Cerebral blood flow regulation following hypoxic attacks and seizures in the newborn piglet

By

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III. Domoki F, Zimmermann A, Toth-Szuki V, Busija DW, Bari F. Acetazolamide induces indomethacin and ischaemia-sensitive pial arteriolar vasodilation in the piglet. *Acta Paediatr.* 2008;**97**:280-4. IF: 1.297

IV. Zimmermann A., Domoki F, Bari F. Seizure-Induced Alterations in cerebrovascular function in the neonate. *Dev Neurosci* . 2008;**371**: In Press IF: 2.691

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List of abbreviations

$[CO]_{CSF}$: carbon monoxide concentration of the cerebrospinal fluid

aCSF: artificial cerebrospinal fluid

AZD: acetazolamide

BRAD: bradykinin

CBF: cerebral blood flow

CerBF: cerebellar cortical blood flow

CNS: central nervous system

CO : carbon monoxide

CO_2 : carbon dioxide

CoBR: cerebral cortical blood flow

CORM: carbon monoxide releasing molecule

COX: cyclooxygenase

CR: cerebrovascular reactivity

CRC: cerebrovascular reserve capacity

H/I: hypoxic/ischemic or hypoxia/ischemia

HO: haeme oxygenase

HR: heart rate

IBU: ibuprofen

ICP: intracranial pressure

INDO: indomethacin

ISO: isoproterenol

L-NAME: N-ω-Nitro-L-arginine methyl ester

MABP: mean arterial blood pressure

NMDA: N-methyl-D-aspartic acid

NOS: nitric oxide synthase

PAD: pial arteriolar diameter

PGs: prostaglandins

RA: room air

Summary

Asphyxia affects approximately 5-10 % of the newborn infants and may have life-long adverse consequences, such as mental retardation and epileptiform seizures. Both hypoxic attacks, including asphyxia and cerebral ischemia, and seizures disrupt the coupling between cerebral metabolic needs and blood supply thereby lead to neuronal damage and impaired cerebrovascular reactivity and function. Although hypoxia is reversed the fastest by ventilation with 100% oxygen (O_2) upon resuscitation, hyperoxygenation may cause additional damage.

We tested if reventilation with room air (RA, 21% O2) or 100% O_2 after asphyxia would differentially affect haemodynamic events and neuronal damage in different brain areas of newborn pigs. We found O_2 toxicity after asphyxia in the hippocampus and cerebellum, but not in the cerebral cortex or basal ganglia. The observed regional differences may be associated with local haemodynamic factors, since reactive hyperemia was significantly higher in the cerebellum than in the cerebral cortex.

Carbon dioxide (CO_2) is a powerful dilator of the cerebral vessels by a mechanism involving cyclooxygenase (COX) metabolites in the newborn. This responsiveness to CO_2/hypercapnia is sensitive to hypoxia/ischemia and is used as a measure of the cerebrovascular function. Acetazolamide (AZD), a carbonic anhydrase inhibitor, produces cerebral vasodilation presumably due to CO_2 retention and acidosis. We examined if cerebrovascular effects of AZD were similar to hypercapnia in the newborn pig. The mechanism of AZD-induced cerebral vasodilation appears to be similar/identical to hypercapnia, since both reactions were abolished by the non-selective COX-inhibitor indomethacin, unaltered by ibuprofen and L-NAME and were significantly attenuated after ischemia/reperfusion.

Cerebrovascular function is protected against seizure-induced damage by enhanced endogenous carbon monoxide (CO) production by heme oxygenase. We hypothesized that exogenous CO derived from the CO releasing molecule-A1 (CORM-A1) exerts protective effects on the cerebral microvasculature. Seizures reduced postictal cerebrovascular responsiveness to physiologically relevant vasodilators (bradykinin, hemin, and isoproterenol), which was prevented by CORM-A1-pretreatment. We provided novel data on the cerebrovascular regulation in the hypoxic and epileptic newborn.

Összefoglalás

Az újszülöttek 5-10%-át érintő perinatális aszfixia súlyos, gyakran egész életen át ható központi idegrendszeri károsodásokat okoz. Az olyan hipoxiás állapotok, mint az aszfixia és az agyi iszkémia valamint a következményes görcsrohamok alatt megszűnik az agyi metabolikus aktivitás és a vérátáramlás egyensúlya, ami az agyi erek funkciózavarához és idegsejt károsodáshoz vezet. A hipoxia mérséklésének legelfogadottabb és leggyorsabb módja a 100% O_2-nel történő lélegeztetés. Egyes megfigyelések azonban arra utalnak, hogy az O_2 túlkínálat súlyosbíthatja az idegrendszeri károsodást.

Újszülött malacokban aszfixiát hoztunk létre és megvizsgáltunk, hogy a szobalevegővel, illetve 100% O_2-nel történő újraélesztés eltérően befolyásolja-e keringési paramétereket és az idegsejtkárosodást a különböző agyterületeken. Eredményeink szerint a 100% O_2 fokozta az aszfixiát követő neuronpusztulást a hippocampusban és a kisagyban, azonban a nagyagykéregben és a bazális ganglionokban nem. Az O_2 iránt érzékeny kisagy területén jelentősen nagyobb mértékű reaktív hiperémia jött létre újraélesztés során, mint a nagyagykéregben. Feltételezzük, hogy a neuronpusztulás súlyosságában tapasztalt regionális különbségek összefüggenek az agyi vérátáramlás helyi jellegzetességeivel.

A CO_2 ciklooxigenáz (COX) metabolitok közvetítésével erőteljes értágító hatást fejt ki az újszülött agyi ereken. Az agyi erek CO_2 iránti válaszkészségét a hipoxia/iszkémia károsítja, ezért ennek mérése az agyi érfunkció indikátoraként használható. A szénsav-anhidráz gátló acetazolamid (AZD) szintén tágítja az agyi ereket, feltehetően a CO_2 felhalmozódásán és acidosis kialakulásán keresztül. Vizsgálatainkban összehasonlítottuk az AZD és a CO_2 agyi erekre gyakorolt hatásait újszülött malacban. Eredményeink alapján valószínű, hogy az AZD és a CO_2 az agyi értágulatot hasonló vagy azonos mechanizmussal hozza létre, mivel mindkét reakció teljesen megszüntethető a nem szelektív COX gátló indometacinnal, jelentősen csökken iszkémia/reperfúziót követően, viszont az ibuprofen vagy L-NAME nem befolyásolja.

Az epilepszia károsítja az agyi erek funkcióját, amely ellen védelmet nyújt a hem-oxigenáz enzim által endogén úton termelt CO. Feltételeztük, hogy az A1 típusú CO felszabadító molekulából (CORM-A1) származó exogén CO szintén protektív hatást fejt ki az agyi erekre. Az epilepszia hatására jelentősen csökkent az agyi erek válaszkészsége olyan különböző, élettanilag fontos vazodilatátorok iránt, mint a bradikinin, hemin és izoproterenol. Ezzel szemben a CORM-A1 előkezelés javította az agyi érreaktivitást az epilepsziát követő időszakban. Kísérleteink új eredményekkel segítik az agyi vérkeringés szabályozásának megismerését hipoxiás és epilepsziás újszülöttekben.

Introduction

It is the time of our first cry when we start breathing air. From this moment, our cardiorespiratory system begins to accommodate to the postnatal conditions to maintain adequate blood and oxygen supply for the body. Nevertheless, approximately 5-10 % of the newborn infants require some assistance to begin breathing in the first minutes after delivery and develop various degrees of asphyxia [1;2]. By definition, asphyxia is the insufficient exchange of the respiratory gases resulting in arterial hypoxia, hypercapnia and metabolic acidosis [3]. Asphyxia is soon accompanied by bradycardia, hypotension and redistribution of the blood flow, thus triggers a hypoxic/ischemic (H/I) insult in the organs including the brain. In survivors, asphyxia and cerebral ischemia often cause life-long neurological consequences, such as epileptiform seizures, cerebral palsy and mental retardation [4]. Clinical seizures affect around 0.3% of term neonates and more than 10% of preterm neonates during the neonatal period. Approximately half of the cases are related to perinatal asphyxia [5]. Prolonged or sustained early-life seizures themselves are harmful to the neonatal central nervous system (CNS) and may further aggravate a preexisting brain damage. Epileptiform seizures interact with brain development, increase the susceptibility to recurrent seizures and possibly lead to behavioural deficits that persist into adulthood [6-9].

Oxygen therapy in the newborn babies

The aim of the resuscitation is to prevent death and adverse neurological sequelae [10]. The success of resuscitation depends on the prompt reversal of hypoxia, of which the fastest way is the ventilation with pure O_2.

O_2 therapy for asphyxiated newborn infants was first introduced in the 1930s in the USA. Ten years later, the link between hypoxia and brain damage was clearly established and a recommendation for the use of O_2 in the nursery room was published at the end of the 1940s [10]. From the early 1950s the toxic nature of O_2, especially to preterm infants, has also been understood [11]. Nevertheless, the use of O_2 in neonates was generally accepted and has only been challenged recently.

According to animal studies and clinical observations, neuronal damage occurs at least in two phases following asphyxia and H/I. Initially, cerebral ATP levels fall rapidly leading to the disruption of the cellular ion homeostasis and an unrestrained glutamate release. The resulting Ca^{2+} influx through N-methyl-D-aspartic acid (NMDA)

receptors triggers a series of biological processes culminating into cell death, collectively termed as excitotoxicity. These processes include extensive activation of degrading enzymes, production of reactive oxygen species (ROS) and altered gene expression. Upon resuscitation after mild to moderate cerebral ischemia, reperfusion and reoxygenation takes place resulting in an initial period of reactive hyperemia with subsequent hypoperfusion. The readministration of O_2 upon reactive hyperemia dramatically increases ROS generation by several enzymes including the mitochondrial electron transport chain, lipooxygenase, cyclooxygenase (COX), cytochrome P450s, xanthine oxidase, NAD(P)H oxidase and other hemoproteins [12]. Thereby reoxygenation/reperfusion after an asphyxic or H/I period leads to secondary injury that aggravates the initial insult [3]. Therefore, limitation of reoxygenation is a reasonable way to prevent at least part of the brain damage.

Indeed, plenty of evidence illustrates that hyperoxygenation during resuscitation is harmful to newborn babies. Hyperoxygenation increases the risk of retinopathy of dysmaturity, chronic lung disease, childhood leukaemia and cancer [11;13;14]. To date, six controlled, randomised or quasy-randomised studies were conducted which compared the effectiveness of resuscitation with room air (RA) to 100% O_2 in depressed neonates [15-20]. According to the meta-analyses of these studies [2;21-23], RA ventilation is at least as effective as O_2 concerning the rate of successful resuscitations and the long-term neurological outcome [24]. Importantly, RA was found significantly superior to O_2 when assessing neonatal mortality and some short-term outcome measures, such as 5 min Apgar scores and the time until the appearance of spontaneous breathing [2;23]. According to the findings, RA ventilation prevents one death for every 20 infants resuscitated, which could be converted to tens of thousands of saved lives annually worldwide.

The beneficial effects of RA compared to O_2 ventilation have also been suggested by a number of animal studies using various models of perinatal asphyxia [25-27]. For instance, RA ventilated piglets had a significantly better early neurological examination score than O_2-resuscitated animals following artificial bilateral pneumothorax. However, the evaluation of differences in cerebral histopathology was compromised by the relatively high variability of the severity of asphyxia and the degree of circulatory shock [28]. Hence, we used a more consistent and controllable model of 10 min of severe asphyxia. We tested if reventilation with RA or O_2 would differentially affect cerebral histopathology and whether the severity of neuronal injury is related to hemodynamic changes.

The cerebrovascular regulation in the newborn brain

The neurological consequences of hypoxic attacks critically depend on the cerebral blood flow (CBF) following the insult, since both hypoperfusion and hyperperfusion may aggravate the initial brain damage. The major characteristics of the cerebrovascular circulation is the maintenance of constant CBF in the face of perfusion pressure changes (pressure autoregulation) and the adjustment of the local CBF to the local metabolic needs of activated neurons [29;30]. The pressure autoregulation is present in healthy term newborns, although functions over a lower and narrower range than in the adult [31;32]. The main regulating factor of the overall CBF is carbon dioxide (CO_2) as it is a powerful dilator of the cerebral vessels. A normal arterial pCO_2 and cerebrovascular responsiveness to CO_2 are essential factors for the adequate blood supply of the CNS [33].

The coupling between the local cerebral metabolic needs and the local CBF is assured by the neurovascular unit. The neurovascular unit is comprised of the neurons, astroglia, pericytes and cerebrovascular endothelial and smooth muscle cells [34]. These cellular elements release a number of vasoactive substances which finally adjust the appropriate cerebrovascular tone. For instance, activated neurons release glutamate, adenosine, protons and nitric oxide (NO) [35;36], astrocytes emit NO, K^+, adenosine, COX products and epoxyeicosatrienoic acids [34;37-41], while endothelial cells produce NO, CO and COX metabolites [42;43].

The relative contribution of the above mediator systems to the cerebrovascular reactions are developmentally regulated. In the early postnatal period, prostanoids appear to play a particularly important role in the cerebrovascular control. According to studies using the non-selective COX-inhibitor, indomethacin, prostanoids 1) maintain the normal cerebrovascular tone [44], 2) set the range of pressure autoregulation [45] and 3) mediate essential cerebrovascular responses to hypercapnia and hypotension in the newborn [46-48]. The neurovascular unit is extremely sensitive to H/I which results in decreased reactivity to multiple vasodilators. Althought the mechanism of H/I cerebrovascular injury is not understood in details, COX system seems to play a dual role. Firstly, H/I rapidly increases the levels of the COX-2 isoenzyme in the cerebral arteries in the newborn piglet [49;50]. The neurovascular axis remains intact if COX-2 is inhibited before the ischemic attack as indicated by preserved pial arteriolar dilation in response to NMDA [51]. The mechanism which injures the neurovascular axis is probably

related to the enhanced production of reactive oxygen species via COX [52]. Secondly, H/I demolishes the COX-dependent cerebrovascular reactions, such as responses to hypercapnia and hypotension [53-58], potentially due to the limited availability of arachidonic acid [59] and consequently, a decreased production of COX products [56].

Hypercapnia and acetazolamide in the assessment of the cerebrovascular function

In clinical practice, cerebrovascular reserve capacity (CRC) is used as an indicator of cerebrovascular function and responsiveness. CRC has been reported to decrease in a number of cerebrovascular diseases [60-64]. In adult patients, CRC is usually determined by the combination of CBF measurements and induced vasodilation with CO_2 inhalation [65] or intravenous (iv) acetazolamide [66;67]. Acetazolamide (AZD), an unsubstituted sulphonamide, is a potent inhibitor of carbonic anhydrase and possesses diuretic and anticonvulsive activity [68]. Carbonic anhydrase catalyzes the rapid conversion of CO_2 to bicarbonate and protons in virtually all tissues of the body including the CNS. AZD, similarly to hypercapnia, prominently increases CBF [69]. Despite its widespread human use, the precise mechanism of AZD-induced CBF increase is unclear. As iv AZD inhibits carbonic anhydrase in both the erythrocytes and brain cells, the vasodilatory effect of AZD is commonly attributed to increased systemic and local CO_2 levels and carbonic acidosis [70]. However, the direct cerebrovascular effect of AZD has also been advocated [71;72].

CRC is not measured routinely in children or newborn infants, however, several studies using neonatal animal models reported the decline of cerebrovascular responsiveness after asphyxia [73;74], brain ischemia [53;75;76] and seizures [77;78]. Optimally, diagnostic and therapeutic interventions in pediatric patients, especially in newborn babies, require no cooperation. In terms of the cooperation, AZD could be advantageous in CRC measurement in newborn babies [61].

Nevertheless, we know very little about the vascular effects of AZD in the neonatal brain. There are only two studies, one in piglets and one in humans where the pronounced effect of AZD on middle cerebral artery flow velocity was described, but no effort was made in either study to identify the mechanism of increased flow [79;80]. Further, pial arteriolar responses to AZD have not been previously determined, characterized or compared to the hypercapnia-induced changes in the piglet model. In the newborn piglet, hypercapnia-induced vasodilation was shown to be an example of

H/I-sensitive [57], endothelium-dependent vascular reaction [81] that requires the presence of cyclooxygenase (COX) metabolites as permissive factors [47] and shows a selective vulnerability to indomethacin but not to other COX inhibitors [82]. Thus, we examined if acetazolamide is an equal substitute of hypercapnia in the assessment of the cerebrovascular endothelial function in the newborn piglet model.

Carbon monoxide and protection of the cerebral vasculature

Recently, a wild range of neuroprotective strategies have been studied in animal models, some of them are already employed in clinical practice [83;84]. The primary aim of these strategies is to stop those cellular pathways that finally lead to neuronal death. An animal study using neuroprotective doses of diazoxide revealed, that diazoxide not only prevents neuronal damage but also protects the endothelium-dependent cerebrovascular reactivity to hypercapnia. This observation indicates that neuroprotective mechanisms can be partially indirect through the protection of the neurovascular unit and the cerebrovascular function [53].

One way to protect the cerebrovascular function is the enhancement of the endogenous protective processes. The enzyme, haeme oxygenase (HO), which produces endogenous CO is not only involved in the CBF regulation, but is also an important cellular defense mechanism against oxidative stress. It converts the prooxidant haeme to iron, CO and bilirubin/biliverdin [85]. Bilirubin is a powerful antioxidant [86], while antiapoptotic, antioxidant, anti-inflammatory, and antiproliferative influences of CO have been described in the respiratory, cardiovascular, and renal systems in vivo and in vitro [87-95]. The neonatal brain and cerebral vessels abundantly expresses the constitutive HO isoform (HO-2) under physiologic conditions [43]. Besides, the expression of the inducible form, HO-1, is elicited by various pathologic stimuli and pharmacologic agents [96]. The cerebrovascular effects of HO/CO system has been extensively studied in the newborn piglet model under physiologic conditions and during epileptiform seizures [43;78;97-99].

Seizures occur when there is a synchronous activation of the neurons due to excessive glutamate release. Epileptiform seizures acutely and profoundly increase the CBF and the cerebral metabolic rate. As seizures progress, cerebral hyperaemia declines, while the metabolism remains high. Increases in CBF are therefore may not be sufficient to match the cerebral metabolic demands throughout the ictal period. Accordingly, seizures deplete cerebral energy sources and lead to the accumulation of

metabolic end/by-products. After seizure onset, cerebral glucose levels rapidly fall, followed by declines in the ATP and phosphocreatinine contents, coinciding with the accumulation of inorganic phosphate, lactate and the evolution of intracellular (IC) acidosis [100-102].

Epileptiform seizures cause both neuronal and cerebrovascular damage as they trigger the disruption of the blood-brain barrier and a loss of cerebral pressure autoregulation [100-102]. Importantly, seizures cause long-term cerebrovascular dysfunction in human patients and animal models, as indicated by symptoms of the postictal state and reduced vasoreactivity to physiologically relevant vasodilators, respectively [77;78;103]. The pharmacological induction of HO-1 was found to be protective against long-term cerebrovascular dysfunction caused by seizures in vivo [78] and glutamate-triggered apoptosis in cultured piglet microvascular endothelial cells [97]. The favourable impact of enhanced HO activity could be either due to the degradation of haeme or the production of CO and/or biliverdin/ bilirubin.

CO is a well-known dilator of the cerebral vessels in the newborn piglet [104;105], but its cerebrovascular protective properties have not been studied selectively. The effects of gaseous CO can be mimicked by a group of pharmacological compounds collectively termed CO-releasing molecules (CORMs) [106-111]. The novel CORM-A1 [sodium boranocarbonate, $Na_2(H_3BCO_2)$] has several advantages compared to other CORMs, as it is water-soluble, spontaneously releases gaseous CO in physiological solutions and its half-life at 37°C is approximately 20 min [106;109]. Moreover, CORM-A1 does not contain heavy metals that may affect ion channel function or induce HO-1 and, thus, stimulate endogenous CO production [109]. Therefore, we used CORM-A1 to test the specific role of CO in the protection against sustained seizure-induced cerebrovascular dysfunction.

The newborn piglet model

The studies described in the current thesis were conducted in newborn piglets. The newborn piglet is a widely accepted, relevant animal model of the term human neonates, since they share numerous anatomical and developmental characteristics. Namely, these similarities between the newborn pig and the term human baby include systemic[112], pulmonary [113] and cerebral circulatory parameters [114], brain morphology and maturational processes [115], as well as cerebral histology [116] and electrophysiology [117]. In piglets, 5-10 minutes of asphyxia induced by turning off the ventilator causes

severe arteriolar hypoxia, hypercapnia and metabolic acidosis, bradycardia, hypotension, a dramatic drop in CBF [118], and an increased production of ROS [119;120]. These profound changes are reversed during reventilation, but the impairment of the cerebrovascular reactivity is sustained for at least one hour [73;121]. Moreover, four hours after an asphyxic insult, the neuronal damage becomes histologically detectable [122]. Based on these characteristics, the newborn piglet is an appropriate model for short-term evaluation of hypoxic-ischemic encephalopathy [123].

Bicuculline-induced seizures in newborn pigs are a well-defined model of neonatal epileptiform seizures. Seizure models are characterized by the induction of seizures without recurrent activity, in contrast with models of epilepsy which are described by chronic epileptic behaviour [124;125]. Infants and children generally have seizures of neocortical origin that is replicated by the $GABA_A$ receptor blocker bicuculline in the newborn piglets, which were documented by increased electroencephalographic amplitude and spectral power within the 1- to 15-Hz frequency range sustained for ~2 h [99]. Anaesthesia, pancuronium paralyzation, and artificial ventilation largely alleviate the systemic effects of seizures allowing a better survival rate and more specific examination of seizure-related cerebral events [126].

Aims of the study:

The mechanisms of the cerebrovascular regulation have been extensively studied both under physiological circumstances and following various hypoxic/ischemic insults in the newborn piglet. However, important questions are still not answered concerning 1) the relationship between hemodynamic changes, oxygenation and neuronal damage, 2) the mechanisms of significant cerebrovascular reactions, 3) and the pathomechanisms of cerebrovascular dysfunction following a pathological insult. In spite of the clinical relevance of the newborn piglet model, the aim of the studies described in this thesis was not to propose therapeutic or diagnostic applications, but to study physiological and pathophysiological mechanism of the cerebrovascular regulation under controlled experimental conditions.

Specifically, these purposes were the following:

I. To test if reventilation with RA or O_2 after 10 min of severe asphyxia would differentially affect (1) cerebral histopathology, (2) blood flow in cerebral and cerebellar cortex (CoBF and CerBF, respectively), and (3) systemic cardiovascular parameters in piglets.
II. To compare hypercapnia- and AZD-induced pial arteriolar and CBF reactions by assessing the effect of (1) indomethacin, (2) ibuprofen (two cyclooxygenase inhibitors), (3) ischemia/reperfusion (I/R) and (4) N-ω-Nitro-L-arginine methyl ester (L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor) on these cerebrovascular responses.
III. To examine the ability of peripherally administered CORM-A1 (1) to deliver CO to the brain, (2) to exert the effects of gaseous CO on the cerebral circulation, and (3) to prevent seizure-induced sustained cerebrovascular dysfunction.

Methods

Animals

Newborn piglets of either sex (0-5 d old, body weight 1-2.5 kg) were used. All procedures were approved by the Animal Care and Use Committee of the University of Szeged or the University of Tennessee Health Science Center.

For acute experiments, the pigs were anaesthetized initially with sodium thiopental (45 mg/kg ip) or a mixture of ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im) then maintained on α-chloralose (30-40 mg/kg iv). The femoral artery and vein were cannulated to monitor arterial pH, blood gases and blood pressure, and to inject drugs and fluids, respectively. The animals were intubated through tracheostomy and artificially ventilated with room air. The body temperature was kept at 37–38°C with a servo-controlled heating pad. At the end of the experiments, anaesthetized pigs were killed with an iv injection of saturated KCl solution.

Cranial window and cerebrovascular reactivity

A closed cranial window and intravital microscopy were used to determine pial arteriolar diameter (PAD). The piglets were placed in the prone position and their heads were fixed in a stereotactic frame. The scalp was incised and removed over the calvaria. A circular craniotomy was made in the left or right parietal bone and the dura was cut and then reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy. For induction of cerebral ischemia, a hollow brass bolt was also inserted into the left frontal cranium rostral to the cranial window. The cranial window and the bolt, if installed, were sealed with bone wax and cemented with cyanoacrylate ester and dental acrylic. The space under the window was filled with artificial cerebrospinal fluid (aCSF). The pial circulation was visualized using an operating microscope equipped with a CCD camera connected to a TV monitor. In each experiment, 1-3 pial arterioles (60-100 μm) were observed and their diameters (PAD) were determined with a video microscaler. When cerebrovascular reactivity (CR) was tested, the maximal PAD achieved in 5-10 min after the application of the stimuli was taken as a response.

Measurement of the cortical blood flow

CoBF or CerBF was monitored using a two-channel laser-Doppler flowmeter. To position the LDF flow probes, two holes were drilled through either the parietal or the occipital bone for the assessment of CoBF or CerBF, respectively. The dura mater was incised and the LDF probes were inserted through plastic cylinders that were secured in place with dental acrylic (Lang Dental, IL, USA). The fibre-tip position was set ~1 mm above the surface of the parietal or cerebellar cortex to obtain a stable LDF signal. Blood pressure and the LDF were recorded online simultaneously during the experiments. Recordings were evaluated offline using Perisoft 1.30: mean arterial blood pressure (MABP), heart rate (HR), CoBF and CerBF (% of baseline) were determined.

Neuropathology scoring

Brains were immersion-fixed in 4% paraformaldehyde. The following areas were sampled: frontal and temporal cortex, cerebellum, hippocampus, basal ganglia, and pons. Tissue blocks were embedded in paraffin, and sections for light microscopy were either double stained with haematoxylin and eosin or triple stained with haematoxylin, eosin and luxol blue to visualize the white matter. Hypoxia-caused neuronal damage was assessed by two observers (dr Gábor Cserni and Rita Bori). Hypoxia-caused neuronal damage was demonstrated by the presence of shrunken hyperchromatic neurons with pyknotic nuclei. The brain sections were evaluated on the basis of a semi-quantitative five-grade scoring system summarized in **Table 1**.

Score	% ratio of damaged cells
5	0
4	<5% (scattered)
3	<33%
2	<66%
1	<100%

Table 1. Neuropathological scoring system

CO detection by gas chromatography-mass spectrometry

CO concentrations of in vitro and in vivo samples were measured. For detection of CO production by the tissue of the cerebral surface, the cortical periarachnoid CSF was collected from the space under the cranial window (0.4ml) through a spout directly into the vials. For measurement of the CO concentrations in vitro solutions, 0,4 ml

samples were pipetted into the vials. The vials also contained 1.3 ml of Krebs buffer and the internal standard, $^{13}C^{18}O$ (1 mM). The sealed vials were heated at 70°C for 10 min and 100 µl head-space gas was collected using a Hamilton syringe. The components of the head-space gas were separated on a molecular sieve-coated capillary column to separate CO from other gases (retention times of 1.5 min for O_2 peak, 2.3 min for N_2 peak, and 6.3 min for CO peak). The CO peak was quantitatively detected on the basis of the peak areas with the mass-to-charge ratios corresponding to $^{12}C^{16}O$ and $^{13}C^{18}O$ [77].

Seizure induction

Epileptic seizures were induced with bicuculline (3 mg/kg ip) in ketamine-acepromazine-anesthetized, ventilated and pancuronium-paralyzed (0.2 mg/kg iv) piglets. Bicuculline was prepared shortly before the experiment. Bicuculline was dissolved in 0.1 N HCl (3 mg/ml), titrated with 1 N NaOH to pH 4–5, and diluted with 5 ml of saline.

For long-term studies of the cerebrovascular function following seizures, we limited the interventions to minimally invasive procedures. The piglets were intubated through the mouth and slightly hyperventilated with a gas mixture of 4% CO_2-21% O_2-75% N_2 to maintain physiological levels of blood gases. A butterfly needle was inserted into the ear vein for administration of pancuronium before seizure induction. Drugs were administered aseptically through a 0.22-µm Millipore syringe filter. The animals were kept on the ventilator for 2 or 3 h until the seizure activity subsided. Body temperature was kept at 37-38 °C. When consciousness was regained, piglets were transferred to the animal care facility and housed in warmed cages with food and drink ad libitum for 2 days.

Experimental procedures

Experimental procedures and endpoints are summarized in **Figure** 1.

I. Cerebral effects of reventilation with 100% O_2 versus RA after asphyxia

The animals were initially divided into three experimental groups (Groups 1-3 n=7, 9 and 8, respectively, **Figure 1**). To induce asphyxia, the artificial ventilation was suspended and the intratracheal tube was clamped for 10 min. The piglets were then reventilated with either RA or O_2 for 1 h. Subsequently, all animals were ventilated with RA for 3 additional hours. During the course of the experiments, 10 min tracings of

arterial blood pressure and CoBF were recorded before, during and after the asphyxia as well as the first 10 min of every hour of survival. Reactive hyperaemia was calculated using the areas under the baseline-normalized LDF flow curves in the initial 10 min of reventilation. Arterial blood gases and pH were measured before and during asphyxia (8^{th} min), and at the end of every hour of survival. At the end of the survival period, the piglets were sacrificed and the brains were removed for neuropathology examinations.

The neuropathological results urged us to include two additional groups reventilated with either RA or O_2 (Groups 4-5, n=5 and 5, **Figure 1**). We used the same experimental protocol as above except that we measured CerBF and removed the brains after 2 h of survival.

II. Comparison of CO_2- and AZD-induced reactions in pial arterioles and CBF

CR to graded hypercapnia was determined at first, then repeated: a) 15 min after iv administration of 1 mg/kg indomethacin (Group 7, n=6) or 30 mg/kg ibuprofen (Group 8, n=6); b) 1 h after 10 min global cerebral ischemia (I/R, Group 9, n=11); or c) 45 min after iv injection of 15 mg/kg L-NAME (Group 10, n=4, **Figure 1**). In all groups, CR to two consecutive doses of AZD (10 and 20 mg/kg iv) was determined after the second hypercapnia challenge and data were collected for 20-25 min intervals. Group 6 (n=11, **Figure 1**) was subjected to a single hypercapnic period which was followed by AZD administration. MABP and CoBF were recorded simultaneously, HR was calculated off-line. PAD was measured at baselines and then in every minute of the hypercapnia and AZD challenges. Arterial pH and blood gases were determined at baselines, 4^{th} min of hypercapnia and 20 min after the administration of AZD.

Graded hypercapnia was elicited by ventilating the animals first with a gas mixture of 5 % CO_2, 21% O_2 and balance N_2 for 5-7 min, then with and 10% CO_2 for an additional 5-7 min. To induce global cerebral ischemia, the intracranial pressure (ICP) was raised above the MABP with the infusion of aCSF. Venous blood was withdrawn as necessary to maintain MABP near normal values. At the end of the ischemic period, the infusion tube was clamped and the ICP was allowed to return to the preischemic levels. The withdrawn and heparinized blood was readministered.

III. Cerebrovascular function following seizures and CORM-A1 treatment

For these experiments, active CORM-A1 (10^{-3} M stock solution) was prepared immediately before use and diluted with either aCSF or physiological saline (1 mg/ml). Inactivated CORM-A1 was prepared by exposure of the CORM-A1 stock solution to open air for 20 h at room temperature to fully decompose the parent compound.

First, we investigated acute cerebrovascular effects of topical and systemic CORM-A1 in newborn pigs. In Group 11 (n=8) CO, then CORM-A1 was applied to the area under the closed cranial window in consecutively increasing concentrations (10^{-7}–10^{-4} M, 10 min each), while in Group 12 (n=5), inactivated CORM-A1 (10^{-7}–10^{-4} M) was administered onto the brain surface (**Figure 1**). In Group 13 (n=5) CORM-A1 (2 mg/kg) was administered systemically two times. First, CORM-A1 was injected iv and followed by a 90 min registration and sampling period. Then the administration of CORM-A1 was repeated ip and measurements were continued for 60 min. PAD and systemic parameters (MABP, HR, blood gases, and hematocrit) were registered, and cortical periarachnoid CSF was collected for CO detection.

Secondly, we compared delayed postictal cerebrovascular reactivity in control and CORM-A1-treated animals. Epileptic seizures were induced by bicuculline, and animals were allowed to recover for 2 days. To test the cerebrovascular function, we applied bradykinin (10^{-5} M), isoproterenol (10^{-6} M), hemin (10-^{6} M) or sodium nitroprusside (10^{-6} M) to the cerebral surface and measured PAD. Animals were randomized to one of the following groups: Group 14 (n=9): control intact piglets, Group 15 (n=6): CORM-A1-treated (2 days after CORM-A1, 2 mg/kg ip) animals, Group 16 (n=7): postictal (2 days after seizure induction) animals, or Group 17 (n=5): CORM-A1-pretreated postictal (2 days after seizures, 2 mg/kg CORM-A1 ip 30 min before seizure induction) animals.

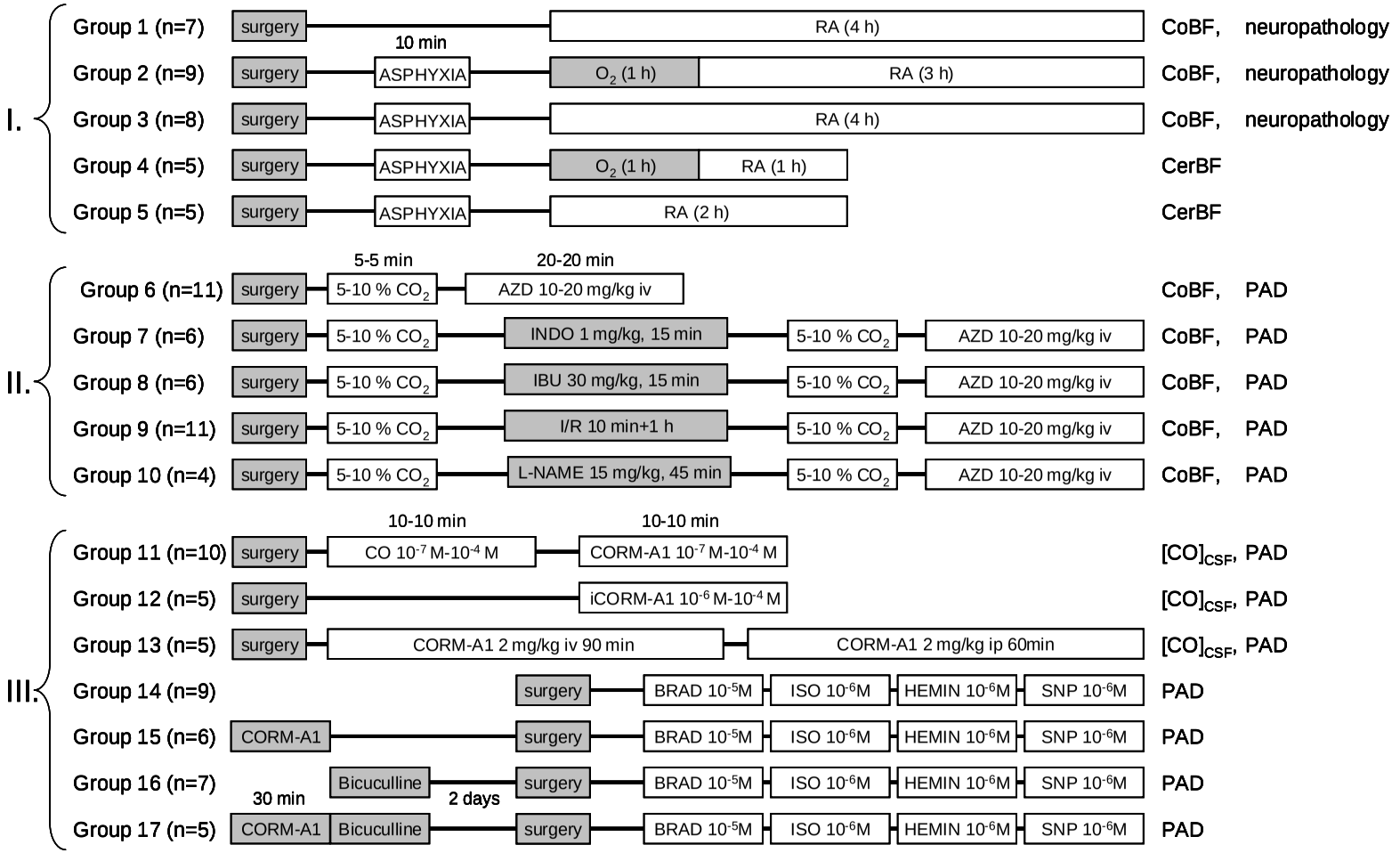

Figure 1 Summary of the experimental protocols. Abbreviations: AZD: acetazolamide, BRAD: bradykinin, CerBF: cerebellar cortical blood flow, CoBF: cerebrocortical blood flow, $[CO]_{CSF}$: carbon monoxide concentration of the cerebrospinal fluid, CORM-A1: CO releasing molecul A1, RA: room air, iCORM-A1: inactivated CORM-A1, I/R: ischemia/reperfusion, IBU: ibuprofen, INDO: indomethacin, ISO: isoproterenol, L-NAME: N-ω-Nitro-L-arginine methyl ester, PAD: pial arteriolar diameter, SNP: sodium nitroprusside

Statistical analysis

Data are expressed as mean±SEM of the absolute values or the percentage of control. MABP, PR, blood gas and LDF data obtained in experimental Groups 1-5 were analysed using two-way repeated measures ANOVA. Neuropathology scores were evaluated with one-way ANOVA on ranks. For post hoc analysis, we used the Student-Newman-Keuls test where appropriate. Reactive hyperaemia data between the cortex and the cerebellum were compared with the t-test. Data collected from Groups 6-10 were analysed with one-way ANOVA, except data of repeated hypercapnia challenges (before and after treatments in the same group) that were analysed using repeated measures ANOVA. For post hoc analysis the Dunnett's test or the Student-Newman-Keuls test was employed. Data collected from Groups 11-17 were analysed by using ANOVA with repeated measures and Tukey-Kramer multiple comparisons test were used to confirm differences among and then between groups, respectively. $P<0.05$ was considered significant in all statistical tests.

Results

I. Cerebral effects of reventilation with 100% O_2 versus RA

1. Physiological parameters

MABP, HR and blood gas values were within the physiological limits in all experimental groups before asphyxia/reventilation. Asphyxia resulted in severe acidosis, hypoxia as well as hypercapnia, which was similar in all groups. Also, asphyxia elicited simultaneous decreases in MABP and HR. Reventilation elicited transient increases in MABP and HR above baseline levels, which returned to around baseline after 10 min (**Figure 2 A and B)**. Reventilation with RA or O_2 did not significantly affect the assessed physiological parameters, except that PaO_2 was significantly higher in the latter groups in the first hour of reventilation (for the table of physiologic parameters, see paper [127]).

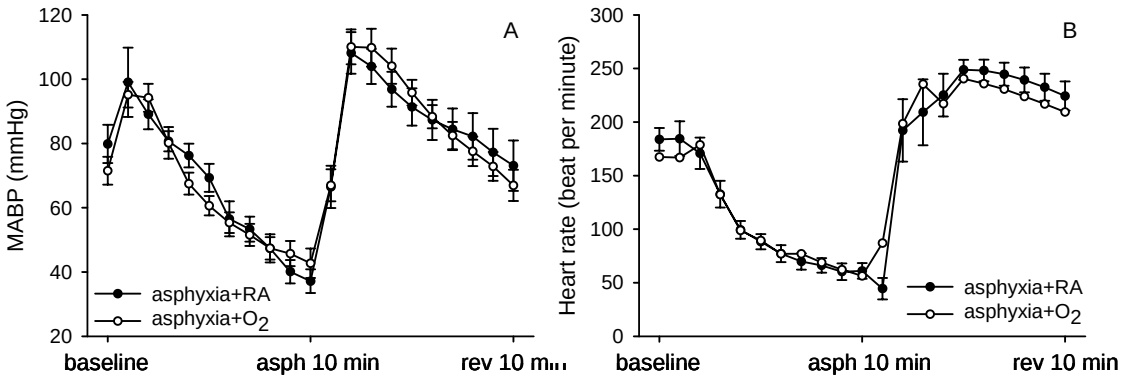

Figure 2 Changes in MABP (A) and heart rate (B) during 10 min asphyxia followed by 10 min of reventilation with oxygen (O_2, Group 2, n=9) or room air (RA, Group 3, n=8). During asphyxia, MABP rose transiently then decreased continuously. Reventilation resulted in prominent but transient MABP elevation that was similar in both RA- or O_2-ventilated groups. Heart rate showed similar changes to MABP during asphyxia/reventilation. Heart rate values were also not significantly different between the treatment groups. Values are presented as mean±SEM. Abbreviations: asph: asphyxia, rev: reventilation.

During the course of asphyxia, CoBF and CerBF gradually decreased by ~80% (Groups 2-5). Reventilation gave rise to reactive hyperaemia, then blood flow returned to around baseline values in the survival period. Reventilation with RA or O_2 did not significantly affect CoBF (**Figure 3**) or CerBF (data not shown); thus, CoBF data from Groups 2 and 3, and CerBF data from Groups 4 and 5 were combined to compare the two regions. Decreases in blood flow were similar in the cortex and the cerebellum

during the course of asphyxia, but during the early reventilation period CerBF displayed a quicker and larger reactive hyperaemia compared to CoBF (**Figure 4 A**). In fact, total reactive hyperaemia was approximately two times larger in the cerebellum than in the cortex (**Figure 4 B**).

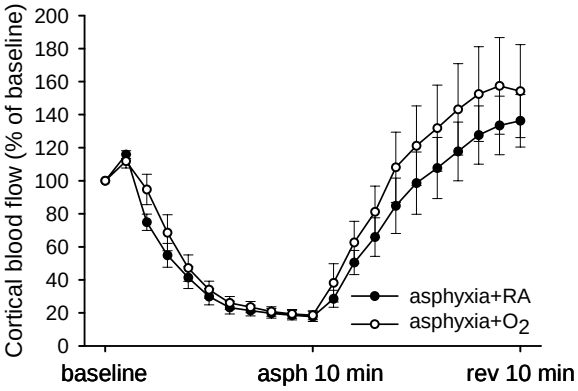

Figure 3 Cortical blood flow (CoBF) measured by laser-Doppler flowmetry during 10 min asphyxia followed by 10 min of reventilation with oxygen (O_2, Group 2, n=9) or room air (RA, Group 3, n=8). By the end of asphyxia, CoBF decreased by ~80%, which was followed by considerable reactive hyperaemia. However, there was no statistical difference between the CoBF responses of the RA- or O_2-reventilated groups. Values are presented as mean±SEM.

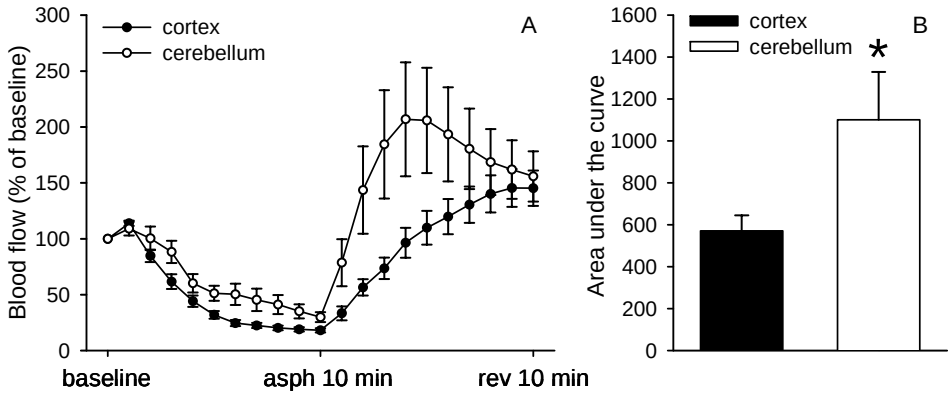

Figure 4 Laser Doppler flowmetry. A: Comparison of cortical (CoBF) with cerebellar blood flow (CerBF) during 10 min asphyxia followed by 10 min of reventilation. Since there was no difference between the room air- or O_2-reventilated groups in either region, data from Groups 2 and 3 (CoBF, n=17) and Groups 4 and 5 (CerBF, n=10) were combined. Asphyxia (asph) resulted in similar decreases in both CoBF and CerBF. However, reactive hyperaemia appeared to be greater during the first 10 min of reventilation (rev) in the cerebellum. B: Comparison of reactive hyperaemia between the cortex and the cerebellum. Reactive hyperaemia was determined by calculating the areas under the baseline-corrected LDF flow curves of CoBF and CerBF. Reactive hyperaemia was found to be significantly greater in the early reperfusion period in the cerebellum. Values are presented as mean±SEM.*$p<0.05$.

2. Neuropathological scores

Neuropathological examinations revealed that O_2 (Group 2) or RA (Group 3) or reventilation after asphyxia differentially affected neuronal lesions in the assessed brain regions (**Figure 5**). Asphyxia caused similar damage in the frontal and the temporal cortices in both reventilation groups. However, compared to the normoxic time controls, O_2 but not RA reventilation resulted in greater neuronal lesions in the hippocampus and the cerebellum. In contrast, only RA but not O_2 reventilation elicited significant damage in the basal ganglia. Asphyxia did not cause significant damage in the pons.

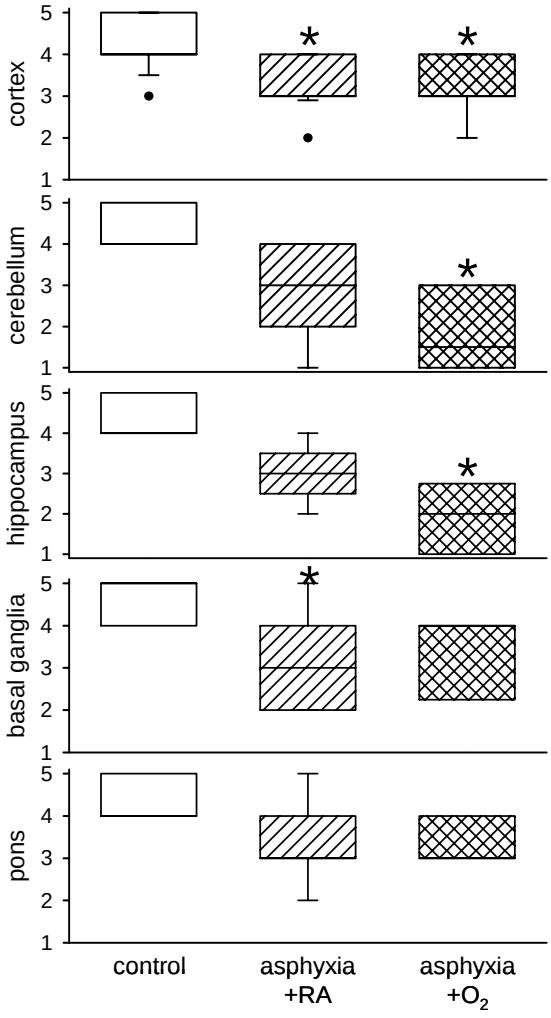

Figure 5 Regional neuropathology scoring in Groups 1-3 (time control, asphyxia and room air reventilation, asphyxia and oxygen reventilation, n=7, 9 and 8, respectively). The graphs show the median values as a line across the boxes. The box plot and the error bars show the 25-75th and the 10-90th percentiles, respectively. Outlying data are indicated as dots. Asphyxia caused similar damage in the frontal and the temporal cortices in both reventilation groups, therefore data from these areas were combined as presented. However, in the hippocampus and the cerebellum, neuropathological scores were significantly worse only in the O_2 reventilation group compared to time controls. In contrast, RA but not O_2 reventilation elicited significant damage in the basal ganglia. In the pons, asphyxia-induced decreases in neuropathology scores did not reach the level of significance. *$p < 0.05$, significantly lower than control values.

II. Comparison of hypercapnia- and AZD-induced pial arteriolar and CBF reactions

1. Physiological parameters

The MABP was in the normal range throughout the experiments and was not affected significantly by the induction of hypercapnia, indomethacin, ibuprofen, and AZD treatment. In group 5, however, L-NAME significantly elevated MABP from 68 ± 4 to 83 ± 6 mmHg. The treatments did not affect baseline arteriolar diameters significantly (data not shown). Graded hypercapnia significantly elevated the arterial pCO_2 levels with simultaneous reductions in arterial pH during repeated challenges in all experimental groups. For instance, in group 1, pCO_2 in response to 5% and 10% CO_2 inhalation was increased from 30.3 ± 1.8 (baseline) to 40.0 ± 1.3*and 57.5 ± 2.8* mmHg, and pH decreased from 7.41 ± 0.03 (baseline) to 7.32 ± 0.01* and 7.17 ± 0.01*, respectively (*$p < 0.05$, significantly different from baseline).

2. Cerebrovascular responses

Hypercapnia resulted in large, concentration-dependent, reversible increases in pial arteriolar diameters and CoBF detected with LDF. Hypercapnia elicited with 10% CO_2 induced significant increases in both PAD and CoBF compared to 5% CO_2 and baseline values (combined data of Groups 6–8, n = 23). Similarly, AZD (10 mg/kg) also significantly augmented PAD and CoBF. Maximal increases were reached at 10–15 min after AZD administration. Additional AZD administration, however, failed to significantly increase the maximal vasodilation/hyperaemia elicited by the first dose. Importantly, maximal AZD-induced vasodilation did not approach the maximal vasodilation induced by 10% CO_2 (**Figure 6**).

In Group 7, indomethacin virtually abolished the pial arteriolar response to hypercapnia, whereas ibuprofen (Group 8) failed to affect the arteriolar dilation. In a similar fashion, AZD-induced vasodilation was also markedly attenuated after indomethacin administration, but was unaffected by ibuprofen. In Group 9, I/R severely attenuated both the hypercapnia- and the AZD-induced arteriolar vasodilation. In Group 10, L-NAME did not alter CR to either CO_2 or AZD (**Figure 7**).

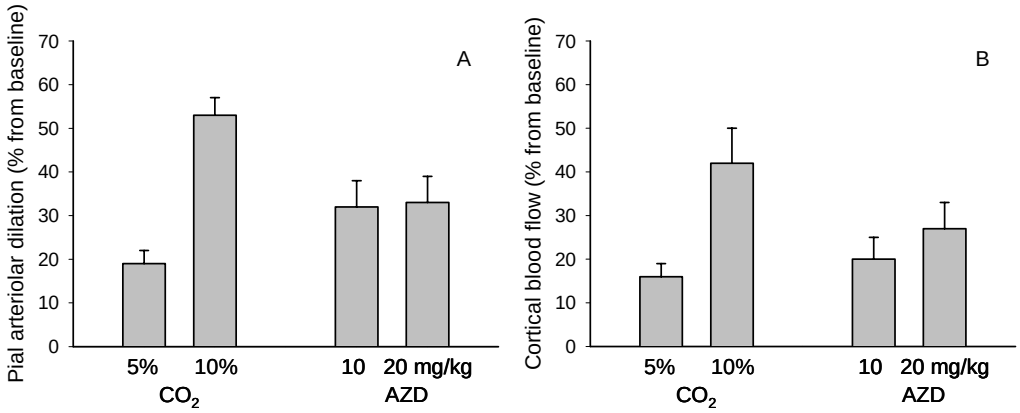

Figure 6 Comparison of hypercapnia and acetazolamide (AZD)-induced pial arteriolar vasodilations and increases in cortical blood flow (CoBF) assessed with laser-Doppler flowmetry. Hypercapnia (data from Groups 6-8, before treatments, n=23) elicits dose-dependent pial arteriolar dilations and CoBF increases. Both doses of AZD (data from Group 6, n=9) also elicited similar increases in both pial arteriolar diameters and CoBF. Values are presented as MEAN±SEM. *p<0.05, significantly larger than vasodilation/CoBF increase to 5% CO_2 as well as AZD.

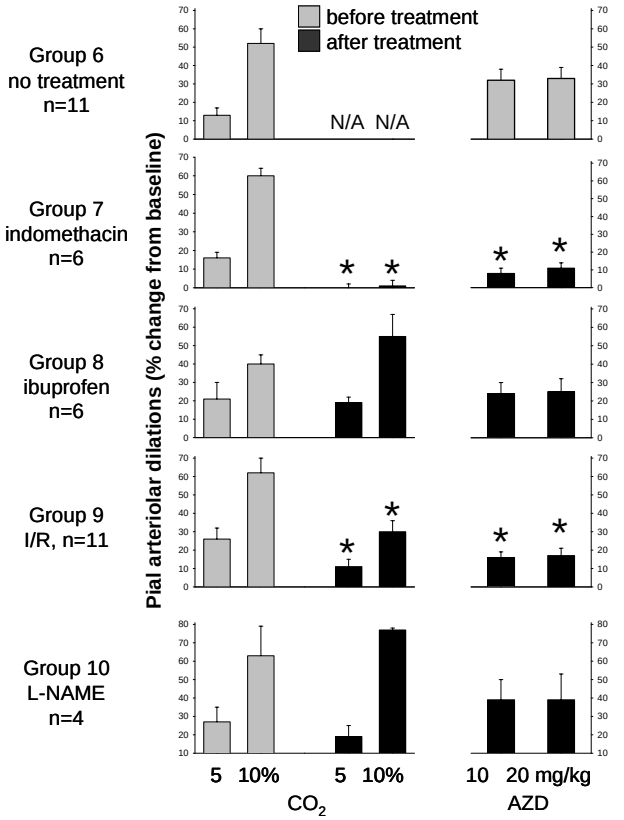

Figure 7 Effect of cyclo-oxygenase inhibitors, ischaemia/ reperfusion (I/R), and N-ω-Nitro-L-arginine methyl ester (L-NAME) on hypercapnia- and acetazolamide (AZD)- induced pial arteriolar vasodilation in piglets. Hypercapnia-induced vasodilation was virtually abolished by indomethacin, attenuated approximately by 50% after I/R, but was unaffected by ibuprofen or L-NAME. AZD-induced vasodilation featured virtually identical sensitivity to all stimuli. Values are presented as mean±SEM. *p < 0.05 significantly smaller than corresponding baseline values.

III. Cerebrovascular function following seizures and CORM-A1 treatment

1. CO release from CORM-A1 in vitro

CORM-A1 dissolved in the physiological Krebs solution (pH 7.4) releases CO in a concentration- and time-dependent manner, as detected by GC-MS. Stable CO release from CORM-A1 was observed during the first 1–2 h in solution and then declined progressively. The half-life ($t_{1/2}$) of CORM-A1 at room temperature (pH 7.4) is ~3 h. After 20 h of exposure to open air at room temperature, CORM-A1 was completely decomposed and incapable of releasing CO (inactivated CORM-A1, CO was below the detection limits). Therefore, CORM-A1 provides a dose- and time-dependent CO release at physiological pH within 5–6 h.

2. Cerebrovascular effects of topically administered CORM-A1

During baseline conditions, CO concentration in cortical periarachnoid CSF in newborn pigs was 94 ± 22 nM. CORM-A1, applied to the brain surface under the cranial window in consecutively increasing concentrations (10^{-6}, 10^{-5}, and 10^{-4} M, 10 min each, Group 11), resulted in 1) a concentration-dependent release of CO into the periarachnoid aCSF **(Figure** 8**)** and 2) a dose-dependent pial arteriolar dilation (**Figure 9**). Both effects were comparable to topically applied gaseous CO. Maximal pial arteriolar dilation (15 ± 2%) was achieved at 10^{-6} M topical CORM-A1 or gaseous CO. In contrast, inactivated CORM-A1 (Group 12) was not capable of releasing CO (100 ± 21, 90 ± 21, 90 ± 23, and 94 ± 22 nM CO under the cranial window at 0, 10^{-6}, 10^{-5}, and 10^{-4} M CORM-A1, respectively) and failed to cause cerebral vasodilation (2 ± 1, 1 ± 1, and 2 ± 2% at 10^{-6}, 10^{-5}, and 10^{-4} M inactivated CORM-A1, respectively).

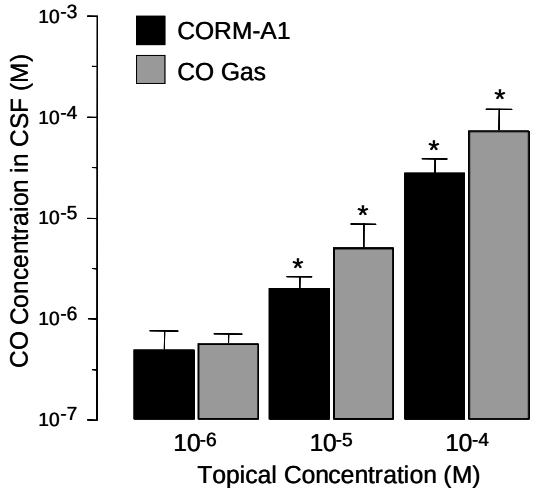

Figure 8 Effects of topical CORM-A1 and CO solutions on CO concentration in the periarachnoid CSF measured by gas chromatography/mass spectrometry (Group 11, n=5). CORM-A1 (10^{-6}M-10^{-4}M, 10 min each) resulted in a concentration-dependent release of CO that was comparable to topically applied gaseous CO. Values are mean±SEM. *p<0.05 compared with the baseline value.

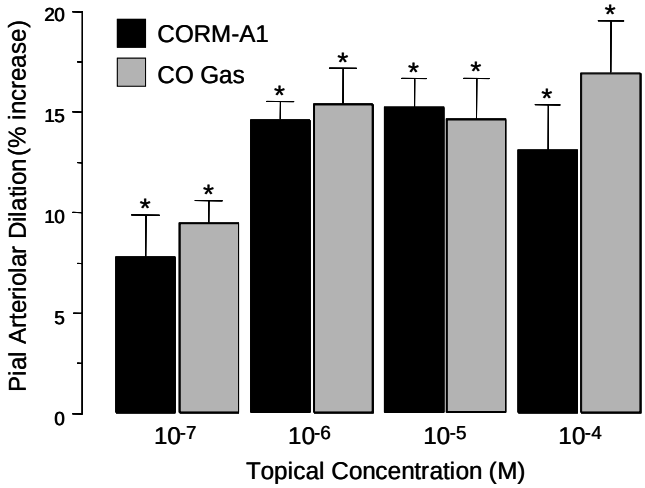

Figure 9 Pial arteriolar dilation caused by topical administration of CORM-A1 and gaseous CO (Group11, n=8) . CORM-A1 (10^{-6}M-10^{-4}M, 10 min each) resulted in a concentration-dependent pial arteriolar dilation that was comparable to the effect of topically applied gaseous CO. Values are mean±SEM. *p<0.05 compared with the baseline values.

3. Acute effects of systemically administered CORM-A1

Systemic CORM-A1 did not affect systemic circulatory parameters, including MABP, HR, and blood gases/oxygenation (Group 13, for physiologic parameters see paper [128]). Systemic (iv or ip administered) CORM-A1 increased CO concentration in cortical periarachnoid CSF (**Figure 10**) and caused vasodilation of pial arterioles (**Figure 11**). Maximal cerebrovascular responses (10–15% dilation, 1.5- to 2-fold cortical CO increase) were observed within 20–40 min of systemic CORM-A1 administration (**Figure 10 and Figure 11**). Iv and ip deliveries of CORM-A1 affected cerebrovascular parameters equally. Brain CO and PAD returned to baseline values within 1–1.5 h of CORM-A1 administration.

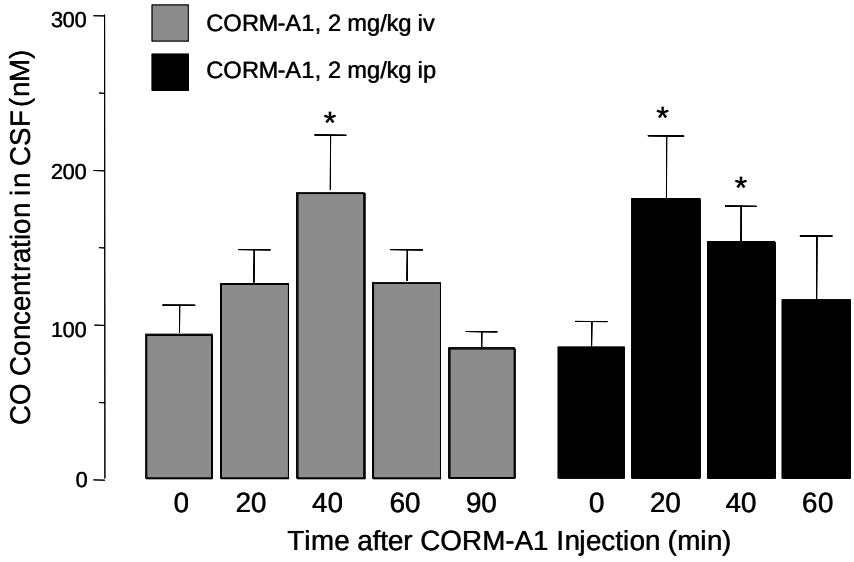

Figure 10 Effects of systemically administered CORM-A1 on CO level in cortical periarachnoid CSF determined by gas chromatography/mass spectrometry (Group 13, n=5). Both intravenous and intraperitoneal CORM-A1 caused a 1.5- to 2-fold increase of the CO concentration in the periarachnoid CSF that was observed within 20–40 min after administration. Values are presented as mean±SEM. *P<0.05 compared with the baseline values.

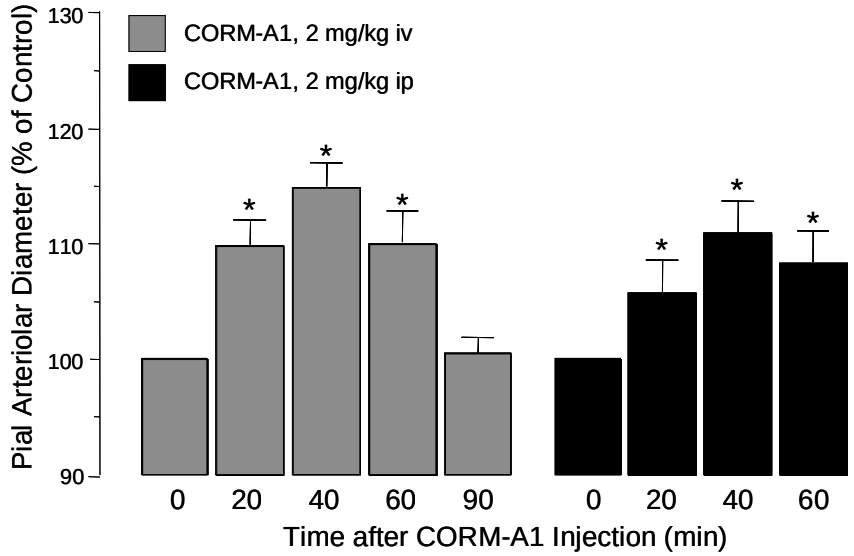

Figure 11. Effects of systemically administered CORM-A1 on pial arteriolar diameter (Group 13, n=5). Both intravenous and intraperitoneal CORM-A1 caused a significant pial arteriolar dilation that reached maximum levels within 20–40 min after administration. Values are presented as mean±SEM. *p<0.05 compared with the baseline values.

4. Long-term effects of systemically administered CORM-A1

In Groups 14-17, MABP, HR, blood gases, and pH were within the physiological range for newborn piglets, and no differences were observed among the experimental groups (for data on physiological variable see paper [128]). Therefore, seizures and/or CORM-A1 treatment had no long-term effects on the systemic

parameters in newborn pigs. At 2 days after epileptic seizures, cerebrovascular responses to bradykinin (10^{-5} M), hemin (10^{-6} M), and isoproterenol (10^{-6} M) were significantly decreased, while those to sodium nitroprusside were not reduced (Group 16, **Figure 12**). When animals were pretreated (30 min before seizures) with systemic CORM-A1 (2 mg/kg ip,), the loss of cerebrovascular reactivity was partially reduced or completely prevented (Group 17; **Figure 12**). CORM-A1 alone had no effects on cerebrovascular responsiveness to all tested vasodilators (Group 15; **Figure 12**).

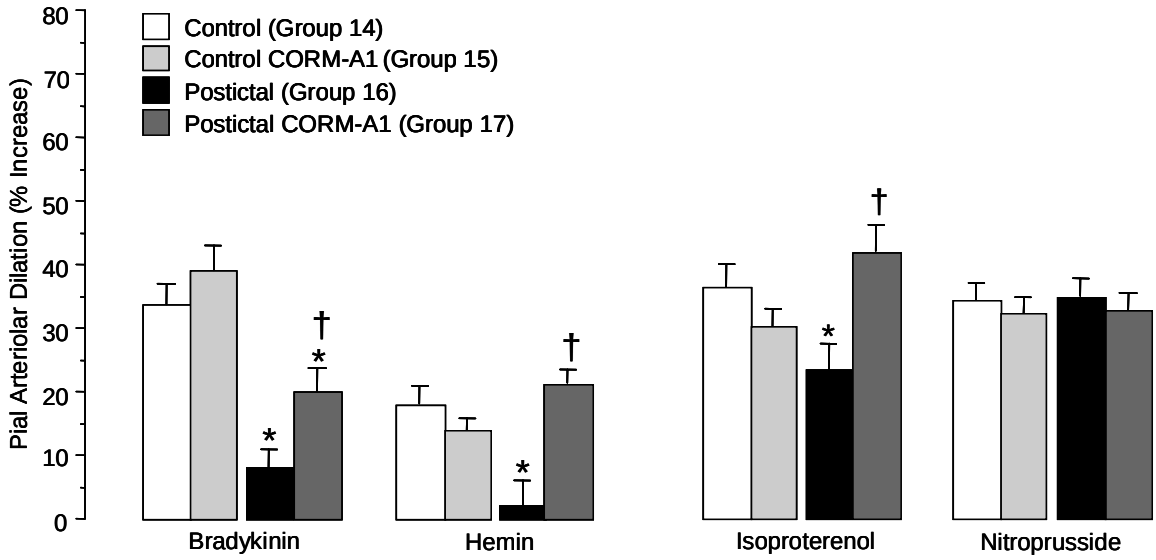

Figure 12 Postictal cerebral vascular reactivity to endothelium-dependent (bradykinin and hemin) and -independent (isoproterenol and sodium nitroprusside) vasodilators during delayed postictal state. Epileptic seizures decrease the cerebral vascular reactivity to bradykinin (10^{-5}M), hemin (10^{-6}M) and isoproterenol (10^{-6}M), but not to sodium nitroprusside (10^{-6}M) (Group 16, n=7) compared to control animals (Group 14, n=9). Treatment with CORM-A1 (2 mg/kg 30 min before seizure induction, significantly improved the postical cerebrovascular responsiveness (Group 17, n=5). CORM-A1 (Group 15, n=6) itself did not exert long-term influence on pial arteriolar reactions. Values are presented as mean±SEM. *$p<0.05$ compared with the baseline values. †$p<0.05$ compared with the corresponding control postictal state values.

Discussion

We confirmed the previously reported findings, that 1) a brief episode of hypoxia/ischemia results in histologically detectable brain damage in several hours in the newborn piglet and 2) the cerebrovascular responsiveness to physiologically relevant stimuli are impaired after I/R and epileptiform seizures.

The major novel findings are the following:

I.) RA or O_2 ventilation following asphyxia had region-specific effects on the early neuropathological changes. Specifically, the cerebellum and the hippocampus, but not the cerebral cortex, were more severely damaged after O_2 reventilation. In contrast, O_2 reventilation alleviated the neuronal injury in the basal ganglia. MABP, HR, blood gases, CoBF and CerBF did not differ significantly between the reventilation groups. Reactive hyperaemia in the O_2 reventilation-sensitive cerebellum was found to be significantly larger than in the cerebral cortex.

II.) AZD increases CoBF through a significant contribution of dilating pial arterioles and the mechanism of AZD-induced vasodilation appears to be virtually identical to hypercapnia-induced caliber changes in the newborn pig. Specifically, AZD-induced vasodilation is attenuated by I/R, abolished by indomethacin but unaffected by ibuprofen or L-NAME similarly to hypercapnia.

III) The systemic administration of CORM-A1 to newborn piglets liberates CO and delivers functionally effective levels of the CO gas to the brain and cerebral microvasculature. Moreover, systemically administered CORM-A1 prevents sustained postictal cerebrovascular injury caused by epileptic seizures and has no effects on arterial blood pressure or HR. These data indicate that systemically administered CORM-A1 can result in vasoactive and cytoprotective effects of CO on the neonatal cerebral circulation.

I. Cerebral effects of reventilation with 100% O_2 versus RA

The detrimental effect of O_2 during resuscitation has been clearly established by a series of both animal and controlled clinical studies [18;19]. Hyperoxygenation has been linked with increased neonatal mortality, the development of retinopathy of dismaturity, chronic lung disease, childhood cancer and adverse neurological outcome [2;11;13;14;23;28]. However, the mechanism of this O_2 toxicity is unclear. Increased availability/delivery of O_2 to the asphyxiated brain could lead to enhanced production of ROS, resulting in

accentuated neuronal damage. Although the general role of ROS in neuronal damage following H/I stress is well known [129;130], it is questionable whether the additional damage seen after O_2 (compared to RA) ventilation can be attributed to ROS. Interestingly, more robust hyperoxaemia achieved with hyperbaric oxygen treatment was actually found to be neuroprotective in a neonatal rat H/I model [131]. Therefore, the existence of O_2 toxicity after neonatal H/I stress may depend critically on a number of different factors besides blood oxygen levels: 1) the severity and duration of asphyxia, 2) specific neuronal vulnerability (ROS-producing/antioxidant systems, ionic homeostasis, metabolic activity, etc.), 3) the presence of additional neurotoxic/neuroprotective factors (acidosis, plasma glucose levels, drugs, etc.), and 4) systemic as well as local haemodynamic factors (MABP, microvascular perfusion, rheology, etc.).

We identified two regions in the newborn piglet brain where the adverse effect of resuscitation with O_2 could be demonstrated by early neuronal damage. The mechanism of increased vulnerability of asphyxiated hippocampal and cerebellar neurons to resuscitation with O_2 is not known. In theory, these neurons are either more susceptible to O_2 or other factors selectively present in these brain areas may result in enhanced O_2 toxicity. We originally hypothesized that local haemodynamic factors may play a role in the development of O_2-induced damage. This assumption was based on the following rationales reported in newborn piglets: 1) Hypoxia and activated oxygen are dilators of the pial arterioles [132-134], while hyperoxia causes vasoconstriction [135]. 2) The cerebrovascular reactivity to hypoxia is retained after H/I [136]. 3) In previous studies, significantly better restoration of CBF following H/I was reported during O_2 reventilation compared to RA, however these CBF events appeared to be at least partially pressure-passive [26;137;138]. Accordingly, we assumed that hyperoxia would either result in limited reperfusion by direct pial arteriolar vasoconstriction or increase the reactive hyperemia via the production of vasodilator ROS.

However, our LDF measurements in the cerebral cortex did not support these assumptions since we found no difference in CoBF between RA- or O_2-ventilated animals. Nor did we found signs of O_2 toxicity in the cerebral cortex. Thus, in a subsequent set of experiments, we aimed to repeat our LDF measurements in a brain region where O_2 toxicity did exist in this experimental model. The cerebellar cortex was chosen since the cerebellum but not the hippocampus could be reached with the LDF

probes without brain trauma. There was also no difference between the CerBF values of RA- or O_2- reventilated animals.

Unfortunately, we cannot exclude the possibility that the failure to detect significant differences between reventilation groups was due to methodological issues. Actually, CoBF levels in the O_2 ventilated groups were slightly above those measured during reventilation with RA. In Groups 6-10 we simultaneously measured the pial arteriolar diameters and the CBF with closed cranial window/intravital microscopy and laser Doppler flowmetry, respectively. Although the diameter changes in response to hypercapnia reflect the changes in CoBF as measured by other methods (e.g. autoradiography or hydrogen clearance), the time-course and the strength of the correlation between the variables strongly depends on which methods are chosen for comparison. CoBF is expected to increase more than threefold in piglets to similar levels of hypercapnia as determined with microspheres [139]. In contrast, we found ~55% increase in PAD and only ~45% elevation in CBF in response to 10% CO_2 indicating that laser Doppler flowmetry have greatly underestimated the increases in CoBF in our experiments. A likely limitation of the laser Doppler flowmetry method in the present study, that the average thickness of the cerebral cortex exceeds 2 mm in the pig [140]; however, the LDF probe monitors tissue perfusion only up to 1-mm deep from the cortical surface.

In spite of the likely technical limitations, we found a striking difference between the reactive hyperaemia of the cerebellar and the cortical microcirculation. In fact, reactive hyperaemia in the early reoxygenation period was almost two times higher in the cerebellar cortex than in the cerebral (parietal) cortex. To the best of our knowledge, our study demonstrates for the first time region-specific differences in the reactive hyperaemia response following H/I stress in piglets. We propose that the more pronounced reactive hyperaemia in the cerebellum may contribute to the observed vulnerability of cerebellar neurons to postasphyxic O_2 ventilation. Reactive hyperaemia coincides with the acute reoxygenation period, when uncontrolled O_2 metabolism leading to immense ROS production is assumed to be the highest. Although blood O_2 content may not be much higher in the O_2- ventilated piglets, the very high CerBF during reactive hyperaemia may deliver enough extra O_2 for the observed toxic effect of O_2. However, a detailed study of regional differences in reactive hyperaemia after cerebral asphyxia is warranted to test this hypothesis.

Interestingly, the basal ganglia were the only region in the piglet brain where O_2 ventilation appeared to have a beneficial effect. This finding suggests that studies focusing on basal ganglia morphology/function may find O_2 ventilation superior to RA. For instance, in neonatal rodent H/I models, the basal ganglia are responsible for a large percent of the lesion, and in fact hyperbaric O_2 ventilation was found to be protective [131]. The mechanism of the beneficial effects of O_2 ventilation on the basal ganglia is also unknown. In the hypoxic/ischemic striatum [138], similar dynamics of reperfusion [137], but a significantly larger release of excitatory amino acids were reported upon RA reventilation compared to O_2. These findings are in accordance with the theory, that indicate, that the vulnerability of the basal ganglia to ROS depend on 1) their location in the neuronal circuitry, 2) the activity the excitatory glutamatergic pathways 3) the resting neuronal activity [141].

In summary, in the neonatal piglet model, we provided morphological evidence that, after asphyxia, O_2 reventilation is not superior to RA in most brain areas. In contrast, in the hippocampus and the cerebellum, significant O_2 toxicity has been observed. Based on our LDF data, local haemodynamic factors, especially the magnitude of reactive hyperaemia, in the immediate reventilation period following HI stress may contribute to the development of O_2 toxicity.

II. Comparison of CO_2- and AZD-induced pial arteriolar and CBF reactions

AZD has long been used in the treatment of various diseases like glaucoma, hydrocephalus, epilepsy, and it is still a first drug of choice in the treatment of acute mountain sickness [142-144]. As a diagnostic tool, AZD has become the first-choice stimulus to determine CRC, because it is more tolerable, gives more consistent results, and does not require patient cooperation as compared with other dilatory stimuli previously used such as hypercapnia or mental tasks [145]. Our present data demonstrate that vasodilation in the pial arterioles substantially contribute to the increased CoBF. The vasodilation of pial arterioles to the doses of AZD (10–20 mg/kg) used in the present study were not dose-dependent, despite being much less than the 50 mg/kg used in a previous piglet study [80]. However, the doses used in the present study are indeed very close to the AZD dose used to elicit maximum CBF increase in adult humans (1000 mg ≈ 12–16 mg/kg) to determine CRC [71;144]. Our data suggest that AZD exerts its maximal effect on the cerebral vasculature in the same dose range in the neonate as

well. Interestingly, AZD could not elicit as large pial arteriolar vasodilation as 10% CO_2 inhalation indicating that the AZD test probably underestimates the true CRC in this experimental setting.

The mechanism of AZD-induced pial arteriolar vasodilation appears to be virtually identical to the mechanism of hypercapnia-induced changes in the present study. Indeed, AZD elicited an increase in pCO_2 by 8–10 mmHg in the artificially ventilated piglets similar to the previous study [80]. This moderate hypercapnia probably contributes to, but does not explain, the robust increase in pial arteriolar diameters to AZD, because inhalation of 5% CO_2 resulted in similar pCO_2 changes but the pial arteriolar response was only ~35% of the response to AZD. AZD elicits carbonic acidosis in the brain independent of pCO_2 changes [146], but passes the blood–brain barrier very slowly [147], especially when compared with the relatively fast changes in pial arteriolar diameters. Therefore, instead of carbonic anhydrases in the brain parenchyma, the major cerebral targets of AZD are likely to be the cerebrovascular endothelial cells outside the blood–brain barrier, where carbonic anhydrase is abundant [148;149].

In the piglet, hypercapnia-induced vasodilation was shown to be attenuated by light-dye endothelium-injury [81]. Our results fully support the concept, that AZD-induced vasodilation is also an endothelium-dependent process, because it showed the same unique vulnerability to indomethacin but not to ibuprofen or L-NAME like hypercapnia did in both the present and previous studies [82]. This finding is important, since asphyxia induces the expression of cerebral carbonic anhydrase mainly in the glial cells and the neurons in several hours [150], which could lead to an enhanced production of cerebral CO_2 and a false overestimation of CRC by AZD. Nevertheless, we found that both AZD- and hypercapnia-induced vasodilations are sensitive to I/R and human studies indicate that these stimulants are equally valid in the measurement of CRC [61;67;151].

We conclude that AZD elicits a significant pial arteriolar vasodilation with a mechanism that requires a functional cerebrovascular endothelium to respond to the carbonic acidosis induced by carbonic anhydrase inhibition. AZD-induced vasodilation can be easily evoked, has less systemic cardiovascular effects than CO_2 inhalation, and thus can be used as a sensitive bioassay to test the efficacy of putative neuroprotective strategies to preserve the integrity of the neurovascular unit in the neonate.

III. Cerebrovascular function following seizures and CORM-A1 treatment

CORMs are designed to liberate CO and elicit its biological activities, including vasodilator effects [87;107;109;110;152-155]. First-generation CORMs, CORM-1 (dimanganese decacarbonyl) and CORM-2 (tricarbonyldichlororuthenium II dimer), contain heavy metals, are soluble in organic solvents, and may require light irradiation for liberation of CO (CORM-1). Water-soluble CORM-3, tricarbonylchloro(glycinato) ruthenium(II), also contains a heavy metal and releases CO rapidly, providing a relatively short-term experimental window [87;107]. Recently introduced water-soluble CORM-A1 contains no heavy metal and releases CO spontaneously and slowly in physiologically buffered solutions [106;109]. Therefore, CORM-A1 is highly suitable for in vitro and in vivo experiments.

In the first part of our experiments, we demonstrated that CORM-A1 elicits the cerebrovascular effects of CO in vivo. In the newborn pigs, gaseous CO dilates cerebral vessels by directly activating large-conductance Ca^{2+} activated K^+ channels on smooth muscle cells [96]. We found that application of CORM-A1 directly to the brain surface elevated CO levels in the periarachnoid CSF and resulted in pial arteriolar dilation similarly to equivalent concentrations of gaseous CO. Systemically administered CORM-A1 also produced cerebrovascular effects, including elevation of CO in the CSF and dilation of pial arterioles, within 20–60 min of injection. These data indicate that CORM-A1 and/or CORM-derived CO, indeed, penetrates the blood-brain barrier by an unknown mechanism. As CO binds strongly to hemoglobin, it is implausible that physically dissolved CO crosses the blood-brain barrier. On the other hand, in isolated pressurized cerebral arterioles from newborn pigs, CORM-1 caused endothelium-dependent dilation [156;157]. Potentially, cerebrovascular endothelial cells take up CORMs and CO is released intracellularly.

We observed no adverse or long-lasting effects of systemic CORM-A1 on cerebrovascular function. CO liberation from CORM-A1 is temperature and pH dependent. According to our in vitro measurements, the half-life ($t_{1/2}$) of CORM-A1 at room temperature and pH 7.4 is ~3h, whereas $t_{1/2}$ of ~20 min has been reported at 37°C [109]. Thus, we estimate that cerebrovascular effects of CORM-A1 are sustained for no longer than 1–1.5 h after a single systemic administration. Indeed, at 2 days after systemic administration of CORM-A1, cerebrovascular reactivity to endothelium-

dependent and -independent vasodilators was not changed. Although data from the adult rat suggest that CORMs mildly decrease MABP [109;110;153], we did not detect acute or delayed effects of iv or ip CORM-A1 on blood pressure, HR, blood gases, pH, or oxygenation.

We found that CORM-A1 exhibits potent cerebrovascular protective effects against seizure-induced dysfunction. Epileptic seizures cause a delayed (2 days) loss of cerebrovascular function in newborn piglets indicative of sustained cerebrovascular injury [78]. In the present study, we confirmed the reduction of cerebrovascular responsiveness to endothelium-dependent (bradykinin and haeme) and endothelium-independent (isoproterenol, but not nitroprusside) vasodilators. Overall, the previous and present data indicate that endothelium-dependent responses are more susceptible to seizure-induced damage, although some cerebrovascular smooth muscle damage may also occur. When CORM-A1 was administered systemically (2 mg/kg iv or ip) shortly before seizures, the loss of cerebrovascular reactivity during the postictal period was partially or completely prevented. Essential roles of HO was reported in protecting the newborn brain from adverse cerebrovascular effects of epileptic seizures [77;78]. Taken together, these data show that CO, formed from an exogenous source (CORM-A1) or endogenously via HO-catalyzed haeme degradation, is protective against seizure-induced cerebrovascular injury.

The mechanism of seizure-induced cerebrovascular dysfunction and the protection afforded by CO is unclear. The relationship between the severity and duration of seizures, the intensity of the ictal brain metabolism, the levels of the accompanying cerebral hyperemia and the development of cerebrovascular dysfunction has not been addressed. The CORM-A1-delivered CO may affect the neurons as endogenous CO produced by cerebral HO has a moderate proconvulsant effect in the newborn pig seizure model [99]. Therefore, systemic CORM-A1 may aggravate epileptic neuronal activation. If cerebrovascular protection were secondary to the effects of CO on neuronal activity, one would expect aggravation of cerebrovascular dysfunction. However, we found that CORM-treated piglets were protected from postictal cerebrovascular dysfunction indicating that the dynamics of neuronal activity and postictal cerebrovascular dysfunction involve independent diverse CO-mediated responses.

Endogenous produced CO substantially contribute to seizure-related hyperemia [77;126]. Seizure-induced cerebral hyperemia remains at submaximal levels [158] and declines

as seizures progress despite the maintained high cerebral metabolic rate. Such insufficient coupling of CBF to the metabolic needs may result in relative hypoperfusion leading to cerebral ischaemia, brain damage and cerebrovascular injury [100-102]. We did not measure the CBF during seizures in CORM-A1-treated pigs. Thus, we cannot exclude the possibility that exogenous CO enhances the blood supply of the ictal brain thereby providing better perfusion of the ictal brain and the protection of the cerebral vessels.

The delayed appearance of the cerebrovascular dysfunction during the postictal period indicates, that apoptosis of cerebrovascular endothelial cells and neuroinflammation are potential factors in its development. Seizures are related to excessive production of excitotoxic and inflammatory mediators. Glutamate and the proinflammatory cytokine TNF-α, are excessively released in the brain during seizures. Glutamate and TNF-α caused apoptosis and increased ROS formation in piglet cerebrovascular endothelial cells were significantly reduced by CORM-A1 [97;159]. ROS production was also enhanced by the epileptic piglet brain [160]. In other vascular beds, antiapoptotic effects of CO have been related to inhibition of ROS generation [94;95;161-164]. Mechanisms by which CO prevents oxidative stress and apoptosis in response to excitotoxicity and inflammation in the cerebral circulation and other vascular beds are not completely understood. Overall, the products of HO activity have antioxidant potential. Biliverdin/bilirubin, along with biliverdin reductase, act as ROS scavengers [86;96]. In contrast, the ability of CO to reduce oxidative stress appears to be related to its ability to block formation of ROS by interacting with the ROS-producing enzymes. CO has a high affinity to haeme and, therefore, may block the action of numerous haeme-containing enzymes, including the mitochondria respiratory chain components cytochrome c, MAPK, and p53 kinase [94;95;161-163;165]. CO uncouples mitochondrial respiration from ATP production by enhancing the proton leakage across the inner mitochondrial membrane and, thus, may act to preserve energy and maintain the cellular antioxidant defense mechanisms [165]. CO has anti-inflammatory properties as well. CORMs inhibited TNF-α production by microglial cells and macrophages [154;166], reduced systemic inflammatory response syndrome after skin burn injury [93] and inhibited the inflammatory response to cytokines in epithelial cells [167]. Epileptic seizures induce inflammation and TNF-α formation in the central nervous system, which might contribute to cerebrovascular damage [168]. Thus, immunomodulation by

CORM-A1 might also contribute to its protective action against seizure-induced cerebrovascular damage.

Overall, we conclude that CORM-A1 provides a pharmacological tool for delivery of CO to the brain and protection against sustained cerebrovascular injury caused by epileptic seizures. CORM-A1 might be the basis of novel pharmacological tools for cerebroprotection.

IV. Conclusions

In the studies presented in this theses, we used multiple experimental conditions in the newborn pig that disrupted the normal CBF regulation. These included hypoxic attacks, such as asphyxia and ischemia, and bicuculline-induced seizures. These approaches are clinically relevant, since asphyxia and cerebral ischemia occur frequently in neonatal patients and often result in the development of seizures. All of these conditions severely uncouple the cerebral metabolic needs and the blood supply either by the critical decline of the CBF or the disproportionate increase of the cerebral metabolism. Hypoxic insults and seizures finally lead to brain damage and cerebrovascular dysfunction. Perinatal brain damage causes life-long devastating consequences for newborn babies, their family and also for the society. Therefore, the protection of the neonatal brain is essential, which could be started promptly after the hypoxic insult by the choice of the most appropriate resuscitation technique.

We conducted our studies on the effects of reventilation with O_2 and RA in asphyxiated piglets in 2004-2005. We found that the use of RA was superior to O_2 as it limited the histological damage in the hippocampus and the cerebellum. That time the guideline of the American Heart Association recommended the use of the highest O_2 concentration which could have been achieved for the resuscitation of the newborn infants [169]. Since then sufficient evidence has accumulated from both animal and human studies that demonstrated the benefits of RA ventilation. Accordingly, the American Academy of Pediatrics and the American Heart Association updated their guidelines [170]. Supplemental oxygen is not routinely needed and resuscitation should be started with RA except in case of central cyanosis or the need of positive pressure ventilation. If resuscitation is started with the use of O_2, its concentration should be reduced as O_2 saturation normalizes [170].

Assuredly the results of the studies performed in newborn piglets cannot be directly extrapolated to newborn babies. Newborn infants are a heterogenous group of

patients concerning their gestational and postnatal age, body weight and underlying diseases. In contrast, newborn piglets are presumably healthy before the experiments, homogenous in terms of the postnatal age and body weight. Their physiological parameters, such as MABP, HR, blood pH and gases and body temperature are controlled throughout the experiments. Importantly, these controlled conditions allow the examination of the specific effects of hypoxic attacks, seizures or other factors. Furthermore, several questions cannot be addressed in clinical conditions because of ethical concerns.

In the piglet model we found that AZD dilates cerebral vessels via virtually identical mechanisms as CO_2 and could be a suitable tool for detection of CRC in the newborn. We also found that the cerebrovascular function during the postictal period can be preserved with CORM-A1 treatment before seizure induction. The examination and protection of the cerebrovascular function could be of clinical importance in the human neonates. Secondary brain damage could be related to abnormal cerebrovascular regulation. Importantly, the protection of the cerebral vasculature could at least partially account for the effect of neuroprotective strategies [53]. The imminence of cerebral ischemia/asphyxia may be predicted in several clinical situations in the perinatal period. Furthermore, seizure activity often occurs after asphyxia in already hospitalized neonates requiring intensive care. Therefore, adequate therapeutic window would be available for the examination of the cerebrovascular function and the administration of protective drugs before the secondary insults in the newborn babies.

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