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

Ph.D. Dissertation

Dr. András Iring

Semmelweis University
Doctoral School of Basic Medicine

Supervisor: Zoltán Benyó, MD, D.Sc.
Consultant: Péter Sándor, MD, D.Sc.

Official Reviewers: Eszter Farkas, MD, Ph.D.
Tamás Terebessy, MD, Ph.D.

Head of the Final Examination Committee:
Attila J Szabó, MD, D.Sc.

Members of the Final Examination Committee:
Pál Riba, MD, Ph.D.
Ádám Dénes, MD, Ph.D.

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1. Abbreviations

2-AG	2-arachidonoyl glycerol
AA	Arachidonic acid
ANOVA	Analysis of variance
BH_4	Tetrahydrobiopterin
BP	Blood pressure
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanosine monophosphate
Ca_v	Voltage operated calcium channel
CB1	Cannabinoid-1
CB2	Cannabinoid-2
CBF	Cerebral Blood Flow
CNS	Central nervous system
CO_2	Carbon dioxide
CoBF	Cerebrocortical Blood Flow
COX	Cyclooxygenase
cpm	Cycle per minute
DAG	Diacylglycerol
EC	Endocannabinoid
EDHF	Endothelium-derived hyperpolarizing factor
ET-1	Endothelin-1
FAAH	Fatty acid amide hydrolase
FFT	Fast Fourier Algorithm
GMP	3',5' guanosine monophosphate
GPCR	G-protein coupled receptors
GTP	Guanosine triphosphate
H/H	Hypoxia and hypercapnia
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
i.v.	Intravenous
ICP	Intracranial pressure
IK_{Ca}	Intermediate calcium-activated potassium channel
IP_3	Inositol-1,4,5-trisphosphate
K_{IR}	Inwardly rectifying K channels
LARG	Leukemia-associated RhoGEF
LD	Laser-Doppler
LDF	Laser-Doppler flux
L-NAME	Nω-nitro-L-arginine methyl ester
LOX	Lipoxygenase
LPA	Lysophosphatidic acid
LPC	1-O-alkyl-lysophosphatidylcholine

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LT	Leukotriene
LX	Lipoxin
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT1	Myosin phosphatase targeting subunit 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
$PaCO_2$	Arterial carbon dioxide tension
PAF	Platelet-activating factor
PaO_2	Arterial oxygen tension
PG	Prostaglandin
PGI_2	Prostacyclin
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cyclic GMP-dependent kinase
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PUFA	ω-3 polyunsaturated fatty acids
RhoGEF	Rho-specific guanine exchange factors
RR	Respiratory rate
S1P	Sphingosine-1-phosphate
SAH	Subarachnoid hemorrhage
SEM	Standard error of the mean
SK_{Ca}	Small calcium-activated potassium channel
THC	Δ^9-tetrahydrocannabinol
TP	Thromboxane
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid type-1
TXA_2	Thromboxane A_2
UTP	Uridine-5'-triphosphate
WHO	World Health Organization

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2. Introduction

The occurrence of cerebrovascular diseases, consisting of ischemic-, hemorrhagic stroke and subarachnoid hemorrhage, are the fourth leading cause of death in the modern world [1]. On average, every 40 seconds someone experiences stroke, and every 4 minutes it causes death [2]. During stroke, the cerebral blood flow is insufficient thus the consequent reduction in oxygen availability (hypoxia) impairs cellular respiration and energy production, resulting in the decreased availability of ATP; subsequently, ATP-dependent ion pumps, such as the Na^+/K^+ ATPase, are unable to maintain the physiological transmembrane ion gradients. The rapid accumulation of Ca^{2+}, due to influx from the extracellular space, results in the activation of lipases, for instance phospholipase A_2, which contributes to the formation of arachidonic acid metabolites [3]. Furthermore, the increased intracellular Ca^{2+} concentration activates, via the Ca^{2+} - calmodulin complex, the neuronal and endothelial nitric oxide synthase (NOS); resulting in the enhanced production of the potent vasodilatory mediator, nitric oxide (NO) [4,5]. The initial burst of NO generation is followed by a gradual exhaustion in the enzymatic activity especially in cases associated with hypertension [6,7], traumatic brain injury [8] and diabetes mellitus [9], resulting in the decreased bioavailability of nitric oxide.

It has been previously observed that under pathophysiological conditions coupled with limited oxygen and NO availability, the vascular reactivity is enhanced [10]; low frequency oscillations in the cerebral blood flow appears, described as vasomotion in isolated vessels, which phenomenon reportedly precedes the appearance of vasospasm [11,12].

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

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2.1. Cerebrovascular disease

2.1.1. Definition and pathogenesis of stroke

Stroke is defined, as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin" [13]. Based on the pathological background of stroke, we can distinguish between ischemic and hemorrhagic origin. The major cause of ischemic stroke (infarction) is the complete occlusion of large cervical and cerebral arteries resulting in infarction of the territory supplied by the occluded artery. This can be due to occlusion at the site of a main atherosclerotic lesion or to embolism originating from other parts of the arterial system, most frequently, from cardiac cavities or valves, or can be due to cardiac rhythm disturbances [14].

Hemorrhagic stroke is spontaneous intracerebral bleeding and is mainly due to arteriolar hypertensive disease. Other potential risk factors are coagulation disorders, vascular malformation within the brain, and diet (such as high alcohol consumption, low blood cholesterol concentration, etc. [14]). Subarachnoid hemorrhage (SAH) is mainly due to the rupture of aneurysms at the bifurcations of large arteries on the inferior surface of the brain. The bleeding occurs between the arachnoid membrane and the pia mater. A serious complication of SAH is vasospasm, in which the blood vessels constrict consequently restricting blood flow, and causing ischemic brain injury (delayed ischemia) [15].

2.1.2. Risk factors of stroke

Risk factors of stroke comprise of inherent biological traits, physiological characteristics, behaviour, social characteristics and environmental features [16,17]. Inherent biological traits consist of characteristics that cannot be altered such as age and sex. Physiological factors predict future occurrence of stroke such as high blood pressure, serum cholesterol levels, blood glucose or plasma fibrinogen concentrations. At population level, behaviour, such as smoking and alcohol consumption are the most important modifiable risk factors. Social characteristics, encompassing social class and ethnical groups, and environmental features such as temperature are also important risk factors.

2.1.3. Incidence and mortality of cerebrovascular diseases

According to WHO´s global mortality report in 2015, 17 million deaths are attributed to cerebrovascular diseases annually, of which 6.24 million deaths are stroke associated. Approximately 795000 individuals experience stroke each year in the United States, 610000 of these are first attacks, and 185000 are recurrent attacks [2]. Of all strokes, 87 % are due to ischemic origin, 10 % is due to intracerebral hemorrhage, whereas 3 % is SAH stroke. Approximately 40000-50000 acute stroke events occur in Hungary annually, from which circa 40 % is fatal within 30 days [18,19].

2.2. Cerebral circulation

The cerebral circulation differs in many aspects from the systemic circulation. Brain is enclosed in the skull that does not allow the expansion of either tissue or extracellular fluid; therefore a tight regulation of cerebral blood flow is required. A unique property of the cerebral circulation is that large arteries account for a greater proportion of vascular resistance in the brain than in other vascular beds. This unusual function of large cerebral arteries facilitates constant blood flow to neuronal tissue and protects the cerebral microcirculation during fluctuations in arterial pressure [20].

2.2.1. Anatomy

The brain is one of the most highly perfused organs in the body receiving approximately 750 milliliters of blood per minute, 15 % of the cardiac output [21]. Blood is supplied by the right and left *internal carotid* and the right and left *vertebral arteries.* The internal carotid arteries principally supply the cerebrum, whereas the two vertebral arteries join distally to form the *basilar artery* [20]. Branches of the vertebral and basilar arteries supply blood for the cerebellum and brain stem. Proximally, the basilar artery joins the two internal carotid arteries and other communicating arteries to form a complete anastomotic ring at the base of the brain known as the *circle of Willis*, from which stems three pairs of main arteries, the *anterior*, *middle*, and *posterior cerebral arteries* (Figure 1), which divide into progressively smaller arteries and arterioles that run along the surface within the pia–arachnoid. These pial vessels give rise to smaller arteries that eventually penetrate into the brain tissue through the Virchow–Robin space that is a continuation of the subarachnoid space. The penetrating arteries become parenchymal arterioles once they penetrate into the brain tissue and supply blood to the

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corresponding regions of the cerebral cortex. The cerebral venous system comprises of superficial cortical veins that are located in the pia matter on the surface of the cortex and drain the cerebral cortex and subcortical white matter. The deep or central veins consist of subependymal veins, internal cerebral veins, basal vein, and the great vein of Galen. Venous outflow is directed via a confluence of sinuses toward the sigmoid sinuses and jugular veins. The cerebellum is drained primarily by two sets of veins, the inferior cerebellar veins and the occipital sinuses. The brain stem is drained by the veins terminating in the inferior and transverse petrosal sinuses [20].

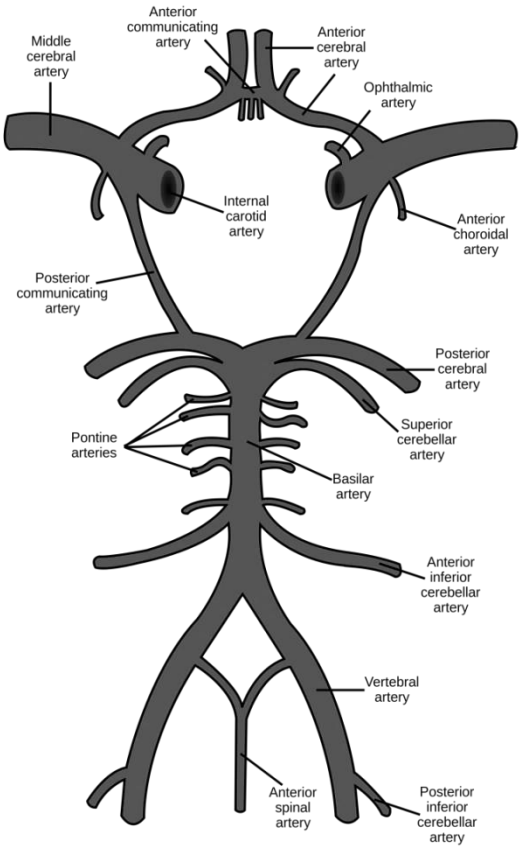

Figure 1. Circle of Willis. Modified after 20th U.S. edition of *Gray's Anatomy of the Human Body* [22].

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2.2.2. Regulation of cerebrovascular tone

Normal neuronal function requires constant supply of glucose and oxygen; therefore, the control of the cerebral blood flow (CBF) is tightly regulated. While any changes in blood flow is directly proportional to the fourth power of radius (Poiseuille´s law), even small changes in lumen diameter have significant effects on cerebral blood flow. Hence, this mechanism is the most effective to control vascular resistance and regulate regional as well as global cerebral blood flow [21]. Essentially, feed-forward mechanisms are responsible in the control of the cerebrovascular tone; the activation of these regulatory pathways precedes the imbalance between oxygen supply and demand, which in turn prevents the loss of function of the neurons. Alterations in the cardiorespiratory system leading to decreased oxygen and glucose transport directly influence the cerebrovascular tone, eliciting compensatory changes in the vascular diameter. The regulatory mechanisms involved in the control of CBF consist mainly of myogenic, metabolic, endothelial and neuronal components (Figure 2) [23].

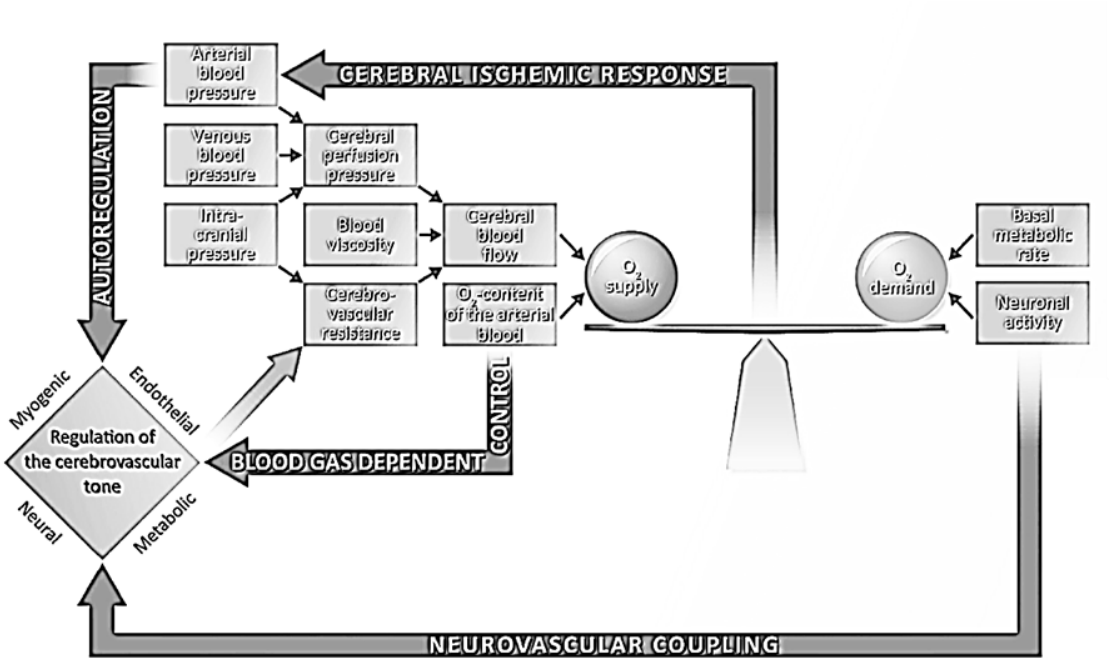

Figure 2. Mechanisms involved in the regulation of the cerebrovascular tone. Modified after Benyó et al; 2016 [23].

2.2.2.1. Autoregulation of cerebral blood flow

Autoregulation is the ability of a given vascular bed to maintain constant blood flow, despite changes in the mean arterial pressure [24]. This feature is well-developed in the brain to supply the necessary nutrients to neurons, since cerebral blood flow is constant when mean arterial pressure changes between approximately 60-160 mmHg, but outside of this range, autoregulation is lost and CBF becomes linearly dependent on mean arterial pressure. Although the exact mechanism underlying cerebral autoregulation eludes us, the endothelium, perivascular innervation and myogenic responses seem to be involved. Autoregulation is highly adaptable, its limits shift to higher values in hypertensive patients [25], but can easily be impaired by various factors such as brain damage due to direct trauma, anoxia, prolonged anesthesia, or hypercapnia [26].

2.2.2.2. Myogenic response

Myogenic response is an intrinsic property of smooth muscle to respond to changes to mechanical load and intravascular pressure [27]. Arteries contract in response to increased intravascular pressure and dilate in response to decreased pressure, thereby limiting changes in blood flow [28,29]. This phenomenon is termed autoregulation. Although myogenic activity is an innate smooth muscle property, it is modulated by the release of vascoactive factors from both endothelium and perivascular nerves and affect vascular resistance. Under physiological, resting conditions resistance arteries and arterioles display a basal tone, called *myogenic tone*, which is a state of partial constriction at a constant pressure [30]. Alteration in tone in response to pressure change is termed *myogenic reactivity* [30]. At excessively high arterial pressure exceeding the autoregulatory pressure range, the vascular tone is lost, and the diameter increases markedly, known as *forced dilation* [30].

2.2.2.2.1. Myogenic tone

Intracellular calcium increase initiates the myogenic response via opening of voltage-dependent calcium channels (Ca_v) due to increased pressure and consequent depolarization of smooth muscle cell membrane [31-33]. The rise in intracellular calcium either from intracellular stores (sarcoplasmic reticulum) or extracellular sources, leads to the activation of calmodulin and consequent phosphorylation of myosin light chain kinase (MLCK) (Figure 3), subsequent phosphorylation of myosin

light chain (MLC) and vasoconstriction. Removal of extracellular calcium abolishes the myogenic response [32,33]. Although the initiation of the myogenic response is clearly calcium dependent, the primary mechanosensor that transduces the change in pressure into depolarization and vasoconstriction is not clear. Stretch-activated cation channels, such as transient receptor potential (TRP) channel 6 and melastatin TRP 4, have been shown to contribute to vascular smooth muscle depolarization [34,35]. Besides TRP channels, chloride channels have been proposed to participate in pressure-induced smooth muscle depolarization resulting in increased entry of calcium through voltage-dependent calcium channels and consequently vasoconstriction [36]. Mechanotransduction initiated by ion channel activity leads to stimulation of actin polymerization, reduction in G-actin content in vascular smooth muscle, formation of contractile stress fibers and increase in force production [37]. Both integrins and TRP channels are linked to the actin cytoskeleton, thereby providing a possible mechanism to transduce pressure into a depolarization and contractile response [38].

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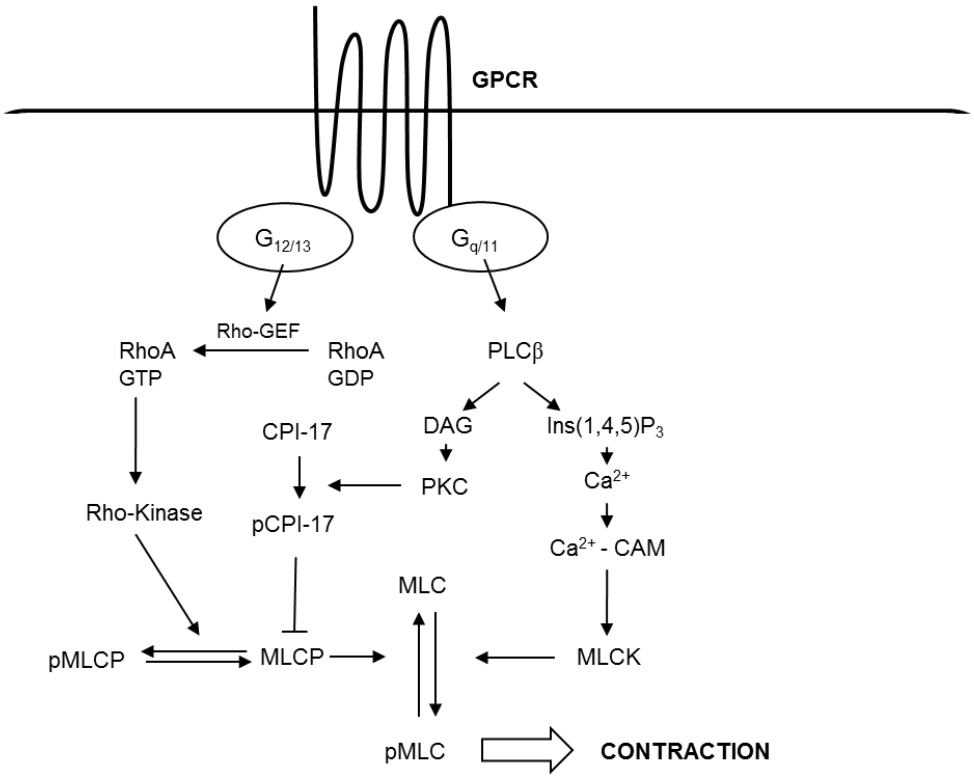

Figure 3. Intracellular signaling underlying vascular smooth muscle cell contraction. Modified after Kauffenstein et al; 2012 [38]. (CAM, Calmodulin; CPI-17, Protein kinase C potentiated Protein phosphatase-1 Inhibitor of 17 kDa; DAG, Diacylglycerol; GPCR, G-protein coupled receptor; PKC, Protein kinase C; PLCβ, Phospholipase Cβ; MLC, Myosin light-chain; MLCK, Myosin light-chain kinase; MLCP, Myosin light-chain phosphatase)

2.2.2.2.2. Myogenic reactivity

Myogenic reactivity on the other hand is modulated by vasoactive stimuli, rather than intravascular pressure. Smooth muscle contraction and relaxation is primarily dependent on the phosphorylation state of MLC and is under dual control by MLCK and myosin phosphatase (MLCP) [39-41]. The classical pathway leading to MLCK activation is mediated through $G_{q/11}$ subtype of G-protein coupled receptors (GPCR). Binding of the ligand leads to phospholipase Cβ (PLCβ) activation and results in inositol-1,4,5-trisphosphate (IP_3) formation. IP_3 binds to its corresponding receptor on the sarcoplasmic reticulum leading to an increase of intracellular free Ca^{2+} concentration.

The complex of Ca^{2+} and calmodulin activates MLCK, which in turn phosphorylates and activates MLC. While the $G_{q/11}$ mediated pathway requires Ca^{2+}, a Ca^{2+} independent regulation also exists, termed calcium sensitization. The pathway is initiated by $G_{12/13}$ stimulation and subsequent activation of Rho-specific guanine exchange factors (RhoGEF), including PDZ-RhoGEF, p115RhoGEF and LARG [42,43], leading to RhoA and Rho-kinase activation and phosphorylation and inactivation of myosin phosphatase [44].

2.2.2.3. Endothelial regulation of tone

Endothelium is a specialized, single cell layer, covering the luminal side of the vessel. It is involved in numerous physiological processes, including regulation of inflammatory and immune responses, thrombosis, adhesion, angiogenesis, and permeability [20]. Also, it creates a barrier between the cerebral extracellular fluid and circulating blood that is permeable to water, gases, and lipid-soluble molecules, but transports selectively glucose and amino acids which are required for neuronal function. This uniquely specialized endothelium forming the blood-brain barrier is phenotypically different from the endothelium localized in the periphery; they form apical tight junction complexes that are highly similar to epithelium rather than endothelium [45,46]. Three integral proteins form tight junctions (claudin, occludin and junction adhesion molecules). Claudins are small phosphoproteins, and they comprise the most abundantly expressed component of tight junctions; claudins on adjacent endothelial cells bind homotypically to form the tight junction, thereby regulating paracellular permeability [47]. Occludins form tight junctions with claudins on neighboring cells; they differ from claudins in that while claudins require accessory proteins (zona occludens proteins and cingulin) to link it to the actin cytoskeleton [48], occludins directly bind to actin [49,50]. Junction adhesion molecules also require zona occludens proteins to couple to actin, although its exact mechanism is not fully characterized [51]. The cerebral endothelium also lacks fenestrations and displays low rate of pinocytosis; both features are unique to cerebral endothelium and important in the formation of a highly restricted and carefully controlled barrier to plasma constituents [20].

Furthermore, the endothelium produces several vasoactive mediators, most importantly nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor

(EDHF) that have a significant influence on vascular tone and consequently on cerebral blood flow.

2.2.2.3.1. Nitric oxide

Nitric oxide, historically termed endothelium-derived relaxant factor [52], is a short half-lived gaseous signaling molecule, produced from L-arginine, oxygen and NADPH by nitric oxide synthase (NOS). The endothelium synthesizes NO that diffuses freely to smooth muscle and interacts with soluble guanylate-cycles to generate cyclic GMP (3',5'-guanosine monophosphate) (cGMP) from guanosine triphosphate (GTP). The soluble cGMP stimulates protein kinase G (PKG) activity, a serine/threonine kinase that promotes the opening of calcium-dependent potassium channels, resulting in hyperpolarization, inhibits PLC activity, consequently causing smooth muscle relaxation and activates K_{ATP} channels, promoting channel opening [53-55]. Furthermore, PKG directly phosphorylates MLCP in a stimulatory fashion, resulting in the dephosphorylation of MLC; additionally, PKG also phosphorylates RhoA, phospholamban and IRAG on inhibitory phosphosites, hence inhibits the activation of ROCK, increases Ca^{2+}-sequestration and decreases Ca^{2+}-release [56]. Three different NOS isoforms are present in the brain; neuronal (nNOS) and endothelial (eNOS) are constitutively expressed in neurons and cerebral endothelium, respectively, and inducible (iNOS) expression is induced under certain pathological conditions. eNOS activity can be stimulated by increase in intracellular calcium [5], the result of either binding of a vasoactive ligand (such as acetylcholine, bradykinin) or shear stress induced phosphorylation cascade [57]. During NO production, free radicals are produced, such as O_2^- and hydrogen peroxide, when lower than optimal concentration of the essential co-factor tetrahydrobiopterin (BH_4) is available. This uncoupling phenomenon results in lower NO bioavailability, generation of reactive oxygen species and endothelial dysfunction [5].

2.2.2.3.2. Prostacyclin

Increase in intracellular calcium induces arachidonic acid metabolism via phospholipase A_2 to produce arachidonic acid lipid precursors that are substrates for cyclooxygenase (COX), lipoxygenases, and cytochrome P450 monooxygenases [for further details please refer to 2.3.1]. Prostacyclin is produced from prostaglandin H_2 and performs its

function through paracrine signaling on prostacyclin receptor activating adenylate cyclase, leading to increased cyclic AMP (cAMP) production and protein kinase A (PKA) stimulation in smooth muscle [58]. In turn PKA phosphorylates MLCK, reducing its activity that consequently leads to smooth muscle relaxation and vasodilation [58].

2.2.2.3.3. Endothelium-derived hyperpolarizing factor

Endothelium-derived hyperpolarizing factor includes vasoactive metabolites that are responsible for the residual vasodilator mechanism after NOS and COX inhibition. Although the chemical nature of EDHF remains elusive, it has been shown that the effect of EDHF on small penetrating parenchymal arteries depends on the activity of small and intermediate calcium-activated potassium (SK_{Ca} and IK_{Ca} respectively) channels [59]. Recent evidence suggests that K^+ released from endothelial cells into the myoendothelial junction activates the Na^+-K^+-ATPase and K_{IR} (inwardly rectifying K channels), and is responsible for the hyperpolarization of smooth muscle (or repolarization in the presence of vasoconstrictor) [60,61]. In the brain, it has been proposed that in addition to K^+, epoxyeicosatrienoic acids, hydrogen peroxide, anandamide, C-type natriuretic peptide and interestingly, ATP may convey EDHF related responses [62].

2.2.2.4. Perivascular nerves and neural-astrocyte regulation

Extracerebral vessels, located at the base and surface of the brain, are innervated by perivascular nerves originating from the superior cervical ganglion, responsible for the sympathetic innervation, the sphenopalatine and otic ganglia, mainly supplying parasympathetic nerves, as well as the trigeminal ganglion [63]. This extrinsic innervation disappears when perforating arteries enter the brain. These parenchymal arterioles have unique properties compared to the microvasculature found in other organs; the endothelial cells are wrapped around by specialized contractile cells, termed pericytes or Rouget cells [64], that are embedded in the basement membrane, separating it from the endothelial cells, and project a sheath around endothelial cells, in which they communicate with the endothelium through either synapse-like direct contact or with paracrine signaling [65]. Pericytes have important homeostatic and hemostatic functions in the brain by stabilizing the maturation of endothelial cells, as well as regulating

capillary blood flow, phagocytosis and clearance of cellular debris, and the permeability of the blood-brain barrier [64,66]. The basal lamina of the cerebral microvasculature is in close connection with astrocytes and neurons that regulate the surrounding microcirculation, hence it is termed intrinsic innervation. Intrinsic efferents originate from subcortical neurons, such as the basal forebrain, the raphe nucleus, locus coreuleus or local cortical interneurons [67,68]; however this type of innervation targets mainly astrocytes rather than parenchymal arterioles [67]. Common feature of both extrinsic- and intrinsic innervation is that nerve endings do not form classical synaptic junctions, but contain varicosities, thereby modulating directly the vascular tone upon stimulation [63]. The main role of the sympathetic innervation, besides the direct effect of vasocontraction, is to shift the upper limit of autoregulation toward higher pressure, thus protecting the brain against high blood pressure. The parasympathetic system causes vasodilation and it appears to be involved in pathological situations, such as ischemia and migraine [69]. Intrinsic innervation is based on the neuronal-astrocytic-vascular tripartite unit, also called neurovascular unit [63]. The innervating fibers sent by the neurons to microvessels and astrocytes contain acetylcholine and nNOS that are responsible for vasodilation and local increase in CBF. Astrocytes are targeted by both noradrenergic and cholinergic neurons, and are responsible for modulating the vascular tone in accordance with neuronal demand [63].

2.2.2.5. Effect of arterial gas tensions

Due to the high metabolic demand of the brain, it is not surprising that reduction in arterial oxygen tension (PaO_2) or increase in arterial carbon dioxide tension ($PaCO_2$) are potent dilators in the cerebral circulation. Hypoxia (<50 mmHg PaO_2) increases CBF, as well as oxygen extraction from blood. Decrease in ATP levels due to hypoxia activates potassium channels (K_{ATP}) causing hyperpolarization in smooth muscle cells and a consequent vasodilation [70,71]. Furthermore, adenosine acting on its A2-type receptor increases adenylyl-cyclase activity and consequent increase in cAMP levels, leading to the activation of PKA [72]. Although direct evidence is lacking, it has been proposed that PKA directly phosphorylates K_{ATP} channels, contributing to the opening of the channel [72]. On the other hand, even small changes in $PaCO_2$ modulate cerebral perfusion. Hypercapnia relaxes cerebrovascular smooth muscle, an increase in $PaCO_2$ over 50 mmHg increases CBF by more than 50%, whereas hypocapnia produces

vasoconstriction [73]. Carbon dioxide induces changes in the extracellular hydrogen ion concentration that directly act as vasoactive mediator on vascular smooth muscle and may also modulate the effects of other vasoactive agents, such as noradrenalin [74]. Vasodilation during hypercapnia is also dependent on nitric oxide formation [75,76], partially due to acidosis that increases the activity of NOS [75] and, as shown more recently, due to direct influence of $PaCO_2$ on NO production [77].

2.3. Lipid mediators

Lipid mediators are bioactive lipids that are synthesized and released to external stimuli, and act on their corresponding receptors as local hormones or autacoids. Structurally they are divided into three categories. *Class 1* includes arachidonic acid derived metabolites, called eicosanoids, such as prostaglandins (PG) and leukotrienes (LT). *Class 2* includes lysophospholipids, including platelet-activating factor (PAF), lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). *Class 3* consists of newly identified anti-inflammatory lipids derived from ω-3 polyunsaturated fatty acids (PUFA) [78].

2.3.1. The arachidonic acid cascade

Arachidonic acid (AA) is a carboxylic acid with a 20-carbon chain that contains four *cis*-double bonds, where the first double bond is situated at the sixth carbon atom from the omega end. Arachidonic acid is released by enzymatic cleavage from membrane phospholipids, mainly phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositides by phospholipase A_2 (PLA_2) or generated from diacylglycerol (DAG) by diacylglycerol lipase.

2.3.1.1. Eicosanoids

Arachidonic acid can be further metabolized via cyclooxygenase (COX) forming prostanoids, lipoxygenase (LOX) resulting in leukotriene formation, or via cytochrome P-450 producing hydroxyeicosatetraenoic acids (HETE) and epoxides. Oxygenated conversion of AA via either constitutive COX-1 or inducible COX-2 produces PGG_2 and then PGH_2. PGH_2 is further metabolized to one of the five major prostanoids that have been identified, namely PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2). They play various biological roles based on their receptor coupled to G protein subunits; PGE_2 has four receptor subtypes (EP_1-EP_4), PGD_2 has two (DP_1-DP_2), and specific receptors bind $PGF_{2\alpha}$, PGI_2 or TXA_2 (FP, IP and TP, respectively) [79]. G_s coupled receptors are IP, DP_1, EP_2 and EP_4 and act as relaxant receptors, on the other hand EP_1, FP and TP signal through G_q subunit leading to increase in intracellular Ca^{2+} concentration and contraction. DP_2 and EP_3 receptors bind $G_{i/o}$ subunit, decreasing adenylate cyclase activity (Table 1) [80]. Prostaglandins were first isolated from the prostate and seminal vesicles, hence the name [81]. Table 2 shows the various

physiological effects of the different metabolites; PGD_2 is released from mast cells and alveolar macrophages, and induces bronchoconstriction and inhibition of platelet-activating factor (PAF). $PGF_{2\alpha}$ is a potent vasoconstrictor also involved in uterine and bronchial smooth muscle contraction. PGE_2 is responsible for maintaining the physiological function of gastric mucosa by regulating the gastric mucosal blood flow, bicarbonate and mucus production. PGI_2 shows anti-aggregatory and vasodilating properties, as well as counteracts thromboxane effects. Thromboxane is mainly produced in the vascular smooth muscle, endothelium and platelets, and as the name indicates, TXA_2 is a very potent aggregating agent; in addition it induces vasoconstriction and bronchoconstriction. TXA_2 is chemically unstable and rapidly metabolized to thromboxane B_2 that is biologically inactive; considering the short half-life of TXA_2, its stable synthetic analog, U-46619, is used in research.

Lipoxygenases convert AA to hydroperoxyeicosatetraenoic acids (HPETE) and HETE. 5-HETE is the precursor substance for leukotriene LTA_4 which can be further metabolized to LTB_4, cysteinyl-LT (LTC_4, LTD_4, and LTE_4) and lipoxins (LX). The effect of LTB_4 is mediated through BLT1 and BLT2 receptors acting on $G_{i/o}$ and G_q, while cyteinyl-LT acts on CysLT1 and CysLT2 receptors via G_q and $G_{i/o}$ mechanisms [80]. Leukotrienes are responsible for inflammatory and hypersensitivity disorders, such as asthma; LTs are released from leukocytes, monocytes and macrophages, cells involved in inflammation. The release of LTs results in bronchoconstriction, vasoconstriction and an increase in vascular permeability. Lipoxins are mainly involved in the resolution of inflammation; the receptor activity inhibits chemotaxis, transmigration, superoxide anion generation and NF-κB activation.

Table 1. Physiological ligands and coupling of eicosanoid receptors

ENDOGENOUS LIGAND	RECEPTOR	COUPLING TO G PROTEIN SUBCLASS
LEUKOTRIENE B_4 (LTB_4)	BLT	$G_{q/11}$, $G_{i/o}$
LTC_4, LTD_4	CysLT1, CysLT2	$G_{q/11}$, $G_{i/o}$
PROSTACYCLIN (PGI_2)	IP	G_s
PROSTAGLANDIN D_2 (PGD_2)	DP_1	G_s
	DP_2	$G_{i/o}$
PROSTAGLANDIN $F_{2\alpha}$ ($PGF_{2\alpha}$)	FP	$G_{q/11}$
PROSTAGLANDIN E_2 (PGE_2)	EP_1	$G_{q/11}$
	EP_2, EP_4	G_s
	EP_3	G_s, $G_{q/11}$, $G_{i/o}$
THROMBOXANE A_2 (TXA_2)	TP	$G_{q/11}$, $G_{12/13}$

Table 2. Physiological effects of major eicosanoids

TISSUE	BIOLOGICAL EFFECTS	EICOSANOIDS
VESSELS	Vasoconstriction	PGF_2, TXA_2, LTC_4, LTD_4
	Vasodilatation	PGI_2, PGE_2, PGD_2
PLATELETS	Anti-aggregation	PGE_1, PGI_2
	Pro-aggregation	TXA_2
BRONCHI	Bronchoconstriction	PGD_2, $PGF_{2\alpha}$, TXA_2, LTC_4, LTD_4
	Bronchodilation	PGE_2, PGI_2
INTESTINES	Nausea, diarrhoea	PGE_1, $PGF_{2\alpha}$
	Motility	PGE_1, $PGF_{2\alpha}$
STOMACH	Inhibition of gastric acid secretion	PGE_2, PGI_2
	Motility	PGE_2, $PGF_{2\alpha}$
UTERUS	Contraction, parturition	PGE_2, PGF_2, TXA_2
KIDNEY	Filtration and renal blood flow	PGH_2, PGE_1, PGI_2
HYPOTHALAMIC AND PITUITARY AXIS	Increase in hypothalamic and pituitary hormone secretion	PGE_1, PGE_2

2.3.1.2. Endocannabinoids

Endocannabinoids are endogenous bioactive ligands for specific G-protein coupled receptors; arachidonoyl ethanolamide (anandamide) [82] acting mainly on cannabinoid receptor 1 (CB1) and 2-arachidonoyl glycerol (2-AG) [83] on CB1 and cannabinoid receptor 2 (CB2). Although the major neurotransmitter systems have been well characterized, the endocannabinoid system remained unknown until the early 1990s

[84]. Originally the effect of Δ^9-tetrahydrocannabinol (THC), produced by Cannabis sativa (marijuana), was believed to act nonspecifically on neural membranes. Emerging pharmacological data indicated specific effect of cannabinoids and in 1990. First CB1 and three years later, CB2 receptor were cloned [85]. Anandamide and 2-AG are synthesized by neurons on demand and undergo depolarization-induced release. Once they are released they are swiftly cleared from the synaptic cleft by reuptake and degraded by fatty acid amide hydrolase (FAAH) that cleaves anandamide into AA and ethanolamine or by monoacylglycerol lipase cleaving 2-AG into AA and glycerol [86]. CB1 receptors are expressed widely in the central nervous system (CNS), as well as in specific peripheral tissues, including the pituitary gland, immune cells, reproductive tissues, gastrointestinal tissue, sympathetic ganglia of the heart, lung and urinary bladder [87]. In contrast, CB2 are expressed mainly in immune cells. Both receptors signal through $G_{i/o}$ subunits, negatively affecting adenylate cyclase activity and positively impacting mitogen-activated protein kinase. Under certain conditions, CB1 can couple to G_s, but its physiological importance is yet to be resolved [87]. To study the physiological role of CB1 and CB2 receptors, selective ligands have been synthetized. SR141716A (rimonabant) is described as a CB1 specific, selective antagonist, while the compound may produce inverse cannabimimetic effect in certain tissues by reducing the constitutive activity of the receptor [87]. The most commonly used selective antagonist (or inverse agonist) against CB2 are SR144528 and AM630. Two structural analogs of SR141716A are marketed, namely AM-251 and AM281, which have been found to be less potent than SR141716A, but have higher affinity to CB1 receptors [87]. Several agonists have been described, of these, HU-210 and WIN55212 are the most widely used in pharmacological studies; both agonists bind CB1 and CB2 equally well. Another possibility to stimulate the endocannabinoid system is to inhibit its rapid clearance by reuptake inhibitor, namely AM-404. Although the exact mechanism of the inhibition is not yet fully elucidated, it has been shown that AM-404 prevents the reuptake of anandamide as well as blocks the metabolism by inhibiting FAAH-mediated hydrolysis; besides AM-404 also blocks COX activity [88]. The role of the endocannabinoid system appears to be the regulation of spontaneous or evoked release of chemical transmitters. Hence neurotransmitter release can be inhibited through presynaptic CB1 receptors, as well as the release of cytokines from immune

cells can be altered via CB1/CB2 receptors. Besides the canonical cannabinoid receptors, other non-cannabinoid receptors and ion channels have been proposed to be activated or inhibited by endocannabinoid ligands (Table 3). One of the G protein-coupled receptors, GPR55, demonstrated activation and Ca^{2+} mobilization when stimulated with anandamide, 2-AG or noladin ether in heterologous overexpression system [89,90]; however the exact physiological relevance of GPR55 is not yet established [84]. Numerous ligand-gated ion channels respond to endocannabinoids; anandamide exhibit allosteric inhibition on serotonin (5-hydroxytryptamine) receptor 3 [91,92], although contradictory evidence has also been published [93]. Nicotinic acetylcholine receptor activation by either nicotine or acetylcholine is antagonized in a non-competitive manner by anandamide and 2-AG, albeit the exact underlying mechanism is still largely unknown [94]. Glycine receptor activation in heterologous expression systems is further enhanced in the presence of anandamide [95,96]; however, contradictory evidence has emerged, where isolated hippocampal neurons exhibited inhibition of glycine receptors in the presence of anandamide or 2-AG, and this effect was independent of CB1 or TRPV1 receptors [97]. Additionally, TRPV1 receptors have been found to increase Ca^{2+} influx when stimulated with anandamide [98].

Table 3. Endogenous endocannabinoid and non-cannabinoid receptors and their putative ligands

TARGET CLASS	TARGET NAME	ENDOGENOUS COMPOUND
GPCR	CB1	anandamide, 2-AG, noladin ether, virodhamine, oleamide
	CB2	anandamide, 2-AG
	GPR55	anandamide, 2-AG, noladin ether, virodhamine
LIGAND-GATED ION CHANNEL	$5\text{-}HT_3$	anandamide
	Nicotinic acetylcholine	anandamide, 2-AG
	Glycine	anandmaide, 2-AG
TRP CHANNEL	TRPV1	anandamide, 2-AG

Endocannabinoids exert their pharmacological actions in the nervous system by modulating neurotransmitter release [99]. Their inhibitory effects on presynaptic Ca^{2+} channels counteract depolarization and the consequent release of neurotransmitters, as well as the facilitatory effect on inwardly rectifying K^+ channels prevents neuronal depolarization and action potential generation. However, in certain regions, endocannabinoids may potentiate neurotransmitter release [99] (Table 4). It has been shown previously that CB1 receptor inhibition enhances acetylcholine and glutamate release in the hippocampus [100,101], which consequently improves working memory in rodents, implicating that endocannabinoids tonically suppress glutamate and acetylcholine release. The analgesic effect of endocannabinoids has been linked to the inhibition of glutamate-mediated neurotransmission in the dorsal root ganglia and periaqueductal grey matter [83]. The neurotransmitter release from the peripheral autonomic nervous system is also modulated by endocannabinoids; activation of prejunctional CB1 receptors induces smooth muscle relaxation, hypotension and bradycardia [102]. The importance of the neuromodulatory action of endocannabinoids in the cardiovascular system depends on whether the animal is conscious or under anaesthesia [103]. Application of anandamide in anaesthetised animals elicits a triphasic response; a rapid, transient bradycardia, followed by a transient increase in blood pressure and then a prolonged hypotensive phase [104]. The initial phase of bradycardia is mediated by TRPV1 receptors [105]; the rapid pressor response during the second phase is associated with TRPV1, NMDA and β_2–adrenoceptor activity [104]. The prolonged hypotensive effect of anandamide is thought to be CB1 receptor dependent [106]. Interestingly, 2-AG induces strong hypotension accompanied with tachycardia; both effects are independent of CB1 receptors, and most likely involve other arachidonic acid metabolites [107]. Conversely, in conscious animals anandamide elicits lasting bradycardia and transient hypotension, followed by lasting increase in blood pressure accompanied with vasoconstriction in renal and mesenteric vascular beds [108,109]. The complex effect of endocannabinoids in conscious state is attributed to the increased level of circulating adrenaline; TRPV1 receptors have limited effect on the hemodynamic regulation in conscious state [109].

In the cerebral vasculature, endocannabinoids act as vasodilators via vasoactive prostanoid release [110]. Furthermore, activation of CB1 receptors in cerebral vascular

smooth muscle cells inhibit L-type Ca^{2+} channels, thereby influencing vasorelaxation [111]; endocannabinoids acting on CB1 receptors expressed in brain endothelial cells counteract the effect of endothelin-1, thus partially reduce cerebral vasoconstriction [112]. Given the observed effects of endocannabinoids on the cerebral vasculature, they are generally considered as neuroprotective agents during traumatic brain injury and stroke events [84]. Indeed, following traumatic injury to the brain, endocannabinoid release is enhanced tenfold [113], and the recovery was further enhanced upon administration of 2-AG; an effect that was dependent on the presence of CB1 receptors. Similarly, after acute stroke events, the circulating plasma levels of endocannabinoids were increased [114]; and they have been shown to protect against ischaemia/reperfusion, and significantly reduce infarct size [115].

Endocannabinoids are also involved in the respiratory regulation, since both the systemic administration of phytocannabinoids [116-119] and endocannabinoids [120-122] demonstrate respiratory depression, which involves CB1 and TRPV1 receptors [122]. Administration of the synthetic cannabinomimetic, WIN55212-2 or CP55940 induced severe cardiovascular depression; however, the observed effects were markedly increased in rats that were spontaneously breathing in comparison to the artificially ventilated group, suggesting that the cardiovascular depression has been further enhanced by the accompanying reduction in respiration and was abrogated by pretreatment with a CB1 receptor antagonist, SR141716A [119,122,123]. The release of endocannabinoids influences the respiratory regulation mainly centrally, since intracisternal injection of WIN55212-2 reduced breath rate and increased the tidal volume in rats in a CB1-dependent manner [120,124]. Additionally, CB1 receptor inhibition reduced the observed reduction of phrenic nerve activity following endocannabinoid injection to the brain stem, indicating the presence of CB1 receptors in the respiratory centers in the medulla and the pivotal role of endocannabinoids in the respiratory regulation [120].

Contradictory with these findings are several studies showing that administration of Δ^9-THC has no effect on the respiratory parameters in humans [125] as well as in primates receiving either Δ^9-THC or WIN55212-2, where both cannabinomimetics failed to influence the respiratory rate, although reduced the tidal volume [126].

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Table 4. Neuromodulatory actions of endocannabinoids in the nervous system. Modified after DiMarzo et al.; 1998 [99]

REGION	NEUROTRANSMITTER	MODULATORY ACTION	ECS INVOLVED	POTENTIAL EFFECT
HIPPOCAMPUS	Glutamate	Inhibition of release	anadamide, 2-AG	Inhibition of LTP
	Acetylcholine	Inhibition of release	anadamide, 2-AG	Inhibition of learning and memory
CEREBELLUM	Glutamate	Inhibition of Ca^{2+} channel	anadamide	Inhibition of motor coordination
CORTEX	Glutamate	Inhibition of Ca^{2+} channel	anadamide, 2-AG	Inhibition of memory and motor function
SPINAL CORD	Glutamate	Inhibition of release	2-AG	Antinociception
BASAL GANGLIA AND SUBSTANTIA NIGRA	GABA	Inhibition of re-uptake	anandamide	Inhibition of locomotor activity
	Dopamine	Inhibition of synthesis/release	anandamide	Inhibition of locomotor activity
	Dopamine	Potentiation	anandamide	Induction of contralateral turning
HYPOTHALAMUS	Dopamine	Potentiation	Not yet identified	Inhibition of prolactin release
PARA-SYMPATHETIC SYSTEM	Acetylcholine	Inhibition of release	Not yet identified	Inhibition of smooth muscle contraction
SYMPATHETIC NERVOUS SYSTEM	Noradrenaline	Inhibition of release	anandamide	Hypotension, bradycardia

2.3.1.3. Lysophospholipids

Lysophospholipids are small bioactive lipid molecules that consist of either a sphingoid base or a glycerol backbone and a polar head group. The first recognized lysophospholipid mediator was PAF, since then S1P and LPA have also been characterized. They behave as auto- or paracrine regulators of angiogenesis, lymphocyte trafficking, development of the nervous system, cancer growth and metastasis, inflammation and arteriosclerosis. PAF is produced from 1-O-alkyl-lysophosphatidylcholine (LPC) by PLA_2 then further acetylated by acyltransferase. It is a potent mediator of leukocyte functions, platelet aggregation and degranulation, as well as anaphylaxis and inflammation. LPA is produced by autotaxin, and acts via LPA receptors (LPA1-6) to regulate brain development, embryo implantation and hair growth, and is also associated with pathological conditions, such as neuropathic pain and pulmonary fibrosis [78,127]. S1P has sphingosine backbone instead of glycerol, and acts via S1P receptors (S1P1-5). It regulates numerous biological processes, such as development of vascular integrity, where S1P stimulates proliferation, survival and migration of endothelial cells and induces formation of adherens junction assembly, thereby decreasing vascular permeability and differentiation of endothelial cells into capillary-like networks. Also, lack of S1P receptors results in profound deafness, as well as anaphylaxis and it has been proposed to be involved in tumor angiogenesis (Table 5) [78].

Table 5. Physiological effects of major lysophospholipids

ENDOGENOUS LIGANDS	RECEPTOR	MAIN TISSUE DISTRIBUTION	SIGNALING	MAIN BIOLOGICAL EFFECT
S1P	$S1P_1$	Ubiquitous	$G_{i/o}$	Migration ↑, proliferation, survival, cell–cell-contacts, angiogenesis, lymphocyte trafficking
	$S1P_2$	Ubiquitous	$G_{i/o}$, G_q, $G_{12/13}$	Migration ↓, vascular development, differentiation of vascular smooth muscle cells
	$S1P_3$	Ubiquitous	$G_{i/o}$, G_q, $G_{12/13}$	Heart rate ↓, vascular development, NO-dependent vasorelaxation
	$S1P_4$	Lymphoid and hematopoietic tissue	$G_{i/o}$, $G_{12/13}$	Proliferation and cytokine secretion in T-cells ↓
	$S1P_5$	Brain, white matter	$G_{i/o}$, $G_{12/13}$	Proliferation, cell rounding
LPA	LPA_1	Ubiquitous	$G_{i/o}$, G_q, $G_{12/13}$	Proliferation, survival, neurite retraction, brain development
	LPA_2	Ubiquitous	$G_{i/o}$, G_q, $G_{12/13}$	Proliferation, survival
	LPA_3	Ubiquitous	$G_{i/o}$, G_q	Implantation and embryo spacing in mice

2.3.1.4. ω-3 polyunsaturated fatty acid derivatives

New class of lipid mediators derived from ω-6 arachidonic acid, such as resolvins, protectins, maresins and lipoxins, have been proposed to be crucial mediators of the resolution of inflammation [78], although *in vivo* relevance and identification of their specific receptor type require further investigation.

3. Statement of Purpose

3.1. Hypersensitivity to Thromboxane Receptor Mediated Cerebral Vasomotion and CBF Oscillations during Acute NO-Deficiency in Rats

Low frequency oscillations [128] of cerebrovascular tone and cerebral blood flow that are independent of the cardiac or respiratory cycles [129] have been observed in diseases associated with endothelial dysfunction. The decreased bioavailability of endothelium-derived nitric oxide under these pathophysiological conditions has been shown to promote vasomotion and oscillation in the cerebral blood flow [12,130]. It has also been shown that nitric oxide synthase inhibition provokes vulnerability to vasomotion that can be triggered by the administration of UTP or U-46619, the chemically stable analogue TXA_2, and is mediated by TP-receptors in isolated cerebral arteries [131]. In the present study we hypothesized that in the absence of NO, hypersensitivity of TP-receptor mediated cerebrovascular signaling contributes to the development of vasomotion and CBF oscillations in the cerebral cortex of rats [132].

3.2. Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation

Endocannabinoids have been implicated in various physiological and pathophysiological processes, such as hypertension, ischemia-reperfusion injury, chemotherapy-induced myocardial injury, hepatic cirrhosis, cardiomyopathy, atherogenesis [133], diabetes [134] and obesity [135]. Regardless of multiple literary evidence published concerning the role of endocannabinoids in the regulation of cerebral blood flow (CBF), the findings were conflicting. It has been shown that Δ^9-THC and anandamide increases CBF in dogs [136], humans [137] and cats [111], but reduces CBF in rats [138]; although, in another study the authors observed that anandamide elicited marked cerebral vasodilatation via CB1 receptor mediated mechanism in rats [139]. To address these contradictory findings and clarify the role of endocannabinoids in the cerebral circulation as well as to overcome the apparent limitation of the published results, namely the utilization of exogenously applied agonists, in the present study the endogenous cannabinoid levels have been regulated via the administration of an EC-reuptake inhibitor (AM-404). Furthermore, we have

tested the effect of a CB1 receptor antagonist/inverse agonist (AM-251) in order to characterize the influence of constitutively active CB1 receptors in the cerebrovascular and cardiovascular regulation under resting conditions [140].

3.3. Cannabinoid-1 Receptor-mediated Respiratory Depression by Endocannabinoids

The endogenous bioactive mediators of Cannabis sativa (marijuana), are anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). While anandamide favors cannabinoid receptor 1 (CB1), 2-AG acts both on CB1 and cannabinoid receptor 2 (CB2) [98]. Both receptors are widely expressed in different tissues, including the respiratory system. Although numerous previous studies have observed that either exogenously applied phytocannabinoids [116] or synthetic or endogenous cannabinomimetics [119-124] elicited marked cardiovascular depression, which were dependent on the respiratory state of the animals, the exact mechanism underlying the influence of ECs on the respiratory regulation is still largely unknown. Anaesthetized, spontaneously breathing rats [119] and cats [116] showed lasting reduction in respiratory rate (RR) upon systemic injection of cannabinomimetics. Central application of the synthetic CB1/CB2 agonist, WIN55212-2, to the cisterna magna induced direct activation of brain stem respiratory regulatory centers, reduced RR and increased the tidal volume in rats [120,124]. Further investigation proposed CB1 to mediate the aforementioned respiratory effects [119], since systemic administration of SR141716A (rimonabant) abolished the respiratory depression. In our previous study the pivotal role of endogenously released ECs have been implicated in the control of breath regulation in a CB1-dependent manner [140], but direct evidence was lacking, because respiratory parameters were not measured. Conflicting with the aforementioned findings are several studies performed in humans [125,141,142] and conscious monkeys [126] that reported no changes in RR and tidal volume upon administration of Δ^9-Tetrahydrocannabinol or WIN55212-2. To unambiguously clarify the role of the endogenous cannabinoid system in respiratory control, in our present study respiratory parameters were recorded using pulse oximetry after enhancing EC levels by administration of an EC-reuptake inhibitor (AM-404). The experiments have been performed both in wild-type control and CB1-deficient (CB1-KO) mice in order to analyze the involvement of CB1 receptors [143].

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4. Experimental Animals, Materials and Methods

4.1. Animals

The experiments were performed in (1) adult male Wistar rats (300-400 g) according to the guidelines of the Hungarian Law of Animal Protection (243/1988) and all procedures were approved by the Semmelweis University Committee on the Ethical Use of Experimental Animals (590/99 Rh); and in (2) CB1-KO mice that were generated as previously described [144] and kindly provided by Dr. Andreas Zimmer (Institute of Molecular Psychiatry, University of Bonn, Germany).

4.2. Materials

The CB1 receptor antagonist/inverse agonist AM-251 (1-(2, 4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide) and the endocannabinoid reuptake inhibitor AM-404 (N-(4-hydroxyphenyl)-5Z, 8Z, 11Z, 14Z-eicosatetrenamide) were obtained from Cayman Chemicals (Ann Arbor, MI, USA); both chemicals were dissolved in 1 ml of vehicle containing ethanol/emulphor/saline (1:1:8; v:v:v). Ketamine (Calypsol) and xylazine (CP-Xylazine) were purchased from Richter Gedeon Plc. (Budapest, Hungary) and CP-Pharma GmbH (Burgdorf, Germany), respectively. The thromboxane A_2 receptor agonist U-46619 (9,11-dideoxy-9α,11α-methanoepoxy Prostaglandin $F_{2\alpha}$), the thromboxane A_2 inhibitor Ozagrel (sodium;(E)-3-[4-(imidazol-1-ylmethyl)phenyl]prop-2-enoate), the inhibitor of nitric oxide synthase L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride), and the specific Rho-kinase inhibitor Y-27632 ((*R*)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl) cyclohexanecarboxamide dihydrochloride), bradykinin, Uridine-5'-triphosphate (UTP), endothelin-1 (ET-1) and Urethane (Carbamic acid ethyl ester) were purchased from Sigma (St. Louis, MO, USA) (Table 6).

Table 6. Biochemical and physiological actions of the chemicals used.

CHEMICAL PRODUCT NAME	BIOCHEMICAL AND PHYSIOLOGICAL ACTION
AM-251	CB1 receptor antagonist/inverse agonist
AM-404	Endocannabinoid reuptake inhibitor
Ketamine	NMDA receptor antagonist, anaestheticum
Xylazine	α2 adrenergic receptor agonist, muscle relaxant
U-46619	Thromboxane A_2 receptor agonist
Ozagrel	Thromboxane A_2 synthesis inhibitor
L-NAME	Nitric oxide synthase inhibitor
UTP	P2Y receptor agonist, vasoconstrictor
Bradykinin	Endothelium-dependent vasodilator
ET-1	Vasoconstrictor
Y-27632	Rho-associated coiled coil forming protein serine/threonine kinase (ROCK) inhibitor
Urethane	Nicotinic acethylcholine receptor, gamma aminobutyric acid and glycine receptor agonist, NMDA receptor antagonist, anaestheticum

4.3. Methods of Measurement

4.3.1. Anesthesia and Interventions

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

Mice were anaesthetized by intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}); the depth of anaesthesia was regularly checked during the experiments by observing the corneal or plantar nociception reflex and additional anaesthetics were administered intraperitoneally when necessary. The animals were spontaneously breathing. Body temperature was recorded by a rectal probe and kept constant between 36.5 – 37.5 °C during the experiments using a heating pad.

4.3.2. Measurement of Cerebrocortical Blood Flow

Cerebrocortical blood flow (CoBF) was measured by laser-Doppler (LD) flowmetry. The head of the rat was fixed in a stereotaxic head holder with the nose 5 mm down from the interaural line. The skull of the parietal region was exposed and the bone was thinned over the parietal cortex on both sides with a microdrill, so that the lamina interna of the skull remained intact. Two LD probes were placed above the thinned skull at a 12° angle to the vertical to provide an optimal view of the cortex (4 mm caudal from bregma, 5 mm lateral from midline). LD flux (LDF) was measured with a two-channel blood flow monitor (MBF3D, Moor Instruments, UK) and was recorded continuously. The LD monitor was calibrated before each individual experiment with a constant movement latex emulsion. The laser light was in the infrared range (780 nm) and penetrated about 1 mm into the brain covering approximately 7 mm^2 of the parietal region, so that the data acquired mostly represented the characteristics of the blood flow in the parietal cortex. Blood pressure (BP) and CoBF were recorded continuously (BIOPAC Systems Inc, Goleta, CA, USA); the heart rate was calculated from the pulsating BP signal.

4.3.3. Measurement of Vascular Tone

The vascular tone of middle cerebral arteries (MCA) supplying the parietal cortex were measured *in vitro*. MCA segments were prepared from adult male Wistar rats sacrificed via exsanguination from the carotid arteries under deep ether anesthesia. The brains were removed and placed in ice-cold modified Krebs solution of the following composition (mM): NaCl, 119; KCl, 4.6; $NaH_2PO_4 \cdot H_2O$, 1.2; $CaCl_2 \cdot 2H_2O$, 1.5; $MgCl_2 \cdot 6H_2O$, 1.2; $NaHCO_3$, 15; glucose, 10. Using a binocular microscope the middle cerebral artery and its large branches (201 ± 14 μm) were dissected and studied in a conventional myograph system (610M, Danish Myo Technology A/S, Aarhus, Denmark) [145]. The segments were transferred into 5-mL organ baths filled with modified Krebs solution and the bath solution was bubbled continuously with a humidified gas mixture (90 % O_2/10 % CO_2). The MCA segments were mounted on 2 L-shaped tungsten wires (50-μm diameter): one wire was fixed to the bath, and the other was fixed to a force transducer.

4.3.4. Arterial Blood Gas Measurement

Arterial blood gas and pH measurements were performed throughout the *in vivo* rat experiments by a Radiometer (Bronshoj, Denmark) ABL-77 analyzer and by the use of a capnograph (Capstar-100, CWE Inc., Ardmore, PA, USA). However, if the onset of capnography resulted in a more than 10 mmHg reduction of the arterial O_2 tension, the device was disconnected and not used in that experiment.

4.3.5. Infrared pulse oximetry

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

4.4. Study Design

4.4.1. Experiments to investigate the role of thromboxane receptors on cerebral vasomotion and CBF oscillations during NO-deficiency:

In our *in vivo* rat experiments we have randomly assigned the animals into four experimental groups. In the control Group I. systemic and cerebral circulatory parameters, as well as blood gas and acid-base values were determined before as well as for 75 minutes after an i.v. bolus injection of 1 ml kg^{-1} vehicle (saline). Thereafter the animals received the thromboxane receptor agonist U-46619 in a dose of 1 mg kg^{-1} i.v., which in preliminary experiments was below the threshold of inducing any systemic or cerebral circulatory changes. Groups IIa., IIb. and IIc. received intravenously first NG-nitro-L-arginine methyl ester (L-NAME) in a dose of 100 mg kg^{-1} for the inhibition of NO synthesis and 75 minutes later 1 mg kg^{-1} U-46619 (Group IIa.), saline (Group IIb.) or 10 mg kg^{-1} of the thromboxane synthase inhibitor ozagrel (Group IIc.). Previous studies have verified that L-NAME and ozagrel in the doses used in our present study effectively inhibit the activity of cerebral NO synthase and thromboxane synthase, respectively [146,147]. The final measurements were performed in all experimental groups 50 minutes after the administration of U-46619, saline or ozagrel.

The role of thromboxane receptors on cerebral vasomotion was also investigated in *in vitro* conditions. These experiments were performed on isolated MCA segments; first each segment was exposed to 124 mmol L^{-1} K^{+} Krebs solution to elicit a reference contraction. After a 30-minute resting period, the functional integrity of the endothelium was tested by application of bradykinin (0.01 to 10 mM) after precontraction induced by 100 mM UTP. Segments that did not exhibit at least 20 % relaxation of the precontraction were considered to have damaged endothelium and were excluded from the study. After a 30-minute resting period, during which the baths were washed several times, the vessels received either 100 mM L-NAME in order to block NO synthesis or saline, the vehicle of L-NAME. Fifteen minutes later the effects of 100 nM U-46619 or 10 nM endothelin-1 (ET-1) were determined on the vascular tension both in intact and NO synthase blocked vessels. The role of Rho – Rho-kinase signaling pathway was tested by administration of 10 mM Y-27632, a specific Rho-kinase inhibitor [148], to NO synthase blocked vessels showing stable vasomotion after administration of U-46619 or ET-1. In additional control experiments intact MCA segments were precontracted with 25 mmol L^{-1} K^{+} Krebs prior to administration of 100 nM U-46619.

4.4.2. Experiments to address the role of endocannabinoids and Cannabinoid-1 (CB1) receptors in cerebrocortical blood flow regulation:

In these studies, we have assigned each animal to one of the following experimental protocols; in the *first protocol* the influence of CB1 receptors on the CoBF under resting conditions was studied. After a 15-min baseline period one experimental group received 1 ml vehicle (containing ethanol/emulphor/saline; 1:1:8; v:v:v); the other was treated with AM-251 (10 mg kg^{-1} i.v. [149]). Blood samples were taken before as well as 1, 2, 4, 8, 16, and 32 minutes after the administration of AM-251 or its vehicle. The *second protocol* was designed to study the effects of enhanced endocannabinoid levels on the systemic and cerebrocortical circulation. Following baseline measurements, the animals received a single dose of 10 mg kg^{-1} AM-404 i.v. (dissolved in the same vehicle as AM-251) in order to inhibit the reuptake of EC [88]. After blood pressure, CoBF, blood gas and acid-base parameters returned to their baseline levels, the animals were randomly divided into two experimental groups receiving intravenously either vehicle or 10 mg kg^{-1} AM-251. Fifteen minutes later the administration of 10 mg kg^{-1} AM-404 was repeated and the measurements were continued for an additional 45 min. With the

third protocol the role of CB1 receptors was studied during controlled hypoxia/hypercapnia (H/H), which was induced in a stepwise manner by the administration of different gas mixtures (10 % O_2–10 % CO_2–80 % N_2 for producing mild H/H, 5 % O_2–20 % CO_2–75 % N_2 for producing moderate H/H and 20 % CO_2–80 % N_2 for producing severe H/H) with a constant flow of 3 L min^{-1} through a 5-ml open chamber connected to the trachea, at atmospheric pressure. CoBF was recorded continuously and its peak values were determined during the 8-min long steps of H/H. After the first mild, moderate and severe H/H challenge, the animals were randomly divided into two experimental groups receiving either vehicle or AM-251 (10 mg kg^{-1} i.v.). Thirty minutes later the three steps of H/H were repeated in both groups and peak values were determined from the continuous recording of CoBF.

4.4.3. Experiments to explore the role of endocannabinoids and Cannabinoid-1 (CB1) receptors in the respiratory regulation:

Wild-type control and genetically modified CB1-KO mice were monitored and parameters of respiratory rate, heart rate, oxygen saturation and breath distension were collected for 10 minutes. Subsequently mice received a single dose of 10 mg kg^{-1} AM-404 or its vehicle intravenously, and respiratory parameters were further recorded for 35 minutes [88].

4.5. Analysis of Data

The Discrete Fourier transform (spectrum) of the time series obtained *in vivo* (CoBF) or *in vitro* (vascular tone) was calculated by Fast Fourier Algorithm (FFT) [150]. The calculations were executed in the Matlab environment which uses an adaptive version of the FFT, called FFTW. The DC (zero frequency) component was eliminated from the spectrum by subtracting the mean value of time series from the samples and this way generating zero-mean time series. In order to identify the largest frequency component of the spectrum the region of interest was gated by an appropriate frequency window.

Statistical analysis was performed using the GraphPad Prism software v.6.07 from GraphPad Software Inc. (La Jolla, CA, USA). Values are presented as mean ± SEM; *n* represents the number of experiments. Statistical analysis was performed using two-way ANOVA for repeated measurements followed by Bonferroni post-hoc test. A *p* value of less than 0.05 was considered to be statistically significant.

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5. Results

5.1. Hypersensitivity to Thromboxane Receptor Mediated Cerebral Vasomotion and CBF Oscillations during Acute NO-Deficiency in Rats

5.1.1. Activation of thromboxane receptors under physiological conditions have no effect on systemic physiological parameters or on cerebrocortical blood flow

In order to characterize the significance of TP-receptor activation in promoting CBF oscillations, we first investigated the effect of the TP-receptor agonist U-46619 in a dose of 1 μg kg^{-1} during physiological conditions. Baseline physiological parameters before administration of 100 mg kg^{-1} L-NAME or its vehicle, saline, were found to be within the normal range in all *in vivo* experimental groups (Table 7.); furthermore, neither i.v. administration of the solvent (saline) nor that of the agonist U-46619 induced any significant changes in acid-base, blood gas or systemic circulatory parameters in the control Group I. (data not shown). Additionally, neither the average CoBF nor its Fourier spectrum changed after the administration of saline or U-46619 in this experimental group (data not shown). These observations confirmed that administration of 1 μg kg^{-1} U-46619 has no significant effect on the systemic and cerebrocortical circulation under physiological conditions.

Table 7. Baseline physiological parameters in the different *in vivo* experimental groups. [132]. Values are mean ± SEM (*n* = 7, 10, 6 and 7 in Groups I, IIa., IIb. and IIc., respectively)

MEASURED VARIABLE	EXPERIMENTAL GROUP			
	I.	IIa.	IIb.	IIc.
Mean Arterial Pressure (mmHg)	97.9 ± 2.4	100.0 ± 2.9	97.7 ± 3.5	107.7 ± 4.0
Heart Rate (bpm)	410 ± 9	408 ± 15	413 ± 8	435 ± 20
$PaCO_2$ (mmHg)	41.0 ± 2.6	43.7 ± 1.5	38.2 ± 1.6	40.0 ± 2.0
O_2 Sat (%)	96.5 ± 0.5	96.5 ± 0.5	96.6 ± 0.3	96.2 ± 0.3
pH	7.34 ± 0.01	7.32 ± 0.02	7.38 ± 0.01	7.37 ± 0.01
Standard Base Excess (mmol/l)	-3.7 ± 1.1	-2.2 ± 0.7	-2.6 ± 0.4	-1.7 ± 1.1

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

In Groups IIa., IIb. and IIc. we have mimicked the pathophysiological state of NO-deficiency by pharmacologically inhibiting NOS activity with 100 mg kg^{-1}

L-NAME. In this challenged state, we have observed no significant changes in acid base or blood gas parameters but we have seen increased systemic blood pressure and decreased heart rate (Figure 4). These changes developed within 25 minutes after L-NAME administration and remained unaltered later even after the intravenous administration of 1 µg kg^{-1} U-46619 (in Group IIa.), 1 ml kg^{-1} saline (in Group IIb.) or 10 mg kg^{-1} ozagrel (in Group IIc.) (data not shown). The CoBF reduced by more than 25 % within the first 25 min after L-NAME administration (Figure 4) but did not change further until the completion of the experiments in any of these experimental groups (data not shown).

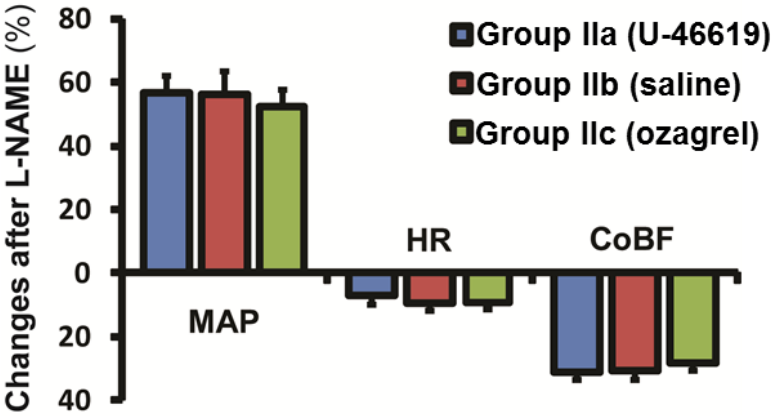

Figure 4. NO synthase blockade increases mean arterial pressure (MAP), while decreases heart rate (HR) and cerebrovascular blood flow (CBF) [132]**.** Data are shown as mean ± SEM, values are normalized to the steady-state pre-injection values and presented as percentual changes (*n* = 10, 6 and 7 in case of MAP and HR and *n* = 20, 12 and 14 in case of CoBF in Groups IIa., IIb., IIc., respectively).

5.1.3. Activation of thromboxane receptors aggravates while inhibition of TXA2 synthesis attenuates vasomotion in the absence of NO

Low frequency CoBF oscillations (4-12 cycles min^{-1}), which were absent under resting conditions, developed after the administration of L-NAME with a dominant frequency of 148 ± 2 mHz and peak magnitude of 5.6 ± 0.5 AU (*n* =46). U-46619 significantly increased while ozagrel decreased the amplitude of these oscillations without changing the dominant frequency (Figure 5A and C, respectively.). In contrast, saline, the vehicle of U-46619 and ozagrel, failed to induce any changes in the magnitude or frequency of CoBF oscillations (Figure 5B).

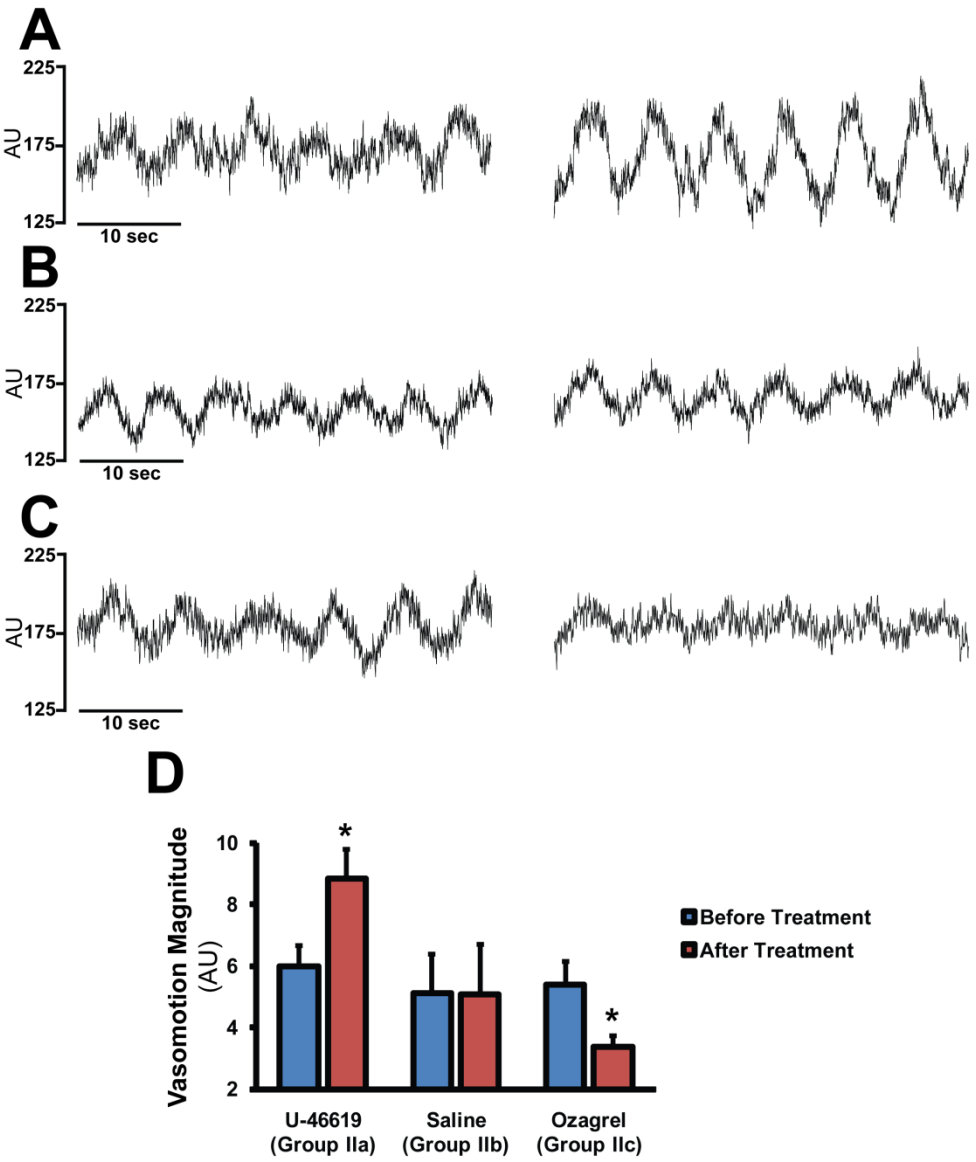

Figure 5. Activation of thromboxane receptors aggravates while inhibition of TXA_2 synthesis attenuates CoBF oscillations developed in the absence of NO. A-C: Original recordings of the cerebrocortical laser-Doppler flux *in vivo* before (left panels) and after (right panels) administration of the TP-receptor agonist U-46619 (A), the thromboxane synthase inhibitor ozagrel (C) or their vehicle (saline) (B) in rats pretreated by the NO synthase inhibitor L-NAME. **D**: Quantitative analysis of slow wave oscillations with discrete Fourier transformation. The peak magnitudes of the power spectra are compared before and after treatments in the three experimental groups [132]. Values are mean ± SEM (n = 20, 12 and 14 in Group IIa, IIb, and IIc, respectively) *p<0.05 *versus* ‘‘Before Treatment’’.

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5.2. Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation

5.2.1. Inhibition of tonic endocannabinoid release and constitutive Cannabinoid-1 receptor activity have no effect on systemic blood pressure and cerebrocortical blood flow

To evaluate the potential influence of tonic EC release and constitutive CB1 receptor activity under resting conditions, blood pressure and CoBF were measured after i.v. administration of the selective CB1 antagonist/inverse agonist AM-251, and were compared to vehicle treated animals. Neither 10 mg kg^{-1} AM-251 nor its vehicle induced any significant change in mean arterial blood pressure (MAP, Figure 6A) or CoBF (Figure 6B) up to 32 minutes after their application. Heart rate, arterial blood gas tensions, acid-base parameters and hematocrit remained unchanged during the observation period (Table 8). These observations indicated that under physiological conditions, constitutive CB1-activity has no significant influence on systemic and cerebrocortical circulation.

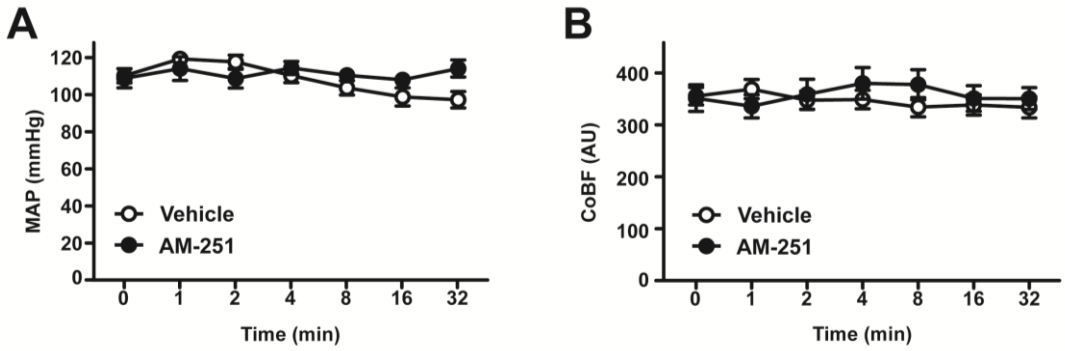

Figure 6. The effects of CB1 receptor blockade on mean arterial pressure (A) and cerebrocortical blood flow (B). Mean arterial pressure (MAP, *n* = 10) and cerebrocortical blood flow (CoBF, *n* = 20) are shown before (0 min) as well as 1, 2, 4, 8, 16 and 32 minutes after intravenous administration of 10 mg kg^{-1} AM-251 (●) or its vehicle (○) in urethane-anaesthetized rats [140]. Values are presented as mean ± SEM. AU, arbitrary unit.

Table 8. Physiological parameters before (0 min) and at several timepoints after the administration of AM-251 or its vehicle [140]. Values are expressed as mean ± SEM (*n* = 22 and 18, in Vehicle and AM-251 treated group, respectively)

MEASURED VARIABLE	TREATMENT	0 MIN	1 MIN	2 MIN	4 MIN	8 MIN	16 MIN	32 MIN
Heart Rate (bpm)	*Vehicle*	420 ± 10	402 ± 7	404 ± 7	404 ± 8	413 ± 10	423 ± 10	396 ± 16
	AM-251	411 ± 11	384 ± 10	379 ± 12	377 ± 10	385 ± 10	404 ± 9	407 ± 12
pH	*Vehicle*	7.40 ± 0.01	7.37 ± 0.01	7.37 ± 0.01	7.38 ± 0.01	7.39 ± 0.01	7.40 ± 0.01	7.41 ± 0.01
	AM-251	7.40 ± 0.01	7.40 ± 0.01	7.41 ± 0.01	7.42 ± 0.02	7.42 ± 0.02	7.40 ± 0.01	7.41 ± 0.01
PaO_2 (mmHg)	*Vehicle*	87.7 ± 2.0	76.3 ± 3.2	76.5 ± 2.7	79.0 ± 2.7	78.4 ± 2.7	81.3 ± 3.2	80.2 ± 3.2
	AM-251	83.6 ± 2.0	82.3 ± 6.5	85.8 ± 2.1	86.9 ± 1.8	85.4 ± 2.7	84.4 ± 2.0	84.4 ± 2.0
$PaCO_2$ (mmHg)	*Vehicle*	42.7 ± 1.0	43.5 ± 1.0	43.0 ± 0.9	41.8 ± 1.2	40.4 ± 1.1	39.6 ± 1.3	38.1 ± 1.7
	AM-251	40.4 ± 1.5	40.2 ± 1.2	37.3 ± 1.7	36.8 ± 2.2	35.7 ± 1.6	37.4 ± 1.2	38.7 ± 1.4
O_2 Saturation (%)	*Vehicle*	96.5 ± 0.2	93.8 ± 0.9	94.2 ± 0.7	94.8 ± 0.5	95.0 ± 0.6	95.5 ± 0.6	95.6 ± 0.6
	AM-251	96.2 ± 0.3	95.2 ± 0.7	96.5 ± 0.3	96.7 ± 0.3	96.5 ± 0.4	96.3 ± 0.3	96.3 ± 0.4
SBE (mmol/l)	*Vehicle*	1.2 ± 0.4	0.1 ± 0.4	-0.3 ± 0.4	-0.6 ± 0.4	-0.2 ± 0.5	-0.1 ± 0.5	-0.5 ± 0.6
	AM-251	0.1 ± 0.7	-0.6 ± 0.5	-0.9 ± 0.5	-0.8 ± 0.4	-1.3 ± 0.3	-1.3 ± 0.4	-1.2 ± 0.3
Hematocrit (%)	*Vehicle*	45.8 ± 1.1	44.1 ± 1.1	43.4 ± 0.9	43.4 ± 1.2	42.9 ± 0.8	43.5 ± 1.2	42.1 ± 1.0
	AM-251	42.8 ± 1.3	40.5 ± 1.0	41.0 ± 0.8	42.6 ± 1.2	42.9 ± 0.9	41.9 ± 1.3	41.1 ± 1.9

5.2.2. Tri-phasic effect of enhanced endocannabinoid release: the initial hypertension and increase in cerebrocortical blood flow is followed by sustained hypotension and decrease in cerebrocortical blood flow

First we tested the effect of enhanced endocannabinoid release on blood pressure and CoBF. Baseline physiological parameters were within normal range in the absence and presence of AM-251 before AM-404 treatment (Table 9). After the i.v. administration of 10 mg kg^{-1} AM-404, a cannabinoid reuptake inhibitor, we observed three distinct phases of BP and CoBF. *Phase I* consisted of marked hypertension (Figures7A and 8A) accompanied by a significant increase of CoBF (Figures 7B and 8B) with minor

changes in arterial blood gas tensions and pH (Figure 9). The BP and CoBF elevations reached their maximum within 0.5 min and thereafter started to return towards their baseline levels until the onset of the second phase with a delay of 1–2 min. During *phase II* CoBF has increased most prominently with a peak at 3.5 min after administration of AM-404 (Figures 7B and 8B), accompanied by increased levels of expired CO_2 (Figure 7C) and BP (Figures 7A and 8A). Blood gas analysis revealed marked hypoxia (Figures 9A and 9C), hypercapnia (Figure 9B) and acidosis (Figure 9D), and therefore, changes in the CoBF and BP were considered to be secondary to the depression of respiration. The *third phase* of changes was dominated by sustained hypotension (Figures 7A and 8A), which reached its maximum at 20 min. During this phase the arterial oxygen tension and saturation normalized (Figures 9A and 9C), whereas the hypercapnia observed in phase II was reverted to a slight hypocapnia (Figure 9B), and the acidic arterial pH returned towards the physiological level (Figure 9D). Interestingly, CoBF showed a significant decrease during phase III (Figure 8B), which was attributed to the reduction of the MAP and the arterial CO_2-tension ($PaCO_2$). BP and CoBF returned to their baseline levels within 45 min after the administration of AM-404.

Table 9. Baseline physiological parameters before the administration of AM-404 in the absence and presence of AM-251 [140]. Values are presented as mean ± SEM (n = 10) *p<0.05 *versus* ‘‘Before AM-251’’

MEASURED VARIABLE	BEFORE AM-251	AFTER AM-251
MAP (mmHg)	112.8 ± 6.5	103.8 ± 3.0
CoBF (AU)	346.2 ± 28.5	362.1 ± 28.7
PaO_2 (mmHg)	86.0 ± 2.9	76.9 ± 2.8 *
$PaCO_2$ (mmHg)	44.8 ± 1.7	38.5 ± 1.5 *
O_2 Saturation (%)	95.9 ± 0.6	94.6 ± 0.6 *
pH	7.38 ± 0.01	7.38 ± 0.01

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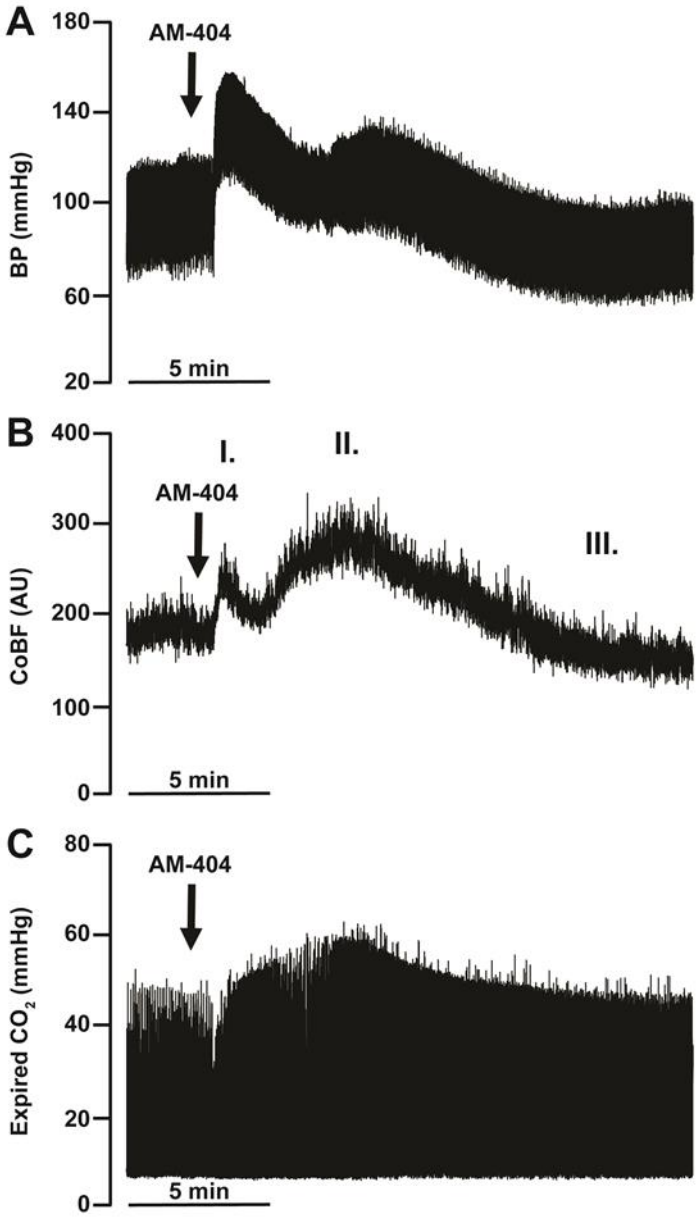

Figure 7. Representative recordings of the effects of endocannabinoid reuptake inhibition on blood pressure (A), cerebrocortical blood flow (B) and expired CO_2 levels (C). After the intravenous administration of 10 mg kg^{-1} AM-404 a transient increase in blood pressure (BP) and cerebrocortical blood flow (CoBF) can be seen (*phase I*). It is followed by a second prominent increase in CoBF (*phase II*) that is accompanied with a rise of expired CO_2. Thereafter a sustained decrease in the BP and CoBF can be seen (*phase III*). The arrows indicate the injection of AM-404 [140].

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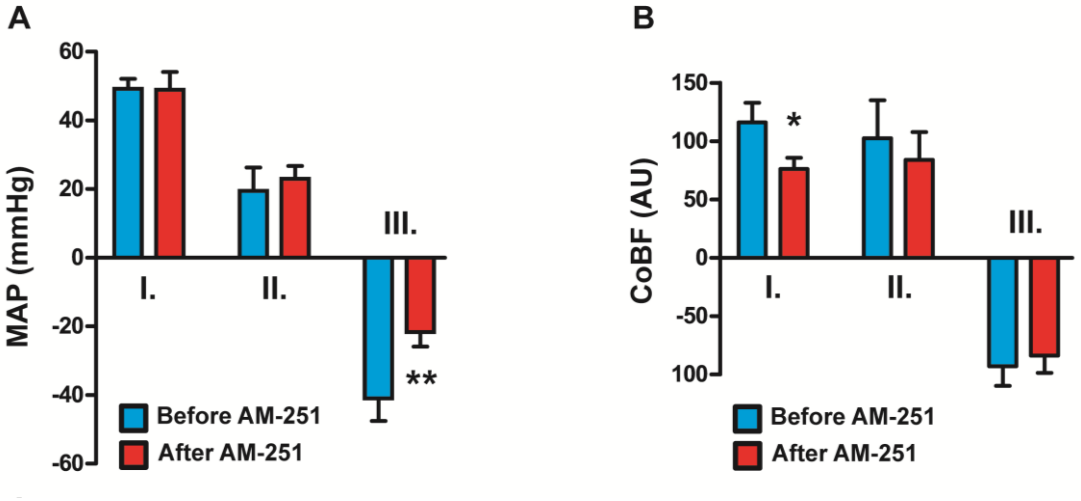

Figure 8. Effects of AM-404 on mean arterial pressure (A) and cerebrocortical blood flow (B) before and after AM-251 treatment. Changes in mean arterial pressure (MAP, *n* = 10) and cerebrocortical blood flow (CoBF, *n* = 20) are shown in phases I, II and III of the AM-404 (10 mg kg^{-1}, i.v.) response before and after treatment with AM-251 (10 mg kg^{-1}, i.v.) [140]. Values are shown as mean ± SEM and are expressed as changes from baseline (see Table 9); *p<0.05, **p<0.01, *versus* ‘‘Before AM-251’’.

After testing the effect of enhanced endocannabinoid release on blood pressure and CoBF, we inhibited CB1 receptors to assess their involvement in the mediation of effects induced by inhibition of EC reuptake. Animals were pretreated with 10 mg kg^{-1} AM-251 or its vehicle, and the administration of AM-404 and subsequent measurements were repeated. During *phase I* the increase of MAP was comparable between the control and CB1-blocked group (Figure 8A), indicating that CB1 receptors are not involved in the transient hypertension induced by enhanced EC levels. However, the increase of the CoBF attenuated significantly in the presence of AM-251 (Figure 8B), implying an improved autoregulation of the cerebral circulation. Administration of AM-251 markedly attenuated hypoxia and hypercapnia as well as the acidosis seen during *phase II* (Figure 9), indicating that the increased EC availability after AM-404 suppresses respiration via CB1 receptor activation. The mild hypertension observed during phase II, and interestingly the increase of CoBF were both resistant to AM-251 treatment (Figure 8), which is surprising if we consider that changes in blood

gas and pH levels were both abolished by CB1 receptor inhibition. Therefore, we hypothesized that in addition to suppressing EC-induced hypoventilation, AM-251 enhanced the reactivity of the cerebrocortical circulation to H/H (further discussed in 6.2.2). Finally the sustained hypotension seen during *phase III* was attenuated after AM-251 treatment (Figure 8A), indicating the involvement of CB1 receptors in mediating the effects of elevated EC levels. The accompanying changes in arterial O_2 tension and saturation as well as the pH were not affected by CB1-blockade, although the mild hypocapnia was attenuated (Figure 9).

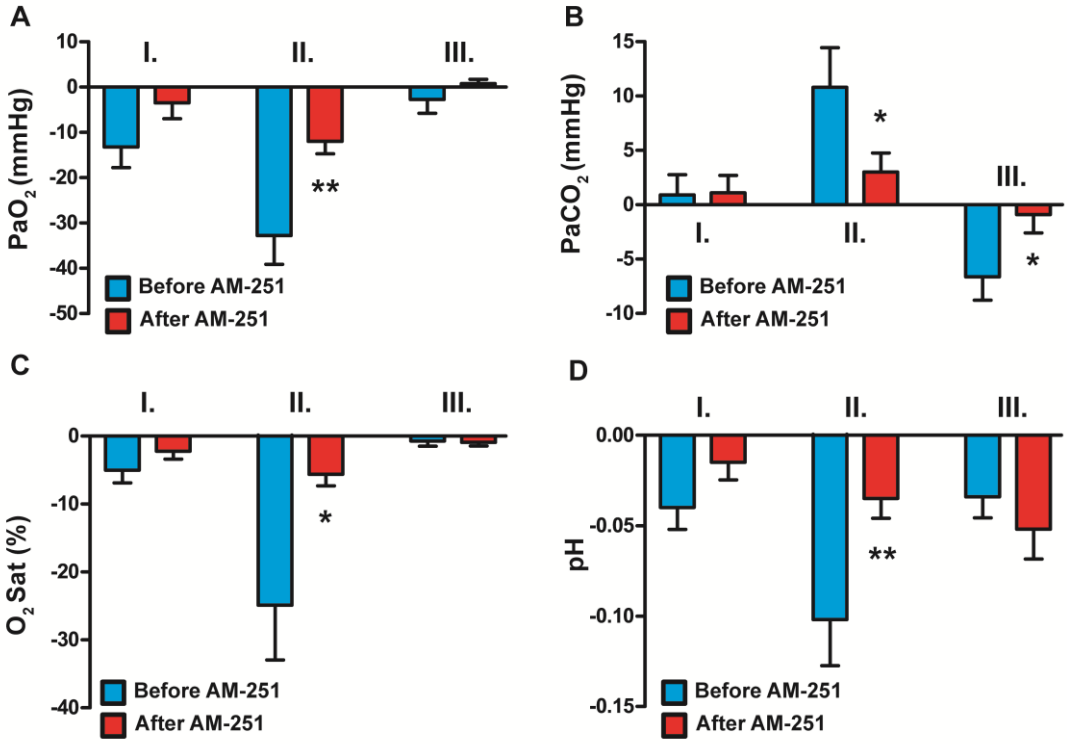

Figure 9. Effects of AM-404 on arterial blood gases and pH before and after AM-251 treatment. Arterial blood-gas and pH changes are shown in phases I, II and III of the AM-404 (10 mg kg^{-1}, i.v.) response before and after treatment with AM-251 (10 mg kg^{-1}, i.v.) [140]. Values are shown as mean ± SEM and are expressed as changes from baseline (see Table 9); *p<0.05, **p<0.01, *versus* ‘‘Before AM-251’’, *n* = 10.

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5.3. CB1 Receptor-mediated Respiratory Depression by Endocannabinoids

5.3.1. Enhanced endogenous cannabinoid levels induce transient respiratory depression and consequent arterial hypoxia in a CB1-dependent manner

Respiratory parameters were within physiological range in both wild-type control and CB1-KO mice. Intravenous administration of 10 mg kg^{-1} AM-404, which inhibits EC reuptake, thus enhancing regional EC levels, induced a transient depression in oxygen saturation (Figure 10A) and respiration rate (Figure 10B) and a concomitant increase in breath distension (Figure 10C) in control mice. Administration of the solvent alone had no effect (data not shown). The rapid onset of respiratory depression started within 1 minute after treatment with AM-404, and reached its maximal level within 4 min, whereas its recovery started after 8 minutes; respiratory parameters gradually recovered to normoxic levels that was completed within 16 minutes. The recovery of oxygen saturation in AM-404 treated wild-type control mice was not complete within the measured experimental period; however, a gradual decrease in the oxygen saturation correlating with the time of anaesthesia could be observed in the vehicle treated control mice (data not shown) and AM-404 treated CB1-KOs (Figure 10A), thus it was considered an endocannabinoid independent effect. Additionally, there was a simultaneous increase in breath distension during respiratory depression in wild-type control animals (Figure 10C), indicating that the lower respiratory rate was associated with larger fluctuations in the central venous pressure and cardiac output. Changes in heart rate were generally unaltered upon AM-404 administration, and were not further analyzed. In contrast to AM-404 evoked respiratory depression in wild-type control mice, CB1-KO mice showed no reduction in oxygen saturation (Figure 10A) and respiration rate (Figure 10B); accompanying changes in breath distension were also absent (Figure 10C).

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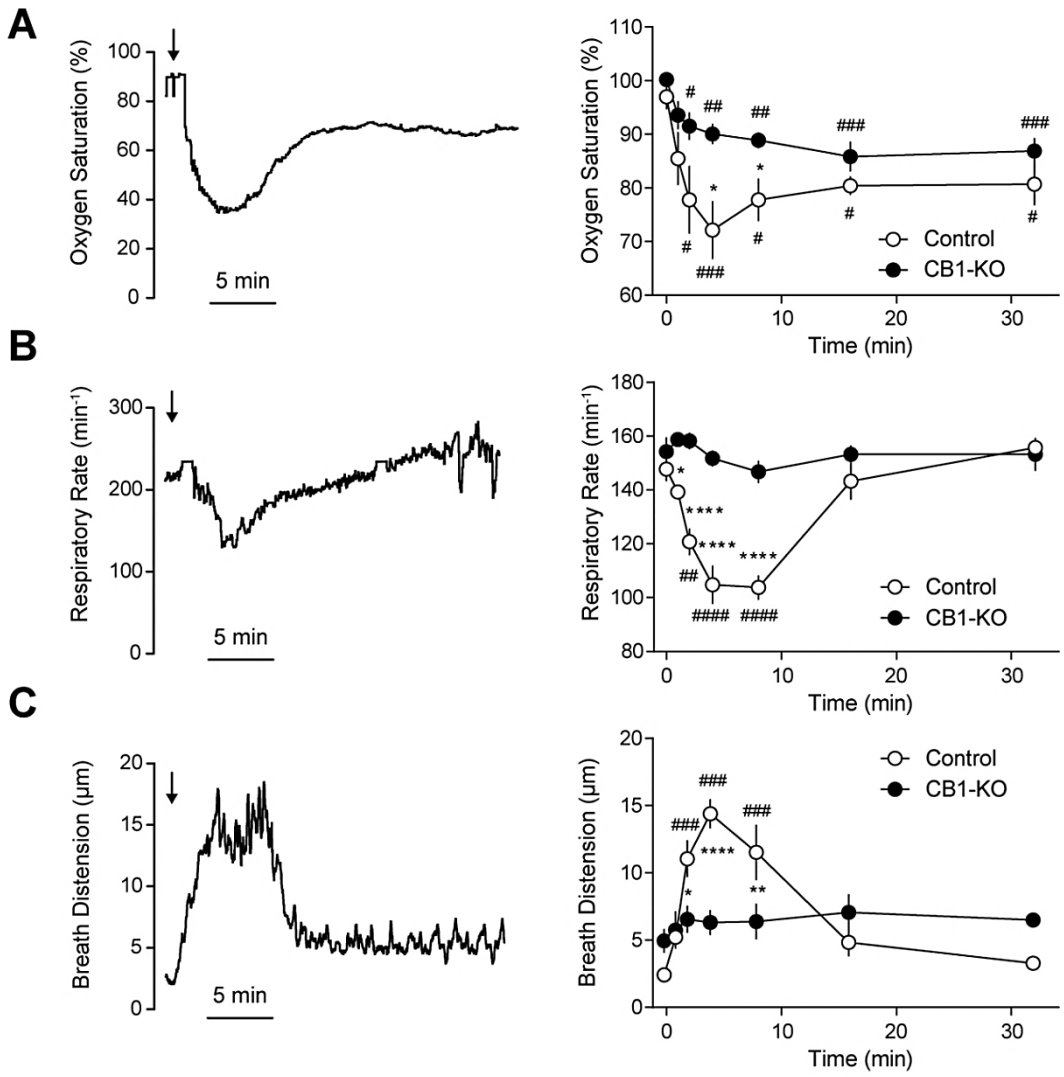

Figure 10. Effect of AM-404 administration on oxygen saturation (A), breath rate (B) and breath distension (C). Representative recordings in wild-type control animals (*left panels*) and averaged results of multiple experiments in wild-type control (○) and CB1-KO (●) mice (*right panels*) of oxygen saturation (A), respiratory rate (B) and breath distension (C) as a function of time. Administration of AM-404 (10 mg kg^{-1}, iv.) is indicated by arrows on original recordings. Physiological parameters were determined before (0 min.) and at 1, 2, 4, 8, 16, and 32 minutes after AM-404 for statistical analysis (*right panels*) [143]. Values are presented as mean ± SEM; *n*= 6-6 in the case of O_2 saturation and 4-4 in the case of respiratory rate and breath distension measurements; **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 control *versus* KO, #*p*<0.05, ##*p*<0.01, ###*p*<0.001, ####*p*<0.0001 *versus* "0 min".

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6. Discussion

6.1. Hypersensitivity to Thromboxane Receptor Mediated Cerebral Vasomotion and CBF Oscillations during Acute NO-Deficiency in Rats

Cerebral vasomotion, the low frequency fluctuation of the cerebral oxygen availability, independent of the cardiac and respiratory cycles, often accompanies pathological incidents, such as SAH, but its relevance and the underlying mechanism is largely unresolved. Our results demonstrate that vasomotion and CBF oscillation during NO-deficiency are enhanced by TP-receptor activation [151]. Numerous pathological condition has been associated with impaired NO release, such as atherosclerosis, diabetes, hypertension, ischemia and SAH. Reduction in the availability of NO under these pathological conditions often accompanies with increased platelet aggregation as well as activation of TP-receptors and consequently the release of TXA_2. The experimental model applied in the present study resembles the features of several cerebrovascular diseases.

During NO-deficiency, ozagrel inhibited L-NAME induced CoBF oscillations indicating the involvement of endogenous TXA_2; however, in order to comprehensively understand the underlying intracellular mechanism, the *in vitro* effect of the TP-receptor agonist U-46619 was tested first on isolated MCA. In accordance with the *in vivo* effect of TP-receptor activation, the administration of 100 nM U-46619 induced a slight (10.9 ± 5.3 %) vasoconstriction, while its vehicle, saline, had no effect on the mean vascular tension; furthermore, no vasomotional activity could be observed in the isolated vessels before or after saline or U-46619, suggesting that TP-receptor activation under physiological conditions fails to induce vasomotion. To resemble the NO-deficient pathophysiological conditions, NO synthesis was inhibited with L-NAME in isolated MCA. In the absence of NO the vascular tone was slightly increased (by 9.5 ± 2.2 %) but did not induce vasomotion; however, the subsequent stimulation of the TP-receptors elicited strong elevation of the mean vascular tone (by 77.2 ± 6.2 %) as well as vasomotion could be observed in 16 out of 19 NOS-blocked MCA with a dominant frequency of 56.1 ± 4.7 mHz. Quantitative analysis of the peak magnitude of the Fourier spectra showed a 5.35-fold increase after administration of U-46619 (Figure 11A). To rule out the possibility that the evoked vasomotional activity was due

to the increased vascular tension, control vessels were immersed in Krebs solution supplemented with 25 mM K^+ prior to treatment with U-46619. Although the mean vascular tension was comparable to that of L-NAME + U-46619 treated (62.3 ± 8.1 % vs. 77.2 ± 6.2 %), no vasomotional activity could be observed in any of the tested vessels [132].

As shown previously [152], inhibition of the NO pathway causes vasoconstriction and reduction in cerebral blood flow. This implies that in the cerebral circulation, constitutive NO-synthesis is necessary to maintain resting basal tone by suppressing TXA_2 synthesis and release [152]. Desensitization of TP-receptors occurs through phosphorylation at Ser^{331} by cyclic GMP-dependent kinase (PKG) [153]; however, NO-deficiency also impairs the signaling pathway that couples TP-receptor activation to smooth muscle contraction. As shown previously, TXA_2 is involved in both $G_{q/11}$ and $G_{12/13}$ mediated vasoconstriction [154,155]; NO reportedly interacts with $G_{12/13}$ pathway mediated calcium sensitization via PKG mediated RhoA [156] and telokin [157] inhibition as well as PKG directly phosphorylates myosin phosphatase targeting subunit (MYPT1) at Ser^{695}, resulting in decreased phosphorylation on the adjacent inhibitory Thr^{696} site [158,159]. Hence, during NO-deficiency, not only TP-receptors, but also downstream signaling pathways are released from tonic inhibition making vascular smooth muscle sensitive to calcium waves, consequently vasoconstriction. To test this hypothesis, NOS blocked vessels showing vasomotion after U-46619 administration, a specific Rho-kinase inhibitor, Y-27632, was administered to explore the involvement of Rho-kinase in the mediation of the vascular responses. Treatment with 10 μM Y-27632 abolished the vasoconstriction and vasomotion (Figure 11), suggesting that besides TXA_2, other $G_{12/13}$ -dependent activator metabolites are involved in the appearance of CoBF oscillations, since inhibition of thromboxane synthesis only partially abolished CoBF oscillations *in vivo*, while blockade of Rho-kinase suppressed completely the vasomotional activity in isolated vessels. In addition, *in vitro* treatment with endothelin-1 (ET-1), which also acts through the RhoA – Rho-kinase dependent pathway in the cerebrovascular bed, evoked vasomotion with a dominant frequency of 48.8 ± 7.3 mHz. The augmentation in the magnitude of the Fourier spectra was equivalent to the increase observed upon TP-receptor activation, which effect was completely abolished after Rho-kinase inhibition (Figure 11B).

In conclusion, pharmacological inhibition of endogenous TXA_2 production or the prevention of TP-receptors hypersensitivity during NO-deficiency abrogates the enhanced vascular reactivity; potentially forestalling the appearance of vasospasm, resulting in better outcome after subarachnoid hemorrhage.

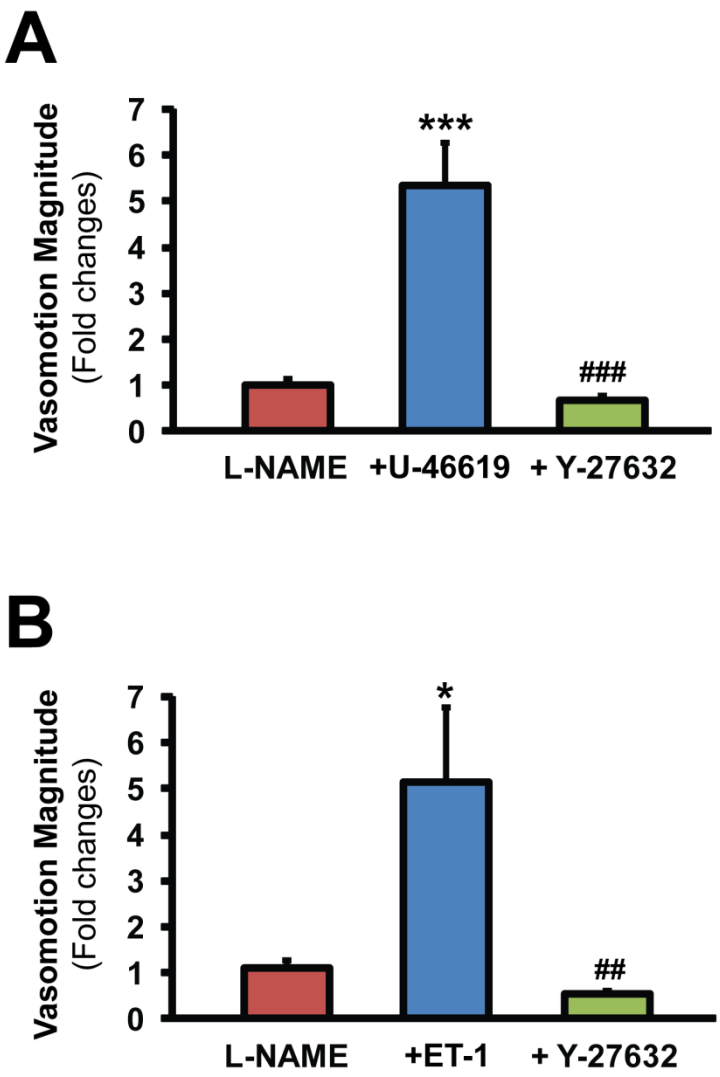

Figure 11. Activation of thromboxane or endothelin receptors induce Rho-kinase dependent vasomotion in NO synthase blocked MCA. Quantitative analysis of slow wave oscillations with discrete Fourier transformation in L-NAME treated vessels before and after the administration of the TP-receptor agonist U-46619 (A) or endothelin-1 (B) followed by the Rho-kinase inhibitor Y-27632 [132]. Values are presented as mean ± SEM fold changes of the peak magnitudes of the power spectra compared to the baseline. *p<0.05, ***p<0.001 *versus* L-NAME, ##<0.01 *versus* ET-1 and ###<0.001 *versus* U-46619 (*n* = 10–20).

6.2. Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation

The effect of endocannabinoids on the cardiovascular system has been extensively studied. Endocannabinoids are involved in the regulation of systemic blood pressure, cardiac output and vascular resistance [23,160,161]. Acute use of marijuana induces tachycardia in humans, while chronic consumption leads to hypotension and bradycardia [162,163]. In mice and rats, potent cannabinomimetics induce CB1 receptor mediated bradycardia, hypotension and depressed cardiac contractility; effects that are partially contributed to the decrease of norepinephrine release from sympathetic nerve terminals and also direct action on the vasculature and myocardium [161]. However, published data on the cerebrovascular effect of the endocannabinoid system are controversial. Elevated CBF has been reported in marijuana smoking humans that directly correlated with plasma Δ^9-THC levels [142]. Similarly, cannabinomimetics increased the cerebral blood perfusion in dogs, humans [137,164], and in anesthetized rats [139]. Conversely, conscious rats showed reduction in CBF after anandamide treatment [138]. These various observations can be attributed to the diverse expression of cannabinoid receptors, and additionally to the wide variety of mechanisms by which endocannabinoids are able to influence the regulation of the cerebrovascular tone. The cerebral vasculature responds with vasodilatation to CB1 receptor stimulation [110], however, cannabinoid receptors on the neurons act as modulators of the synaptic transmission, thereby adjusting the cerebral metabolic demand, and consequently decreasing CBF indirectly [23]. Considering that the observed effects were of exogenously applied phyto- or endocannabinoids that does not necessarily reproduce physiological conditions, a different approach was used in the current study, where the endocannabinoid system activity was enhanced by administration of the cannabinoid reuptake inhibitor, AM-404.

6.2.1. Influence of constitutive endocannabinoid release and CB1 receptor activation on systemic and cerebrocortical circulation

The constitutive activity of CB1 receptors has been reported in various studies [165]. Therefore, to study the involvement of constitutive CB1 receptor activity on the cerebrovascular circulation, an inverse agonist/antagonist, AM-251, was tested under

physiological conditions. Administration of 10 mg kg^{-1} AM-251 that had been shown previously to effectively inhibit CB1 receptor activity *in vivo* [149], failed to induce any significant changes in the observed systemic and cerebral circulatory parameters, suggesting the absence of constitutive influence of CB1 receptors on the cardiovascular system under physiological conditions. This finding is in accordance with reports on CB1 receptor knockout mice [166], although neither the data presented in the current study nor the published results exclude the possible involvement of several backup regulatory mechanisms that may take over the function of CB1 receptor mediated pathways in case of pharmacological blockade or genetic absence.

6.2.2. Influence of enhanced endocannabinoid levels and consequent CB1 receptor activation on systemic and cerebrocortical circulation

To test the effect of increased EC levels on the systemic and cerebrocortical circulation, AM-404, a cannabinoid reuptake inhibitor, was administered and subsequent changes were recorded. Although the exact mechanism of action of AM-404 has been debated, while it is not solely involved in the inhibition of cellular uptake, but is also a substrate for fatty acid amide hydrolase [167] as well as a transient receptor potential vanilloid type-1 (TRPV1) channel activator [168], it has been shown to increase the endogenous levels of anandamide in the brain [88]. Three different phases of systemic and cerebral circulatory responses could be distinguished after administration of 10 mg kg^{-1} AM-404.

Phase I consisted of a transient hypertension, consistent with published results after i.v. anandamide administration in mice [105] and rats [169,170], indicating that EC enhancement via reuptake inhibition recapitulate the previously observed effect of anandamide to elevate the blood pressure. However, the transient hypertension was resistant to CB1 receptor inhibition, indicating that this phenomenon is not mediated by CB1 recptors. Similar observations were reported in TRPV1-receptor deficient mouse model [105], proposing, together with the published observation of anandamide mediated TRPV1 activation [171], the most likely mechanism behind the transient hypertension. Accompanying the increase in mean arterial pressure, CoBF also increased markedly, probably due to the hypertension exceeding the upper limit of cerebral autoregulation. However, CB1 receptor inhibition resulted in significant

reduction of the cortical hyperemia, indicating an influence of CB1 receptors on the autoregulation of cerebral circulation.

During *phase II*, pronounced respiratory depression was observed leading to changes of the arterial blood gas tensions and pH, consequently increasing CoBF. The respiratory changes were sensitive to CB1 receptor inhibition, consistent with a previous study using AM-281 after anandamide treatment in rats [122], as well as the respiratory depression induced by synthetic CB1 agonists WIN-55212-2 and CP-55940 that could be blocked by SR-141716A [119]. Injection of WIN-55212-2 to the cisterna magna or rostral ventrolateral medulla oblongata [120,124] of rats suppressed respiration suggesting that central CB1 receptors negatively modulate respiratory control circuits. The marked increase in CoBF accompanying the respiratory changes was surprisingly resistant to CB1 receptor inhibition, although AM-251 suppressed arterial blood gas and pH changes related to enhanced EC levels. In order to explain this discrepancy, the possible involvement of AM-251 in the enhancement of CoBF reactivity was hypothesized. To determine the involvement of CB1 receptors during the respiratory depression and their effect on the CoBF, closely regulated hypoxia and hypercapnia were induced and changes in the CoBF were evaluated before and after AM-251 administration. This experimental setup allowed us to observe the influence of CB1 receptor inhibition on the CoBF independent of the blood gas changes, while they were kept constant and changes secondary in nature to the respiratory depression could be ruled out. In our study we have shown that inhalation of three different gas mixtures, which induced reproducible degree of mild or moderate hypoxia and hypercapnia, CoBF elevation was amplified (by 28.1 ± 8.8 % and 39.4 ± 10.0 %, respectively) whereas the peak CoBF during severe H/H was unaffected after treatment with AM-251 compared to its control (Figure 12B).

It has been shown that cerebrovascular CB1 receptors have vasodilatory influence on cerebral vessels [110], suggesting the involvement of receptors extravascular in nature; both neurons and astrocytes express CB1 receptors [172,173], regulating neuronal NOS activity and influencing vascular tone. KCl-induced neuronal NOS activity is impaired in the presence of CB1-agonists in cerebellar granule cells without influencing basal NO-production [174]. However, pertussis toxin or SR141716A treatment reversed the effect of CB1-activation and increased NO production that was additive with KCl

stimulation [174]. It has also been published that CB1 receptors have inhibitory effect on the metabolic activity of neurons and astrocytes [175], consequently influencing release of NO and vasoactive metabolites. Furthermore, the H/H-induced cerebrovascular responses involve perivascular sympathetic nerves [176,177] that are modulated by the EC system [178].

Although the exact mechanism of the CB1 receptor involvement in H/H-induced cerebrocortical hyperemia is unclear, the presented results support that during mild and moderate H/H, inhibition of CB1 receptors enhanced CoBF responses, indicating that EC system has an inhibitory role in H/H induced vasorelaxation.

Phase III could be characterized with a sustained hypotension, in agreement with published data describing the sustained hypotensive effect of phyto- and endocannabinoids [179], as well as synthetic analogues [133], although the receptor involved mediating this effect is unclear. AM-251 treatment decreased the reduction in blood pressure, however, did not completely abolish it, suggesting that CB1 receptors are involved, but not the sole receptors mediating the depressor effect of AM-404. During this phase, reduction in CoBF could be observed, most likely due to the decrease in the arterial CO_2-tension as well as the reduction of the mean arterial blood pressure close to the lower limit of cerebral autoregulation. The attenuation of hypocapnia observed after AM-251 treatment is most likely due to the decrease of respiratory depression found during *phase II*, nevertheless, the possibility that CB1 receptor inhibition influenced cerebral autoregulation at lower BP levels cannot be excluded.

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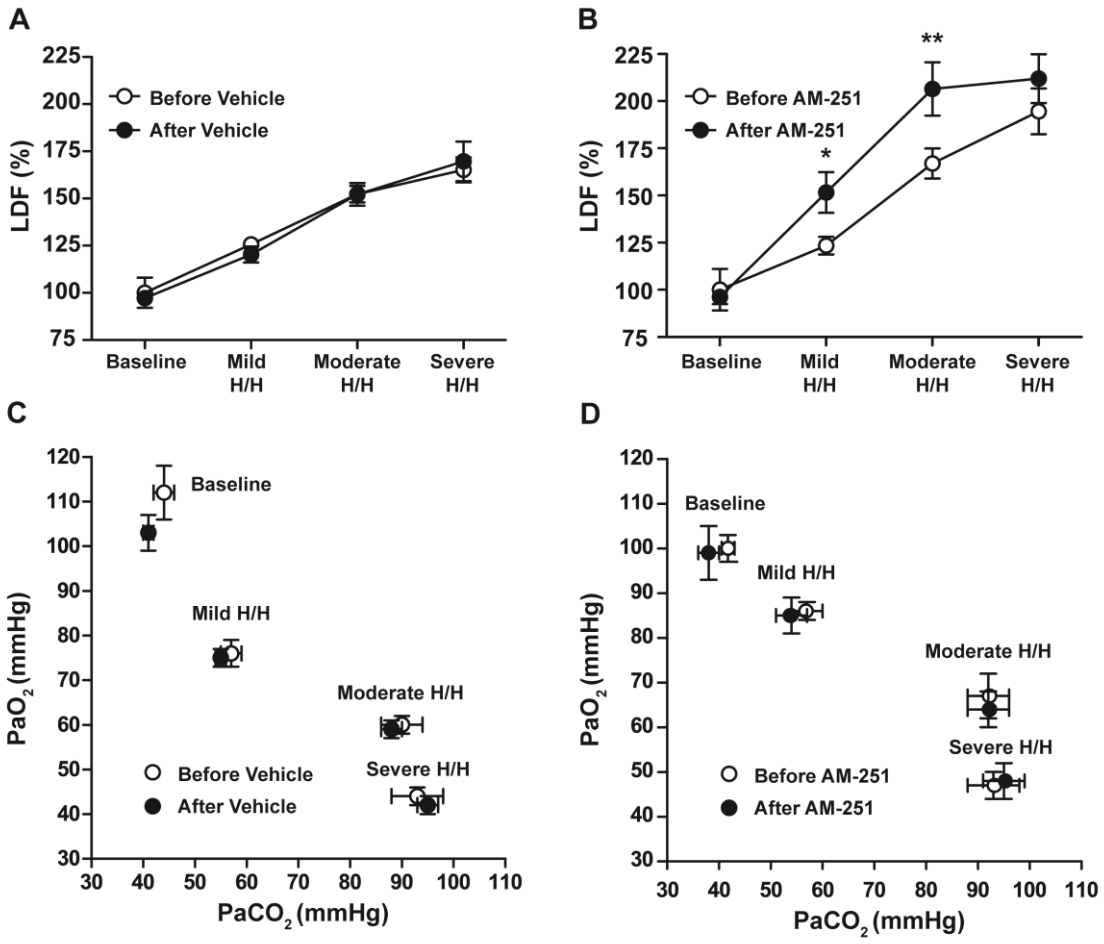

Figure 12. Cerebrocortical blood flow and arterial blood gas tensions during stepwise hypoxia/hypercapnia. Cerebrocortical blood flow (A and B, expressed as percentage of baseline levels) and arterial blood-gas tensions (C and D) are shown before (Baseline) and during mild, moderate and severe hypoxia/hypercapnia before (○) and after (●) intravenous injection of 10 mg kg^{-1} AM-251 or its vehicle [140]. Values are mean ± SEM, *p<0.05, **p<0.01 *versus* ‘‘Before AM-251’’, *n* = 4–12.

6.3. CB1 Receptor-mediated Respiratory Depression by Endocannabinoids

Previous studies utilized exogenous cannabinomimetics to investigate the role of cannabinoids and endocannabinoids in the physiological control of respiration; however, this approach may not necessarily mimic the physiological stimulation of the EC system. Compared to previous studies, the administration of a cannabinoid reuptake inhibitor, AM-404, at a concentration shown previously to enhance ECs [88] has enabled us to analyze the effect of the activated EC system on the regulation of respiration by more closely modeling the physiological concentration and distribution of ECs. Administration of AM-404 to wild-type control mice induced a rapid, transient reduction in respiratory rate and oxygen saturation; in agreement with previous published results, where intracisternal [124] or intramedullar [120] injection of WIN55212-2, a synthetic CB1 and CB2 receptor agonist, induced reduction in respiratory rate and minute volume, and an increase in tidal volume. The recent and previous findings implicate a pivotal role for the cannabinoid receptors located in the brainstem in the control of respiration.

Concomitant to the activated EC system evoked respiratory depression; a simultaneous increase in breath distension was present in wild-type mice. Breath distension reflects the change in blood volume in peripheral vessels that are due to local blood pressure changes generated by the fluctuation of intrathoracic pressure associated with breathing [180], and is generally considered as an indicator of increased breathing effort [181,182]. Cannabinoids have been shown to induce bronchodilation [183,184], suggesting that the accompanying increase in breath distension during elevation of EC levels is not the result of the increased airway resistance; instead it correlates with increased respiratory workload, particularly deeper breath intake. Additionally, previous studies also reported increased tidal volume during EC administration [124]. Hence, the displayed changes in breath distension in wild-type control mice are most likely a compensatory mechanism to counteract the strong reduction in breath rate [143].

Albeit our previous work [140] lacked direct evidence proving conclusively the involvement of endogenously released ECs in the control of breath regulation; analyzing the effects of the elevated EC system in mice proved critical to unambiguously demonstrate the involvement of CB1 receptors in the respiratory regulation. Indeed, administration of AM-404 in a dose that has been shown to increase

endogenous cannabinoid levels [88], recapitulated the transient respiratory depression in wild-type control mice [143]. Treatment of genetically modified animals lacking the CB1 gene (CB1-KO) with AM-404, to enhance the EC-system, failed to demonstrate suppression of breath rate and also did not show the consequent reduction in oxygen saturation. Conclusively, the lack of the respiratory depression in CB1-KO mice indicates that the presence of CB1 receptors is integral to the observed effects.

The importance of phyto- and endocannabinoids are increasing in the medicinal treatment of acute and chronic pain, diabetes and obesity, cerebral ischaemia, epilepsy, cancer and inflammation, but the medical application is severely thwarted by adverse side effects [185,186]. Acute application induces elevation in heart rate, as well as decrease in subjective alertness and a decrease in motor stability [185]; chronic usage has reportedly led to increased prevalence of respiratory symptoms [185], and more importantly to memory disorders. A proposed approach is aimed towards targeting selectively the peripheral CB1 receptors, when the therapeutic value is related to receptors expressed on the periphery, thus reducing the psychoactive effects [186,187]. Another promising concept is the enhancement of the endocannabinoid system by inhibition of the hydrolysis, consequently blocking the degradation of anandamide and 2-AG [186,188], which would potentially improve analgesia, while reducing side-effects. Administration of the endocannabinoid reuptake inhibitor, AM-404 inhibits the clearance and reuptake of ECs, therefore leads to the enhancement of the physiological levels of endogenously released cannabinoids, closely mimicking the effect of the prevention of hydrolysis. Our finding that the augmented endocannabinoid elevation promotes marked respiratory depression suggests a potential limitation of endocannabinoid reuptake and hydrolysis inhibitors in pharmacological applications [143].

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7. Conclusions

7.1. Hypersensitivity to Thromboxane Receptor Mediated Cerebral Vasomotion and CBF Oscillations during Acute NO-Deficiency in Rats

The involvement of TP-receptor activation was explored in the appearance of cerebral blood flow oscillations under physiological conditions as well as after impaired NO synthesis. U-46619 elicited TP-receptor activation in control animals failed to evoke CBF oscillations; however, inhibition of NO-synthase by L-NAME resulted in increased blood pressure and reduction in CBF accompanied by oscillations in the blood flow that was further enhanced after TP-receptor agonist treatment. TXA_2 synthesis inhibition with ozagrel abrogated CBF oscillations *in vivo*. U-46619 treatment induced weak contraction in isolated middle cerebral arteries, although in the absence of NO, sustained vasomotion could be observed that was sensitive to the Rho-kinase inhibitor, Y-27632. These results indicate that under pathophysiological conditions associated with NO-deficiency, such as subarachnoid hemorrhage, the hypersensitivity of TP-receptor – Rho-kinase pathway augments the development of vasomotion, potentially leading to the propagation of vasospasm.

7.2. Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation

The involvement of CB1 receptor in the cardiovascular and cerebral blood flow regulation was investigated under physiological conditions and during increased endocannabinoid levels. Inhibition of CB1 receptors under resting conditions with AM-251 induced no changes in the systemic or cerebral circulation, indicating that CB1 receptor mediated mechanisms have limited influence on circulation under physiological conditions. During enhanced endocannabinoid activation that induced triphasic responses, the transient hypertension was CB1-independent; however, the sustained hypotension observed during *phase III* was sensitive to CB1 receptor blockade. Furthermore, the marked respiratory depression observed during *phase II* proved to be CB1-sensitive.

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7.3. CB1 Receptor-mediated Respiratory Depression by Endocannabinoids

The influence of increased levels of endogenously produced endocannabinoids on the respiratory regulation was explored; while published evidence was inadequate in proving undoubtedly the involvement of CB1 receptors on respiratory control. In order to characterize the influence of elevated EC levels on respiratory control, direct measurement of the respiratory parameters with pulse oximetry was used after enhancing endocannabinoid levels by administrating AM-404. Treatment with the cannabinoid reuptake inhibitor, but not with the solvent, induced transient reduction in respiratory rate accompanied with depression in the arterial oxygen saturation and a concomitant increase in breath distension in wild-type control mice. However, CB1-deficient mice exhibited no alterations in the measured respiratory parameters after AM-404 administration; indicating that the endocannabinoid system has a pivotal role in the physiological control of respiration by regulating the respiratory rate and consequently influencing arterial oxygen saturation, and this mechanism is entirely dependent on CB1 receptors.

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8. Summary

Arachidonic acid metabolites are involved in various cerebrovascular diseases associated with endothelial dysfunction. The aim of the present experiments was to investigate the role of thromboxane and endocannabinoids in the regulation of the cerebral circulation. To study the role of thromboxane-receptors (TP) in the regulation of the cerebrovascular tone, the influence of TP-receptor activation was tested both under physiological conditions and in the absence of NO. In our *in vivo* studies, TP-receptor stimulation under baseline conditions failed to induce any changes in the cerebral blood flow (CBF). However, CBF oscillations, induced by pharmacological inhibition of NO synthase, showed further increase by TP-receptor activation and were severely inhibited after blockade of either TP-receptors or the Rho-kinase pathway. These findings implicate that the hypersensitivity of TP-receptor – Rho-kinase pathway contributes to the development of CBF oscillations, and may lead to vasospasms in pathophysiological conditions when NO availability is limited. In the second part of our experiments, the involvement of endocannabinoids (ECs) in cerebral blood flow regulation was explored. In order to model more closely the physiological distribution and release of ECs, a cannabinoid reuptake inhibitor was administered. Treatment with a CB1 receptor antagonist/inverse agonist under physiological state had no effect on basal circulatory and cerebrovascular parameters, indicating that CB1 receptors have minimal influence under resting conditions; however, enhancement of the EC system induced a tri-phasic response, where certain changes proved to be CB1 receptor mediated effects. Hypertension during *phase I* was resistant to CB1-inhibition, whereas the concomitant CBF-increase was attenuated. Conversely, the sustained hypotension during *phase III* was CB1 receptor dependent, whereas the CBF-decrease was not. During *phase II*, pronounced respiratory depression developed that was CB1 receptor dependent. To further elucidate the role of ECs on the physiological respiratory control, respiratory parameters were directly measured during EC system activation. Treatment with cannabinoid reuptake inhibitor elicited a transient decrease in respiration rate, a concomitant decrease in arterial oxygen saturation and a compensatory increase in breath distension. On the contrary, CB1-deficient mice showed no alterations in the measured respiratory parameters after EC system activation; suggesting that ECs influence the physiological control of respiration in a CB1 receptor dependent manner.

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9. Összefoglalás

Az agyi keringészavarok során kialakuló endothelialis diszfunkció az agyszövetben arachidonsav származékok felszabadulásához vezet. Ezen endogén vegyületcsoport agyi keringésre gyakorolt hatásai és a különböző cerebrovaszkuláris kórállapotok patho-mechanizmusában játszott szerepe nem teljesen tisztázott. A felszabaduló tromboxán A_2 az agyi értónus szabályozásában betöltött szerepének alaposabb megismerése érdekében az aktivált tromboxán receptorok (TP) hatását vizsgáltuk élettani, valamint nitrogén-monoxid (NO) hiányos állapotban. Élettani körülmények között a TP-receptor stimuláció nem volt hatással az agykéreg lokális szöveti véráramlására (CoBF), ellenben akut NO szintáz gátlás hatására CoBF oszcilláció jelentkezett, melyet TP-receptor aktiváció tovább erősített és TP-receptor vagy Rho-kináz gátlás jelentősen gátolt. Ezen megfigyelések arra utalnak, hogy a TP-receptor - Rho-kináz útvonal hozzájárul a CoBF oszcillációk megjelenéséhez NO szegény kórélettani körülmények között, mely vazospazmus kialakulásához vezethet. Kísérleteink második csoportjában az endokannabinoid rendszer (EC) és a kannabinoid-1 receptorok (CB1) szisztémás- és agyi keringés szabályozásban játszott szerepét vizsgáltuk. Korábbi közlemények a kannabioidok agyi véráramlás növelő hatásáról számoltak be. Ezek a kísérletek azonban különböző *exogén* kannabinomimetikumok hatását vizsgálták, így nem feltétlenül reprezentálják a regionálisan eltérő koncentrációiktól függő, *endogén* kannabinoid molekulák hatásait. Nyugalmi körülmények között a CB1 receptorok gátlása nem volt hatással a szisztémás és CoBF paraméterekre, ellenben az EC rendszer farmakológiai aktivációja trifázisos válaszreakcióval járt. Az *első fázis* során megfigyelt hipertenzió CB1 független volt, ellenben a társuló CoBF emelkedés CB1 függőnek tűnt. Ezzel szemben a *harmadik fázisban* jelentkező hipotónia bizonyult CB1 függőnek, míg a CoBF csökkenés nem. A *második fázis* során erőteljes légzésdepresszió jelentkezett, mely hatás CB1 receptor érzékeny volt. Az endokannabinoidok fiziológiás légzés-szabályozásban betöltött szerepét vizsgálva megállapítottuk, hogy az EC felszabadulást követő átmeneti légzésdepresszióhoz arteriás oxigén szaturáció csökkenés, és a légzés mélységének kompenzatorikus növekedése társul. Genetikailag módosított, CB1 receptor hiányos egereken végzett kísérleteinkben ezzel szemben az EC rendszer aktivitásának fokozását követően nem alakult ki légzésdepresszió, arra utalva, hogy az EC rendszer CB1 receptorok közvetítésével befolyásolja a légzésszabályozást.

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11. Publications

11.1. Publications directly related to the thesis

Horvath B, Lenzser G, Benyo B, Nemeth T, Benko R, **Iring A**, Herman P, Komjati K, Lacza Z, Sandor P, Benyo Z.

Hypersensitivity to thromboxane receptor mediated cerebral vasomotion and CBF oscillations during acute NO-deficiency in rats.

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Iring A[1], Ruisanchez E[1], Leszl-Ishiguro M[1], Horvath B, Benko R, Lacza Z, Jarai Z, Sandor P, Di Marzo V, Pacher P, Benyo Z.

Role of endocannabinoids and cannabinoid-1 receptors in cerebrocortical blood flow regulation.

PLOS ONE 8:(1) (2013)

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[1]*shared first authorship*

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