerebrovascular dilation to glutamate in piglets (Leffler et al. 2005).

2.3. Endocannabinoids and the cerebral circulation

Endocannabinoids are lipid mediators, isolated from brain and peripheral tissues, which include amides, esters and ethers of long chain polyunsaturated fatty acids (Battista et al. 2004). Endocannabinoids include N-arachidonylethanolamine (anandamide), 2-arachidonoylglycerol (2-AG), 2-arachidonoylglyceryl ether (noladin ether) and virodhamine, also called inverted anandamide, because the arachidonic acid and ethanolamine is joined together by an ester bond instead of the amide bond of anandamide (Battista et al. 2004).

2.3.1 N-arachidonylethanolamine (anandamide)

The first endocannabinoid to be isolated was the lipid soluble eicosanoid derivative arachidonylethanolamide also known as anandamide (ANA) from pig brain (Devane et al. 1992). The sites of ANA synthesis include neurons (Di Marzo et al. 1994 and Cadas

et al. 1996), neuroblastoma, leukocytes, retina (Bisogno et al. 1999), circulating and tumorous macrophages (Di Marzo et al. 1999), vascular tissue (Mechoulam et al. 1998). The endocannabinoids are synthesized enzymatically in neurons and are released from them upon stimulation (Bisogno et al. 1997 and Stella et al. 1997). The biosynthesis of ANA was first thought to involve the enzymatic condensation of arachidonic acid (AA) and ethanolamine (Deutsch et al. 1993 and Devane et al. 1994). Current opinion on ANA synthesis is that it is formed from membrane phospholipids via two independent synthetic pathways.

The first pathway involves the transacylation of the amino group of phosphatidylethanolamine with arachidonate (Hillard et al. 1997) followed by a D-type phosphodiesterase activity on the resulting N-arachidonylphosphatidylethanolamide (NAPE) (Di Marzo et al. 1999 and Schmid et al. 1990). This pathway does not generate a large amount of ANA, because the tissue level of NAPE is relatively low. The level of AA esterified at the 1-position of glycerophospholipids is usually very low (Sugiura et al. 2002).

The second pathway involves the direct N-acylation of ethanolamine catalyzed by the reverse reaction of ANA amidohydrolase/fatty acid amide hydrolase (FAAH). A significant amount of ANA is formed via this pathway if concentrations of AA and ethanolamine reach high levels, co-localized at certain sites within the cell (Sugiura et al. 2002).

It has been proposed that ANA is synthesized within the cell membrane and acts on the same cell or neighboring cells as an autocrine or a paracrine mediator (Di Marzo et al. 1999). ANA, unlike other classical neurotransmitters, is not stored in vesicles (Cadas et al. 1996, 1997), but is produced and immediately released from neurons upon demand (Di Marzo et al. 1999 and Piomelli et al. 1998). Upon stimulation with depolarizing agents, an enhanced ANA release was reported (Di Marzo et al. 1994 and Giuffrida et al. 1999). In the human and rat neocortical tissue, K^+ depolarization induced release of ANA (Steffens et al. 2003).

ANA, with its poor water solubility does not have an extensive free diffusion in the extracellular space. Once cleaved from NAPE, ANA is immediately expelled out of the cell membrane with the assistance of a membrane transporter such as P-glycoprotein (Goutopoulos et al. 2002) or a lipid-binding protein such as lipocalin

(Piomelli et al. 1998). Such a lipid-binding protein may also facilitate the diffusion of ANA through aqueous extracellular medium to its sites of action (Goutopoulos et al. 2002). Thus ANA is paracrine in nature, formed near its site of action, with very low levels of ANA detected in serum, plasma and cerebrospinal fluid. The tissue levels of ANA are generally low (in the order of pmol/g tissue) (Sugiura et al. 2002). For example, ANA is found in the human brain at the following levels: 100 pmol/g in the hippocampus, 75 pmol/g in the thalamus, 60 pmol/g in the cerebellum, and 55 pmol/g in the striatum (Martin et al. 1999). The concentration of ANA in cerebellar granule cells treated in hypoxic conditions was shown to increase (Hillard et al. 1997). Such concentration increases occur in living tissue under certain conditions, including hypoxia and brain injury (Goutopoulos et al. 2002).

The degradation of ANA occurs in two steps. First, it is transported into the cell via the anandamide transporter (AT), a protein with high affinity and specificity for ANA involving a carrier-mediated process (Beltramo et al. 1997). The facilitated uptake of ANA into cells is an important part of the inactivation process, as shown by the observation that the inhibition of the AT protein with selective blockers, for example linvanil and AM404, produced a more pronounced ANA action (Calignano et al. 1997). Once inside the cell, ANA is hydrolyzed by FAAH, with the products being AA and ethanolamine (Maccarrone et al. 2000). FAAH is membrane associated and shows significant specificity for ANA (Lang et al. 1999). FAAH activity is important in maintaining the driving force for ANA transmembrane movement and accumulation inside cells, while transmembrane movement of ANA depends on a concentration gradient and a significant portion of the intracellular ANA is sequestered by a protein or lipid compartment, which does not equilibrate with the extracellular pool (Hillard et al. 2003). Several studies showed that the inhibition of FAAH results in the enhancement of ANA action. The inhibition of FAAH with methyl-arachidonyl-fluorophosphonate caused a significant increase in the ANA-induced NO release from leech and mussel ganglia, which was blocked by CB1 receptor antagonist SR141716A (Stefano et al. 1998). Likewise, in the presence of methyl-arachidonyl-fluorophosphonate, ANA caused a greater NO release, demonstrating the presence of FAAH in human saphenous vein, internal thoracic artery, and right atrium tissue segments (Bilfinger et al. 1998).

ANA, other than being substrate of FAAH, may also be a substrate of lipoxygenase and be oxidized to 12-hydroxy-anandamide, which may bind to cannabinoid receptors found in the brain, acting as a functional cannabinoid agonist (Ueda et al. 2000). The hydroperoxides generated from ANA by lipoxygenase are potent inhibitors of FAAH, suggesting that these oxygenated metabolites of ANA may well influence the endocannabinoid system (Battista et al. 2004).

2.3.2 2-arachidonoylglycerol (2-AG)

Somewhat resembling the chemical structure of ANA, 2-arachidonoylglycerol (2-AG) is a member of the monoacylglycerol family, having esterified AA at the 2-position of the glycerol backbone. 2-AG was first isolated from canine gut (Mechoulam et al. 1995), and from rat brain (Sugiura et al. 1995). 2-AG was found to be released in a Ca^{2+} -dependent manner and to reach concentrations 170 times higher than those of ANA in the brain (Stella et al. 1997). The rat brain contains 3.25 nmol/g tissue of 2-AG, a level about 800 times higher than that of ANA in the same tissue (Sugiura et al. 2002).

2-AG exhibits several cannabimimetic activities similar to those of ANA. 2-AG activates both CB-1 and CB-2 receptors (Sugiura et al. 2002). However, unlike ANA, which binds to the CB-2 receptor without activating it, 2-AG binds as well as activates the CB-2 receptor (Sugiura et al. 1998). Another difference between ANA and 2-AG is that 2-AG, unlike ANA, does not bind to the vanilloid receptor (Sugiura et al. 2002).

One pathway of 2-AG biosynthesis involves rapid hydrolysis of inositol phospholipids by PLC and subsequent hydrolysis of the resulting 1,2-diacylglycerol (Prescott et al. 1983) (Figure 3.) After electrical stimulation of Schaffer collaterals, 2-AG was released from rat hippocampal brain slices (Stella et al. 1997). It was confirmed by Sugiura et al. that this pathway is important for the depolarization-induced generation of 2-AG in rat synaptosomes (Sugiura et al. 2002).

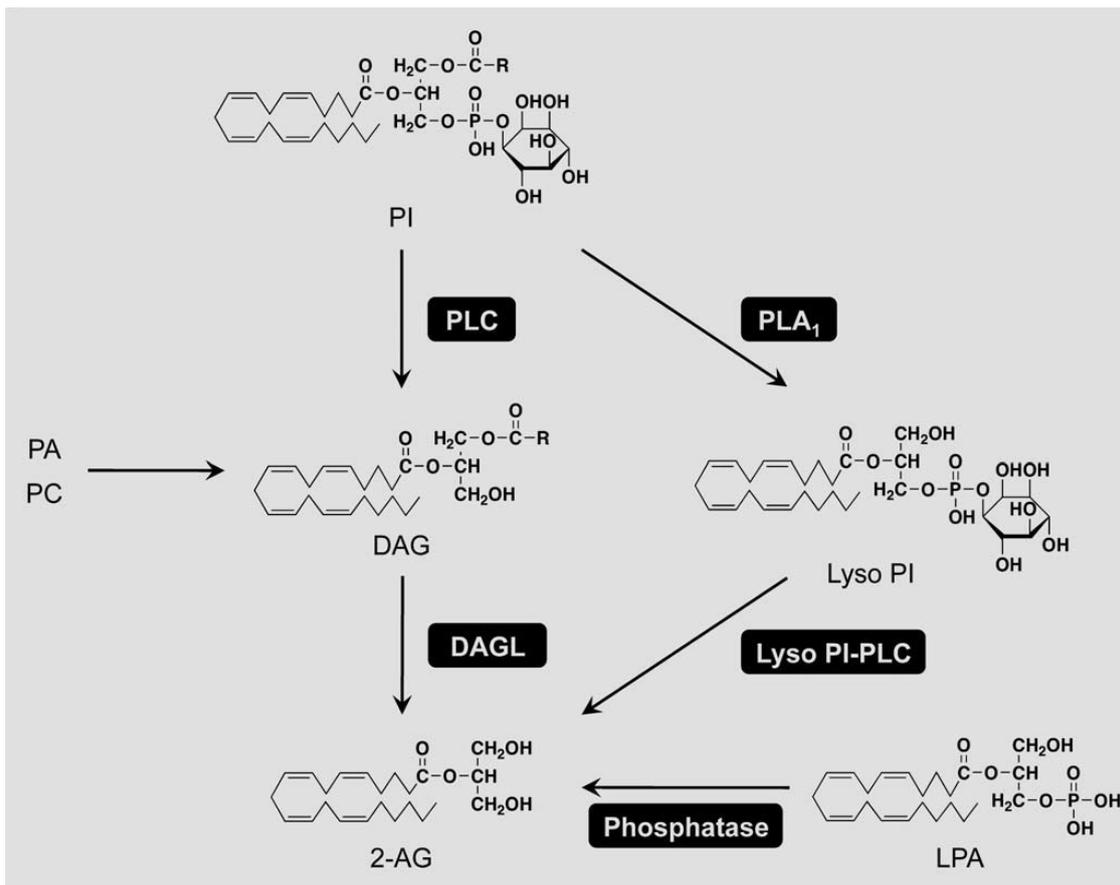


Figure 3: The biosynthetic pathways of 2-AG (Ueda et al., 2011)

The second pathway of 2-AG biosynthesis involves hydrolysis of phosphatidylinositol (PI) by PLA that generates lysophosphatidylinositol (LPI), which in turn is hydrolyzed to 2-AG by a lysophospholipase C (Piomelli et al. 1998), which is localized in the synaptosomes (Tsutsumi et al.1995). It is therefore possible that this specific enzyme may be involved in the generation of 2-AG in synapses.

Besides these two main pathways of 2-AG biosynthesis, there are several possible routes for the generation of 2-AG, which differ from one another depending on the type of tissue and cell and the type of stimulus (Sugiura et al. 2002). One route is the rapid enzymatic conversion of an AA-containing lysophosphatidic acid (2-arachidonoyl-sn-glycero-3-phosphate) to 2-AG shown in rat brain (Nakane et al. 2002). Another route involves the biosynthetic precursor 2-arachidonoyl-phosphatid acid in the generation of 2-AG in intact mouse neuroblastoma cells stimulated with ionomycin (Bisogno et al. 1999). Similar to ANA, 2-AG is synthesized within the cell membrane, is not stored in vesicles (Cadas et al. 1997 and Di Marzo et al. 1999) and is produced and released

immediately from neurons upon demand (Di Marzo et al. 1999 and Piomelli et al. 1998).

The re-uptake mechanism of 2-AG is mediated by the same facilitated transport as that of ANA, via the AT (Di Marzo et al. 1999, Piomelli et al. 1999 and Maccarrone et al. 2000). Based on studies involving radio-labeled ANA and 2-AG uptake by rat glioma cells, it was concluded that once inside the cell, 2-AG undergoes a rapid and efficient intracellular metabolism, which prevents a sustained accumulation in the cells (Bisogno et al. 2001). In many tissues, 2-AG is thought to be only an intermediate of a signaling pathway that generates 1,2-diacylglycerol and AA, both known as signaling molecules (Goutopoulos et al. 2002). However, in the brain, 2-AG may have regulatory roles, for it escapes immediate metabolism and accumulates in response to stimuli-generated Ca^{2+} surges (Stella et al. 1997). These differences may be due to various tissues expressing and containing different levels of metabolizing isoenzymes. One metabolizing enzyme, for instance is the FAAH, which recognizes and hydrolyzes 2-AG as well (Goparaju et al. 1999, Di Marzo et al. 1999 and Lang et al. 1999). Another possible metabolizing enzyme is a specific hydrolase, monoacylglycerol lipase, which breaks down monoacylglycerols including 2-AG (Goparaju et al. 1999 and Di Marzo et al. 1999). 2-AG may also undergo direct esterification into membrane phosphoglycerides and hydrolysis to AA and glycerol. AA produced from 2-AG hydrolysis is also esterified into phospholipids (Di Marzo et al. 1999).

Recently, the cross-talk of ANA and 2-AG signaling was proposed at striatal synapses, where ANA acted as inhibitor of 2-AG biosynthesis instead of competing for CB1 receptors (Katona and Freund, 2008). It is known that ANA also inhibits 2-AG uptake by competing for the same site on the membrane transporter, and intracellular 2-AG hydrolysis (Bisogno et al. 2001). By contrast, 2-AG is likely to inhibit ANA uptake by interfering only with the AT (Bisogno et al. 2001).

2.3.3 Cannabinoid receptors

2.3.3.1 The CB1 receptor

Specific cannabinoid binding sites were first described in rat brain by Devane et al. in 1988, using a radioactively labeled synthetic cannabinoid compound. Two years later, Matsuda et al. reported the cloning of a cDNA encoding a cannabinoid receptor from rat brain cDNA library and named the receptor cannabinoid 1 (CB1) receptor (Matsuda et

al. 1990). ANA binds to CB1 receptors in the brain with high affinity (with a binding coefficient of $K=40$ nM) and mimics the effects of plant-derived cannabinoids, for instance Δ^9 -THC (Devane et al. 1992). However, in CNS synapses, 2-AG may be a more suitable candidate as an endogenous ligand of CB1 receptors (Katona and Freund 2008). The CB1 receptor has been found elsewhere in the central nervous system as well as in the periphery, in both neural and non-neural tissues. For instance, CB1 receptors were demonstrated in pial vessels, smooth muscle cells derived from cerebral microvessels, and peripheral vascular endothelium (Sugiura et al. 2002). In the periphery, CB1 receptor is found in the retina, adrenal glands, bone marrow, heart, lungs, prostate, thymus, tonsils, spleen, lymphocytes, phagocytes, smooth muscle, vascular endothelium, peripheral neurons (e.g. in the gut), kidneys, uterus, and sperm cells (Goutopoulos et al. 2002).

Various mammalian brain autoradiographic studies have shown that CB1 receptors are found in the putamen, cerebellum, hippocampus, cerebral cortex, and spinal chord. In the central nervous system, CB1 receptor density is the highest in the basal ganglia, substantia nigra pars reticulata, entopeduncular nucleus and the external segment of the globus pallidus (Herkenham et al. 1990 and Gatley et al. 1998).

The CB1 receptors, with a sequence of 472 amino acids is a member of the G protein coupled receptor (GPCR) family and contains seven hydrophobic transmembrane domains (Matsuda et al. 1990 and Gerard et al. 1991). The CB1 receptor is the most abundant G protein-coupled receptor in the brain, and it is found at GABAergic, glutamatergic, cholinergic, noradrenergic, and serotonergic CNS synapses (Katona and Freund 2008). The CB1 receptor seems to consistently reside on the presynaptic side of the synapse (Katona and Freund 2008). Retrograde endocannabinoid signaling involves messengers that are released from the somatodendritic domain of neurons and then retrograde modify release properties of afferent axon terminals or regulate activity in nearby glial processes (Katona and Freund 2012). The activation of metabotropic glutamate receptors cause 2-AG release, which activates presynaptic CB1 receptors, resulting in the attenuation of neurotransmitter release (Katona and Freund 2008). The activation of CB1 receptors on hippocampal neurons almost entirely blocked neurotransmitter release from both glutamatergic and GABAergic axon terminals (Katona et al. 1999, Katona and Freund 2008).

In different brain regions, different G proteins or second messengers that couple to CB1 receptors may mediate different physiological effects (Howlett et al. 1999). The diverse intracellular effector mechanisms following CB1 receptor activation may explain the variety of responses to cannabimimetics observed in different types of cells. In bipolar cells of the retina, for instance, cannabinoid receptor agonists caused a drastic decrease in the amplitude of the current through voltage-sensitive L type Ca^{2+} channels (Straiker et al. 1999).

The G proteins of the $G_{i/o}$ family are sensitive to the pertussis toxin, that ribosylates and inhibits G proteins of the G_i and G_o subtype (Gebremedhin et al. 1999). The binding of ANA to the CB1 receptor interacts with the G protein of the $G_{i/o}$ family, and thereby inhibits the adenylyl cyclase, causing the reduction of cAMP levels. (Howlett et al. 1998, Vogel et al. 1993 and Holland et al. 1999). This causes inhibition of voltage-gated N-type Ca^{2+} channels (Felder et al. 1993) and stimulates voltage-sensitive K^+ current and inwardly rectifying K^+ current by activating K_{ir} channels (Deadwyler et al. 1993 and Mackie et al. 1995). The interaction of the CB1 receptor with the $G_{i/o}$ protein also activates the mitogen activated protein kinase (Bouaboula et al. 1995).

Recently, it was shown that the CB1 receptor, like other G-protein-coupled receptors, can adopt different affinity states, which may represent multiple conformations that can be stabilized by different ligands, leading to different signaling events (Turu and Hunyady, 2010).

Endocannabinoids may also have a role in the regulation of the neuroendocrine system. The mRNA of the CB1 receptor was observed via in situ hybridization in the pituitary gland (Wenger et al. 1999 and Gonzalez et al. 1999). Δ^9 -THC and other natural and synthetic cannabinoid molecules were shown to decrease prolactin and gonadotropin secretion (Wenger et al. 1995), and to increase the adrenocorticotrop hormone release in the anterior lobe of the pituitary gland (Gonzalez et al. 1999). Anandamide has similar actions suggesting that it may also act as a neurotransmitter and neuromodulator in the reproductive system (Wenger et al. 1995).

2.3.3.2 The CB2 receptor

The cannabinoid 2 (CB2) receptor is primarily located in the periphery, mainly in tissues of the immune system (Munro et al. 1993 and Galicque et al. 1995). Molecular characterization of the CB2 receptor was performed in the macrophages of the marginal

zone of the spleen. The amino acid sequence of the CB2 receptor was found to show 44% homology to the CB1 receptor (Munro et al. 1993). The distribution of CB2 receptors include cells associated with the immune system such as leukocytes and mast cells, in the spleen, thymus and tonsils (Matsuda et al. 1997). Later, the CB2 receptor was identified in peripheral nerve endings (Griffin et al. 1997). Various immune cells contain not only CB2 but also CB1 receptors (Cabral et al. 1999). The localization of CB2 receptors in the immune system suggests that this receptor may have an immunomodulatory role. It should be mentioned that macrophages have the ability to synthesize and metabolize endogenous cannabinoid molecules (Di Marzo et al. 1996, Wagner et al. 1997, and Varga et al. 1998). These data show that cannabinoids may have significant roles in the regulation of the immune system.

Some of the signal transduction pathways coupled to CB2 receptors are common with those coupled to CB1 receptors. Examples include the inhibition of adenylyl cyclase and the stimulation of mitogen activated protein kinase. An important difference between the signal transductions coupled to CB2 and to CB1 receptors is that until now, CB2 receptors have not been shown to affect ion channels (Pertwee, 1997). It should be noted that an alternatively spliced form of the human CB1 receptor, CB1A has been characterized, although this is thought to be extremely rare, with its shorter mRNA poorly transcribed in humans and not being expressed in rat or mouse (Howlett, 2000).

2.3.3.3 The putative CB3 receptor (GPR55 receptor)

Recently, the orphan G protein coupled receptor, GPR55, was presented as one of the missing candidate cannabinoid receptor subtypes (Johns et al. 2007 and Ryberg et al. 2007). The ability of GPR55 to recognize cannabinoids was first described in yeast expression system in a Glaxo Smith Kline patent, where the CB1 antagonists AM251 and SR141716A acted as agonists in micromolar concentrations (Brown and Wise 2001; Brown and Hiley 2009). However, the classification of the GPR55 as a cannabinoid receptor is still controversial. Oka et al. reported that GPR55 is not a typical cannabinoid receptor since numerous endogenous and synthetic cannabinoids had no effect on GPR55 activity while endogenous lipid lysophosphatidylinositol (LPI) and its 2-arachidonyl derivatives stimulated the receptor, as a result of their abilities to phosphorylate the extracellular regulated kinase and induce calcium signaling (Oka et al. 2007 and Oka et al. 2009). In addition, the potent CB1 and CB2 receptor agonist

WIN55212-2 does not activate GPR55. Until this controversy is resolved, GPR55 can be regarded as an atypical cannabinoid receptor (Sharir and Abood 2010).

2.3.3.4 The VR1 receptor

Anandamide, structurally related to capsaicin and olvanil (N-vanillyloleamide) (compounds that all have an amide bond and an aliphatic side chain), may bind to vanilloid 1 (VR1) receptors, located on perivascular sensory neurons (Zygmunt et al. 1999). Olvanil is able to bind to the anandamide transporter and to the CB1 receptor, which suggests that capsaicin-like molecules and endocannabinoids are able to recognize identical proteins (Di Marzo et al. 1999). Capsaicin and olvanil activate a subpopulation of primary sensory neurons, which can then become refractory to subsequent stimuli (desensitization). As such nerves mediate vasodilation, it has been proposed that anandamide and capsaicin may act as endogenous VR1 receptor modulators and thereby participate in the regulation of nociception, visceral reflexes, and local vasodilation. Anandamide induces mesenteric vasodilation by activating VR1 receptor, thereby causing release of CGRP, a known vasodilator neuropeptide (Zygmunt et al. 1999). The endothelium-independent component of the vasorelaxant effect of anandamide in rabbit aortic rings is inhibited by the VR1 receptor antagonist capsazepine (Mukhopadhyay et al. 2002). This also indicates existence of activation sites of anandamide distinct from CB1 and CB2 receptors, notably in this case, the VR1 receptor.

2.3.3.5 A putative novel "endothelial anandamide" receptor

In 1999, J arai and colleagues demonstrated that anandamide-induced mesenteric vasodilation persists in mice deficient either in CB1 receptors or in both CB1 and CB2 receptors. In rat mesenteric vessels anandamide caused relaxation partly via an endothelial anandamide receptor, sensitive to CB1 receptor antagonist SRI41716A and to the removal of endothelium (Jarai et al. 1999). Subsequently, they demonstrated that the endothelial cannabinoid receptor is also activated by the neurobehaviorally inactive cannabinoid compound "abnormal cannabidiol" (abn cbd) which, similar to anandamide, causes vasorelaxation that remains unchanged in mice lacking both CB1 and CB2 receptors, and that is inhibited by cannabidiol.

In mesenteric arteries capsazepine did not affect abn cbd-induced vasodilation at a

concentration that inhibited the effect of capsaicin, thus ruling out a role for VR1 receptors in the endothelium-dependent effect of abn cbd (Offertaler et al. 2003).

Further characterization of the "endothelial cannabinoid" receptor lead to the finding, that the synthetic cannabinoid ligands abn cbd and O1918 act as a selective agonist and a selective silent antagonist of a vascular endothelial receptor, respectively (Offertaler et al. 2003). As O1918 also inhibits the mesenteric vasorelaxant effect of anandamide, it was concluded that the same endothelial receptor is the site of anandamide action. Neither abn cbd, nor O1918 binds to CB1 or CB2 receptors, indicating that this novel "endothelial anandamide receptor" is distinct from CB1 and CB2 receptors.

This novel receptor is also involved in the relaxation of isolated human pulmonary arteries elicited by abnormal cannabidiol (Kozłowska et al. 2007) and virodhamine (Kozłowska et al. 2008). In addition, this O1918-sensitive novel receptor was shown to be involved in the delayed hypotension induced by anandamide in anesthetized rats (Zakrzewska et al. 2010).

In the rabbit isolated aortic ring preparation, anandamide was shown to interact with a nonCB1/CB2 endothelial receptor coupled to G_i/G_o and NOS, supported by inhibition of the vasorelaxant effect by pertussis toxin and by NOS inhibitor nitro-L-arginine-methyl ester (L-NAME), respectively (Mukhopadhyay et al. 2002). Pertussis toxin was shown to inhibit the abn cbd-induced endothelium-dependent vasodilation in rat mesenteric artery segments, while abn cbd-induced mesenteric vasorelaxation was shown to be independent of endothelial NO, as L-NAME did not inhibit abn cbd caused vasodilation (Offertaler et al. 2003). This confirmed the involvement of G_i/G_o in the abn cbd induced vasodilation.

Regarding the signal transduction mechanisms coupled to G_i/G_o , it has been shown that abn cbd induces phosphorylation of p42/44 MAP kinase and protein kinase Akt in human umbilical vein endothelial cells, which is inhibited by pertussis toxin and the "endothelial anandamide" receptor antagonist O1918. Another possible effector mechanism coupled to this receptor is the involvement of endothelial K^+ channels, as the vasorelaxant effect is inhibited by charybdotoxin, an inhibitor of K_{Ca} channels and delayed rectifier K^+ channels (Offertaler et al. 2003). This may be possibly related to an EDHF release (Edwards et al. 1998).

2.3.3.6 Other putative cannabinoid receptors

In the central nervous system, other receptors of endocannabinoids may exist, which are distinct from the ones discussed above. Anandamide and the synthetic cannabinoid agonist WIN55,212 2 have been shown to stimulate guanosine triphosphate labeling in brain plasma membrane preparations (Breivogel et al. 2001) and to inhibit glutamatergic synaptic transmission in hippocampal slices (Hajos et al. 2001) in preparations obtained from CB1-receptor deficient mice. The possibility that these receptors are different from the above discussed "endothelial anandamide" receptor is supported by observations that these neuronal sites seem to be uniquely sensitive to activation by WIN55,212 (Breivogel et al. 2001 and Hajos et al. 2001), which was found to be devoid of vasodilator activity in rat mesenteric arteries (Wagner et al. 2001) and in rat aortic rings (Mukhopadhyay et al. 2002).

In rat hippocampal slices, it was observed that abn cbd does not inhibit glutamatergic excitatory postsynaptic potentials (Offertaler et al. 2003). In addition, since Δ^9 -THC induces a CB1/CB2 receptor independent release of CGRP from sensory nerve terminals (Zygmunt et al. 2002) and cannot induce mesenteric vasorelaxation (Wagner et al. 2001), it is likely that this site also differs from the "endothelial anandamide" receptor. Therefore it is reasonable to predict the existence of yet unidentified cannabinoid receptors in the central nervous system and in other tissues.

2.3.4 Cerebrovascular and hemodynamic effects of endocannabinoids

The cardiovascular actions of cannabinoids have been studied extensively since the identification of the biologically active constituents of marijuana (termed later as phytocannabinoids) in the 1960s, and the discovery of ECs in the early 1990s (Pacher and Steffens 2009, Kunos et al. 2000, Randall et al. 2002, Montecucco and Di Marzo 2012). Cannabinoids play a role in the control of systemic cardiovascular parameters such as blood pressure or cardiac output, as well as in the regulation of the regional vascular resistance and the blood supply to different organs and tissues including the cerebral circulation. Batkai et al. showed in hypertensive adult rats that endocannabinoids tonically suppress cardiac contractility and that enhancing the CB1-mediated effects of endogenous anandamide by blocking its hydrolysis can normalize blood pressure (Batkai et al., 2004). Upon its intravenous bolus injection into anesthetized rats and mice, anandamide was found to elicit a triphasic blood pressure

response and bradycardia (Pacher et al. 2005). The first vagally mediated phase of the response consists of a precipitous drop in heart rate and blood pressure that lasts for a few seconds only. This vagal component is followed by a brief pressor response, which persists in the presence of α -adrenergic blockade and also in rats in which sympathetic tone is abolished by pithing, and is thus not sympathetically mediated (Varga et al. 1995). This pressor component is also unaffected by CB1 receptor antagonists and it persists in CB1 knockout mice (Járai et al. 1999; Pacher et al. 2004), indicating the lack of involvement of CB1 receptors. The third, and most prominent, phase in the effect of anandamide is hypotension associated with moderate bradycardia that last about 2–10 minutes, which is absent in conscious normotensive rats (Stein et al. 1996; Lake et al. 1997a), but is present and more prolonged in conscious, spontaneously hypertensive rats (Lake et al. 1997b; Bátkaï et al. 2004). Since sympathetic tone is known to be low in conscious, undisturbed normotensive rats (Carruba et al. 1987), these observations support that anandamide-induced hypotension and bradycardia involves a sympatho-inhibitory mechanism.

Literature on the cerebrovascular effects of cannabinoids is controversial. Marijuana smoking has been reported to elevate CBF in correlation with the increasing plasma levels of Δ 9-THC (Mathew et al. 1992). In accordance, Δ 9-THC was able to increase the cerebral blood perfusion of dogs and humans (Beaconsfield et al. 1972, Mathew et al. 1999, Mathew et al. 2002). According to previous observations CB1 receptors mediate vasorelaxation in cerebral vessels (Ellis et al. 1995, Gebremedhin et al. 1999, Wagner et al. 2001). Both anandamide and the CB1 receptor agonist HU-210 resulted in enhancement of the CBF in anesthetized rats, effects which could be prevented by the CB1 receptor antagonist SR141716A (Wagner et al. 2001).

In contrast, conscious rats responded with a reduction of the CBF upon intravenous administration of Δ 9-THC or anandamide (Stein et al. 1998 and Bloom et al. 1997). These discrepancies are not surprising, if we consider the variety of mechanisms by which cannabinoids may influence cerebral circulation. One obvious target is the cerebral vasculature itself, which appears to respond with vasodilation in a CB1-dependent manner (Ellis et al. 1995, Gebremedhin et al. 1999, Wagner et al. 2001). The cerebral circulation, however, is tightly regulated by neuronal mechanisms (Sándor 1999, Hamel 2006, Ruisanchez et al. 2012), and in these pathways endocannabinoids

may act as modulators of the synaptic transmission. CB1 receptors were identified on peripheral sympathetic neuronal endings, which mediate the inhibition of NE release from sympathetic nerve terminals (Wagner et al. 1998), thereby affecting cerebral circulation. Support for this comes from several studies. First, mRNA for the CB1 receptor is detected in a sympathetic ganglion, the superior cervical ganglion of the rat, which would be expected if a receptor was present on sympathetic nerve terminals (Ishac et al., 1996). Second, the CB1 receptor agonist Win 55212-2 decreases the spillover of NE into the plasma in pithed rabbits with continuously stimulated sympathetic neuronal activity (Niederhoffer and Szabo, 1999).

In addition, endocannabinoids may have indirect effects on cerebral circulation by influencing the metabolic demand of neurons, or respiration. It was observed in rhesus monkeys that WIN55212, anandamide and Δ -9-THC all suppress respiratory function, notably decrease tidal volume, while not affecting respiratory frequency, which could be reversed with SR141716A (Jeffrey AV et al. 1998).

The endocannabinoids anandamide (ANA) and 2-arachydonyl glycerol (2-AG) activate the CB1 cannabinoid receptors located on pial vessels, smooth muscle cells derived from cerebral microvessels, and human brain endothelium thereby contributing to endothelium dependent relaxation (Chen et al., 2000). In vascular endothelial cells, ANA increases intracellular Ca^{2+} levels, sufficient to activate K_{Ca} channels (Mombouli et al. 1999). ANA causes a concentration-dependent release of Ca^{2+} from intracellular stores without triggering capacitative Ca^{2+} entry, contrary to histamine or the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (Mombouli et al. 1999). The mobilization of cytosolic Ca^{2+} from intracellular stores evoked by ANA was impaired by 10 mM caffeine and 5 μM of the CB1-receptor antagonist SR141716A (Mombouli et al. 1999). Similarly, vascular SMC potassium channels may also be activated to cause vasorelaxation. It is important, however, that different tissues show varying results concerning this possibility. The various potassium channel inhibitors, namely apamin, charybdotoxin, ciclazindol and glibenclamide were shown to have no effect on ANA-induced vasorelaxation in the guinea-pig basilar artery (Dong et al., 2000 and Petersson et al., 1997). The lack of effect of potassium channel blockers on ANA-induced relaxation suggests that EDHF and ANA are distinct chemical factors.

Endocannabinoids may also interact with several different pathways that regulate cerebral circulation, for instance the NO pathway, prostanoids, and endothelin-1 (ET-1). ET-1 is a strong vasoconstrictor, synthesized by the endothelium, following activation by arginine-vasopressin, adrenalin or thrombin. ET-1 acts on smooth muscle endothelin ETA receptors, thus activating PLC, producing inozitol-triphosphate 3, which causes an increase in intracellular Ca^{2+} levels (Dogulu et al., 2003). 2-AG was shown to reduce the ET-1-stimulated Ca^{2+} mobilization in human cerebral capillaries and human cerebral microvessels, which was reversed by the cannabinoid CB1 receptor antagonist, SR141716, indicating a possible role of CB1 receptor in the interaction of 2-AG with ET-1 (Chen et al., 2000). However, it was also observed that the signal transduction pathway for the 2-AG modulation of ET-1 stimulated Ca^{2+} mobilization was independent of NOS, COX and lipoxygenase activity, thus not mediated by cGMP or cAMP kinases, and instead mediated by G protein, inozitol phosphate hydrolysis and PKC, indicating that 2-AG affects the pathway distal to the CB1 receptor (Chen et al., 2000).

In support of the potential interaction of 2-AG and ET-1 in the regulation of endothelium-dependent vascular activity, is the observation that inhibitors of K_{Ca} channels or K^+ ions, modulate the 2-AG-induced reduction of ET-1-stimulated Ca^{2+} mobilization, with these channels being involved in vasorelaxation induced by the yet unknown EDHF. These studies indicate the presence of a functional interaction between 2-AG and ET-1 and show the importance of an endogenous cannabinoid as balancing the effects of ET-1 in several pathological conditions such as hypertension, arteriosclerosis and haemorrhagic shock (Chen et al., 2000).

ANA was also shown to interact with the prostanoid system, thus also taking part in the local regulation of cerebral circulation. Experimental evidence suggests a possible activation of phospholipase enzymes PLA, PLC, or PLD as a result of CB1 receptor activation (Felder et al. 1995). Cannabinoids were shown to induce free AA mobilization (Burstein et al. 1994). Also, ANA is degraded by the enzyme FAAH to ethanolamine and AA, and the latter stimulates prostaglandin formation and is known to be a dilator of cerebral arterioles (Ellis et al., 1990). In addition, it was shown that ANA-induced vasodilation of rabbit cerebral arterioles was inhibited by indomethacin, a

COX inhibitor, which gives the possibility of a COX-dependent mechanism to mediate ANA-induced vasodilation (Ellis et al., 1995).

In support of its interaction with the prostanoid system, ANA was shown to activate CB1 receptors located on astrocytes, which are known to play active roles in regulating both neuronal and cerebrovascular function (Shivachar et al. 1996). Cannabinoid receptors may be linked to astrocyte signaling, namely to AA release, substrate for various eicosanoids, that affects cerebrovascular activity. Astrocytes are also capable of metabolizing exogenous AA via COX, lipoxygenase, and cytochrome P450 epoxygenase pathways (Shivachar et al. 1996). Furthermore, prostaglandins, epoxyeicosatrienoic acids, AA, K⁺ ions, all of which may be released by astrocytes, are candidate members of communication between astrocyte end-feet and vascular smooth muscle, taking part in the flow-metabolism coupling (Koehler et al. 2006).

Both anandamide and 2-AG are oxygenated by COX-2 at postsynaptic sites (Katona and Freund 2012). The resulting prostanoids, for example prostaglandin E₂ glycerol ester, increase neurotransmitter release from axon terminals, which is opposite to the effect of 2-AG in the retrograde signaling, and thus COX-2 may be an important molecular switch to change the direction of synaptic retrograde signaling (Katona and Freund, 2012).

ANA also interacts with the NO system. Previous studies have shown that endocannabinoids may induce NO release via the constitutive endothelial NOS, by activating the cannabinoid CB1 receptor (Stefano et al., 1998, and Fimiani et al., 1999). ANA also stimulates the activity and expression of the inducible endothelial NOS which is blocked by the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) (Maccarrone et al., 2000). ANA-induced NO release was sensitive to the selective antagonist of CB1 receptor, the SR141716A, suggesting the involvement of CB1 receptors in this signaling pathway (Maccarrone et al., 2000). In addition, in vivo blocking the reuptake of ANA with the AT protein inhibitor AM404 suppressed ex vivo NO production in sciatic nerve homogenates, indicating that ECs may negatively regulate NO release from nitroxidergic nerves (Costa et al. 2006).

The interaction of the endocannabinoid system with the NO system also includes the observation that NO enhances the uptake of 2-AG by cells (Bisogno et al., 2001), and

uptake of ANA by human umbilical vein endothelial cells (HUVEC), by potentiating the activity of the anandamide transporter (AT) (Maccarrone et al., 2000). In general, the NO system may be part of a physiological regulation of endocannabinoid uptake, possibly linked to the activation of CB1 receptor (Maccarrone et al., 2000). This may be a new mechanism, through which ANA and 2-AG can limit their CB1 mediated actions.

In guinea-pig isolated basilar arteries, ANA was shown to evoke a complete relaxation in endothelium-denuded arterial segments, which failed to respond to ACh, suggesting that vasodilator response to ANA may also involve endothelium-independent mechanisms (Zygmunt et al., 1999, Ishioka and Bukoski, 1999). This suggests that ANA may be involved in the EDHF-mediated relaxation in some tissues, but not in others, and also shows that EDHF is not a single substance, but rather a common mechanism that acts through different substances in the various tissues.

3. THE AIMS OF OUR INVESTIGATIONS

As mentioned above, 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 heme oxygenase pathway and the endocannabinoid system on cerebrocortical blood flow (CBF) under resting conditions as well as during combined hypoxia and hypercapnia.

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 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.

4. MATERIALS AND METHODS

4.1 Experimental animals, anaesthesia and surgical procedures

The experiments were carried out on 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). The experimental animals were obtained from Toxi-Coop Kft. (Dunakeszi, Hungary.) During the *in vivo* experiments, the animals were 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.

4.2 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 (aPCO₂, aPO₂ and O₂-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 °C with a controlled heating pad.

4.3 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 (Moor Instruments, Devon, UK). LD flowmetry is a method of continuous and noninvasive measuring of the tissue blood flow, utilizing the Doppler shift of laser light as the information carrier (Fabricius et al., 1997). In experimental medicine, laser-Doppler flowmetry has been used in the study of spontaneous rhythmical variations as well as in the study of spatial and temporal

fluctuations in human skin blood flow. In our experiments, the skull of the rat was fixed in a stereotaxic head holder with the nose 5 mm down from the interaural line, 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 probes were placed above both holes of the thinned skull at a 12°-angle to the vertical, to provide an optimal view of the cortex (4 mm caudal from the bregma, 5 mm lateral from the midline). LD flow (LDF) was measured with a two-channel LDF monitor (Moor Instruments) and recorded continuously (Lacza et al. 2000). The 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 mm² of the parietal region, so that the data acquired represented the characteristics of the parietal cortex (Haberl et al. 1989). The two LD flow data sets gained from the parietal region of both hemispheres of the animal were evaluated and treated as independent measurements.

4.4 Experimental Protocols

4.4.1 The influence of HO pathway on cerebrocortical blood flow

4.4.1.1 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. 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, CA, USA), according to Johnson FK et al. 2006. 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 remaining matched aliquots after being incubated at 37°C for 60 min. The differences between the incubated and cold vials were expressed as $\mu\text{mol CO generated/kg of wet tissue per hour}$. ZnDPBG induced a reduction of cerebral HO activity from 4.58 ± 0.87 to $2.47 \pm 0.36 \mu\text{mol/kg per hour}$ ($p = 0.025$).

In order to investigate the interaction of NO and CO 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-nitro-L-arginine methyl ester) in a dosage of 50 mg/kg, 30 minutes prior to the administration of saline ($n=5$) or ZnDPBG ($n=6$), respectively. In this case, the CBF of the L-NAME treated rats was determined before as well as 15, 30, and 45 minutes after the administration of saline or ZnDPBG.

4.4.1.2 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% O₂-20% CO₂-75% N₂ for producing moderate H/H and 20% CO₂-80% N₂ for producing severe H/H) with a constant flow rate of 3 L/min into a 5 mL chamber connected to the trachea at atmospheric pressure. 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. (For the CBF values before and after the H/H, please see Figure 4). 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.

4.4.2 Influence of endocannabinoids on cerebral circulation

4.4.2.1 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 emulphor 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.

4.4.2.2 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. Since one side of the chamber was open, the pressure in the chamber was not different from the atmospheric pressure. 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% O₂-10% CO₂-80% N₂, which produced arterial pO₂ of 75-85 mmHg and pCO₂ of 50-60 mmHg. In the second step, during moderate H/H, animals inhaled a gas mixture of 5% O₂-20% CO₂-75% N₂, which produced arterial pO₂ of 55-65 mmHg and pCO₂ of 80-90 mmHg. In the third step, during severe H/H, animals inhaled gas mixtures of 0% O₂-20% CO₂-80% N₂, which produced arterial pO₂ of 45-50 mmHg and pCO₂ 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.

4.5 Drugs and chemicals

ZnDPBG (zinc deuteroporphyrin 2,4-bis glycol) was purchased from Frontier Scientific, Logan, UT, USA, and AM251 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4methyl-N-1-piperidiny-1H-pyrazole-3-carboxamide) from Cayman Chemicals, Ann Arbor, MI, USA. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.6 Statistical analysis

Statistical analysis was performed using Student's t-test for comparison 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.

5. RESULTS

5.1 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) (Table I). Neither ZnDPBG nor its vehicle induced any significant changes in the mean arterial blood pressure (Figure 4.) or in the heart rate (data not shown). In contrast, administration of ZnDPBG but not its vehicle resulted in a marked increase of the CBF (Figure 5.).

	Treatment	0 min	15 min	30 min	45 min
Hemoglobin conc. (mg/ml)	<i>Vehicle</i>	15.1 ± 0.3	14.5 ± 0.3	14.5 ± 0.4	14.5 ± 0.4
	<i>ZnDPBG</i>	16.2 ± 0.3	16.0 ± 0.3	15.9 ± 0.3	16.0 ± 0.3
pH	<i>Vehicle</i>	7.34 ± 0.01	7.35 ± 0.01	7.34 ± 0.01	7.33 ± 0.01
	<i>ZnDPBG</i>	7.36 ± 0.02	7.36 ± 0.02	7.36 ± 0.02	7.35 ± 0.02
PaO₂ (mmHg)	<i>Vehicle</i>	89.9 ± 2.7	89.6 ± 2.4	89.9 ± 2.7	89.3 ± 2.9
	<i>ZnDPBG</i>	94.6 ± 3.3	93.8 ± 3.1	92.5 ± 3.6	89.5 ± 3.5
PaCO₂ (mmHg)	<i>Vehicle</i>	44.2 ± 1.4	42.6 ± 1.2	41.9 ± 1.2	42.9 ± 0.7
	<i>ZnDPBG</i>	42.9 ± 1.4	41.4 ± 1.6	41.6 ± 1.8	42.0 ± 2.0
O₂ Saturation (%)	<i>Vehicle</i>	95.8 ± 0.4	96.0 ± 0.3	95.9 ± 0.4	95.8 ± 0.3
	<i>ZnDPBG</i>	96.5 ± 0.4	96.5 ± 0.4	96.3 ± 0.5	95.9 ± 0.5
Standard Base Excess (mmol/l)	<i>Vehicle</i>	-2.0 ± 0.7	-2.3 ± 0.7	-2.7 ± 0.7	-2.9 ± 0.5
	<i>ZnDPBG</i>	-1.0 ± 0.8	-1.4 ± 0.9	-1.4 ± 0.9	-1.6 ± 0.8

Table I: Physiological parameters of the arterial blood before (0 min) and at 15, 30 and 45 min after the intraperitoneal administration of 45 µmol/kg zinc deuteroporphyrin 2,4 bis glycol (ZnDPBG) or its vehicle (saline) in anesthetized rats (n=9-9). There were no significant differences between the vehicle-treated and ZnDPBG-treated groups or within one of the two experimental groups at different timepoints.

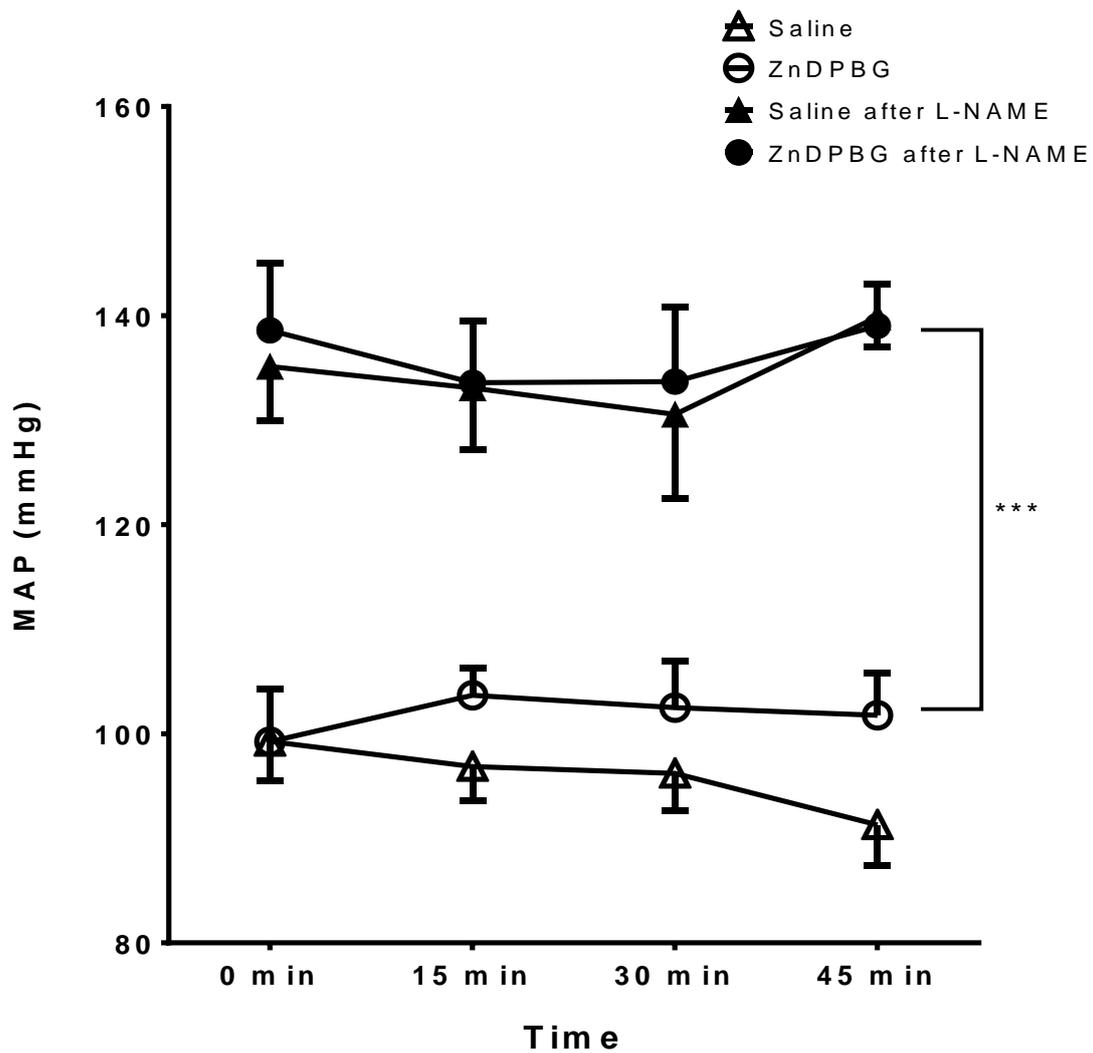


Figure 4: Mean arterial blood pressure (MAP) are shown before (0 min) as well as 15, 30, 45 min after intraperitoneal injection of saline (triangles, n=9) or 45 $\mu\text{mol/kg}$ zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) (circles, n=9) in control rats that did not receive N-nitro-L-arginine methyl ester (L-NAME) (open symbols, n=5 for saline after L-NAME and n=6 for ZnDPBG after L-NAME) or in L-NAME pre-treated (filled symbols) urethane-anaesthetized rats. Values are presented as mean \pm SEM. At all four timepoints, there was a significant (***) increase of MAP in L-NAME pre-treated animals, compared to rats with intact NO synthesis.

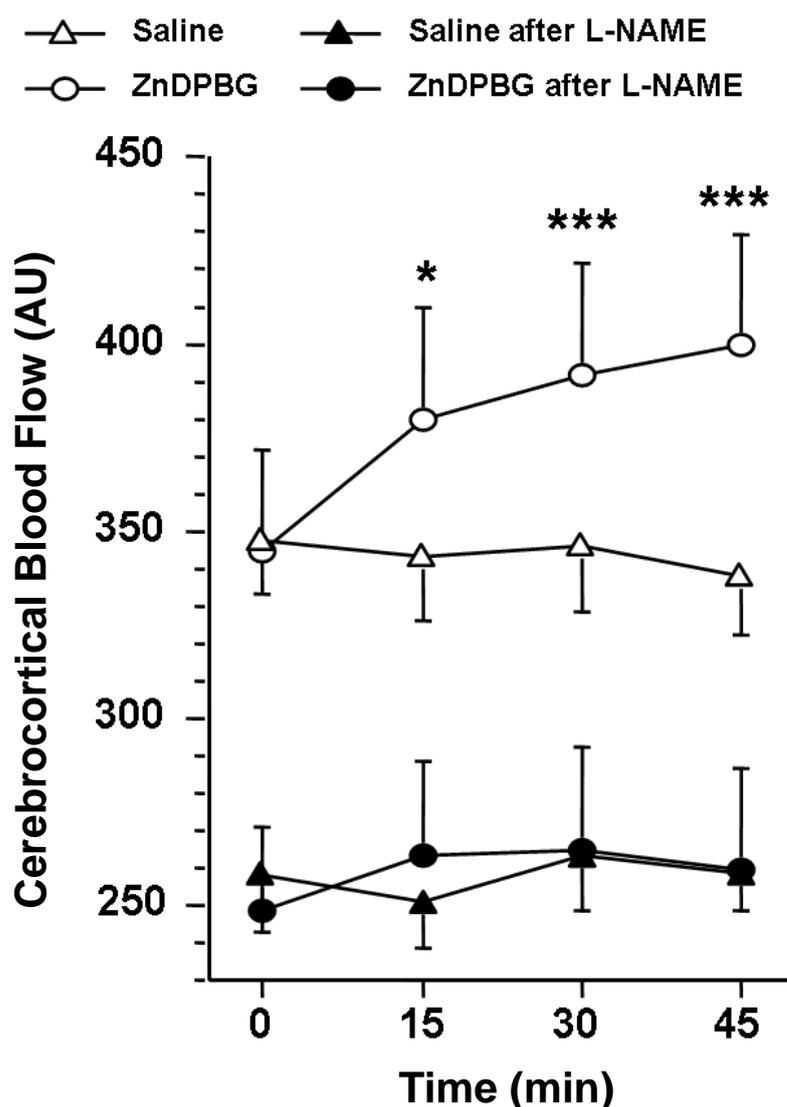


Figure 5: Cerebrocortical blood flow (CBF) before (0 min) as well as 15, 30, 45 min after intraperitoneal injection of saline (triangles) or 45 micromol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG; circles) in control (open symbols) or in N-nitro-L-arginine methyl ester (L-NAME) pretreated (filled symbols) rats. * $p=0.016$, *** $p<0.001$ vs. '0 min.' with repeated measures analysis of variance and Tukey's post hoc test, $n=10-16$. 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 (Table II). In L-NAME pre-treated animals, neither ZnDPBG nor its vehicle induced any significant changes in

the mean arterial blood pressure (Figure 4.) or arterial blood gas and acid-base parameters (Table II.). Most importantly, L-NAME prevented the enhancement of the CBF after inhibition of the endogenous CO production by administration of ZnDPBG (Figure 5.).

	Treatment	0 min	15 min	30 min	45 min
Hemoglobin conc. (mg/ml)	<i>L-NAME</i> + <i>Vehicle</i>	16.2 ± 0.4	16.4 ± 0.4	16.4 ± 0.3	16.2 ± 0.4
	<i>L-NAME</i> + <i>ZnDPBG</i>	16.1 ± 0.4	16.0 ± 0.4	16.0 ± 0.3	15.8 ± 0.4
pH	<i>L-NAME</i> + <i>Vehicle</i>	7.35 ± 0.02	7.36 ± 0.02	7.35 ± 0.02	7.34 ± 0.02
	<i>L-NAME</i> + <i>ZnDPBG</i>	7.36 ± 0.01	7.34 ± 0.01	7.33 ± 0.01	7.35 ± 0.02
PaO₂ (mmHg)	<i>L-NAME</i> + <i>Vehicle</i>	99.2 ± 5.1	96.6 ± 3.6	99.9 ± 7.6	102.2 ± 7.2
	<i>L-NAME</i> + <i>ZnDPBG</i>	95.6 ± 4.3	95.9 ± 2.4	100.9 ± 6.3	106.2 ± 6.9
PaCO₂ (mmHg)	<i>L-NAME</i> + <i>Vehicle</i>	45.5 ± 1.8	43.9 ± 1.9	44.4 ± 1.6	44.6 ± 2.4
	<i>L-NAME</i> + <i>ZnDPBG</i>	44.8 ± 1.7	44.3 ± 2.8	43.3 ± 2.9	44.7 ± 3.2
O₂ Saturation (%)	<i>L-NAME</i> + <i>Vehicle</i>	95.5 ± 0.9	95.5 ± 0.9	95.2 ± 0.9	95.6 ± 1.1
	<i>L-NAME</i> + <i>ZnDPBG</i>	96.0 ± 0.7	96.0 ± 0.7	96.0 ± 0.8	96.4 ± 1.1
Standard Base Excess (mmol/l)	<i>L-NAME</i> + <i>Vehicle</i>	0.1 ± 0.6	-0.9 ± 0.9	-1.0 ± 0.7	0.2 ± 0.9
	<i>L-NAME</i> + <i>ZnDPBG</i>	-0.4 ± 0.7	-1.5 ± 0.9	-1.8 ± 0.9	-0.6 ± 1.3

Table II: Physiological parameters of the arterial blood before (0 min) and after (at 15, 30 and 45 min) administration of zinc deuteroporphyrin 2,4-bis glycol or its vehicle (saline) in N-nitro-L-arginine methyl ester pre-treated animals. (n=5-6)

5.2 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, PaO₂ was reduced to 60-65 mmHg, PaCO₂ was increased to 80-85 mmHg (Figure 6.) 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 7A.). During severe H/H, PaO₂ was reduced to 45-50 mmHg, PaCO₂ was increased to 95-100 mmHg (Figure 6.) and pH decreased to 7.05-7.10; CBF increased in both experimental groups by ~65% (Figure 7B.), whereas MAP remained unchanged (data not shown).

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 6.), without affecting MAP (data not shown). Most importantly, neither ZnDPBG nor saline treatment had any effect on CBF responses to moderate (Figure 7A.) or severe (Figure 7.B.) H/H, when compared to the pretreatment values.

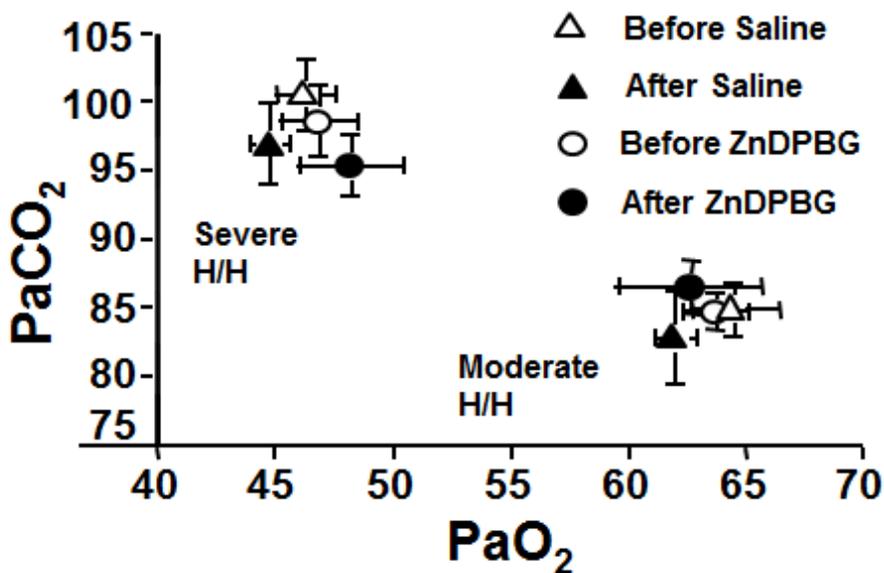


Figure 6: Arterial blood-gas tensions during mild and severe H/H before (open symbols) and after (filled symbols) intraperitoneal injection of saline (triangles) or 45 μmol/kg zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG; circles), (n=8-8).

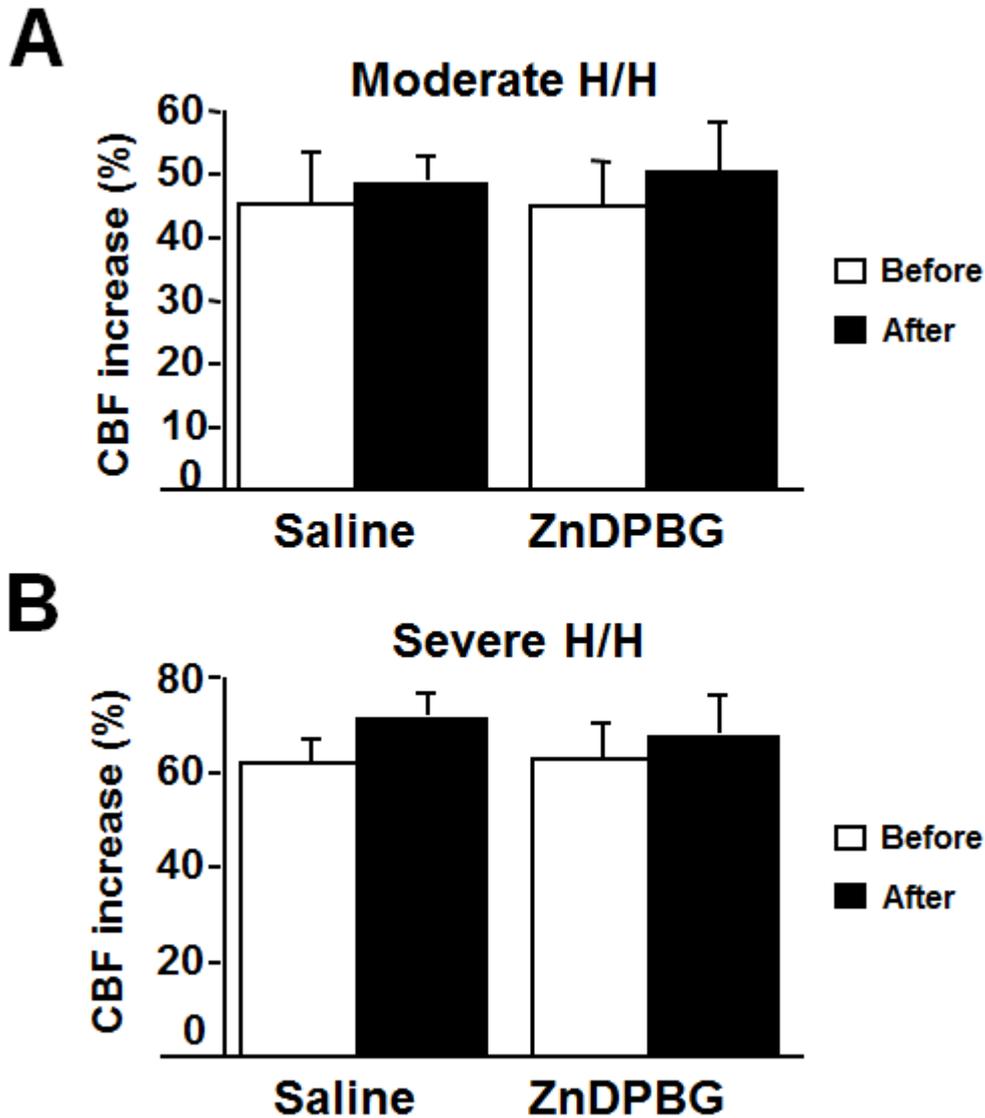


Figure 7: Changes of the cerebrocortical blood flow (CBF) during moderate (A) and severe (B) hypoxia/hypercapnia (H/H) before (open bars) and after (filled bars) intraperitoneal injection of saline or 45 $\mu\text{mol/kg}$ zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), (n=16-16).

5.3 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/inverse agonist AM251 in a dose of 10 mg/kg, which had been shown to be effective in blocking CB1 receptors in previous studies (Gatley et al. 1996; Van der Stelt et al. 2001). Vehicle-treated animals served as controls. Neither AM251 nor its vehicle induced any

significant changes in the mean arterial blood pressure MAP (Figure 8A.), heart rate HR (Figure 8B.) or CBF (Figure 8C.) up to 45 minutes after their administration. Furthermore, arterial blood gas tensions and acid-base parameters remained unchanged during the observation period (Table III.). 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.

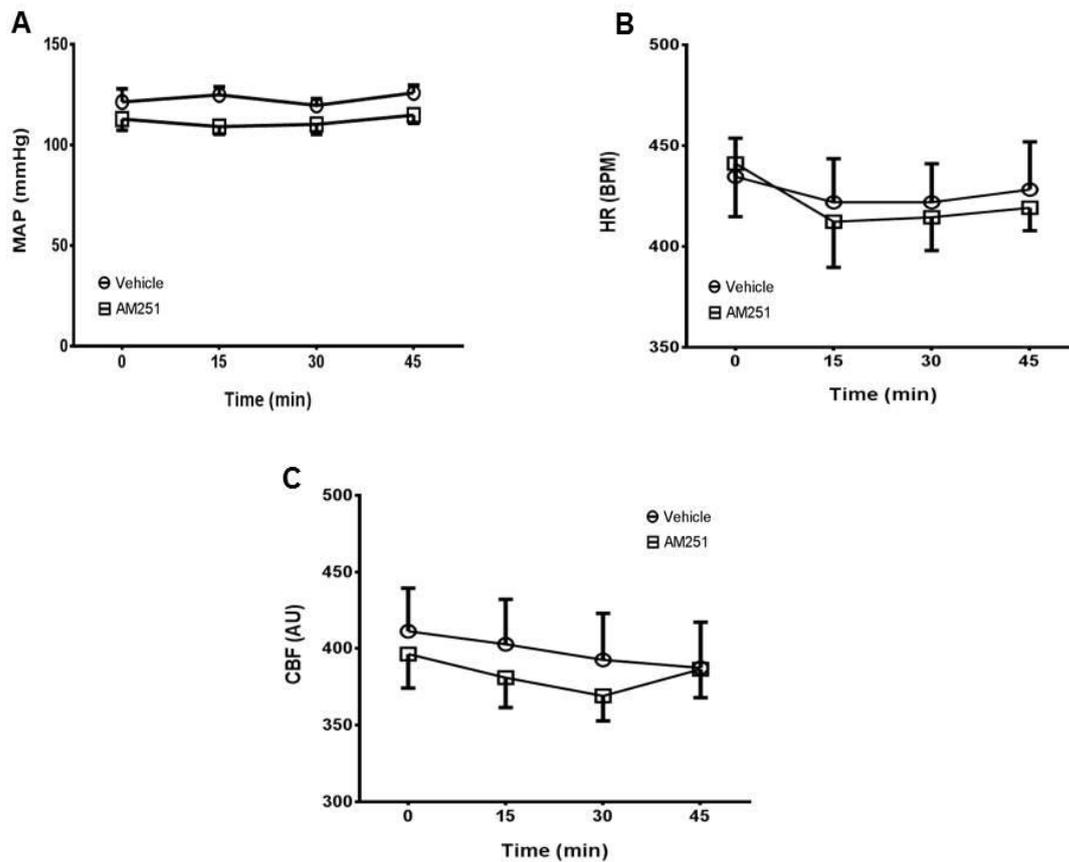


Figure 8: Mean arterial blood pressure (MAP, panel A), heart rate (HR, panel B) and cerebrocortical blood flow (CBF, panel C) are shown before (0 minute) as well as 15, 30, 45 minutes after intravenous administration of 10 mg/kg of the CB 1 receptor antagonist AM251 (open squares, n=7) or its vehicle (open circles, n=5) in urethane-anesthetized rats. Values are presented as mean \pm SEM. No significant change of the measured variables was found at any given time points after the administration of vehicle or AM251. AU, arbitrary unit.

	Treatment	0 min	15 min	30 min	45 min
pH	<i>Vehicle</i>	7.40 ± 0.01	7.36 ± 0.01	7.36 ± 0.01	7.37 ± 0.01
	<i>AM251</i>	7.40 ± 0.01	7.37 ± 0.01	7.39 ± 0.01	7.39 ± 0.01
Arterial O₂ (PaO₂) (mmHg)	<i>Vehicle</i>	91.0 ± 2.5	82.2 ± 3.8	83.2 ± 2.4	85.4 ± 3.7
	<i>AM251</i>	86.3 ± 1.3	80.3 ± 2.6	86.3 ± 1.6	86.1 ± 1.3
Arterial CO₂ pressure (PaCO₂) (mmHg)	<i>Vehicle</i>	40.6 ± 1.4	42.4 ± 1.9	42.2 ± 2.1	41.2 ± 1.7
	<i>AM251</i>	40.4 ± 1.7	41.6 ± 0.7	38.4 ± 0.7	37.1 ± 1.1
O₂ Saturation (%)	<i>Vehicle</i>	97.5 ± 0.2	95.2 ± 0.2	95.4 ± 0.3	95.8 ± 0.4
	<i>AM251</i>	96.6 ± 0.2	95.3 ± 0.4	96.5 ± 0.3	96.5 ± 0.2

Table III: Arterial blood gas and acid-base parameters before (0 min) and after (15, 30, and 45 min) the intravenous administration of 10 mg/kg AM251 (n=7) or its vehicle (n=5)

There were no significant differences between the vehicle-treated and AM251 treated groups or within one of the two experimental groups at different timepoints.

5.4 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 AM251 or its vehicle, and determined the changes in CBF. Inhalation of three different gas mixtures containing decreased O₂- and increased CO₂-content (as compared to air) induced reproducible levels of hypoxia and hypercapnia before and after the intravenous administration of 10 mg/kg AM251

(Figure 9B.) or its vehicle (Figure 9A.), without significant changes in the BP (data not shown). The H/H-induced enhancement of CBF was identical before and after the administration of the vehicle (Figure 10A.). In contrast, AM251 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 10B.).

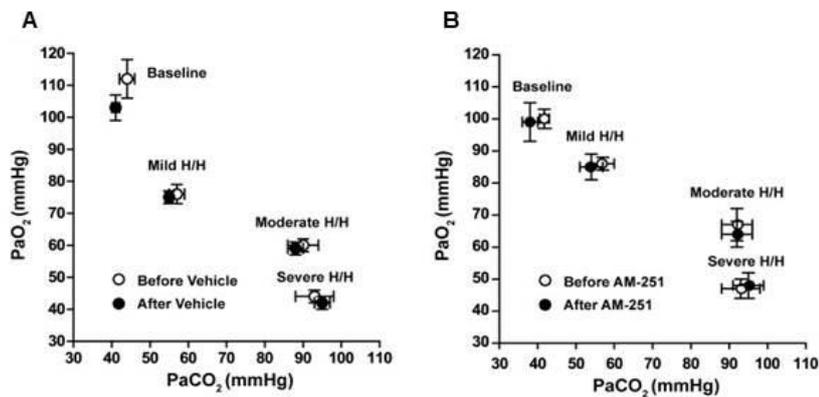


Figure 9: Inhalation of three different gas mixtures containing decreased O₂- and increased CO₂-content (as compared to air) induced reproducible levels of hypoxia and hypercapnia before and after the administration of AM251 (Figure 9B.) or its vehicle (Figure 9A.).

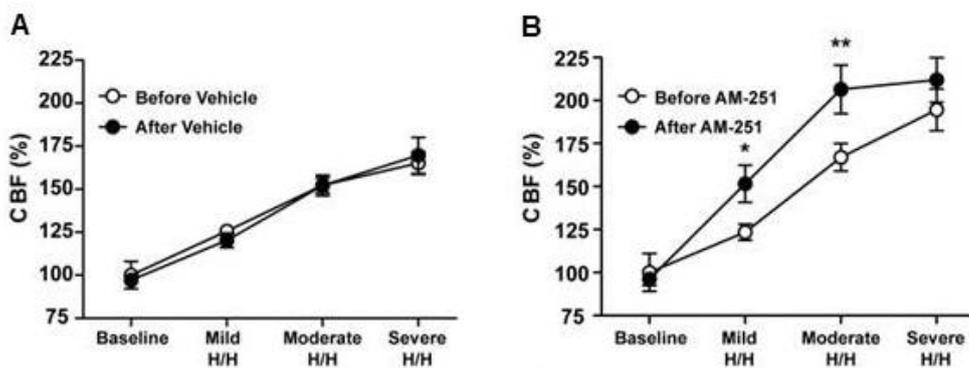


Figure 10: The hypoxia/hypercapnia (H/H) induced enhancement of the cerebrocortical blood flow (CBF) was identical before and after the administration of the vehicle of AM251 (Figure 10A.). In contrast, AM251 resulted in markedly increased CBF changes during mild and moderate H/H (by $28.1 \pm 8.8\%$ and $39.4 \pm 10.0\%$, respectively) without significantly influencing the peak CBF during severe H/H (Figure 10B.).

6. DISCUSSION

6.1 Role of heme oxygenase metabolites in the regulation of the cerebral circulation during resting conditions and during hypoxia and hypercapnia

Our present study provides evidence for an interaction between the HO and NOS pathways in the regulation of the cerebrocortical blood flow. We show clearly, that administration of the HO-inhibitor ZnDPBG causes an increase in the CBF under physiological (normoxic/normocapnic) resting conditions. ZnDPBG had been previously shown to effectively inhibit HO activity in the rat brain (Johnson RA et al. 1995). In addition, ZnDPBG was shown to effectively inhibit HO activity in human and rat brain microsomes (Appleton et al. 1999, Chernick et al. 1989). In our studies ZnDPBG was preferred to other metalloporphyrins, because it has been shown that its ip. administration in the dose used in our experiments induced identical cardiovascular effects to those observed after topical administration of the drug into the nucleus tractus solitarii (NTS) of rats. Furthermore, microinjection of CO into NTS reversed the effects of the ip. applied ZnDPBG, indicating that ZnDPBG when ip. injected in this dose, crosses the blood-brain barrier and inhibits the cerebral HO activity in rats (Johnson et al. 1997).

The CBF-increase after administration of ZnDPBG and its inhibition with L-NAME pretreatment indicate that constitutive CO release tonically suppresses NO production and consequently reduces blood flow in the cerebral cortex. Several recent studies suggest that its interaction with NOS may modulate significantly the overall vascular effects of CO (Johnson et al. 2002 and Ndisang et. al. 2004). Furthermore, L-NAME was shown to augment both the reduction of renal blood flow and the contraction of isolated renal interlobular arteries in response to the HO inhibitor stannous mesoporphyrin (Rodriguez et al. 2003). Since endogenous CO was shown to relax smooth muscle cells via activation of sGC and large-conductance K_{Ca} channels (Johnson RA et al. 1995), it appears that this mediator, depending on the mechanism of its action, may induce both vasoconstriction and vasodilation.

Our findings should be interpreted in light of the recent, independent study by Ishikawa et al. This study demonstrated that HO-2 is co-localized with eNOS in the cerebrovascular endothelium and with nNOS in neurons and arachnoid trabecular cells

providing the anatomical basis for interactions between the CO- and NO-generating pathways (Ishikawa et al. 2005). Furthermore, tricarbonyldichlororuthenium (II) dimer, a CO-releasing molecule, reduced NO-release from cultured endothelial cells. In accordance, HO blockade by zinc protoporphyrin IX (ZnPPIX) induced CO- and L-NAME-reversible increase of the cerebrovascular and perivascular NO-production by 70-80%, which effect is comparable to the ZnDPBG-induced 67% increase of the NOS activity in the rat hypothalamus observed in our previous study (Horváth et al. 2003). Most importantly, ZnPPIX induced a dose-dependent increase of the pial arteriolar diameter, which could be prevented by co-administration of CO or L-NAME, indicating the involvement of NO in mediating the vasodilation. Our findings confirm and extend these observations by providing direct evidence for the significance of the CO-NOS interaction at the level of cerebrocortical blood perfusion.

In our previous study (Horváth et al. 2003), ZnDPBG failed to relax the isolated middle cerebral artery while Ishikawa et al. (Ishikawa et al. 2005) reported marked pial arteriolar dilatation in response to ZnPP in rats. Since 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 non-vascular CO release or augmentation of non-vascular NO release plays an important role in the interaction leading to the NO-mediated cerebral vasodilation and hyperemia after inhibition of HO.

The second part of our study showed that the HO – CO pathway does not play a significant role in the regulation of the acute hyperemic response to hypoxia or hypercapnia in adult rats. However, it was also found that HO blockade significantly inhibited the pial arteriolar dilation in response to hypoxia or hypercapnia in newborn piglets (Carratu et al. 2003 and Leffler et al. 1999). Since the regulation of the CBF during hypoxia or hypercapnia shows marked changes during maturation (Armstead et al. 2005), the contribution of the HO pathway may likely be different in adult animals as in our study. Furthermore, we cannot exclude the possibility that endogenous CO by itself would facilitate the hyperemic response to H/H also in adult rats, but its simultaneous inhibitory influence on the synthesis of NO, a well established mediator of cerebral vasodilation during hypoxia and hypercapnia, masks this effect. Further studies may clarify this hypothesis and investigate the role of endogenous CO in the adaptation

of the cerebral circulation to chronic hypoxia where the expression of HO-1 is increased in the brain (Chang et al. 2005, Mazza et al. 2001).

6.2 Role of endocannabinoids in the regulation of the cerebral circulation during resting conditions and during hypoxia and hypercapnia

Several studies have described marked changes of the cerebral circulation after administration of cannabinoid compounds as it has been summarized in the “Introduction”. The effects of exogenously applied phyto- or endocannabinoids, however, may hardly resemble the functions of an endogenous control system. We were able to overcome these limitations, by focusing on the changes elicited by suppressed activity of the EC system. We used AM251, which works both as an antagonist and as an inverse agonist at CB1-receptors (Pertwee RG 2005, and Hanlon and Vanderah 2010). In our experiments, we did not observe any significant effect of AM251 on the systemic or cerebral circulation. It appears that CB1-receptors have no constitutive influence on the cardiovascular system under steady-state resting conditions, at least in healthy normotensive rats, which is consistent with reports on the normal hemodynamic profile of CB1-knockout mice (Mukhopadhyay et al. 2010 and Rajesh et al. 2012). CB1-receptors have been reported to tonically modulate various physiological functions either by their constitutive activity or by mediating the effects of constitutively released ECs (Pertwee 2005, Hanlon and Vanderah 2010). It is well known that both systemic BP and CBF are vital parameters of homeostasis, and therefore several backup regulatory mechanisms are involved in their maintenance. For this reason we cannot exclude the possibility that CB1-mediated pathways do contribute to steady-state BP- or CBF-regulation and when they are blocked pharmacologically or genetically, other control mechanisms may take over their function.

The last part of our study was devoted to investigate the role of CB1-receptors in H/H-induced CBF rise. In spite of the fact that the enhancement of CBF during H/H was the first well-described reaction of the cerebral circulation, its mechanism is still poorly understood. In the present study we found that blockade of CB1-receptors enhances CBF responses to H/H. Since it is well established that both neurons and astrocytes abundantly express CB1-receptors (Freund et al. 2003, Stella 2010), a CB1-mediated modulation of neuronal nitric oxide synthase activity may explain our observations,

indicating that ECs play an inhibitory role in the CBF response to H/H. In addition, in previous studies AM251 was reported to inhibit basal G-protein-activity in rat cerebellar membranes (Savinainen et al. 2003) and to enhance electrically evoked glutamate release from rat cerebellar neurons (Kreitzer et al. 2001).

CB1-agonists inhibit K^+ -induced NOS-activation in cerebellar granule neurons without influencing the basal NO-release from these cells, and the CB1-antagonist rimonabant both reversed the effect of CB1-activation and produced an increase in NOS activity that was additive with K^+ (Hillard et al. 1999). Furthermore, CB1-receptors reportedly inhibit both glutamatergic transmission (Freund et al. 2003) and the metabolic activity of neurons and astrocytes (Duarte et al. 2012), effects that may influence the release of NO and other vasoactive mediators and consequently alter CBF. Astrocytes are potential oxygen-sensors of this control system, since it is well established that hypoxia suppresses glutamate uptake by astrocytes (Vangeison and Rempe 2009), which may result in activation of the glutamate receptor-mediated release of vasoactive mediators from neuronal elements of the neurovascular unit. Moreover, it is well documented that the cerebrovascular responses to H/H are modulated by sympathetic perivascular nerves (Busija and Heistad 1984, Deshmukh et al. 1972, Harper et al. 1972, Wagerle et al. 1986, Goplerud et al. 1991), a pathway that may be modulated via prejunctional CB1-receptors. NE, ATP and neuropeptide Y are costored and coreleased from sympathetic vesicles in sympathetic nerves and prejunctional CB1 receptors inhibit these releases (Randall et al. 2004, Ralevic and Kendall 2009). Whatever is the exact mechanism by which CB1-receptors modulate the H/H-induced CBF rise, our results appear to support the pivotal role of neuronal regulation of CBF during H/H. With regard to the potential physiological role of CB1 receptor-mediated inhibition of H/H-induced cerebral hyperemia it is likely that it functions as a negative feed-back mechanism. It is well established that hypoxia and hypercapnia, especially if they are sustained, may result in the development of brain edema (Wilson et.al. 2009; Adeva et.al. 2012) . Although the pathomechanism of H/H-induced brain edema is complex (Yang and Rosenberg 2011), cerebral vasodilation and the consequent increase of the hydrostatic pressure in brain capillaries is likely to play a key role in it. Therefore, the CB1 receptor-mediated attenuation of H/H-induced cerebral vasodilation may represent a protective mechanism against the development of brain edema during hypoxia and hypercapnia.

7. 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 examined the interaction between the HO and NOS pathways in the regulation of the cerebrocortical blood flow under resting conditions. The results of this 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 inverse 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.

8. SUMMARY

The cerebral vasoregulation incorporates various local and global control mechanisms, involving participation of neuronal and vascular elements. Cerebrovascular regulatory 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. Specifically, we investigated the involvement of the heme oxygenase (HO) – carbon monoxide (CO) pathway and the endocannabinoid system in the cerebral vasoregulation under resting (normoxic/normocapnic) physiological conditions and during hypoxia and hypercapnia (H/H). Cerebrocortical blood flow (CBF) was measured by laser Doppler flowmetry in anesthetized adult male Wistar rats. First, the role of the HO pathway and its interaction with the nitric oxide synthase (NOS) pathway were studied in the regulation of CBF under normoxic/normocapnic conditions. CBF increased after inhibition of HO, and this effect was reversed by NOS-blockade, suggesting that constitutive CO release tonically suppresses NO production and consequently reduces CBF. In order to analyze the role of the HO – CO pathway in the CBF regulation during H/H we developed an experimental protocol for reproducible, controlled, stepwise H/H in anesthetized rats by administration of different gas mixtures, resulting in CBF increase. Surprisingly, however, HO blockade failed to alter the cerebrocortical hyperemia, indicating that the HO – CO pathway, albeit influences NO release, does not contribute to the CBF regulation during H/H. We also investigated the effects of endocannabinoids and CB1 cannabinoid receptors on the CBF under resting physiological conditions and during H/H. We did not observe any significant effect of CB1 blockade by AM251 on the cerebrocortical circulation under normoxic/normocapnic conditions. In contrast, AM251 enhanced significantly the CBF rise to H/H, indicating the importance of the CB1 receptors in modulating the response. We propose that the CB1 receptor-mediated attenuation of cerebral vasodilation may represent a negative feed-back mechanism against the development of brain edema and increased intracranial pressure during H/H.

9. ÖSSZEFOGLALÁS

Az agyi vérkeringés szabályozása igen komplex folyamat, melyben idegi és vaszkuláris tényezők egyaránt fontos szerepet játszanak. Annak ellenére, hogy vizsgálata évtizedek óta intenzív kutatások tárgyát képezi, mind a mai napig hiányosak az ismereteink olyan alapvető szabályozási funkciók tekintetében, mint véráramlás és metabolizmus kapcsoltsága, ill. a hypoxia és hypercapnia (H/H) hatására kialakuló agyi vazodilatáció mechanizmusa. Feltételeztük, hogy az újonnan felfedezett vazoaktív mediátorok agyi vérkeringésre kifejtett hatásainak feltérképezése közelebb vihet a még nyitott kérdések tisztázásához. Kutatásaink során a hem oxigenáz (HO) – szénmonoxid (CO) rendszer és az endogén cannabinoid mediátorok hatásait vizsgáltuk az agyi vérkeringésre nyugalmi (normoxiás/normokapniás) állapotban valamint H/H során. Felnőtt hím Wistar patkányok agykérgi véráramlását lézer Doppler módszerrel mértük. Először a HO útvonal szerepét és ennek a nitrogén monoxid szintáz (NOS) rendszerrel való kölcsönhatását vizsgáltuk normoxiás/normokapniás állapotban. Kimutattuk, hogy a HO enzim(ek) gátlása agykérgi véráramlás-fokozódást okoz, mely hatást az NO közvetíti. Ez arra utal, hogy a konstitutív CO felszabadulás tónusosan gátolja az NO szintézisét, és ezzel csökkenti az agykérgi véráramlást. Mivel a NOS közrejátszik az agyi véráramlás szabályozásában H/H során, kíváncsiak lettünk a HO rendszer szerepére ebben a folyamatban. E vizsgálatok kivitelezésére kifejlesztettünk egy olyan kísérleti protokollt, mely során reprodukálhatóan, kontrolláltan és lépcsőzetes módon H/H-t alakítottunk ki altatott patkányokban különböző összetételű gázkeverékek belélegeztetésével. Azt találtuk, hogy a HO rendszer, annak ellenére, hogy hatással van a NOS aktivitására, nem befolyásolja szignifikánsan a H/H során kialakuló agykérgi véráramlás-fokozódást. Ezt követően az endocannabinoidok és a CB1 cannabinoid receptorok szerepét vizsgáltuk az agyi vérkeringés szabályozásában. Azt találtuk, hogy a CB1 antagonistá AM251 nem befolyásolta az agykérgi véráramlást nyugalmi, normoxiás/normocapniás állapotban. Meglepő módon azonban AM251 hatására fokozódott a H/H során kialakuló agykérgi véráramlás-fokozódás, jelezve a CB1 receptorok szerepét a folyamatban. Eredményeink arra engednek következtetni, hogy az endocannabinoidok CB1 receptorok által közvetített módon egy negatív visszacsatolós szabályozást valósítanak meg H/H során, melynek célja a túlzott vazodilatáció nyomán kialakuló ödéma-képződés és intracraniális nyomás-emelkedés megelőzése lehet.

10. LIST OF REFERENCES

Adeva MM, Souto G, Donapetry C, Portals M, Rodriguez A and Lamas D. (2012) Brain edema in diseases of different etiology. *Neurochem Int*, 61: 166-174.

Appleton SD, Chretien ML, McLaughlin BE, Vreman HJ, Stevenson DK, Brien JF, Nakatsu K, Maurice DH and Marks GS. (1999) Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations. *Drug Metab Dispos*, 27: 1214-1219.

Armstead WM. (2005) Age and cerebral circulation. *Pathophysiology*, 12: 5-15.

Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA and Newman EA. (2010) Glial and neuronal control of brain blood flow. *Nature*, 468: 232-243.

Bakalova RA, Matsuura T and Kanno I. (2002) Cyclooxygenase-pathway participates in the regulation of regional cerebral blood flow in response to neuronal activation under normo- and hypercapnia. *Prostaglandins Leukot Essent Fatty Acids*, 67(6): 379-388.

Baranano DE and Snyder SH. (2001) Neural roles for heme oxygenase: contrast to nitric oxide synthase. *Proc Nat Acad Sci USA*, 98: 10996-11002.

Bari F, Thore CR, Louis TM and Busija DW. (1998) Inhibitory effects of hypoxia and adenosine on N-methyl-D-aspartate-induced pial arteriolar dilation in piglets. *Brain Res*, 780: 237-244.

Bátkai S, Pacher P, Osei-Hyiaman D, Radaeva S, Liu J, Harvey-White J, Offertáler L, Mackie K, Rudd MA, Bukoski RD and Kunos G. (2004) Endocannabinoids acting at cannabinoid-1 receptors regulate cardiovascular function in hypertension. *Circulation*. 110: 1996–2002.

Battista N, Fezza F and Maccarrone M. (2004) Endocannabinoids and their involvement in the neurovascular system. *Curr Neurovasc Res* 1: 129-140.

Beaconsfield P, Carpi A, Cartoni C, De Basso P and Rainsbury R. (1972) Effect of delta-9-tetrahydrocannabinol on cerebral circulation and function. *Lancet*, 300: 1146.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A and Piomelli D. (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science*, 277: 1094-1097.

Bilfinger TV, Salzet M, Fimiani C, Deutsch DG, Tramu G and Stefano GB. (1998) Pharmacological evidence for anandamide amidase in human cardiac and vascular tissues. *Int J Cardiol*, 64: 15-22.

Billar TR, Curran RD, Harbrecht BG, Stadler J, Williams DL, Ochoa JB, Di Silvio M, Simmons RL, and Murray SA. (1992) *Am J Physiol*, 262: C 1077-C1082.

Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L and Di Marzo V. (1997) Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. *Biochem J*, 322: 671-677.

Bisogno T, Melck D, De Petrocellis L and Di Marzo V. (1999) Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. *J Neurochem*, 72: 2113-2119.

Bisogno T, Maccarrone M, de Petrocellis L, Jarrahian A, Finazzi-Agro A, Hillard C and Di Marzo V. (2001) The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. *Eur J Biochem*, 268: 1982-1989.

Blood AB, Hunter CJ and Power GG. (2003) Adenosine mediates decreased cerebral metabolic rate and increased cerebral blood flow during acute moderate hypoxia in the near-term fetal sheep. *J Physiol*, 553.3: 935-945.

Bloom AS, Tershner S, Fuller SA, Stein EA. (1997) Cannabinoid-induced alterations in regional cerebral blood flow in the rat. *Pharmacol Biochem Behav*, 57: 625-631.

Bohlen HG and Harper SL. (1984) Evidence of myogenic vascular control in the rat cerebral cortex. *Circ Res* 55: 554-559.

Bonev AD, Jaggar JH, Rubart M and Nelson MT. (1997) Activators of protein kinase C decrease Ca spark frequency in smooth muscle cells from cerebral arteries. *Am J Physiol* 273: C2090-2095.

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P. (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J*, 312: 637-641.

Brayden JE. (2002) Functional roles of K_{ATP} channels in vascular smooth muscle. *Clin Exp Pharmacol and Physiol*, 29: 312-316.

Breivogel CS, Griffin G, Di Marzo V and Martin BR. (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol*, 60: 155-163.

Brown A and Wise A. (2001) Identification of modulators of GPR55 activity. USPTO. WO0186305, GlaxoSmithKline

Brown AJ and Hiley CR. (2009) Is GPR55 an anandamide receptor? *Vitam Horm*, 81: 111-137.

Burstein S, Budrow J, Debatis M, Hunter SA and Subramanian A. (1994) Phospholipase participation in cannabinoid-induced release of free arachidonic acid. *Biochem Pharmacol*, 48: 1253-1264.

Busija DW, Heistad DD. (1984) Effects of activation of sympathetic nerves on cerebral blood flow during hypercapnia in cats and rabbits. *J Physiol*, 347: 35-45.

Cabral HR and Novak IT. (1999) Autologous rosette formation by human blood monocyte-derived macrophages and lymphocytes. *Am J Hematol*, 60: 285-288.

Cadas H, Gaillet S, Beltramo M, Venance L and Piomelli D. (1996) Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J Neurosci*, 16: 3934-3942.

Cadas H, di Tomaso E and Piomelli D. (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci*, 17: 1226-1242.

Calignano A, La Rana G, Beltramo M, Makriyannis A and Piomelli D. (1997) Potentiation of anandamide hypotension by the transport inhibitor, AM404. *Eur J Pharmacol*, 337: R1-R2.

Carratu P, Pourcyrus M, Fedinec A, Leffler CW, Parfenova H. (2003) Endogenous heme oxygenase prevents impairment of cerebral vascular functions caused by seizures. *Am J Physiol Heart Circ Physiol*, 285: H1148-H1157.

Carruba MO, Bondiolotti G, Picotti GB, Catteruccia N, Da Prada M. (1987) Effects of diethyl ether, halothane, ketamine and urethane on sympathetic activity in the rat. *Eur J Pharmacol* 134:15-24.

Chang EF, Claus CP, Vreman HJ, Wong RJ Noble-Haeusslein LJ. (2005) Heme regulation in traumatic brain injury: relevance to the adult and developing brain. *J Cereb Blood Flow Metab*, 25: 1401-1417.

Chen Y, McCarron RM, Ohara Y, Bembry J, Azzam N, Lenz FA, Shohami E, Mechoulam R and Spatz M. (2000) Human brain capillary endothelium: 2-arachidonoglycerol (endocannabinoid) interacts with endothelin-1. *Circ Res*, 87: 323-327.

Chernick RJ, Martasek P, Levere RD, Margreiter R and Abraham NG. (1989) Sensitivity of human tissue heme oxygenase to a new synthetic metalloporphyrin. *Hepatology*, 10: 365-369.

Cho HG, Shin HK, Shin YW, Lee JH and Hong KW. (2003) Role of nitric oxide in the CBF autoregulation during acute stage after subarachnoid haemorrhage in rat pial artery. *Fundam Clin Pharmacol*, 17(5): 563-573.

Chrissobolis S, Ziogas J, Chu Y, Faraci FM and Sobey CG. (2000) Role of inwardly rectifying K⁺ channels in K⁺-induced cerebral vasodilatation in vivo. *Am J Physiol Heart Circ Physiol*, 279: H2704-H2712.

Chrissobolis S and Sobey CG. (2002) Inhibitory effects of protein kinase C on inwardly rectifying K⁺ and ATP-sensitive K⁺ channel-mediated responses of the basilar artery. *Stroke*, 33: 1692-1697.

Chrissobolis S and Sobey CG. (2003) Inwardly rectifying potassium channels in the regulation of vascular tone. *Current Drug Targets*, 4: 281-289.

Chrissobolis S and Sobey CG. (2004) Influence of gender on K⁺-induced cerebral vasodilatation. *Stroke* 35: 747-752.

Costa B, Siniscalco D, Trovato AE, Comelli F, Sotgiu ML, Colleoni M, Maione S, Rossi F and Giagnoni G. (2006) AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain. *Br J Pharmacol*, 148: 1022-1032.

Deadwyler SA, Hampson RE, Bennett BA, Edwards TA, Mu J, Pacheco MA, Ward SJ and Childers SR. (1993) Cannabinoids modulate potassium current in cultured hippocampal neurons. *Receptors Channels*, 1: 121-134.

Deshmukh VD, Harper AM, Rowan JO, Jennett WB. (1972) Experimental studies on the influence of the sympathetic nervous system on cerebral blood-flow. *Br J Surg*, 59: 897.

Deutsch D and Chin SA. (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol*, 46: 791-796.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, 258: 1946-1949.

Devane WA and Axelrod J. (1994) Enzymatic synthesis of anandamide, an endogenous ligand for the cannabinoid receptor, by brain membranes. *Proc Natl Acad Sci*, 91: 6698-6701.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC and Piomelli D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*, 372: 686-691.

Di Marzo V, De Petrocellis L, Sepe N and Buono A. (1996) Biosynthesis of anandamide and related acylethanolamides in mouse J774 macrophages and N18 neuroblastoma cells. *Biochem J*, 316: 977-984.

Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA and Kunos G. (1999) Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *Eur J Biochem*, 264: 258-267.

Dogulu FH, Ozogul C, Akpek S, Kurt G, Emmez H, Ercan S and Baykaner MK. (2003) Intra-arterial simultaneous administration of anandamide attenuates endothelin-1 induced vasospasm in rabbit basilar arteries. *Acta Neurochir Wien*, 145: 579-582.

Dong H, Jiang Y, Cole WC and Triggle CR. (2000) Comparison of the pharmacological properties of EDHF-mediated vasorelaxation in guinea-pig cerebral and mesenteric resistance vessels. *Brit J Pharmacol*, 130: 1983-1991.

Dunn KM and Nelson MT. (2010) Potassium channels and neurovascular coupling. *Circ J*, 74: 608-616.

Duarte JM, Ferreira SG, Carvalho RA, Cunha RA, Köfalvi A. (2012) CB(1) receptor activation inhibits neuronal and astrocytic intermediary metabolism in the rat hippocampus. *Neurochem Int*, 60: 1-8.

Durante W, Kroll MH, Christodoulides N, Peyton KJ and Schafer AI. (1997) Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res*, 80: 557-564.

Edvinsson L, Erlinge D, Ekman R and Thulin T. (1992) Sensory nerve terminal activity in severe hypertension as reflected by circulating calcitonin gene-related peptide (CGRP) and substance P. *Blood Press*, 4: 223-229.

Edvinsson L. (2001) Sensory nerves in man and their role in primary headaches. *Cephalalgia*, 21(7): 761-764.

Edwards G, Dora KA, Gardener MJ, Garland CJ and Weston AH. (1998) K⁺ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*, 396: 269-272.

Ellis EF, Police RJ, Yancey LM, McKinney JS and Amruthesh SC. (1990) Dilation of cerebral arterioles by cytochrome P-450 metabolites of arachidonic acid. *Am J Physiol*, 259: 1171-1177.

Ellis EF, Moore SF, Willoughby KA. (1995) Anandamide and delta 9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am J Physiol*, 269: H1859-H1864.

Fabricius M, Akgoren N, Dirnagl U and Lauritzen M. (1997) Laminar analysis of cerebral blood flow in cortex of rats by laser-Doppler flowmetry: a pilot study. *Journal of Cerebral Blood Flow and Metabolism* 17:1326-1336.

Falk JE. (1964) Porphyrins and metalloporphyrins. *Journal of basic microbiology*, 4 (5): 398.

Faraci FM, Breese KR and Heistad DD. (1994) Cerebral vasodilation during hypercapnia. Role of glibenclamide-sensitive potassium channels and nitric oxide. *Stroke*, 25: 1679-1683.

Faraci FM, Sobey CG, Chrissobolis S, Lund DD, Heistad DD and Weintraub NL. (2001) Arachidonate dilates basilar artery by lipoxygenase-dependent mechanism and activation of K⁺ channels. *Am J Physiol Regulatory Integrative Comp Physiol*, 281: R246-R253.

Fasia L, Karava V and Siafaka-Kapadi A. (2003) Uptake and metabolism of [3H]anandamide by rabbit platelets. Lack of transporter? *Eur J Biochem*, 270: 3498-3506.

Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K and Devane WA. (1993) Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc Natl Acad Sci*, 90: 7656-7660.

Figley CR, Stroman PW. (2011) The role(s) of astrocytes and astrocyte activity in neurometabolism, neurovascular coupling, and the production of functional neuroimaging signals. *Eur J Neurosci*, 33: 577-588

Filosa JA, Bonev AD, Straub SV, Meredith AL, Wilkerson MK, Aldrich RW and Nelson MT. (2006) Local potassium signalling couples neuronal activity to vasodilation in the brain. *Nature neuroscience*, 9(11): 1397-1402.

Fimiani C, Liberty T, Aquirre AJ, Amin I, Ali N and Stefano GB. (1999) Opiate, cannabinoid, and eicosanoid signaling converges on common intracellular pathways nitric oxide coupling. *Prostaglandins Other Lipid Mediat*, 57: 23-34.

Freund TF, Katona I, Piomelli D. (2003) Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev*, 83: 1017-1066.

Friebe A, Schultz G and Koesling D. (1996) Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *EMBO Journal* 15: 6863-6868.

Galieque S, Mary S, Marchland J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem*, 232: 54-61.

Gatley SJ, Gifford AN, Volkow ND, Lan R, Makriyannis A. (1996) ¹²³I labeled AM251: a radioiodinated ligand which binds in vivo to mouse brain cannabinoid CB1 receptors. *Eur J Pharmacol*, 307: 331-338.

Gatley SJ, Lan R, Volkow ND, Pappas N, King P, Wong CT, Gifford AN, Pyatt B, Dewey SL and Makriyannis A. (1998) Imaging the brain marijuana receptor: development of a radioligand that binds to cannabinoid CB1 receptors in vivo. *J Neurochem*, 70: 417-423.

Gebremedhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR. (1999) Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca²⁺ channel current. *Am J Physiol*, 276: H2085-H2093.

Giuffrida A, Parsons LH, Kerr TM, de Fonesca RF, Navarro M and Piomelli D. (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nat Neurosci* 2: 358-363.

Goadsby PJ. (1994) Nitric oxide is not the sole determinant of hypercapnic or metabolically driven vasodilation in the cerebral circulation. *J Auton Nerv Syst* 49: S67-72.

Golanov EV, Christensen JRC and Reis DJ. (2001) Neurons of a limited subthalamic area mediate elevations in cortical cerebral blood flow evoked by hypoxia and excitation of the rostral ventrolateral medulla. *J Neuroscience* 21(11): 4032-4041.

Golech SA, McCarron RM, Chen Y, Bembry J, Lenz F, Mechoulam R, Shohami E and Spatz M. (2004) Human brain endothelium: coexpression and function of vanilloid and endocannabinoid receptors. *Molecular Brain Research* 132: 87-92.

Gonzalez S, Manzanares J, Berrendero F, Wenger T, Corchero J, Bisogno T, Romero J, Fuentes JA, Di Marzo V, Ramos JA and Fernandez-Ruiz J. (1999) Identification of endocannabinoids and cannabinoid CB(1) receptor mRNA in the pituitary gland. *J Neuroendocrinol* 70: 137-145.

Goparaju SK, Ueda N, Taniguchi K and Yamamoto S. (1999) Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem Pharmacol* 57: 417-423.

Goplerud JM, Wagerle LC, Delivoria-Papadopoulos M. (1991) Sympathetic nerve modulation of regional cerebral blood flow during asphyxia in newborn piglets. *Am J Physiol* 260: H1575-H1580.

Goutopoulos A and Makriyannis A. (2002) From cannabis to cannabinergics: new therapeutic opportunities. *Pharmacol Therap* 95: 103-117.

Graser T, Vedernikov YP and Li DS. (1990) Study on the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein. *Biomed Biochim Acta* 49: 293-296.

Griffin G, Fernando SR, Ross RA, McKay NG, Ashford MLJ, Shire D, Huffman JW, Yu S, Lainton JA and Pertwee RG. (1997) Evidence for the presence of CB2-like cannabinoid receptors on peripheral nerve terminals. *Eur J Pharmacol* 339: 53-61.

Haberl RL, Heizer ML, Marmarou A and Ellis EF. (1989) Laser Doppler assessment of brain microcirculation: effect of systemic alterations. *Am J Physiol* 256: H1247- 1254.

Hajos N, Ledent C and Freund TF. (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience* 106: 1-4.

Hamel E. (2006) Perivascular nerves and the regulation of cerebrovascular tone. *J Appl Physiol* 100: 1059-1064.

Hanlon KE, Vanderah TW. (2010) Constitutive activity at the cannabinoid CB(1) receptor and behavioral responses. *Methods Enzymol* 484: 3-30

Harper AM, Deshmukh VD, Rowan JO, Jennett WB. (1972) The influence of sympathetic nervous activity on cerebral blood flow. *Arch Neurol* 27: 1-6.10

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, De Costa BR and Rice KC. (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci* 87: 1932-1936.

Hillard CJ and Campbell WB. (1997) Biochemistry and pharmacology of arachidonylethanolamide, a putative endogenous cannabinoid. *J Lipid Res* 38: 2383-2398.

Hillard CJ, Muthian S, Kearns CS. (1999) Effects of CB1 cannabinoid receptor activation on cerebellar granule cell nitric oxide synthase activity. *FEBS Lett* 459: 277-281

Hillard CJ and Jarrahian A. (2003) Cellular accumulation of anandamide: consensus and controversy. *Br J Pharmacol* 140: 802-808.

Hoff JT, MacKenzie ET and Harper AM. (1977) Responses of the cerebral circulation to hypercapnia and hypoxia after 7th cranial nerve transection in baboons. *Circ Res* 40(3): 258-262. *Physiol Neurobiol* 150(2-3): 301-306.

Holland M, John Challiss RA, Standen NB, and Boyle JP. (1999) Cannabinoid CB1 receptors fail to cause relaxation, but couple via Gi/Go to the inhibition of adenylyl cyclase in carotid artery smooth muscle. *Br J Pharmacol* 128: 597-604.

Horváth B, Hrabák A, Káldi K, Sándor P, Benyó Z. (2003) Contribution of the heme oxygenase pathway to the maintenance of the hypothalamic blood flow during diminished nitric oxide synthesis. *J Cereb Blood Flow Metab* 23: 653-657.

Horváth B, Hortobágyi L, Lenzser G, Schweer H, Hrabák A, Sándor P and Benyó Z. (2008) Carbon monoxide-prostaglandin E2 interaction in the hypothalamic circulation. *Neuroreport* 19(16): 1601-1604.

Howlett AC. (1998) The CB1 cannabinoid receptor in the brain. *Neurobiol Dis* 6: 405-416.

Howlett AC, Mukhopadhyay S, Shim JY and Welsh WJ. (1999) Signal transduction of eicosanoid CB1 receptor ligands. *Life Sci* 65: 617-625.

Howlett AC. (2000) "Cannabinoid receptors" in: *The IUPHAR Compendium of Receptor Characterization and Classification*, 2nd edition, 129-138.

Hunter CJ, Blood AB, White CR, Pearce WJ and Power GG. (2003) Role of nitric oxide in hypoxic cerebral vasodilatation in the ovine fetus. *J Physiol* 549: 625-633.

Iadecola C, Beitz AJ, Renno W, Xu X, Mayer B, Zhang F. (1993) Nitric oxide synthase-containing neural processes on large cerebral arteries and cerebral microvessels. *Brain Res* 606:148-155

Ilch CP and Golanov EV. (2004) Cerebrovasodilation evoked by stimulation of subthalamic vasodilator area and hypoxia depends upon the integrity of cortical neurons in the rat. *Neurosci Lett* 368: 92-95.

Imuta N, Hori O, Kitao Y, Tabata Y, Yoshimoto T, Matsuyama T and Ogawa S. (2007) Hypoxia-mediated induction of heme oxygenase type I and carbon monoxide release from astrocytes protects nearby cerebral neurons from hypoxia-mediated apoptosis. *Antioxid Redox Signal* 9(5): 543-552.

Ishac EJ, Jiang L, Lake KD, Varga K, Abood ME, Kunos G. (1996) Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br J Pharmacol* 118:2023–2028.

Ishikawa M, Kajimura M, Adachi T, Maruyama K, Makino N, Goda N, Yamaguchi T, Sekizuka E and Suematsu M. (2005) Carbon monoxide from heme oxygenase-2 is a tonic regulator against NO-dependent vasodilatation in the adult rat cerebral microcirculation. *Circ Res*; 97: e104-e114.

Ishioka N and Bukoski RD. (1999) A role for N-arachidonylethanolamine (anandamide) as the mediator of sensory nerve-dependent Ca²⁺-induced relaxation. *J Pharmacol Exp Ther* 289: 245-250.

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova DES and Cheng X. (2002) Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca²⁺ sparks to Ca²⁺-activated K⁺ channels. *Circ res* 91: 610-617.

Jansen-Olesen I, Mortensen CH, El-Bariaki N and Ploug KB. (2005) Characterization of KATP-channels in rat basilar and middle cerebral arteries: Studies of vasomotor responses and mRNA expression. *Eur J Pharmacol* 523: 109-118.

Jarai Z, Wagner JA, Varga K, Lake K, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, Razdan RK, Zimmer A and Kunos G. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci* 96: 14136-14141.

Johns DG, Behm DJ, Walker DJ, Ao Z, Shapland EM, Daniels DA, Riddick M, Dowell S, Staton PC, Green P, Shabon U, Bao W, Aiyar N, Yue TL, Brown AJ, Morrison AD and Douglas SA. (2007) The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects. *Br J Pharmacol* 152: 825-831.

Johnson RA, Lavesa M, Deseyn K, Scholer MJ and Nasjletti A. (1996) Heme oxygenase substrates acutely lower blood pressure in hypertensive rats. *Am J Physiol* 271: H1132-H1138.

Johnson FK, Teran FJ, Prieto-Carrasquero M, Johnson RA. (2002) Vascular effects of a heme oxygenase inhibitor are enhanced in the absence of nitric oxide. *Am J Hypertens*; 15: 1074-1080.

Johnson FK and Johnson RA. (2003) Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. *Am J Physiol Regul Integr Comp Physiol* 285: R536-R541.

Johnson FK, Johnson RA, Durante W, Jackson KE, Stevenson BK, Peyton KJ. (2006) Metabolic syndrome increases endogenous carbon monoxide production to promote hypertension and endothelial dysfunction in obese Zucker rats. *Am J Physiol Regul Integr Comp Physiol* 290: R601- R608.

Johnson RA, Lavesa M, Askari B, Abraham NG, Nasjletti A. (1995) A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats. *Hypertension* 25: 166-169.

Johnson RA, Colombari E, Colombari DS, Lavesa M, Talman WT and Nasjletti A. (1997) Role of endogenous carbon monoxide in central regulation of arterial pressure. *Hypertension* 30: 962-967.

Kanu A, Gilpin D, Fedinec AL and Leffler CW. (2006) Cyclooxygenase products stimulate carbon monoxide production by piglet cerebral microvessels. *Exp Biol Med* 231: 181-185

Kanu A and Leffler CW. (2007) Carbon monoxide and Ca²⁺-activated K⁺ channels in cerebral arteriolar responses to glutamate and hypoxia in newborn pigs. *Am J Physiol Heart Circ Physiol* 293(5): H3193-200.

Katona I, Sperl agh B, S ik A, K afalvi A, V izi ES, Mackie K and Freund TF. (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J Neurosci* 19: 4544-4558.

Katona I and Freund TF. (2008) Endocannabinoid signalling as a synaptic circuit breaker in neurological disease. *Nat Med* 14: 923-930.

Katona I and Freund TF. (2012) Multiple functions of endocannabinoid signalling in the brain. *Annu Rev Neurosci* 35: 529-558.

Kim YM, Bergonia HA, Muller C, Pitt BR, Watkins WD and Lancaster JR. (1995) Loss and degradation of enzyme-bound heme induced by cellular nitric oxide synthesis. *J Biol Chem* 270: 5710-5713.

Koehler RC and Traystman RJ. (2002) Cerebrovascular effects of carbon monoxide. *Antiox redox signal* 4: 279-290.

Koehler RC, Gebremedhin D and Harder DR. (2006) Role of astrocytes in cerebrovascular regulation. *J Appl Physiol* 100: 307-317.

Koehler RC, Roman RJ, Harder DR (2009) Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci* 32: 160-169.

Kozłowska H, Baranowska M, Schlicker E, Kozłowski M, Laudanski J, Malinowska B (2007). Identification of the vasodilatory endothelial cannabinoid receptor in the human pulmonary artery. *J Hypertens* 25: 2240-2248.

Kozłowska H, Baranowska M, Schlicker E, Kozłowski M, Laudanski J, Malinowska B (2008). Virodhamine relaxes the human pulmonary through the endothelial cannabinoid receptor and indirectly through a COX product. *Br J Pharmacol* 155: 1034-1042.

Kreitzer AC, Regehr WG (2001) Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* 29: 717-727.

Kunos G, Járαι Z, Varga K, Liu J, Wang L and Wagner JA. (2000) Cardiovascular effects of endocannabinoids—the plot thickens. *Prostaglandins Other Lipid Mediat* 61: 71-84.

Kutty RK and Maines MD. (1984) Effects of induction of heme oxygenase by cobalt and tin on the in vivo degradation of myoglobin. *Biochem Pharmacol* 18: 2924-2926.

Lacza Z, Erdos B, Gorlach C, Wahl M, Sandor P and Benyo Z. (2000) the cerebrocortical microcirculatory effect of nitric oxide synthase blockade is dependent upon baseline red blood cell flow in the rat. *Neurosci Lett* 291: 65-68.

Lake KD, Compton DR, Varga K, Martin BR, Kunos G. (1997a) Cannabinoid-induced hypotension and bradycardia in rats mediated by CB1-like cannabinoid receptors. *J Pharmacol Exp Ther* 281:1030–1037.

Lake KD, Martin BR, Kunos G, Varga K. Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. (1997b) *Hypertension* 29:1204–1210.

Lang W, Qin C, Lin S, Khanolkar AD, Goutopoulos A, Fan P, Abouzid K, Meng Z, Biegel D and Makriyannis A. (1999) Substrate specificity and stereoselectivity of rat brain microsomal anandamide amidohydrolase. *J Med Chem* 42: 896-902.

Lavi S, Egbarya R, Lavi R and Jacob G. (2003) Role of nitric oxide in the regulation of cerebral blood flow in humans. *Circulation* 107: 1901-1905.

Lee TJF, Zhang W and Sarwinski S.(2000) Presynaptic β_2 adrenoceptors mediate nicotine induced NOergic neurogenic vasodilation in the pig cerebral arteries. *Am J Physiol Heart Circ Physiol* 279: H808-H816.

Lee TJF. (2002) Sympathetic modulation of nitrenergic neurogenic vasodilation in cerebral arteries. *Jpn J Pharmacol* 88(1): 26-31.

Leffler CW, Nasjletti A, Yu C, Johnson RA, Ferdinac AL and Walker N. (1999) Carbon monoxide and cerebral microvascular tone in newborn pigs. *Am J Physiol Heart Circ Physiol* 276: H1641-H1646.

Leffler CW, Nasjletti A, Johnson RA and Ferdinec AL. (2001) Contributions of prostacyclin and nitric oxide to carbon monoxide-induced cerebrovascular dilation in piglets. *Am J Physiol Heart Circ Physiol* 280: H1490-H1495.

Leffler CW, Balabanova L, Fedinec AL, Waters CM, Parfenova H. (2003a) Mechanism of glutamate stimulation of CO production in cerebral microvessels. *Am J Physiol Heart Circ Physiol* 285: H74-H80.

Leffler CW, Balabanova L, Sullivan CD, Wang X, Fedinec AL, Parfenova H. (2003b) Regulation of CO production in cerebral microvessels of newborn pigs. *Am J Physiol Heart Circ Physiol* 285: H292-H297.

Leffler CW, Balabanova L, Fedinec AL, Parfenova H. (2005) Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. *Am J Physiol Heart Circ Physiol* 289: H1442-H1447.

Leffler CW, Parfenova H and Jaggar JH. (2011) Carbon monoxide as an endogenous vascular modulator. *Am J Physiol Heart Circ Physiol* 301: H1-H11.

Li A, Xi Q, Umstot ES, Bellner L, Schwartzman ML, Jaggar JH and Leffler CW. (2008) Astrocyte-derived CO is a diffusible messenger that mediates glutamate-induced cerebral arteriolar dilation by activating smooth muscle cell K_{Ca} channels. *Circ Res* 102(2): 234-241.

Lindauer U, Kunz A, Schuh-Hofer S, Vogt J, Dreier JP, Dirnagl U. (2001) Nitric oxide from perivascular nerves modulates cerebral arterial pH reactivity. *Am J Physiol Heart Circ Physiol* 281:H1353–H1363.

Lindauer U, Vogt J, Schuh-Hofer S, Dreier JP and Dirnagl U. (2003) Cerebrovascular vasodilation to extraluminal acidosis occurs via combined activation of ATP-sensitive and Ca²⁺-activated potassium channels. *J Cereb Blood Flow Metab* 23: 1227-1238.

Linden I-B, Tokola O, Karlsson M and Tenhunen R. (1987) Fate of haem after parenteral administration of haem arginate to rabbits. *J Pharmacy Pharmacol* 39: 96-102.

Liu QH, Zheng YM, Korde AS, Li XQ, Ma J, Takeshima H and Wang YX. (2009) Protein kinase C- ϵ regulates local calcium signalling in airway smooth muscle cells. *Am J Respir Cell Mol Biol* 40: 663-671.

Luckhoff A and Busse R. (1990) Activators of potassium channels enhance calcium influx into endothelial cells as a consequence of potassium currents. *Naunyn Schmiedeberg's Arch Pharmacol* 342: 94-99.

Maccarrone M, Lorenzon T, Bari M, Melino G, and Finazzi-Agro A. (2000) Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J Biol Chem* 275: 31938-31945.

Mackie K, Lai Y, Westenbroek R and Mitchell R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci* 15: 6552-6561.

Maines MD. (1984) New developments in the regulation of heme metabolism and their implications. *Crit Rev Toxicol* 12: 241-314.

Maines MD. (2000) The heme oxygenase system and its functions in the brain. *Cell Mol Biol* 46: 573-585.

Makino R, Matsuda H, Obayashi E, Shiro Y, Iizuka T and Hori H. (1999) EPR characterization of axial bond in metal center of native and cobalt-substituted guanylate cyclase. *J Biol Chem* 274: 7714-7723.

Mancuso C, Pistritto G, Tringali G, Grossman AB, Preziosi P and Navarra P. (1997) Evidence that carbon monoxide stimulates prostaglandin endoperoxide synthase activity in rat hypothalamic explants and in primary cultures of rat hypothalamic astrocytes. *Brain Res Mol Brain Res* 45: 294-300.

Martin BR, Mechoulam R and Razdan RK. (1999) Discovery and characterization of endogenous cannabinoids. *Life Sci* 65: 573-595.

Mathew RJ, Wilson WH, Humphreys DF, Lowe JV, Wiethe KE (1992) Regional cerebral blood flow after marijuana smoking. *J Cereb Blood Flow Metab* 12: 750-758.

Mathew RJ, Wilson WH, Chiu NY, Turkington TG, Degrado TR and Coleman RE. (1999) Regional cerebral blood flow and depersonalization after tetrahydrocannabinol administration. *Acta Psychiatr Scand* 100: 67-75.

Mathew RJ, Wilson WH, Turkington TG, Hawk TC, Coleman RE, DeGrado TR and Provenzale J. (2002) Time course of tetrahydrocannabinol-induced changes in regional cerebral blood flow measured with positron emission tomography. *Psychiatry Res* 116: 173-185.

Matsuda LA, Lolait SJ, Browstein MJ, Young AC and Bonner TI. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *346*: 561-564.

Matsuda LA. (1997) Molecular aspects of cannabinoid receptors. *Crit Rev Neurobiol* 11: 143-166.

Matsuoka A, Stuehr DJ, Olson JS, Clark P and Ikeda-Saito M. (1994) L-arginine and calmodulin regulation of the heme iron reactivity in neuronal nitric oxide synthase. *J Biol Chem* 269: 20335-20339.

Mazza E, Thakkar-Varia S, Tozzi CA, Neubauer JA. (2001) Expression of heme oxygenase in the oxygen-sensing regions of the rostral ventrolateral medulla. *J Appl Physiol*; 91: 379-385.

McCarron JG, Crichton CA, Langton PD, MacKenzie A and Smith GL. (1997) Myogenic contraction by modulation of voltage-dependent calcium currents in isolated rat cerebral arteries. *J Physiol* 498: 371-379.

McCoubrey Jr WK, Huang TJ and Maines MD. (1997) Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 247: 725-732.

McMillan K and Masters BS. (1995) Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415. *Biochem* 34: 3686-3693.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR and Compton DR. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 50: 83-90.

Mechoulam R, Fride E, Ben-Shabat S, Meiri U and Horowitz M. (1998) Carbachol, an acetylcholine receptor agonist, enhances production in rat aorta of 2-arachidonoyl glycerol, a hypotensive endocannabinoid. *Eur J Pharmacol* 362: R1-R3.

Mombouli JV, Schaeffer G, Holzmann S, Kostner GM and Graier WF. (1999) Anandamide-induced mobilization of cytosolic Ca²⁺ in endothelial cells. *Br J Pharmacol* 126: 1593-1600.

Montandon G, Kinkead R, Lomenech AM, Bairam A and Guenard H. (2006) Heterogeneity of brainstem blood flow response to hypoxia in the anesthetized rat. *Respir Physiol Neurobiol* 150:301-6.

Montecot C, Seylaz J and Pinard E. (1998) Carbon monoxide regulates cerebral blood flow in epileptic seizures but not in hypercapnia. *NeuroReport* 9: 2341-2346.

Montecucco F, Di Marzo V (2012) At the heart of the matter: the endocannabinoid system in cardiovascular function and dysfunction. *Trends Pharmacol Sci* 33: 331-340

Moore MR, McColl KEL, Rimington C and Goldberg A. (1987) Disorders of porphyrin metabolism. Plenum Medical Book, 374.

Motterlini R, Hidalgo A, Sammut I, Shah KA, Mohammed S, Srai K and Green CJ. (1996) A precursor of the nitric oxide donor SIN-1 modulates the stress protein heme oxygenase-1 in rat liver. *Biochem Biophys Res Commun* 225: 167-172.

Mukhopadhyay S, Chapnick BM and Howlett AC. (2002) Anandamide-induced vasorelaxation in rabbit aortic rings has two components: G protein dependent and independent. *Am J Physiol Heart Circ Physiol* 282: 2046-2054.

Mukhopadhyay P, Rajesh M, Bátkai S, Patel V, Kashiwaya Y, Liaudet L, Evgenov OV, Mackie K, Haskó G and Pacher P. (2010) CB1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes. *Cardiovasc Res* 85: 773-784.

Munro S, Thomas KL and Abu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365: 61-65.

Nakane S, Oka S, Arai S, Waku K, Ishima Y, Tokumura A and Sugiura T. (2002) 2-Arachidonoyl-sn-glycero-3-phosphate, an arachidonic acid-containing lysophosphatidic

acid: occurrence and rapid enzymatic conversion to 2-arachidonoyl-sn-glycerol, a cannabinoid receptor ligand, in rat brain. *Arch Biochem Biophys* 402: 51-58.

Nakahata K, Kinoshita H, Hirano Y, Kimoto Y, Iranami H and Hatano Y. (2003) Mild hypercapnia induces vasodilation via adenosine triphosphate-sensitive K channels in parenchymal microvessels of the rat cerebral cortex. *Anesthesiol* 99: 1333-1339.

Ndisang JF, Tabien HE, Wang R. (2004) Carbon monoxide and hypertension. *J Hypertens* 22: 1057-1074.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ and Lederer WJ. (1995) Relaxation of arterial smooth muscle by calcium sparks. *Science* 270: 633-637.

Nguyen T, Winn HR and Janigro D. (2000) ATP-sensitive potassium channels may participate in the coupling of neuronal activity and cerebrovascular tone. *Am J Physiol Heart Circ Physiol* 278: H878- H885.

Niederhoffer N and Szabo B. (1999) Effect of the cannabinoid receptor agonist WIN55212-2 on sympathetic cardiovascular regulation. *Br J Pharmacol* 126:457-466.

Nishida N, Blood AB, Hunter CJ, Bragg S, Williams J, Pearce WJ and Power GG. (2006) Role of prostanoids in the regulation of cerebral blood flow during normoxia and hypoxia in the fetal sheep. *Pediatric Res* 60(5): 524-529.

Niwa K, Haensel C, Ross ME and Iadecola C. (2001) Cyclooxygenase-1 participates in selected vasodilator responses of the cerebral circulation. *Circ Res* 88: 600-608.

Offertaler L, Mo F, Batkai S, Liu J, Begg M, Razdan RK, Martin BR, Bukoski RD and Kunos G. (2003) Selective ligands and cellular effectors of a G protein-coupled endothelial cannabinoid receptor. *Mol Pharmacol* 63: 699-705.

Oka S, Nakajima K, Yamashita A, Kishimoto S and Sugiura T. (2007) Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochem Biophys Res Commun* 362: 928-934.

Oka S, Toshida T, Maruyama K, Nakajima K, Yamashita A and Sugiura T. (2009) 2-Arachidonoyl-sn-glycero-3-phosphoinositol: A possible Natural Ligand for GPR55. *J Biochem* 145: 13-20.

Pacher P, Bátkai S, Kunos G. (2004) Haemodynamic profile and responsiveness to anandamide of TRPV1receptor knock-out mice. *J Physiol* 558:647–657.

Pacher P, Bátkai S, Kunos G. (2005) Cardiovascular pharmacology of cannabinoids. *Handb Exp Pharmacol* 168: 599–625.

Pacher P, Steffens S. (2009) The emerging role of the endocannabinoid system in cardiovascular disease. *Semin Immunopathol* 31: 63-77.

Panerai RB. (2008) Cerebral autoregulation: From models to clinical applications. *Cardiovasc Eng* 8: 42-59.

Parfenova H, Hsu P and Leffler CW. (1995) Dilator prostanoid-induced cyclic AMP formation and release by cerebral microvascular smooth muscle cells: inhibition by indomethacin. *J Pharmacol Exp Ther* 272: 44-52.

Parfenova H, Massie V and Leffler CW. (2000) Developmental changes in endothelial derived vasorelaxant factors in cerebral circulation. *Am J Physiol* 278: H780-H788.

Parfenova H, Neff RA, Alonso JS, Shlopov BV, Jamal CN, Sarkisova SA and Leffler CW. (2001) Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate. *Am J Physiol* 281: 1954-1963.

Parfenova H, Ferdinac A, Leffler CW. (2003) Ionotropic glutamate receptors in cerebral microvascular endothelium are functionally linked to heme oxygenase. *J Cereb Blood Flow Metab* 23: 190-197.

Pearce W. (2006) Hypoxic regulation of the fetal cerebral circulation. *J Appl Physiol* 100: 731-738.

Pertwee RG. (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* 74: 129-180.

Pertwee RG (2005) Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci* 76: 1307-1324

Petersson J, Zygmunt PM and Hogestatt ED. (1997) Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br J Pharmacol* 120: 1344-1350.

Phillis JW. (2004) Adenosine and adenine nucleotides as regulators of cerebral blood flow: roles of acidosis, cell swelling, and KATP channels. *Crit Rev Neurobiol* 16(4): 237-270.

Phillis JW, Lungu CL, Barbu DE and Regan MHO. (2004) Adenosine's role in hypercapnia-evoked cerebral vasodilation in the rat. *Neurosci Lett* 365: 6-9.

Piomelli D, Beltramo M, Giuffrida A and Stella N. (1998) Endogenous cannabinoid signaling. *Neurobiol Dis* 5: 462-473.

Piomelli D, Beltramo M, Glasnapp S, Lin SY, Goutopoulos A, Xie XQ and Makriyannis A. (1999) Structural determinants for recognition and translocation by the anandamide transporter. *Proc Natl Acad Sci* 96: 5802-5807.

Ploug KB, Edvinsson L, Olesen J and Jansen-Olesen I. (2006) Pharmacological and molecular comparison of KATP channels in rat basilar and middle cerebral arteries. *Eur J Pharmacol* 553: 254-262.

Pourcyrous M, Bada HS, Parfenova H, Daley ML, Korones SB and Leffler CW. (2002) Cerebrovasodilatory contribution of endogenous carbon monoxide during seizures in newborn pigs. *Pediatr Res* 51: 579-585.

Prescott SM and Majerus PW. (1983) Characterization of 1,2-diacylglycerol hydrolysis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate. *J Biol Chem* 258: 764-769.

Raff H and Jankowski B. (1994) Inhibition of aldosterone release by hypoxia in vitro: interaction with carbon monoxide. *J Appl Physiol* 76: 689-693.

Rajesh M, Bátikai S, Kechrid M, Mukhopadhyay P, Lee WS, Horváth B, Holovac E, Cinar R, Liaudet L, Mackie K, Haskó G and Pacher P. (2012) Cannabinoid 1 receptor promotes cardiac dysfunction, oxidative stress, inflammation, and fibrosis in diabetic cardiomyopathy. *Diabetes* 61: 716-727.

Ralevic V and Kendall DA. (2009) Cannabinoid modulation of perivascular sympathetic and sensory neurotransmission. *Curr Vasc Pharmacol* 7: 15-25.

Randall MD, Harris D, Kendall DA, Ralevic V. (2002) Cardiovascular effects of cannabinoids. *Pharmacol Ther* 95: 191-202.

Randall MD, Kendall DA, O'Sullivan S. (2004) The complexities of the cardiovascular actions of cannabinoids. *Br J Pharmacol* 142: 20-26.

Rodriguez F, Zhang F, Dinocca S, Nasjletti A. (2003) Nitric oxide synthesis influences the renal vascular response to heme oxygenase inhibition. *Am J Physiol Renal Physiol*; 284

Roy CS and Sherrington CS. (1890) On the regulation of the blood supply of the brain. *J Physiol* 11: 85-158.

Ruisanchez E, Cselenyák A, Papp RS, Németh T, Káldi K, Sandor P, Benyó Z. (2012) Perivascular expression and potent vasoconstrictor effect of dynorphin a in cerebral arteries. *PLoS One* 7: e37798

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* 152: 1092-1101.

Sandor P, Komjáti K, Reirich M, Nyáry I. (1994) Major role of nitric oxide in the mediation regional CO₂ responsiveness of the cerebral and spinal cord vessels of the cat. *J Cereb Blood Flow Metab* 14: 49-58.

Sándor P. (1999) Nervous control of the cerebrovascular system: doubts and facts. *Neurochem Int* 35: 237-259.

Santa N, Kitazono T, Ago T, Ooboshi H, Kamouchi M, Wakisaka M, Ibayashi S and Iida M. (2003) ATP-sensitive potassium channels mediate dilatation of basilar artery in response to intracellular acidification in vivo. *Stroke* 34: 1276-1280.

Savinainen JR, Saario SM, Niemi R, Jarvinen T, Laitinen JT. (2003) An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A₁ and cannabinoid CB₁ receptors. *Br J Pharmacol* 140: 1451-1459

Schmid HH, Schmid PC and Natarajan V. (1990) N-acylated glycerophospholipids and their derivatives. *Prog Lipid Res* 29: 1-43

Schmidt HH. (1992) NO., CO and .OH. Endogenous soluble guanylyl cyclase-activating factors. *FEBS Letters* 307: 102-107.

Schwedt TJ and Dodick DW. (2009) Advanced neuroimaging of migraine. *Lancet Neurol* 8: 560-568.

Sharir H and Abood ME. (2010) Pharmacological characterization of GPR55, a putative cannabinoid receptor. *Pharmacol Ther.* 126: 301-313.

Shivachar AC, Martin BR and Ellis EF. (1996) Anandamide- and delta9-tetrahydrocannabinol-evoked arachidonic acid mobilization and blockade by SR141716A [N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride]. *Biochem Pharmacol* 51: 669-676.

Spicuzza L, Porta C, Bramanti A, Maffei M, Casucci G, Casiraghi N and Bernardi L. (2005) Interaction between central-peripheral chemoreflexes and cerebro-cardiovascular control. *Clin Auton Res* 15: 373-381

Stefano GB, Salzet B and Salzet M. (1997) Identification and characterization of the leech CNS cannabinoid receptor: coupling to nitric oxide release. *Brain Res* 753: 219-224.

Stefano GB, Rialas CM, Deutsch DG and Salzet M. (1998) Anandamide amidase inhibition enhances anandamide-stimulated nitric oxide release in invertebrate neural tissues. *Brain Res* 793: 341-345.

Steffens M, Feuerstein TJ, van Velthoven V, Schnierle P and Knorle R. (2003) Quantitative measurement of depolarization-induced anandamide release in human and rat neocortex. *Naunyn-Schmiedeberg's Arch Pharmacol* 368: 432-436.

Stein EA, Fuller SA, Edgmond WS and Campbell WB. (1996) Physiological and behavioural effects of the endogenous cannabinoid, arachidonylethanolamide (anandamide), in the rat. *Br J Pharmacol* 119:107–114.

Stein EA, Fuller SA, Edgemond WS and Campbell WB. (1998) Selective effects of the endogenous cannabinoid arachidonylethanolamide (anandamide) on regional cerebral blood flow in the rat. *Neuropsychopharmacology* 19: 481-491

Stella N, Schweitzer P and Piomelli D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* 388: 773-778.

Stella N (2010) Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia* 58: 1017-1030

Stone and Marletta. (1998) Synergistic activation of soluble guanylate cyclase by YC-1 and carbon monoxide: implications for the role of cleavage of the iron-histidine bond during activation by nitric oxide. *Chem Biol* 5: 255-261.

Straiker A, Stella N, Piomelli D, Mackie K, Karten HJ and Maguire G. (1999) Cannabinoid CB1 receptors and ligands in vertebrate retina: localization and function of an endogenous signaling system. *Proc Natl Acad Sci* 96: 14565-14570.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A and Waku K. (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215: 89-97.

Sugiura T, Kodaka T, Nakane S, Kishimoto S, Kondo S and Waku K. (1998) Detection of an endogenous cannabimimetic molecule, 2-arachidonoylglycerol, and cannabinoid CB1 receptor mRNA in human vascular cells: is 2-arachidonoylglycerol a possible vasomodulator? *Biochem Biophys Res Commun* 243: 838-843

Sugiura T Kobayashi Y, Oka S and Waku K. (2002) Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids* 66: 173-192.

Tenhunen R, Marver HS, and Schmid R. (1969) Microsomal heme oxygenase. Characterization of the enzyme. *J Biol Chem* 244: 6388-6394.

Tsutsumi T, Kobayashi T, Miyashita M, Watanabe S, Homma Y and Okuyama H. (1995) A lysophosphoinositide-specific phospholipase C distinct from other phospholipase C families in rat brain. *Arch Biochem Biophys* 317: 331-336.

Turu G and Hunyady L. (2010) Signal transduction of the CB1 cannabinoid receptor. *J Mol Endocrinol* 44: 75-85.

Ueda N, Puffenbarger RA, Yamamoto S and Deutsch DG. (2000) The fatty acid amide hydrolase (FAAH). *Chem Phys Lipids* 108: 107-121.

Ueda N, Yamanaka K and Yamamoto S. (2001) Purification and characterization of an acid amidase selective for N-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. *J Biol Chem* 276: 35552-35557.

Van der Stelt M, Veldhuis WB, van Haften GW, Fezza F, Bisogno T, Bar PR, Veldink GA, Vliegthart JF, Di Marzo V and Nicolay K. (2001) Exogenous anandamide protects rat brain against acute neuronal injury in vivo. *J Neurosci* 21: 8765-8771.

Vangeison G, Rempe DA. (2009) The Janus-faced effects of hypoxia on astrocyte function. *Neuroscientist* 15: 579-588

Varga K, Lake K, Martin BR, Kunos. (1995) G Novel antagonist implicates the CB1 cannabinoid receptor in the hypotensive action of anandamide. *Eur J Pharmacol*: 278:279-283.

Varga K, Wagner JA, Bridgen DT and Kunos G. (1998) Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J* 12: 1035-1044.

Vigne P, Feolde E, Ladoux A, Duval D and Frelin C. (1995) Contributions of NO synthase and heme oxygenase to cGMP formation by cytokine and hemin treated brain capillary endothelial cells. *Biochem Biophys Res Commun* 214: 1-5.

Vivian JA, Kishioka S, Butelman ER, Broadbear J, Lee KO and Woods JA. (1998) Analgesic, respiratory and heart rate effects of cannabinoid and opioid agonists in rhesus monkeys: antagonist effects of SR141716A. *J Exp Pharmacol Exp Ther* 286: 697-703.

Vogel Z, Barg J, Levy R, Saya D, Helman E and Mechoulam R. (1993) Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *J Neurochem* 61: 352-355.

Vogel KM, Hu S, Spiro TG, Dierks EA, Yu AE and Burstyn JN. (1999) Variable forms of soluble guanylyl cyclase: protein-ligand interactions and the issue of activation by carbon monoxide. *J Biol Inorg Chem* 4: 804-813.

Vreman HJ, Lee OK and Stevenson DK. (1991) In vitro and in vivo characteristics of a heme oxygenase inhibitor: ZnBG. *Am J Med Sciences* 302: 335-341.

Vreman HJ, Wong RJ, Sanesi CA, Dennery PA and Stevenson DK. (1998) Simultaneous production of carbon monoxide and thiobarbituric acid reactive substances in rat tissue preparations by an iron ascorbate system. *Canad J Physiol Pharmacol* 76: 1057-1065.

Wada A, Ohnishi T, Nonaka Y, Okamoto M and Yamano T. (1985) Synthesis of aldosterone by a reconstituted system of cytochrome P-450_{11β} from bovine adrenocortical mitochondria. *J Biochem* 98: 245-256.

Wagerle LC, Kumar SP, Delivoria-Papadopoulos M. (1986) Effect of sympathetic nerve stimulation on cerebral blood flow in newborn piglets. *Pediatr Res* 20: 131-135.

Wagner JA, Varga K, Ellis EF, Rzigalinski BA, Martin BR and Kunos G. (1997) Activation of peripheral CB1 cannabinoid receptors in haemorrhagic shock. *Nature* 390: 518-521.

Wagner JA, Varga K and Kunos G. (1998) Cardiovascular actions of cannabinoids and their generation during shock. *J Mol Med* 76: 824-836.

Wagner JA, J rai Z, B tkai S, Kunos G. (2001) Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB₁ receptors. *Eur J Pharmacol* 423: 203-210.

Wenger T, Toth BE and Martin BR. (1995) Effects of anandamide (endogen cannabinoid) on anterior pituitary hormone secretion in adult ovariectomized rats. *Life Sci* 56: 2057-2063.

Wenger T, Fernandez- Ruiz JJ and Ramos JA. (1999) Immunocytochemical demonstration of CB1 cannabinoid receptors in the anterior lobe of the pituitary gland. *J Neuroendocrinol* 11: 873-878.

White KA and Marletta MA. (1992) Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31: 6627-6631.

Willis AP and Leffler CW. (2001) Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *Am J Physiol Heart Circ Physiol* 281: H2366-H2377.

Wilson MH, Newman S and Imray CH. (2009) The cerebral effects of ascent to high altitudes. *Lancet Neurol* 8: 175-191.

Winestone JS, Bonner C and Leffler CW. (2003) Carbon monoxide as an attenuator of vasoconstriction in piglet cerebral arterioles. *Biol Med* 228: 46-50.

Wolff DG. (1976) The formation of carbon monoxide during peroxidation of microsomal lipids. *Biochem Biophys Communications* 73: 850-857.

Wu X and Davis MJ. (2001) Characterization of stretch-activated cation current in coronary smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 280: H1751-61.

Xi Q, Tcheranova D, Parfenova H, Horowitz B, Leffler CW, Jaggar JH. (2004) Carbon monoxide activates KCa channels in newborn cerebral arteriole smooth muscle cells by increasing the apparent Ca²⁺-sensitivity of α -subunits. *Am J Physiol Heart Circ Physiol* 286: H610-H618.

Xi Q, Umstot E, Zhao G, Narayanan D, Leffler CW and Jaggar JH. (2010) Glutamate regulates Ca²⁺ signals in smooth muscle cells of newborn piglet brain slice arterioles through astrocyte – and heme oxygenase-dependent mechanisms. *Am J Physiol Heart Circ Physiol* 298: H562-H569.

Xi Q, Tcheranova D, Basuroy S, Parfenova H, Jaggar JH and Leffler CW. (2011) Glutamate-stimulated, astrocyte-derived carbon monoxide production is intracellular calcium concentration –dependent in piglets (Abstract) *FASEB J* 24: 1b556.

Yang Y and Rosenberg GA. (2011) Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. *Stroke*, 42: 3323-3328.

Zakhary R, Gaine SP, Dinerman JL, Ruat M, Flavahan NA and Snyder SH. (1996) Heme oxygenase 2: Endothelial and neuronal localization and the role in endothelium-dependent relaxation. *Proc Natl Acad Sci USA* 93: 795-798.

Zakrzaska A, Schlicker E, Baranowska M, Kozłowska H, Kwolek G. (2010) A cannabinoid receptor, sensitive to O-1918, is involved in the delayed hypotension induced by anandamide in anaesthetized rats. *Br J Pharmacol* 160: 574-584.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, DiMarzo V, Julius D and Hogestatt ED. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400: 452-457.

Zygmunt PM, Andersson DA and Hogestatt ED. (2002) Delta 9-tetrahydrocannabinol and cannabinol activate capsaicin-sensitive sensory nerves via a CB1 and CB2 cannabinoid receptor-independent mechanism. *J Neurosci* 22: 4720-4727.

11. LIST OF PUBLICATIONS

Articles

1. Leszl-Ishiguro M, Horváth B, Johnson RA, Johnson FK, Lenzsér G, Hermán P, Horváth EM, Benyó Z. (2007) Influence of the heme oxygenase pathway on cerebrocortical blood flow. *NeuroReport* 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. (2013) Role of endocannabinoids and cannabinoid-1 receptors in cerebrocortical blood flow regulation. *PLoS One* 8(1): e53390. **(IF: 3.730)**

*These authors contributed equally to the publication.

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