Preservation of neuronal-vascular reactivity to N-methyl-D-aspartate after ischemia in newborn pigs

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A thesis for the Degree of
DOCTOR OF PHILOSOPHY
(Ph. D.)

In the Department of Physiology, Faculty of Medicine,
University of Szeged

March 2001
Szeged

Papers related to the subject of this thesis

I. Domoki, F., R. Veltkamp, F.Bari, T. M. Louis, and D. W. Busija. Cerebrovascular reactivity remains intact after cortical depolarization in newborn piglets. *Pediatr Res* 45:834-837, 1999.

II. Veltkamp, R., F. Domoki, F. Bari, and D. W. Busija. Potassium channel activators protect the N-methyl-D-aspartate-induced cerebral vascular dilation after combined hypoxia and ischemia in piglets. *Stroke* 29:837-843, 1998

III. Domoki, F., J. V. Perciaccante, R. Veltkamp, F. Bari, and D. W. Busija. Mitochondrial potassium channel opener diazoxide preserves neuronal-vascular function after cerebral ischemia in newborn pigs. *Stroke* 30:2713-2719, 1999.

IV. Veltkamp, R., F. Domoki, F. Bari, T. M. Louis, and D. W. Busija. Inhibitors of protein synthesis preserve the N-methyl-D-aspartate-induced cerebral arteriolar dilation after ischemia in piglets. *Stroke* 30:148-152, 1999.

V. Domoki, F, J. V. Perciaccante, R. Veltkamp, G. Robins, F. Bari, T. M. Louis, and D. W. Busija. Cycloheximide inhibits cyclooxygenase(COX)-dependent arteriolar dilation and prostaglandin synthetic capacity in piglet cerebral cortex. *Am J Physiol* 277: H1113-H1118, 1999.

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AA: arachidonic acid

AD: anoxic depolarization

Act-D: Actinomycin D

aCSF. Artificial cerebrospinal fluid

CBF: cerebral blood flow

CGRP: calcitonin gene-related peptide

CHX: cycloheximide

COX: cyclooxygenase

DIAZ: diazoxide

H/I: hypoxia/ischemia

5-HD: 5-hydroxy-decanoate

I/R: ischemia/reperfusion

ICP: intracranial pressure

IPC: ischemic preconditioning

K_{ATP}: ATP-sensitive potassium channel

K_{Ca}: calcium-sensitive potassium channel

KCO: potassium channel opener

MCA: middle cerebral artery

NMDA: N-methyl-D-aspartate

PG: prostaglandin

ROS: reactive oxygen species

SOD superoxide dismutase

SNP: sodium nitroprusside

VSM: vascular smooth muscle

Introduction

Stroke- an all-inclusive term for acute failure of cerebrovascular circulation- is a leading cause of death in humans. When considered separately from other cardiovascular diseases, stroke ranks as the third leading cause of death, behind diseases of the heart and cancer (Center for Disease Control). In the United States, each year about 600,000 people suffer a new or recurrent stroke. About 500,000 of these are first attacks, and 100,000 are recurrent attacks. (Framingham Heart Study). Stroke is also a leading cause of serious, long-term disability in the United States, affecting about 4,500,000 stroke survivors (2,200,000 males and 2,300,000 females) who are alive today.

The morbidity and thus the importance of stroke in the pediatric age group (<14 years old) seems to be minor: about 0.002-0.003% of children undergo some sort of global or focal hypoxic-ischemic insult or intracranial hemorrhage, and mortality is much less (Roach, 1993). However, during the perinatal period morbidity is approximately 0.05-0.1%, and may affect as much as 20-30% of premature infants (<35 gestational weeks) (Allan, 1990). Thus, neonatal stroke probably affects several million newborns worldwide. Neonatal stroke may result in severe, life-long neurological dysfunction that has an immense impact on both the health care system and the families providing care for such disabled children. In Germany alone, conservative estimates of cost of care to these children exceed 1 billion German marks/year (Berger and Garnier, 1999). Therefore, development of reasonable and successful therapies to diminish neural damage following stroke in newborns is of great importance. Compared to adult-onset stroke, there are some unique aspects that can be utilized in the therapy of neonatal stroke. First, in developed countries, virtually all babies are delivered in hospitals and may have instant access to adequate medical care in case of stroke. Second, babies at risk may be identified before the actual insult occurs, thus a low-risk effective pretreatment may prevent damage from a subsequent stroke. Third, the central nervous system of the newborn has a considerably larger plasticity compared to adults; a successful therapy reducing tissue damage may offer better functional recovery.

Transient cerebral ischemia/reperfusion (I/R) induces neuronal injury both via insufficient cerebral blood flow (CBF) during the ischemic phase and reperfusion injury in the postischemic phase. Among the pathophysiological processes triggered by I/R, impaired cerebrovascular reactivity appears to play a significant role. Inadequate CBF regulation after I/R may worsen metabolic challenges of already compromised cells establishing a vicious circle. Therefore, preservation of cerebrovascular responsiveness after I/R is a promising area

of research to develop treatments that may complement other neuroprotective treatments (Hossmann, 1997). To obtain such strategies, we need to answer fundamental questions concerning the regulation of neonatal cerebrovascular circulation under physiological and pathophysiological conditions.

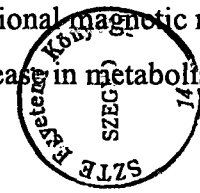
Overview of the cerebral circulation in the neonate

Total CBF in the neonate similarly to adults is determined primarily by two factors: (1) perfusion pressure, and (2) cerebrovascular resistance. Perfusion pressure is the difference between mean arterial pressure and mean venous pressure. Although perfusion pressure does not fluctuate significantly under physiological conditions, CBF is in fact very well maintained over a wide perfusion pressure range by a directly proportional change in vascular resistance. This feature of the cerebral circulation called cerebral autoregulation is present in both adults and neonates. The presence of autoregulation in neonates however, appears to be dependent on gestational age, and may be abolished by perinatal asphyxia (Younkin et al., 1987; Lou, 1988; Pryds et al., 1990). Clinical and ethical concerns however, limit the study of autoregulation in human babies. Studies in experimental neonatal animal models like in newborn piglets, lambs, and puppies revealed that significant differences exist between the regulatory mechanisms of newborns and adults. The plateau phase of the perfusion pressure-CBF relationship is shifted to the left, newborn animals for instance can maintain adequate CBF when arterial pressure is as low as 30-35 mmHg in contrast to 60-65 mmHg in adults (Ashwal et al., 1984; Leffler et al., 1986; Pasternak and Groothuis, 1985).

Cerebrovascular resistance is determined by the factors described by Poisseuille's law: (1) blood viscosity, and (2) vascular geometry including number, length, and caliber of vessels. Indeed, vascular radius has the biggest impact on vascular resistance given that resistance is inversely proportional to 4^{th} power of vascular radius, and this is the only regulated factor under physiological conditions. Conceivably, microvessels, especially pial and intracerebral arterioles are expected to contribute to the greatest extent to cerebrovascular resistance, due to their small diameters and relatively small numbers. Experimental data on the contribution of different vascular segments are scarce, but they indicate a significant involvement of large cerebral arteries in the regulation of cerebrovascular resistance (Heistad and Kontos, 1979). Studies in cats revealed that approximately 40% of total cerebrovascular resistance derives from arteries larger than 250-400 μm, and 60% of total resistance is contributed by pial and intraparenchymal arterioles (Faraci et al., 1987). Vascular diameter is

actively determined by the tone of vascular smooth muscle (VSM). Therefore, the regulation of CBF simply depends on the combined effect of all vasodilator and vasoconstrictor stimuli on the VSM. Most of these stimuli are of chemical nature, and released from different cellular origin: neurons, glia, endothelial cells, perivascular nerve endings, and the VSM cells themselves. In addition to the vasoactive substances, VSM tone is also influenced by mechanical stretch, as well as electrical stimuli that can travel between the individual VSM cells through gap junctions. Unfortunately, not only the seemingly endless list of vasoactive substances but also the complex interactions between the regulatory cells and humoral mechanisms are responsible for the failure to provide undisputed *in vivo* evidence on the physiological role to most mechanisms. Limited studies in humans clarified the existence of basic cerebrovascular responses, but most systematic studies were carried out in animal models. These studies concluded, that many if not all cerebrovascular responses appear to be dependent on the species, the brain region studied, and clearly also on the brain developmental stage of the individual. These developmental differences can be of several qualities. First, some stimuli may differ in the amplitude of their effects on the cerebral circulation. For instance, stimulation of sympathetic adrenergic nerves results in large decreases in CBF in newborn piglets (Busija et al., 1985; Wagerle et al., 1986), but only minor changes in adult pigs (Lee and al., 1981). Second, many stimuli evoke similar changes in brain perfusion in both adults and neonates, albeit via different mechanisms. A very good example is the special role that prostaglandins and thromboxanes (prostanoids) play in the cerebrovascular response to various stimuli. Prostanoids mediate vasodilation to hypotension (Leffler et al., 1986; Leffler and Busija 1987), and have permissive effect on the vasodilation to hypercapnia in piglets (Leffler et al., 1993, 1994a, 1994b). In adult animals of many species these mechanisms are independent of prostanoids (Traystman, 1997). Third, some stimuli may elicit the opposite effect in newborns compared to adults. One striking example that acethylcholine induces vasoconstriction of the pial arterioles in piglets via prostanoids in contrast to the usual vasorelaxation seen in adults (Wagerle and Busija, 1989,1990).

For over one hundred years maybe the most captivating hypothesis on the regulation of CBF that the brain is able to match its energy supply to meet the metabolic needs required by its local activity via corresponding changes in regional CBF (rCBF) (Roy and Sherrington, 1890). This "neurovascular coupling" is now widely utilized in different functional neural imaging techniques, and became a major method to study the function of the human brain. In fact, positron emission tomography and functional magnetic resonance imaging demonstrate not the increase in activity but rather the increase in metabolism and/or rCBF, by measuring



glucose utilization and oxygen consumption. (Phelps et al., 1979; Frackowiak et al., 1980) or the degree of hemoglobin oxygenation (Ogawa et al., 1992), respectively both in humans and laboratory animals. Therefore the use of these techniques relies on the tight and fast coupling between neuronal activity-induced increase in cerebral metabolism and rCBF. Tight CBF-metabolism coupling has clinical relevance in stroke pathophysiology, since it prevents not only relative ischemia in activated regions but also luxury perfusion in regions of decreasing activation. Overperfusion may elevate tissue PaO_2 that could elicit oxidative damage of proteins and lipid membranes. Brain ischemia seems to uncouple CBF from metabolism, and may result in relative ischemic and/or overperfused areas during reperfusion (Kogure et al., 1980). Either of these derangements may elicit further neuronal damage.

Despite its significance, there is no equivocal understanding of this remarkable phenomenon. Neuronal activity may be signaled to cerebral resistance vessels by a number of possibilities. Neurons release a variety of substances upon activation including potassium ions, cellular metabolites and neurotransmitters. These substances may directly affect vascular smooth muscle, but may also induce release of specific vasoactive agents from other neurons, glia, and autonomic or sensory nerve endings. Actually, there may be no single mechanism to be identified. From this confusing variety the present paper focuses at one major possible local regulatory mechanism: the effect of excitatory amino acids on the regulation of CBF.

The mechanism of NMDA-induced pial arteriolar vasodilation

Excitatory amino acids glutamate and aspartate are the most common excitatory transmitters in the mammalian brain. In addition to their well-known transmitter role, glutamate and aspartate may participate in the mediation of CBF increases in response to neuronal activation. This hypothesis has been based on the pilot experiments by Busija and Leffler (1989), who reported dose-dependent pial arteriolar vasodilation to topical glutamate application using intravital microscopy in neonatal pigs. However, later glutamate receptor stimulation was found to dilate cerebral vessels in all species studied including both newborn (piglet, sheep) (Northington et al., 1995), as well as adult (rat, rabbit,) animals (Faraci and Breese, 1993). The effect of glutamate is largely mediated by N-methyl-D-aspartate (NMDA) receptors (Meng et al., 1995) in piglets. These receptors can be selectively activated by the subtype-specific analogue NMDA thus more specific data on this system could be obtained. Since NMDA receptors are associated with a wide range of physiological and pathological

functions, a whole series of studies was undertaken to explore the mechanism of NMDA receptor-mediated vascular dilation, and the effect of hypoxia/ischemia (H/I) on this neuronal-vascular phenomenon.

Glutamate and NMDA elicit a marked, dose-dependent pial arteriolar vasodilation. Compared to glutamate, NMDA induces more consistent and larger vasodilation probably due to the smaller elimination by glia. NMDA-induced vasodilation may be repeated several times during a single experiment, the vascular responses are essentially identical both in amplitude and dynamics upon repeated NMDA application (Busija and Meng, 1993a). This result suggests that the NMDA doses used are not affecting neuronal excitability and there is no tachyphylaxis. This feature of the response also enabled us to use repeated measurement experimental design to assess the mechanism of the response with pharmacology or the effect of H/I on vascular reactivity to NMDA.

The exact mechanism of NMDA-related vascular changes is still unclear in some details, but considerable evidence indicates a unique action, requiring complex interactions between neurons and cerebral blood vessels. Neurons must have a key role in NMDA-induced vascular dilation since neither cerebral blood vessels nor glial cells express NMDA receptors (Beart et al., 1988; Morley et al. 1998). Accordingly, cerebral vessels *ex situ* are not responding to NMDA (Wendling et al., 1996). Our current hypothesis on the mechanism of NMDA-induced vasodilation includes: 1) Activation of neuronal NMDA receptors, 2) inflow of Na^+ and Ca^{2+} ions resulting in membrane depolarisation and action potentials, 3) subsequent activation of nNOS positive neurons via unidentified monosynaptic or polysynaptic local neuronal connections, 4) activation of nNOS by increased intracellular Ca^{2+} levels, 5) release and diffusion of NO from the neuropil to the blood vessels, and 6) arteriolar dilation due to NO actions on vascular smooth muscle. This hypothesis is based on the following findings. 1) In the piglet, NMDA-induced pial vasodilation is sensitive to inhibition of nitric oxide (NO) synthesis by L-nitroarginine-methyl-esther, but also by 7-nitroindazole, a selective inhibitor of neuronal NO synthase (nNOS) (Bari et al., 1996a). 2) Recently, we have been able to show that NMDA application resulted in significant increases of NO metabolites in the cerebrospinal fluid over the stimulated pial surface (unpublished data). 3) Double immunostaining indicated that NMDA receptor immunoreactive neurons were much more numerous than nNOS positive neurons in the piglet cerebral cortex (Bari et al., 1998a). Further, nNOS and NMDA receptors do not seem to coexist in the same neurons. 4) Tetrodotoxin suppresses NMDA-induced vasodilation (Faraci and Breese, 1993; Yang and Chang, 1998), also indicative that NMDA receptor positive neurons are different from those

releasing the NO upon NMDA application. Finally, 5) other possible vasodilator mechanisms have been tested with negative results: inhibition of endothelial function, prostanoid and epoxyeicosatrienoic acid (EET) biosynthesis did not alter NMDA-induced vasodilation (Busija and Leffler, 1989; our unpublished observations).

Unfortunately, unambiguous data on the physiological relevance in the CBF-metabolism coupling of this complex sequence are lacking. In anesthetized piglets, pharmacological blockade of NMDA receptors by MK801 does not affect pial arteriolar diameters (Meng et al., 1995). However, physiological glutamate-mediated vasoregulation may be more pronounced in the close vicinity of neurons than on distant arterioles of the pial surface. Indeed, decreases in baseline intraparenchymal vessel diameters have been reported after NMDA receptor inhibition in the hippocampus (Fargus and Lee, 1997). Several studies supported the involvement of NMDA receptors in the regulation of CBF. For instance, NMDA-receptor inhibition prevented nociceptive stimulation-induced increases in rCBF in the trigeminal nucleus in cats (Goadsby and Classey, 2000). Also, in a focal cerebral ischemia model in rats, MK801 was shown to decrease CBF by 30% in the unaffected hemisphere, however, MK801 did not affect CBF on the ischemic side (Park et al., 1989). There is an important limitation in the interpretation of such results. Namely, direct inhibition of NMDA receptors will also likely to alter neuronal activity *per se*. In addition, one can speculate that NMDA-induced vasodilation may be rather involved in the dynamic changes of CBF related to local activity changes that are largely suppressed during anesthesia. Fortunately, a recent PET study yielded compelling evidence suggesting the importance of NMDA receptors in the neuronal activity – rCBF coupling in conscious rhesus monkeys (Tsukada et al., 1998). In that study, D-cycloserin –a partial agonist of the strychnine-insensitive glycine site of the NMDA receptor- significantly recovered the rCBF increase in the somatosensory cortex to vibrotactile stimulation abolished by scopolamine, without affecting neuronal activity. In conclusion, NMDA receptor activity appears to affect activity-dependent CBF changes either directly via neuronal NO release or increased metabolism resulting in increased rCBF via a different albeit undisclosed metabolism-flow coupling mechanism.

Effect of anoxic stress on cerebrovascular reactivity to NMDA

The effect of different types of anoxic stress (hypoxia, ischemia, asphyxia) on cerebrovascular mechanisms to numerous stimuli has been extensively studied in piglets. In

general, selective impairment of some specific vasodilatory but not vasoconstrictor mechanisms was reported. More specifically, 20 min of global cerebral ischemia followed by 2h reperfusion virtually abolished pial arteriolar dilation to hypercapnia, reversed vasodilation to vasoconstriction to arterial hypotension, but had no effect on vasodilation evoked by PGE_2, and isoproterenol or vasoconstriction by norepinephrin (Leffler et al., 1989a-1989b-1989c). This duration of ischemia, however, is almost certainly too long to be clinically relevant, in fact piglets underwent to 20 min of global cerebral ischemia have never regained consciousness up to 24h post ischemia (Leffler et al., 1989a). Therefore, later studies used 10 min global cerebral ischemia to study the short-term effect of I/R on pial arteriolar reactivity. This duration also attenuated hypercapnia-induced pial arteriolar vasodilation, but cerebrovascular reactivity gradually recovered within 2-4 hours after ischemia (Bari et al., 1998b). Similar attenuation of cerebrovascular reactivity was observed with calcitonin gene-related peptide (CGRP) (Louis et al., 1996), iloprost a stable prostacyclin analogue, and ATP-sensitive potassium channel (K_{ATP}) opener aprikalim (Bari et al., 1996c). Interestingly, oxytocin-induced vasodilation was found to be reversed to constriction by I/R indicating that I/R preferentially targets dilator mechanisms (Bari et al., 1997a). However, 10 min ischemia did not affect pial arteriolar responses to many dilator agents such as arterial hypoxia, adenosine (Bari et al., 1998b), and NO donor sodium nitroprusside (SNP) (Busija et al., 1996a).

NMDA-induced vasodilation was demonstrated to be very sensitive to anoxic stress. During arterial hypoxia NMDA-induced vasodilation is diminished (Bari et al., 1998a), at least in part because oxygen is a substrate for NO synthesis. However, reoxygenation does not restore normal responsiveness to NMDA. Not only 10 min of asphyxia (Busija and Meng, 1993a) or global cerebral ischemia (Busija et al., 1996a) but also short periods (5 min) of hypoxia (Bari et al., 1996b) attenuate NMDA-induced vasodilation for 1-2 hours, then cerebrovascular reactivity gradually returns in 2-4 hours. Disturbed oxygen availability but not hypercapnia and acidosis seem to play major role in the pathogenesis of decreased arteriolar responses to NMDA after hypoxia. The impaired vascular response is related to the severity of anoxic stress. 15 min of hypoxia has a greater effect than 5 min hypoxia in attenuating the NMDA-induced vascular sequence (Bari et al., 1996b). However, hypercapnia resulting in severe respiratory acidosis does not affect vascular responses to NMDA. Several studies elucidated that the attenuation of NMDA-induced vasodilation after anoxic stress cannot be attributed to general deterioration of neuronal and vascular function, but rather due to the selective damage to NMDA receptor containing neurons, or the NMDA

receptors themselves. This hypothesis is based mainly on the following findings. 1) Pial arterioles show normal dilations to exogenous NO but not to NMDA after I/R indicating that VSM function remains intact. 2) Cortical nNOS enzyme levels and activity do not change after H/I (Beasley et al., 1998; Busija et al., 1996). 3) Kainate also activates neuronal glutamate (kainate) receptors; actions of both NO and prostanoids mediate the vasodilation (Bari et al., 1997b). This sequence is very similar to that of NMDA. However, kainate-induced vasodilation is independent of activation of NMDA receptors. Further, kainate-induced vasodilation is not affected by I/R (Bari et al., 1997b), indicating that not all neuronal-vascular response is damaged by H/I. Finally, 4) the assumption that the attenuation of NMDA-induced vascular dilation after H/I is due to a decrease in the number of functional NMDA receptors is also corroborated by the findings that upregulation of NMDA receptors results in an enhancement of NMDA-induced increases in CBF without affecting basal CBF (Weiss et al., 1996).

Role of reactive oxygen species (ROS) in the attenuation of NMDA-induced vasodilation after anoxic stress

The pathomechanism of decreased neuronal-vascular responsiveness to NMDA after H/I is only partly understood. All available evidence indicates the involvement of ROS in the attenuation of NMDA-induced vasodilation after H/I. This notion is supported mostly by experiments where different pharmacological interventions preserved the cerebrovascular responsiveness to NMDA. Thus, local treatment with superoxide dismutase (SOD) during hypoxia and reoxygenation (Bari et al., 1996b), or systemic pretreatment with oxypurinol prior to asphyxia (Busija and Meng, 1993a) preserved NMDA-induced vasodilation by scavenging ROS produced in the reoxygenation/reventilation period. Importantly, these studies also found NMDA-induced vasodilation unaltered when the piglets were pretreated with cyclooxygenase (COX) inhibitor indomethacin prior the anoxic stress. COX has been demonstrated be the major source of superoxide anions both after hypoxic hypoxia, asphyxia/reventilation and global cerebral ischemia/reperfusion in the piglet (McGowan et al., 1994; Pourcyrous et al., 1993; Armstead et al., 1988). In the latter study, indomethacin was able to suppress SOD-inhibitable nitroblue tetrazolium reduction to similar extent as the radical scavenger oxypurinol. During H/I, arachidonic acid (AA) is released by activated phospholipases (Abe et al., 1987; Umemura et al., 1992), but further metabolism is inhibited by the lack of oxygen shown by unaltered superoxide production during anoxia (Pourcyrous

et al., 1993). In contrast, reoxygenation results in excessive AA metabolism by COX that produces PGH_2 and superoxide anions in an equimolar concentration. The superoxide production in this uncontrolled reaction may exceed the antioxidant capacity and result in oxidative damage to membrane lipids and proteins including the NMDA receptor itself (Graham et al., 1996; Marro et al., 1998). In addition to NMDA, other ischemia-sensitive vascular mechanisms could also be preserved by inhibiting ROS production by indomethacin, such as pial arteriolar dilation to CGRP (Louis et al., 1996), prostacyclin, and K_{ATP} channel opener aprikalim (Bari et al., 1996c). Not only microvascular reactivity, but also altered blood-brain barrier transport by I/R can be preserved with indomethacin (Zuckerman et al., 1994) similarly to SOD and catalase (Armstead et al., 1992), indicating an overall effect of COX-produced ROS on microvascular pathology elicited by anoxic stress. In summary, ROS, especially COX-derived superoxide radicals seem to play a major role in the attenuation of NMDA-induced vasodilation.

Ligand-gated potassium channels in the brain and the cerebral circulation

Recently considerable attention has been focused at potassium channels of neurons and cerebral vessels for at least three different reasons: (1) potassium conductance determined by potassium channels have a major influence on VSM tone, and a number of vasoactive stimuli were shown to mediate their effect via potassium channels; (2) neuronal potassium channels affect cell excitability, calcium homeostasis and neurotransmitter release through their effect on membrane potential, and all these factors may be crucial in survival of neurons after anoxic stress; and (3) K_{ATP} have been shown to be involved in the mechanism of ischemic preconditioning (IPC) in the myocardium. IPC is a phenomenon whereby exposure to a brief ischemic period protects the cells from the damage inflicted by a subsequent longer anoxic stress. IPC can be induced in the brain by global cerebral ischemia (Kitagawa et al., 1990), and various other stimuli (Prass et al., 1998), but the potential involvement of K_{ATP} channels in the development of neuronal IPC is not known.

In the VSM of cerebral vessels four different classes of potassium channels have been found: (1) Ca^{2+}-sensitive (K_{Ca}), (2) K_{ATP}, (3) voltage-gated delayed rectifier, and (4) inwardly rectifying potassium channels (Faraci and Sobey, 1998). Activation of these channels results in increased potassium conductance with subsequent outward potassium current down the electrochemical gradient of potassium resulting in hyperpolarization. Since

the estimated "resting" membrane potential of cerebral VSM cells is about –36-46 mV and the equilibrium potential for potassium measures around –85 mV, little changes in potassium conductance change VSM membrane potential significantly. Changes in VSM membrane potential are determining free intracellular Ca^{2+} levels via affecting the activity of voltage-gated Ca^{2+}-channels thus regulating VSM contraction. The most numerous potassium channels in VSM are believed to be the K_{Ca} channels estimated around 10^4 channels present per cell (Nelson and Quayle, 1995). These channels are activated by increases in intracellular Ca^{2+}. One important physiological function of these channels to limit vasoconstriction elicited by increased Ca^{2+} levels originating from either extra- or intracellular sources. The role of K_{Ca} channels in the regulation of pial arteriolar caliber has been investigated in piglets (Bari et al. 1997c). Selective activation of K_{Ca} channels with NS1619 produces dose-dependent vasodilation. However, selective inhibition of K_{Ca} channels by charybdotoxin does not affect baseline arteriolar diameters indicating that these channels are quiescent at rest similarly to other microvascular beds (Jackson and Blair, 1998). Nevertheless, charybdotoxin does inhibit vascular dilation to activators of adenylate cyclase, and cAMP analogues. Importantly, K_{Ca} channel activators successfully dilate pial arterioles 1h after 10 min of global cerebral ischemia, thus this vasodilatory mechanisms may be utilized to dilate cerebral resistance vessels in an effort to increase brain perfusion after stroke (Bari et al., 1997c). K_{Ca} channels mediate vasodilation induced by ROS, and thus mechanism appears to play a role in the mechanism of reactive hyperemia in the reperfusion period (Sobey et al., 1997).

Similarly to K_{Ca}, K_{ATP} channels can also be found in cerebral microvessels albeit much fewer in numbers compared to K_{Ca}. A crucial feature of these channels that their activity may somewhat reflects the metabolic state of the cell: ATP inhibits channel activity. The K_{ATP} channel activator aprikalim is a potent dilator of pial arterioles in piglets, but the selective inhibitor glibenclamide does not cause vasoconstriction, indicating that K_{ATP} channels probably do not determine resting VSM tone (Bari et al., 1996c). However, pial arteriolar responses to various stimuli are sensitive to inhibition by glibenclamide. In piglets, K_{ATP} channels mediate CGRP and prostacyclin-induced arteriolar dilations. In contrast to K_{Ca}, K_{ATP} channels were found to be vulnerable to ischemic stress, aprikalim, CGRP, and iloprost –induced vasodilation was severely inhibited up to 4 h after 10 min of global ischemia (Bari et al., 1996c; Louis et al., 1996).

All types of potassium channels, including K_{Ca} and K_{ATP} channels have been identified also on neurons. However, the physiologic role of these channels on neuronal function is unclear. Activation of these channels by potassium channel openers (KCO-s) has produced neuronal hyperpolarization. Importantly, in some *in vivo* or *in vitro* experimental settings, KCO-s employed protective effects both on neuronal function and survival. Perhaps this effect may not be so surprising since KCO-induced neuronal hyperpolarization may prevent or delay the development of anoxic depolarization (AD). AD is characterized by the loss of transmembrane potential due to metabolic energy failure. The duration of AD correlates with the extent of hypoxic neuronal damage (Balestrino et al. 1989) indicating its importance in the development of neuronal injury after anoxic stress. AD may trigger several potentially neurotoxic events such as Na^+ and Ca^{2+} loading, intracellular edema formation, together with subsequent glutamate release, ROS generation, and activation of the apoptotic cascade. Therefore, KCO-s may represent a class of potential neuroprotective agents that prevent uncontrolled ion fluxes in compromised neurons similarly to inhibitors of voltage-gated sodium and calcium channels ad well as ionotropic glutamate receptor antagonists.

K_{ATP} channels appear to play a key role in the antiischemic effect of IPC in the heart. The induction of IPC is a multi-step process involving redundant extracellular mediators, signal transduction mechanisms and complex intracellular cascade of kinases (Cohen et al., 2000). Nevertheless, K_{ATP}-s probably serve as the final common pathway in the generation of IPC. KCO-s mimic IPC (Gross and Fryer, 1999; Liu et al., 1998; Garlid et al., 1997; Baines et al, 1999), and the protection by IPC is blocked by K_{ATP} inhibitors in dogs (Auchampach et al., 1992; Gross and Auchampach, 1992) and pigs (Shulz et al., 1994). The exact mechanism of this remarkable effect has not been elucidated, but paradoxically sarcolemmal K_{ATP} channels may not participate (Liu et al., 1998). However, recent findings strongly indicate the involvement of mitochondrial K_{ATP} (mitoK_{ATP}) channels in the mediation of IPC in the heart. MitoK_{ATP} channels differ from the sarcolemmal ones in their pharmacological profile. MitoK_{ATP} channels can be selectively activated with diazoxide (DIAZ), or inhibited with 5-hydroxy-decanoate (5-HD) (Garlid et al., 1996a, 1997). Indeed, DIAZ preserves post-ischemic cardiac function and reduces infarct size in rabbits independently of sarcolemmal K_{ATP} channels (Liu et al., 1998). It is not clear why activation of mitoK_{ATP} channels is cardioprotective, or this antiischemic effect of mitoK_{ATP} channels would occur in the brain.

Neuroprotection by protein synthesis inhibitors

Suppressed protein synthesis is a substantial feature of hypoxic neuronal damage (Hossmann, 1996a). Long-lasting inhibition of cellular protein synthesis may directly contribute to neuronal loss conceivably because injured cells are unable to repair their proteom. Also, the recovery of NMDA-induced vasodilation after H/I may involve replenishment of proteins damaged by ROS. Therefore, pharmacological inhibition of protein synthesis may delay the restoration of NMDA-induced vasodilation after H/I. Paradoxically, several studies provided evidence that protein synthesis inhibitors are beneficial when administered shortly before or after cerebral ischemia in different experimental conditions. For instance, postischemic administration of the translation inhibitor cycloheximide (CHX) prevented delayed neuronal cell death in the hippocampal CA1 region (Goto et al., 1990). This effect of CHX was attributed to the inhibition of apoptotic gene expression required for cell death. More interestingly, other studies showed that CHX pretreatment reduces infarct size in transient focal ischemia models in rats (Linnik et al., 1993; Du et al., 1996). In an elegant study, Aronowski et al. (1997) convincingly demonstrated that the neuroprotective effect of CHX was to preserve those brain areas that were lost to reperfusion injury. More specifically, transient focal ischemia evoked by middle cerebral artery (MCA) occlusion produced bigger infarcts than permanent MCA occlusion. However, after CHX administration transient ischemia resulted in same infarct size as seen after permanent MCA occlusion. The authors concluded that a short-lived transiently expressed noxious/killer protein would be responsible for the neuronal death in the areas of reperfusion injury that was blocked by CHX.

Rationale of the present studies

NMDA elicits a complex neuronal-vascular sequence that is severely attenuated by anoxic stress via ROS. We wanted to further investigate the pathomechanism of altered cerebrovascular reactivity to NMDA after I/R, since it is a likely factor in the physiological regulation of rCBF. In addition, pharmacological preservation of this response after I/R would indicate intact cerebrovascular responsiveness that directly or indirectly imply a neuroprotective action. Based on the findings described in the previous sections we wanted to test the following specific hypotheses:

1. We wanted to determine if depolarization of the cerebral cortex *per se* affects vascular responses to NMDA. Cortical depolarisation (CD) also occurs during anoxia as well as cortical spreading depression (CSD), a transient CD that is accompanied by tremendous increases in metabolism and CBF. Diminished cerebrovascular responses have been reported after single CSD-s even when cerebral oxygenation was unchallenged in cats and rats (Lauritzen et al., 1984; Wahl et al., 1987; Piper et al. 1991), but not in rabbits (Busija and Meng, 1993b). Moreover, CSD-like transient CD-s called periinfarct depolarizations were suggested to play a role in the developing neuronal injury after focal ischemia (Hossmann, 1996b). Thus, we tested the hypothesis if CD induced by topical KCl reduced NMDA-induced vasodilation.

2. We also addressed if pretreatment with potassium channel openers (KCO-s) such as aprikalim or NS1619 (activators of K_{ATP} or K_{Ca}, respectively), and other vasodilators like CGRP, or papaverine prior to H/I preserved NMDA-induced vasodilation.

3. The results from experiments described previously urged us to search for other targets of KCO-s than plasmolemmal potassium channels. We investigated if selective activation of mitochondrial K_{ATP} channels by diazoxide (DIAZ) preserved the NMDA-induced vascular response after global ischemia. We also tested if DIAZ affected NMDA-induced vasodilation, and if the effect of DIAZ could be antagonized with 5-hydroxy-decanoate (5-HD).

4. We assessed if transcription inhibitor Actinomycin-D (Act-D) and CHX affected NMDA-induced vasodilation. We then also explored whether Act-D and/or CHX pretreatment would preserve NMDA-induced vasodilation after ischemia.

5. The protective effect of protein synthesis inhibitors on NMDA-induced vasodilation after ischemia was similar to cyclooxygenase inhibitors. Therefore, we tested if CHX had a short-term effect on cortical COX-activity. Therefore, we tested if CHX affected COX-dependent pial arteriolar responses to hypercapnia and arterial hypotension, and cortical metabolism of exogenous AA.

Materials and methods

The results of the papers related to the subject of this thesis have been derived from experiments on 183 piglets. For the sake of clarity and brevity, only those procedures as well as experimental groups and designs shall be described in detail that are crucial for the interpretation of results. The reader is referred to the original papers attached for any additional detail.

The piglet as an experimental model

The neonatal piglet is a widely accepted and popular model to study the cerebral circulation as well as the cardiorespiratory and endocrine systems (Book and Bustad, 1974). At birth, the size and brain developmental stage are similar to humans, and the physical development in the first 2 weeks parallels that of the first month of life of the human newborn (De Roth and Downie, 1978). Concerning the cerebral circulation neonatal piglets feature remarkably similar cerebrovascular responses as compared to human babies (Busija, 1994).

The closed cranial window technique

Essentially all data in these studies were obtained using the closed cranial window technique and intravital microscopy described below. The history of this experimental approach dates back to the 1920-s, and Forbes (1928) published the first detailed description of the method. We have used the closed cranial window technique described previously by Levasseur et al. (1975) with slight adaptation to the neonatal piglet. This method has numerous advantages far outweighing the technical limitations in assessing the pial microcirculation *in vivo*. The observer is able to continuously observe the pial circulation. Changes in pial arteriolar diameter can be measured which vessel segment makes up a significant portion of total cerebral vascular resistance. However, it is important to note, that these vessels are not the only determinants of cerebrovascular conductance. Thus, vasodilation or vasoconstriction upstream or downstream affects flow through these pial vessels. In conclusion, pial arteriolar diameter measurements are excellent to study microvascular reactivity. Pial arteriolar diameter changes do relate but may not always exactly predict changes in CBF as described by Poiseuille's law. On the other hand, the 4^{th} power relationship between vascular radius and blood flow may result in statistically undetectable changes in vascular diameter when changes

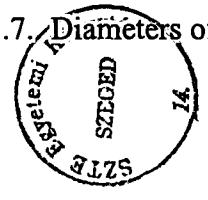
in CBF do not exceed 5-10% (cf. 1-3% changes in diameter). This relative "insensitivity" of the method is mainly due to the technical limitation of measurement accuracy, and considerable biological variability. Sealing and cementing "closing" the cranial window has further advantages: (1) the integrity of the skull thus normal intracranial pressure (ICP) is restored. (2) The space between the pial surface and the glass window may be filled with artificial cerebrospinal fluid (aCSF) providing physiological environment for the pial vessels observed. (3) It also allows application of drugs locally to the brain surface circumventing their systemic effects on cerebrovascular circulation, and aCSF samples can be collected to determine concentrations of endogenous vasoactive substances. (4) Finally, the mechanical stability of the surgical preparation enabled us to induce selective and well-controlled anoxic stress by producing global cerebral ischemia via elevated ICP.

Animals

Newborn piglets of either sex (1-7 days old, body weight 1-2 kg) were used. All procedures were approved by the Institution Animal Care and Use Committee. The animals were anesthetized with sodium thiopental (30-40 mg/kg ip) followed by intravenous injection of α-chloralose (75 mg/kg). Supplemental doses of α-chloralose were given to maintain a stable level of anesthesia. The right femoral artery and vein were catheterized to record blood pressure and to administer drugs and fluids, respectively. The piglets were intubated via tracheotomy and artificially ventilated with room air. The ventilation rate (~20/min) and tidal volume (~20 ml) were adjusted to maintain arterial blood gas values and pH in the physiological range. Body temperature was maintained at 37-38 °C by a water-circulating heating pad. Body temperature, arterial pH and blood gases were also in the normal ranges, and did not vary significantly among different groups.

The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (19 mm in diameter) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax and cemented with cyanoacrylate ester and dental acrylic.

The closed window was filled with artificial cerebrospinal fluid (aCSF) warmed to 37°C and equilibrated with 6% O_2 and 6.5% CO_2 in balance N_2 to give pH=7.33, pCO_2=46 mmHg, and pO_2=43 mmHg. The aCSF consisted of (mmol/L): NaCl 132, KCl 2.9, $CaCl_2$ 1.2, $MgCl_2$ 1.4, $NaHCO_3$ 24.6, urea 6.7 and glucose 3.7. Diameters of pial arterioles were



measured using a microscope (Wild M36, Switzerland) equipped with a video camera (Panasonic, Japan) and a video micro scaler (IV-550, For-A-Co. Newton, Mass., USA). At the end of the experiments the animals were killed while anesthetized with an intravenous bolus of 5% KCl.

General protocol to study the effects of drugs and anoxic stress on NMDA-induced vasodilation

These studies were based on the same principle (Figure 1.). We determined NMDA-induced vasodilation before, and 1-4 h after a physiologic, pharmacological or combined physiologic and pharmacological challenge. First, we obtained stable baseline arteriolar diameters, by gently flushing the cranial window with aCSF several times. We examined the responses of cerebral arterioles (baseline diameter ≈ 100 μm) to different doses of NMDA (10, 50, 100 μmol/L). The NMDA, and all other drugs were dissolved in aCSF and administered topically through the injectable ports of the cranial window onto the brain surface with single application unless otherwise stated. Arteriolar diameters were measured continuously for 5-7 min for each dose of NMDA. Typically, maximal steady state vasodilation was observed 2-4 min after NMDA application. Then the window was flushed with aCSF and the arteriolar diameters returned to baseline values. The same procedure has been repeated after the following stimuli.

Cortical Depolarization

CD was achieved by single topical application of 1mol/L KCl dissolved in aCSF for 3 min. After CD the cranial window was infused several times with aCSF until arteriolar diameters returned to baseline. To demonstrate CD, in some animals extracellular DC potential was recorded. The DC potential was derived from the potential between a silver/silver-chloride electrode placed on the frontoparietal cortex 5 mm anterior to the cranial window and a grounded reference electrode placed in the subcutaneous connective tissue of the scalp, amplified (EXT-MC, Experimetria Ltd., Hungary), and stored with an on-line data acquisition software.

Combined cerebral hypoxia/ischemia (H/I)

Cerebral hypoxia was induced by artificial ventilation with 8.5% O_2 balance N_2 over 10 min. Arterial blood gases were measured 8 min after hypoxia was started. After 10 min of hypoxia ventilation with room air was restored, but hypoxia immediately was followed by cerebral ischemia as described below.

Global cerebral ischemia

To induce global cerebral ischemia, a 3-mm hole was made by an electric drill with a toothless bit, and the dura was exposed. A hollow brass bolt was inserted in the left frontal cranium rostral to the cranial window and secured in place with cyanoacrylate ester and dental acrylic. Cerebral ischemia was produced by infusion of aCSF to raise ICP above arterial pressure. Ischemia was verified by the cessation of blood flow in the observed vessels. Previously, using microspheres CBF was shown to be virtually zero (<1ml/min/100g) in all brain areas examined during the ischemic period (Beasley et al., 1998). When this type of anoxia was induced in unanesthetized piglets, within 5 seconds all animals became unresponsive to tactile and auditory stimuli, and the pupils were fixed and dilated (Leffler et al., 1989a). Venous blood was withdrawn as necessary to overcome the Cushing response and maintain mean arterial blood pressure near normal values. At the end of the ischemic period the infusion tube was clamped and the ICP could return to preischemic values. The heparinized blood was reinfused intravenously.

Experimental groups

Group 1 (n=9): NMDA-induced vasodilation was assessed before and 1 hour after 3 min of CD. (Figure 1.)

Groups 2-6 (n=7,6,7,6,5, respectively) NMDA-induced vasodilation was assessed before and 1 h after combined H/I. Different groups received topical pretreatment of aCSF, NS1619 (10μmol/L), aprikalim (1μmol/L), calcitonin gene-related peptide (CGRP, 1μmol/L), and papaverin (10 μmol/L), respectively for 10 min prior H/I. Arteriolar responses to these drugs were recorded during this period. The drugs were washed away just before the beginning of hypoxia.

Groups 7-11 (n=8,8,8,12,5) NMDA-induced vasodilation was assessed before and 1 h after 10 min of global cerebral ischemia. Different groups received the following pretreatments: none, topical diazoxide (DIAZ, 1,5,10μmol/L), and 10μmol/L DIAZ coapplied with the antagonist 5-hydroxy decanoate (5-HD), respectively

Groups 12-16 (n=7,5,5,5,5, respectively) NMDA-induced vasodilation was assessed before and 1, 2, and 4 h after 10 min of global cerebral ischemia. Different groups received the following pretreatment: none, topical Act-D (1,10 μmol/L) for 15 min washed away just before the onset of ischemia, and iv injection of CHX (0.3,1 mg/kg, dissolved in saline) given 20 min prior to ischemia, respectively.

Groups 17-18 (n=4,6) In these groups NMDA-induced vasodilation was assessed before and 1 h after different drug treatments in the absence of anoxic stress. Group 17 received topical DIAZ (10μmol/L) for 10 min, Group 18 first topical Act-D (10μmol/L) for 15 min, and after that trial iv CHX (1 mg/kg), respectively.

Groups 19-21 (n=22, not shown on Figure 1) Responses of cerebral arterioles to COX-dependent (arterial hypercapnia, arterial hypotension), and COX-independent (SNP) stimuli were examined before and 20 min after CHX treatment. Different groups received iv vehicle, and CHX (0.3, 1mg/kg) respectively. Hypercapnia was elicited by artificially ventilating the animal with a gas mixture containing (5% or 10% CO_2, 21% O_2, balance N_2). Arterial hypotension was induced by withdrawing venous blood to yield approximately 25% and 40% decreases in MAP, respectively. Following the measurements the heparinized blood was reinfused. SNP (1, 10 μmol/L) dissolved in aCSF was administered topically. Arteriolar diameters were measured continuously for 5 min for each stimulus, until steady state values were obtained. Typically, we obtained data for two different stimuli in each animal. With this protocol we obtained similar arteriolar responses to hypercapnia, hypotension, and SNP to previous experience with these challenges. These doses of hypercapnia, hypotension, and SNP were chosen to provide medium and large increases in vascular diameters. Between different stimuli the window was flushed with aCSF, and the arteriolar diameters returned to baseline values. Twenty min after treatment, challenges of hypercapnia, arterial hypotension and SNP were repeated according to the procedure described above.

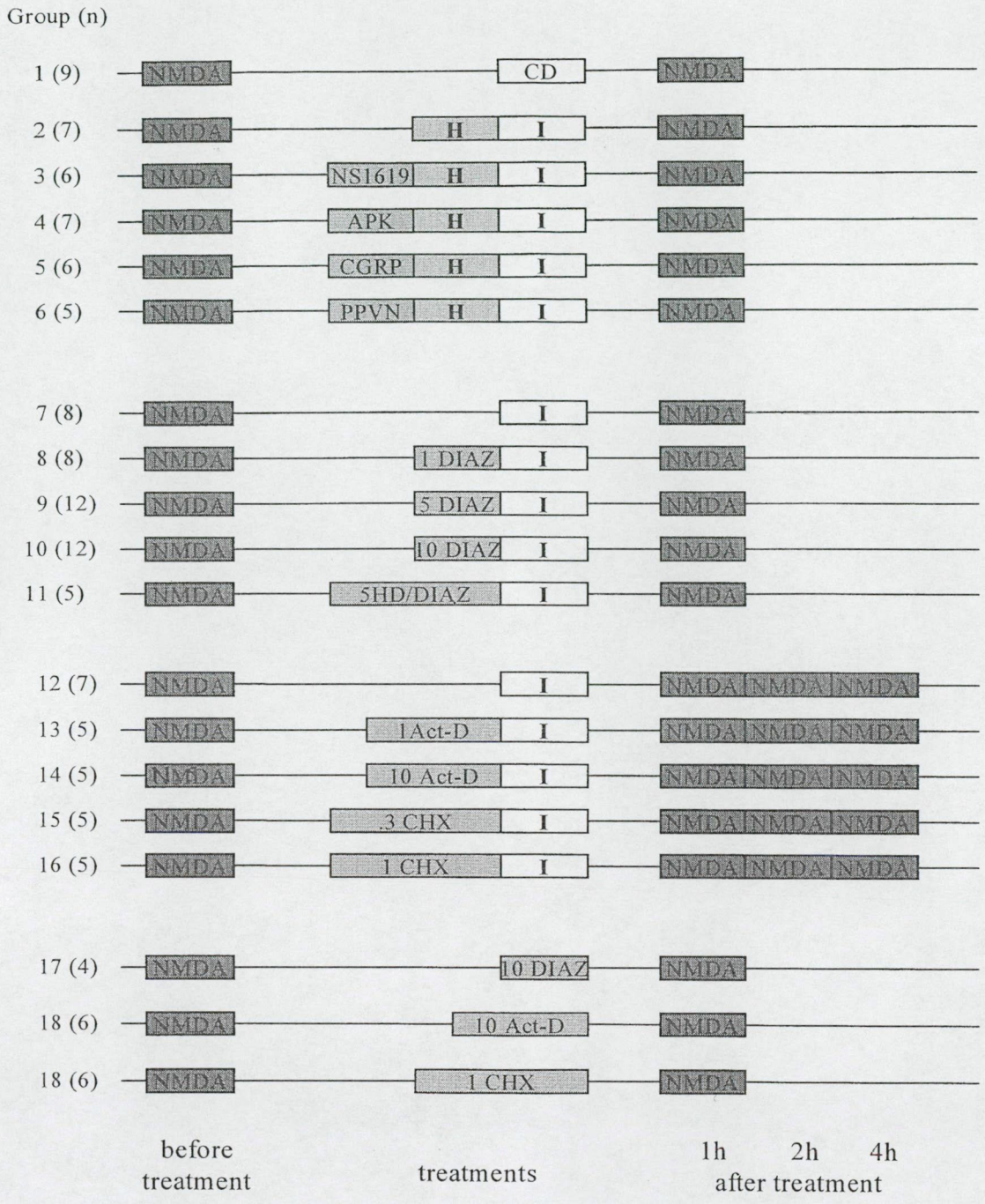


Figure 1. Experimental protocols to study N-methyl-D-aspartate (NMDA)-induced vasodilation before and after various physiological and/or pharmacological manipulations using repeated measurement experimental design. Pial arteriolar vasodilation to NMDA (10-100 μmol/L) was detected using closed cranial window/intravital microscopy before and 1-4h after treatments. All drugs were applied topically onto the pial surface for 10min, except cycloheximide (CHX) that was given intravenously 20 min before. CD: cortical depolarization (3min) by 1 mol/L KCl; H: 10 min hypoxia (ventilation with 8.5% O_2 in N_2); I: 10min global ischemia by elevated intracranial pressure; NS1619: selective activator of Ca^{2+}-

sensitive potassium channels; APK: aprikalim, selective activator of ATP-sensitive potassium channels channels; CGRP: calcitonin gene-related peptide; 1-10DIAZ: diazoxide (1-10 μmol/L), selective activator of mitochondrial ATP-sensitive potassium channels; 5HD/DIAZ: coapplication of 100 μmol/L 5-hydroxy-decanoate - selective inhibitor of mitochondrial ATP-sensitive potassium channels - with 10 μmol/L diazoxide; 1-10 Act-D: 1-10 μmol/L Actinomycin-D, inhibitor of gene transcription; .3-1CHX: 0.3-1 mg/kg cycloheximide, inhibitor of protein translation.

Group 22 (n=9) We determined cortical conversion of exogenous AA to $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$. AA (1, 10, and 20 μg/ml) dissolved in aCSF was administered onto the brain surface through the injectable ports of the cranial window. Each dose of AA was applied on the brain surface for 10 min, then the cranial window was gently flushed and the effluent aCSF (~300 μl) was collected and frozen. AA was applied with 1 hr intervals 2 times before, then 20 min and 1 hr after CHX (1 mg/kg, iv) treatment. Typically, we applied AA 3 times in each animal. Since the data obtained from these animals did not differ significantly, we combined these data as shown in the Results. From the aCSF samples concentrations of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were determined using ELISA kits (Oxford Biomedical Research Inc., Oxford, Mich., USA).

Chemicals & Statistics

The drugs used in these studies were: SIGMA: NMDA, DIAZ, papaverine, SNP, AA, CHX; Rhone-Roulenc-Rohrer: aprikalim; Research Biochemical International: NS1619, CGRP, 5-HD; and Calbiochem: Act-D.

Data are expressed as mean ± standard error of the mean (SEM). Pial arteriolar diameter data (absolute and percent change) were analyzed using repeated measures analysis of variance (ANOVA). Percent preservations of preischemic vasodilation data between different experimental groups were analyzed using one-way ANOVA. For pairwise comparisons the Student-Newman-Keuls test was used where appropriate. P values of <0.05 were considered as statistically significant.

Results

Effect of CD on cerebrovascular reactivity to NMDA.

In all animals topical application of 1mol/L KCl resulted in a large reduction of diameters of pial arterioles from 105±3 to 48±4 μm (n=27). Also, there was a negative deflection in the extracellular DC potential (max 5-7 mV) indicating CD. After 3 min the KCl was washed out by repeated infusion of aCSF and arteriolar diameters returned to baseline within 30-40 min.

In Group 1, NMDA resulted in a dose-dependent arteriolar vasodilation. The baseline diameters were very similar (102±2 *versus* 99±3 μm, before and after CD). Only at the highest dose the absolute change in diameter was slightly (~10%) decreased. However the percent changes were intact (9±1 *versus* 8±1 at 10 μmol/L, 19±2 *versus* 18±3 at 50 μmol/L, and 29±2 *versus* 26±3 at 100 μmol/L NMDA). Thus, prior CD did not significantly affect the NMDA-induced vasodilation. Similarly, CD did not affect other ischemia-sensitive mechanisms like pial arteriolar dilations to hypercapnia, CGRP, or aprikalim (data not shown, see details in Appendix I).

Effect of anoxic stress on NMDA-induced vasodilation

Global cerebral ischemia or combined H/I did not alter baseline arteriolar diameters significantly. However, both types of anoxic stress severely attenuated NMDA-induced vasodilation as previously reported. For instance, in Group 7 and in Group 12 10 min ischemia reduced vascular responses to 100 μmol/L NMDA by ~50%, percent changes from baseline (before *versus* 1 h after ischemia) were 38±5% *versus* 16±4%, and 40±4% *versus* 20±4%, respectively (see also Figure 2.). Combined H/I damaged NMDA-induced vasodilation even more; in Group 2 percent changes from baseline (before *versus* 1 h after H/I) to 100 μmol/L NMDA were 33±4% *versus* 7±2%. The attenuation of NMDA-induced vasodilation by ischemia was transient, arteriolar dilations to NMDA gradually returned in 4h after ischemia. In Group 12 percent changes from baseline (before *versus* 1, 2, and 4 h after ischemia) to 100 μmol/L NMDA were 40±4% *versus* 20±4%, 27±5%, and 34±5%, respectively.

Effects of KCO-s on pial arteriolar diameters

Pretreatment with NS1619 and aprikalim elicited pial arteriolar vasodilation. Percent changes from baseline were 15±4% (Group 3), and 26±7% (Group 4). However, DIAZ did not affect pial vascular diameters significantly. Typically, there was only a transient dilation immediately upon application of DIAZ unlike the maintained dilation elicited by NS1619 or aprikalim. Percent changes from baseline diameters were: in Group 8, no vasoactivity was observed; in Group 9, 2±1%, in Group 10, 9±3% but vascular diameters quickly returned to baseline values in 2-3 min, and none of these changes were significantly different from baseline values.

Preservation of NMDA-induced vasodilation after anoxic stress by KCO-s

The KCO-s used in these studies did not affect NMDA-induced vasodilation directly. For instance in Group 17, 10 μmol/L DIAZ did not potentiate or attenuate vascular dilations to NMDA 1 hr after DIAZ treatment. Baseline arteriolar diameters were 100±2μm before and 100±6μm 1 hr after DIAZ treatment. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 hr after DIAZ treatment) were 3±1% *versus* 4±1%, 28±7% *versus* 26±9%, and 50±8% *versus* 47±8%, respectively. In contrast, KCO-s preserved NMDA-induced vasodilation 1h after anoxic stress.

In Group 3 (NS1619)-, baseline arteriolar diameters were 103±3μm before and 104±3μm 1 h after H/I. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 h after H/I) were 9±2% *versus* 6±4%, 19±6% *versus* 21±5%, and 35±3% *versus* 31±5%, respectively.

In Group 4 (aprikalim), baseline arteriolar diameters were 101±4μm before and 104±4μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 h after H/I) were 6±2% *versus* 8±2%, 22±6% *versus* 15±3%, and 41±5 *versus* 32±6%, respectively.

DIAZ also employed dose-dependent effect on preservation of NMDA-induced vasodilation after ischemia. In Group 8 (1 μmol/L DIAZ), decreases in pial arterial responsiveness to NMDA were similar to those observed in Group 7. In Group 8, baseline arteriolar diameters were 102±3μm before and 104±3μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 h

after ischemia) were 5±2% *versus* 3±1%, 20±7% *versus* 8±2%, and 38±5% *versus* 19±3%, respectively.

In contrast, in Groups 9-10 we found a dose-dependent preservation of pial vascular responses to NMDA. More specifically, in Group 9, baseline arteriolar diameters were 95±3μm before and 95±4μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 hr after ischemia) were, respectively: 4±0% *versus* 7±2%, 30±10% *versus* 23±6%, and 45±6% *versus* 37±3%. In Group 10, baseline arteriolar diameters were 102±6μm before and 106±5μm 1 hr after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were, respectively: 7±1% *versus* 6±2%, 28±5% *versus* 24±4%, and 36±5% *versus* 32±4%. Therefore, pretreatment with 10 μmol/L DIAZ resulted in virtually full preservation of pial arteriolar responses to NMDA 1 h after I/R compared to preischemic values.

Topical application of K_{ATP} antagonist 5-HD, and coapplication of 5-HD with DIAZ did not alter pial arteriolar diameters. Also, 5-HD treatment did not affect pial arteriolar responses to NMDA (data not shown, see details in Appendix V.). However, pretreatment with 5-HD and DIAZ abolished the protection on NMDA-induced vasodilation achieved by DIAZ alone. In Group 11, baseline arteriolar diameters were 90±6μm before and 92±6μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were, respectively: 3±1% *versus* 0±0%, 40±12% *versus* 19±6%, and 61±7% *versus* 33±5%. Interestingly, coapplication of 5-HD with aprikalim did not block the vasodilation elicited by aprikalim (data not shown, see details in Appendix III.).

Effects of CGRP and papaverine on NMDA-induced vasodilation after H/I

CGRP and papaverine dilated pial arterioles to similar extent as NS1619 and aprikalim. Percent changes from baseline in Group 5 (CGRP) and Group 6 (papaverine) were 15±4% and 20±2%, respectively. However, pretreatment with either CGRP or papaverine did not have protective effect on NMDA-induced vasodilation following H/I, attenuation of the response was similar to non-treated controls (Group 2).

In Group 5, baseline arteriolar diameters were 104±4μm before and 112±7μm 1 h after H/I. Percent changes in pial arteriolar diameter from baseline to 10, 50, and 100 μmol/L

NMDA (before *versus* 1 h after H/I) were 6±2% *versus* 0±0%, 22±5% *versus* 2±1%, and 28±7% *versus* 2±1%, respectively.

In Group 6, baseline arteriolar diameters were 102±3μm before and 102±2μm 1 h after H/I. Percent changes in pial arteriolar diameter from baseline to 10, and 50, μmol/L NMDA (before *versus* 1 h after H/I) were 7±1% *versus* 3±1%, and 38±4% *versus* 16±6%, respectively.

Preservation of NMDA-induced vasodilation after I/R by protein synthesis inhibitors

In normoxic conditions, neither Act-D nor CHX influenced NMDA-induced vasodilation significantly in the absence of ischemia. In Group 18, percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after Act-D) were, respectively: 7±3% *versus* 8±1%, and 37±6% *versus* 35±4%. Also in Group 18, percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after CHX) were, respectively: 7±3% *versus* 5±2%, and 37±6% *versus* 30±1%. However, both Act-D and CHX pretreatment resulted in largely (smaller dose), or completely (higher dose) preserved pial arteriolar vasodilation to NMDA after I/R.

Thus, in Group 13 (1 μmol/L Act-D), baseline arteriolar diameters were 98±4μm before and 101±5μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were 6±2% *versus* 6±2%, and 51±9% *versus* 39±10%, respectively.

In Group 14 (10 μmol/L Act-D), baseline arteriolar diameters were 103±1μm before and 103±1μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were 7±2% *versus* 7±2%, and 38±4% *versus* 35±4% respectively.

Similarly, in Group 15 (0.3 mg/kg CHX), baseline arteriolar diameters were 110±5μm before and 111±5μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were 8±2% *versus* 8±1%, and 39±4% *versus* 28±6% respectively.

Finally, in Group 16 (1 mg/kg CHX), baseline arteriolar diameters were 101±5μm before and 99±6μm 1 h after ischemia. Percent changes in pial arteriolar diameter from baseline to 10, and 100 μmol/L NMDA (before *versus* 1 h after ischemia) were 10±2% *versus* 6±2%, and 37±7% *versus* 35±6% respectively. NMDA-induced vasodilation was also

essentially identical to preischemic values in Groups 13-16 at 2 and 4 h after ischemia (data not shown, see details in Appendix IV).

Effect of CHX on hypercapnia-induced vasodilation

First exposure to graded hypercapnia resulted in a concentration dependent increase in pial arteriolar diameters in accordance with the elevated pCO_2 levels in all groups of animals.

In control animals (Group 19, n=5), repeated exposure to high pCO_2 levels elicited essentially identical vasodilation in pial arterioles (Figure 3.). Baseline arteriolar diameters were 112±11μm before and 113±11μm 20 min after vehicle (saline) injection. Percent changes in pial arteriolar diameter from baseline to 5%, and 10% CO_2 (before *versus* 20 min after treatment) were 27±5% *versus* 26±5%, and 46±5% *versus* 44±5% respectively.

However, in the groups treated with either 0.3 or 1.0 mg/kg CHX iv, vasodilation was significantly reduced to either level of hypercapnia (Figure 3). In Group 20 (n=6) baseline arteriolar diameters were 102±5μm before and 107±5μm 20 min after CHX treatment. Percent changes in pial arteriolar diameter from baseline to 5%, and 10% CO_2 (before *versus* 20 min after CHX) were, respectively: 26±5% *versus* 15±4%, and 42±8% *versus* 31±6%.

The attenuation of the response was bigger in the group treated with the higher dose of CHX, especially at the lower level of hypercapnia (Figure 3). Thus, in Group 21 (n=8) baseline arteriolar diameters were 93±3μm before and 94±5μm 20 min after CHX treatment. Percent changes in pial arteriolar diameter from baseline to 5%, and 10% CO_2 (before *versus* 20 min after CHX) were 22±2% *versus* 10±2%, and 49±5% *versus* 31±3%, respectively.

Effect of CHX on arterial hypotension-induced vasodilation

Graded arterial hypotension (25% and 40% reduction in MAP, Grade 1 and Grade 2, respectively) resulted in a dose-dependent increase in pial arteriolar diameters in accordance with decreased arterial blood pressure levels. In (control) Group 19 (n=5), pial arteriolar responses in response to stimulation by arterial hypotension were unaltered (Figure 3). Baseline arteriolar diameters were 108±10μm before and 109±8μm 20 min after vehicle. Percent changes in pial arteriolar diameter from baseline to hypotension (Grade 1 and Grade 2, before *versus* 20 min after vehicle) were 20±4% *versus* 19±4%, and 31±5% *versus* 33±4%, respectively.

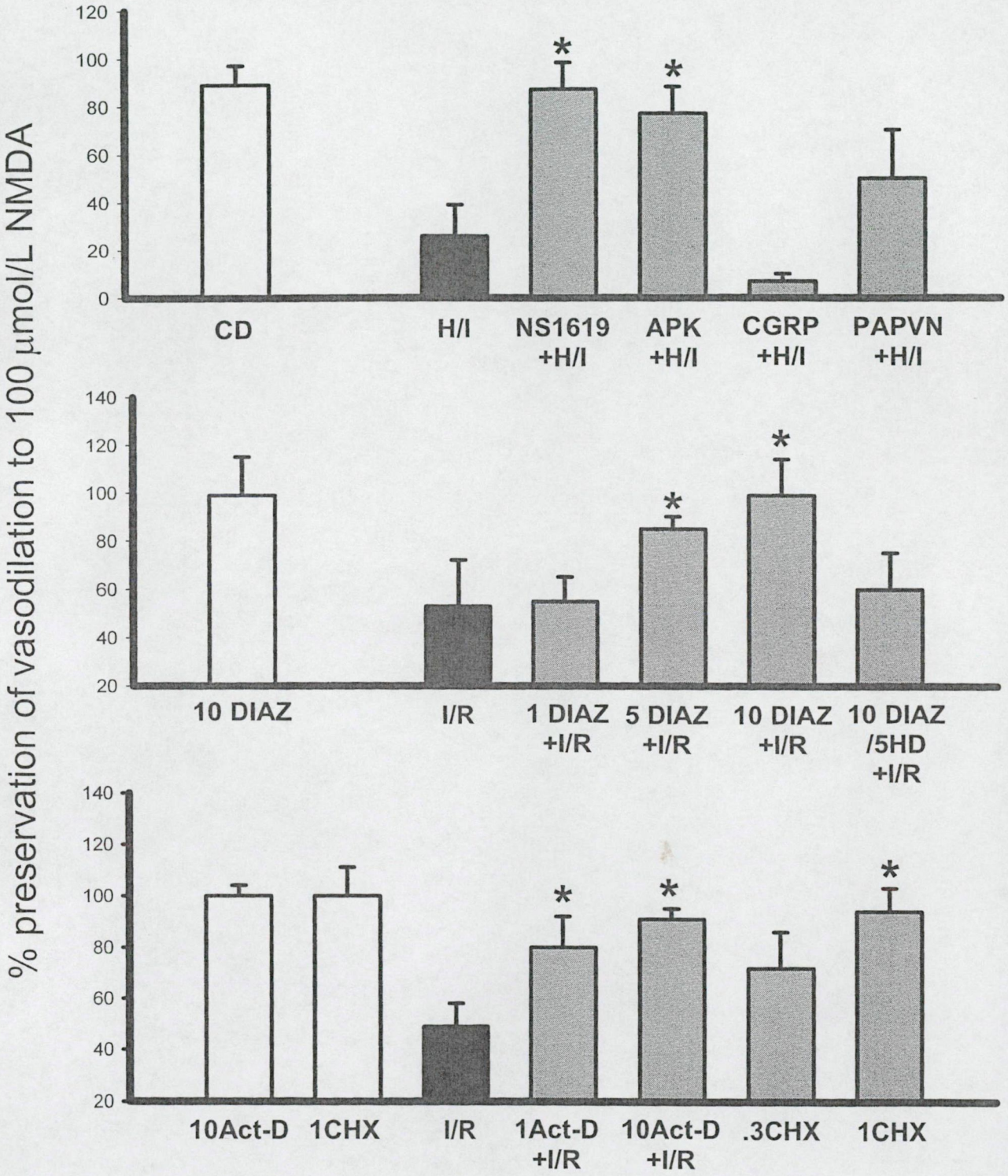

% preservation of vasodilation to 100 µmol/L NMDA
120
100
80
60
40
20
0
CD
H/I
NS1619 +H/I
APK +H/I
CGRP +H/I
PAPVN +H/I
140
120
100
80
60
40
20
10 DIAZ
I/R
1 DIAZ +I/R
5 DIAZ +I/R
10 DIAZ +I/R
10 DIAZ /5HD +I/R
140
120
100
80
60
40
20
10Act-D
1CHX
I/R
1Act-D +I/R
10Act-D +I/R
.3CHX
1CHX

Figure 2. Preservation of neuronal-vascular reactivity to 100μmol/L N-methyl-D-aspartate (NMDA). Data are expressed as %preservation of arteriolar dilation to NMDA 1h after treatments with various drugs and physiologic challenges. Thus, 100% means unaltered responsiveness to NMDA.
Cortical depolarization (CD) does not affect NMDA-induced vasodilation (n=9, white bar, upper panel). However, 10 min of total global cerebral ischemia followed by reperfusion (I/R) significantly decreased arteriolar responses by roughly 50% in both groups receiving I/R only (n=8+7, black bars, upper and middle panels). A more severe stress like 10min severe hypoxia immediately followed by 10 min ischemia and then reperfusion (H/I) resulted in even greater attenuation (approximately 70%) of NMDA-induced vascular responses (n=7, black bar, upper panel).
Pretreatment with potassium channel openers preserved neuronal-vascular responsiveness to NMDA after either anoxic stress, I/R or H/I (gray bars, upper and middle panels). More specifically, aprikalim (APK), and NS1619 - selective activators of Ca^{2+} and ATP-sensitive potassium channels (K_{ATP})-, preserved NMDA-induced vasodilation after H/I (n=6, 7, respectively; *= significantly different from corresponding untreated H/I control group, $p<0.05$).
Diazoxide (10 μmol/L, 10DIAZ), *per se* did not affect NMDA-induced response (n=4, white bar, middle panel). However, DIAZ, in a selective dose range (1-10 μmol /L) for mitochondrial K_{ATP} (mitoK_{ATP}) channels, produced dose-dependent preservation of the NMDA-induced sequence after I/R (1-10DIAZ+I/R). The highest dose of DIAZ used yielded full preservation (n=8,12,12, respectively for 1, 5, and 10 DIAZ, gray bars, middle panel, *= significantly different from corresponding untreated I/R control group, $p<0.05$). This effect was blocked by coapplication of DIAZ with 5-hydroxy-decanoate (10 DIAZ/5HD+I/R, n=5,), a selective inhibitor of mitoK_{ATP} channels.
Pretreatment with other vasodilators like calcitonin gene-related peptide (CGRP) or papaverine (PAPVN), was not effective in protecting the response after H/I (n=6, 5, respectively, gray bars, upper panel).
Inhibitors of protein synthesis, the transcription inhibitor Actinomycin-D (10 μmol/L, 10Act-D), and the translation inhibitor cycloheximide (1mg/kg, 1CHX) did not affect NMDA-induced vasodilation *per se* (n=6, 6, respectively, white bars, lower panel.
However, both doses of Act-D (1, and 10 μmol/L: 1Act-D and 10Act-D), and CHX (0.3-1 mg/kg: .3CHX and 1CHX) employed dose-related preservation of NMDA-induced arteriolar responses after I/R (n=5,5,5,5 respectively, gray bars, lower panel, *= significantly different from corresponding untreated I/R control group, $p<0.05$).

The vasodilator response to arterial hypotension was also largely retained in Group 20 treated with 0.3 mg/kg CHX (n=6, Figure 3). Baseline arteriolar diameters were 104±3μm before and 106±6μm 20 min after CHX. Percent changes in pial arteriolar diameter from baseline to hypotension (Grade 1 and Grade 2, before *versus* 20 min after CHX) were 10±1% *versus* 7±1%, and 21±4% *versus* 19±4%, respectively.

However, arteriolar responsiveness to arterial hypotension was severely reduced in the animals treated with 1 mg/kg CHX in Group 21 (n=6, Figure 3). Baseline arteriolar diameters were 96±3μm before and 104±12μm 20 min after CHX. Percent changes in pial arteriolar

diameter from baseline to hypotension (Grade 1 and Grade 2, before *versus* 20 min after CHX) were 12±3% *versus* 3±1%, and 26±5% *versus* 6±4%, respectively.

However, in Group 21 (n=7) SNP induced dose-dependent pial arteriolar vasodilation that was unaffected by treatment with 1mg/kg CHX. Baseline arteriolar diameters were 112±6μm before and 118±7μm 20 min after CHX. Percent changes in pial arteriolar diameter from baseline to SNP (1 and 10 μmol/L, before *versus* 20 min after CHX) were 21±4% *versus* 19±4%, and 38±5% *versus* 37±5%, respectively.

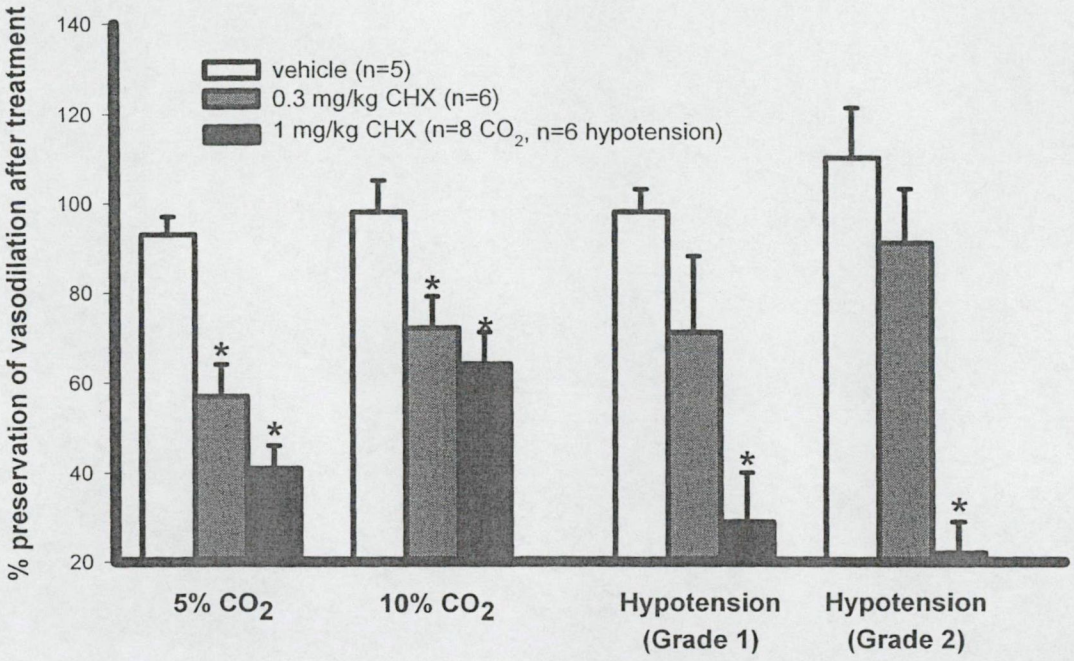


Figure 3. Effect of CHX treatment on COX-dependent vascular responses. Data are plotted as the percent preservation of vascular dilation to arterial hypercapnia and hypotension after CHX treatment.

In the vehicle treated control group pial arteriolar responses to inhalation of 5-10% CO_2 (5%CO_2 and 10% CO_2, respectively) were repeatable and unaltered. Similarly pial arteriolar responsiveness to decreased mean arterial pressure by 25 and 40%[Hypotension (Grade 1 and Grade 2, respectively)] were also fully preserved.

In contrast, treatment with 0.3-1 mg/kg CHX significantly reduced vasodilation to both doses of hypercapnia (*$p<0.05$, significantly different from control group). The attenuation of the vascular response to arterial hypotension was also elicited by CHX, especially the higher dose of CHX almost abolished arteriolar dilation (attenuation ~70-80%, *$p<0.05$, significantly different from control group).

Effect of CHX on cortical AA metabolism

In Group 22, topical application of AA onto the brain surface elicited a dose dependent increase in the concentrations of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ in the aCSF. Repeated application of AA resulted in similar changes in aCSF PG levels (Figure 4) CHX attenuated baseline as well as AA-stimulated $PGF_{2\alpha}$ levels as soon as 20 minutes following CHX administration. Baseline and AA-stimulated (1, 10, and 20 μg/mL) $PGF_{2\alpha}$ concentrations before *versus* 20 min after CHX were 3.94±0.55 *versus* 1.64±0.42, 5.10±0.76 *versus* 1.93±0.32, 14.28±3.04 *versus* 5.90±1.26 and 14.85±2.00 *versus* 6.74±1.11 ng/mL (n=9), respectively. The inhibition of $PGF_{2\alpha}$ synthesis lasted at least as long as 1 hour after CHX administration (Figure 4). Similarly, AA-stimulated 6-keto-$PGF1_{\alpha}$ levels were also significantly reduced 20 min after administration of CHX. Baseline and AA-stimulated (1, 10, and 20 μg/mL) 6-keto-$PGF_{1\alpha}$ concentrations before *versus* 20 min after CHX were 1.74±0.20 *versus* 1.51±0.37 (not significant), 2.29±0.23 *versus* 1.41±0.29, 5.65±0.60 *versus* 3.48±0.49 and 6.83±0.77 *versus* 3.90±0.48 ng/mL (n=7), respectively.

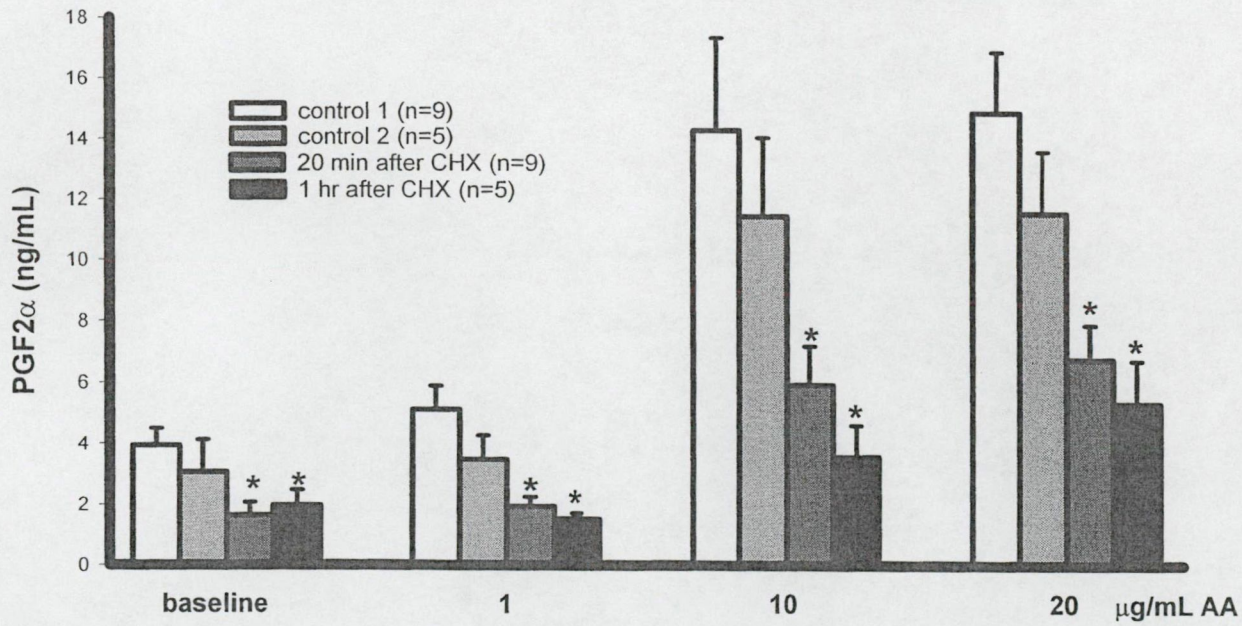


Figure 4. Effect of cycloheximide (CHX) treatment on conversion of exogenous arachidonic acid (AA) to $PGF_{2\alpha}$. Topical application of 1-20 μg/ml AA resulted in concentration-dependent, reproducible increases in prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) concentrations in the artificial cerebrospinal fluid (control 1 and control 2). In contrast, 20 min after CHX (1mg/kg,) treatment, baseline and AA-stimulated $PGF_{2\alpha}$ levels were significantly decreased compared to control values. At 1 hr after CHX treatment we obtained similar results, there was a trend towards further inhibition of $PGF_{2\alpha}$ synthesis. *$p<0.05$, significantly different from respective control values.

Discussion

The major findings of the studies were the following:

1 CD does not change pial arteriolar responsiveness to NMDA, and other vasodilatory stimuli such as hypercapnia, aprikalim, CGRP, or forskolin. Since neuronal-vascular reactivity is unaltered by CD, CD probably does not play a direct role *per se* in the attenuation of NMDA-induced vasodilation after anoxic stress accompanied by anoxic CD.

2 We have shown that activation of K_{ATP} or K_{Ca} by selective KCO-s for a short period immediately before combined H/I preserves pial arteriolar dilation to NMDA. In contrast to the KCO-s, CGRP and papaverine failed to preserve the NMDA vascular sequence after H/I.

3 We demonstrated that the selective mitoK_{ATP} opener DIAZ did not directly influence, but dose dependently preserved NMDA-induced pial arteriolar vasodilation after I/R in piglets. In fact, we presented evidence for the first time showing an *in vivo* protective effect of DIAZ after I/R in the central nervous system

4 We found that inhibitors of protein synthesis (Act-D and CHX) preserved NMDA-induced neuronal-vascular sequence after I/R, but they did not directly affect the vascular response in normoxic conditions.

5 We also demonstrated for the first time *in vivo* that CHX rapidly inhibits COX-activity in the piglet cerebral cortex. More specifically, cerebrovascular reactivity to COX-dependent vasodilatory stimuli like hypercapnia and arterial hypotension were diminished within 20 min of CHX administration, but COX-independent vasodilation to SNP remained unaffected. Similarly to COX-dependent vascular responses, cerebral cortical PG-synthesizing capacity from exogenous AA is largely reduced shortly after CHX treatment

NMDA-induced vasodilation is a complex neuronal-vascular function, and its physiological importance in CBF-metabolism coupling is poorly known. However, we believe that the findings of these studies yield new important information on the pathophysiology of neonatal neuronal injury after ischemic stress. Our approach to assess neuronal injury or protection was not based on traditional measures such as recovery of EEG activity or determination of infarct size. Rather we established NMDA-induced vasodilation

as an assay of short-term changes of neuronal function. NMDA-induced vasodilation can be regarded as a prototype of an ischemia-sensitive neuronal-vascular sequence. Preservation of this sequence after ischemia must involve spared function of many neurons, those bearing functional NMDA receptors, and nNOS+ neurons in mono- or multisynaptic connection with the former cells, that need to produce sufficient NO for the pial arteriolar response.

However, we need to address an important concern: can preservation of NMDA-receptor function be regarded as neuroprotective? In the last decade, glutamate-induced neuronal cell death e.g. excitotoxicity has been widely accepted as a major mechanism of cellular injury after anoxic stress. The excitotoxicity concept states that ischemia results in a single cascade of events consisting of 1) excessive synaptic glutamate release during I/R, 2) "overexcitation" of ionotropic glutamate receptors permeable to Na^+ and Ca^{2+}, and 3) development of AD, cellular swelling, and intracellular Ca^{2+} overload culminating in neuronal cell death. Three influential discoveries supplied the strongest evidence supporting the excitotoxicity hypothesis. 1) Extracellular glutamate increases during ischemia (Benveniste et al., 1984). 2) Glutamate receptor antagonists protect cultured neurons against both anoxia and glutamate-induced cell death (Rothman, 1984), (3) Glutamate receptor antagonists are also neuroprotective *in vivo* after ischemia (Simon et al., 1984). According to the excitotoxicity concept, preservation of NMDA receptor function in the reperfusion phase would contribute to neuronal injury.

We believe, however, that the classic excitotoxic concept may not apply to *in vivo* conditions necessarily since it conflicts with convincing experimental data. 1) Increased extracellular glutamate levels by glutamate uptake inhibitors do not enhance the development of AD in a global ischemia model in rats (Obrenovitch et al., 1998). 2) Also, NMDA receptor antagonists MK-801 and 2-amino-phosphonoheptanoate do not delay the development of AD (Marranes et al., 1989; Xie et al., 1995). 3) Efficacy of NMDA receptor antagonists to prevent neuronal death after I/R may not indicate the specific or unique involvement of these ligand-gated cation channels in the pathomechanism of injury. In fact, the neuroprotective capacity of different cation channel blockers appears to be redundant and interchangeable to some extent. For instance, NMDA receptor antagonists prevent neuronal cultures from injury induced by veratridine – a Na^+ channel activator (Pauwels et al., 1989). Reciprocally, excitotoxic injury was alleviated by voltage-gated Na^+ channel antagonist tetrodotoxin in hippocampal neurons (Ogure et al, 1988), and also by Ca^{2+}-channel antagonist in cultured retinal ganglion cells (Sucher et al., 1991).

Therefore, preservation of NMDA receptor activity by no means condemns the neurons to die by excitotoxic events. Instead, we believe that preservation of NMDA-induced vasodilation indicates: 1) functional protection of an important and complex ligand-gated cation channel, and 2) implies less ROS-inflicted cellular damage, and maybe better general neuronal viability after anoxic stress.

KCO-s preserve NMDA-induced vasodilation via actions on plasmolemmal and mitochondrial potassium channels

We observed significant increases in pial arteriolar diameters in response to local application of aprikalim and NS1619, indicating that the doses used were effective to induce vascular smooth muscle (VSM) hyperpolarization. Thus, the first obvious mechanism of the protective effect of these KCO-s was that not the specific activation of K^+ channels but the preischemic hyperemia was protective. This possible mechanism has been rejected based on our findings using alternative vasodilatory substances before I/R. Specifically, CGRP and papaverine although induced vasodilation comparable to KCO-s, were unable to preserve the NMDA vascular sequence after H/I. Interestingly, CGRP elicits vasodilation largely via activation of K_{ATP}. In addition, VSM components of NMDA-induced vasodilation are resistant to the effects of H/I. In summary, these results indicate that the protective effect of KCO-s is independent of their effect on VSM K^+ channels. Thus, the preserved NMDA-induced vasodilation must be attributed to actions of KCO-s on neuronal K^+ channels.

However, the third KCO, DIAZ was used in a dose that activates the mitoK_{ATP}-channels (1-10 μmol/L). We did not test directly if only mitoK_{ATP} were activated by DIAZ, but fortunately a good indication of selective activation could be the absence of significant vasodilation accompanied by drug application. The sarcolemmal potassium channels directly mediate the vasodilatory effect of KCO-s on cerebral arterioles. Administration of 5-10 μmol/L DIAZ elicited only 2-9% arteriolar dilation, and the response was transient, did not last for more than 1-2 min. In contrast, we found that the general K_{ATP} opener aprikalim (10 μmol/L) elicits ~60-70% increases in vascular diameters, and the vasodilation does not wane. Moreover, the dose-dependent effect of DIAZ on preservation of the NMDA-induced vasodilation after I/R was inhibited by a selective K_{ATP} antagonist 5-HD. 5-HD was found to be selective for mitoK_{ATP} channels at least in some experimental designs (Garlid et al., 1997; Liu et al., 1998; Sato et al., 1998). Also, in our experimental model 5-HD did not inhibit the vasodilation induced by aprikalim suggesting minor effects on plasmolemmal K_{ATP} channels.

These observations, together with those of the literature make us conclude that the protective effect of DIAZ on neuronal-vascular function after I/R is most probably mediated by activation of mitoK_{ATP}-s. The mechanism by which activation of mitoK_{ATP}-s may lead to increased resistance to I/R remains to be clarified. The duration of global cerebral ischemia (10 min) used in the present study has been thought to cause only reversible mitochondrial alterations, mitochondria have been shown to recover full function in one-two hours after reperfusion (Rechncrona et al., 1979; Sims, 1991). Thus, the attenuation of the NMDA-mediated cerebral arteriolar response is not likely due to energy failure by inhibited mitochondrial function. This notion also supported by the resistance of kainate-induced vasodilation to I/R (Bari et al., 1997b).

All available evidence indicates the involvement of ROS in the attenuation of NMDA-induced vasodilation, thus reduction of ROS production by KCO-s appears to be a feasible mechanism. Based on our findings we believe the protection employed by KCO-s may be mediated via 2 different sets of potassium channels, at plasmolemmal and/or mitochondrial sites. Activation of plasmolemmal ligand-gated K^+ channels (K_{ATP}, K_{Ca}) results in potassium efflux and membrane hyperpolarization approaching the equilibrium potential for potassium. Unfortunately, we do not possess direct evidence that could link the activation of K^+ channels to decreased ROS production in our experimental model.

However, we do have circumstantial evidence, that the protective effect of KCO-s may not be explained simply by their action on the membrane potential. We demonstrated, that CD, e.g. loss of membrane potential in the cortical neurons does not attenuate NMDA-induced vasodilation if cerebral blood flow and metabolism is not compromised. Therefore, KCO-s cannot simply preserve responsiveness because the neurons do not depolarize during ischemia. Neuronal hyperpolarization by KCO-s may prevent events that are subsequent to anoxic depolarization and result in ROS production in the early reperfusion phase. One major factor is the accumulation of free cytosolic Ca^{2+} in the depolarized neurons. The influx of Ca^{2+} through voltage gated channels or ionotropic glutamate receptors may be sufficiently reduced by KCO-s.

Elevated intracellular Ca^{2+} levels contribute to ROS production at least through 2 mechanisms. First, Ca^{2+} activates phospholipaseA_2 that yields AA during ischemia to be processed by COX during the reperfusion phase. This mechanism appears to be the most important source of extracellularly detected ROS in the piglet (Armstead et al., 1988,1989). Second, cytosolic Ca^{2+} may enter and accumulate in mitochondria disrupting the electron transport chain leading to ROS production as described below in detail.

Activation of mitochondrial K_{ATP} channels (mitoK_{ATP}) may represent another possibility how KCO-s affect intracellular calcium homeostasis. MitoK_{ATP}-s have been found in the inner membrane of mitochondria (Inoue et al., 1991), and represent a pharmacologically distinct population of K_{ATP} (Garlid et al., 1996a). MitoK_{ATP}-s appear to control the activity of the electron transport chain via regulating mitochondrial matrix volume by regulated K^+ uptake. In isolated mitochondria, KCO-s induce slight swelling, partially dissipate the transmembrane potential ($\Delta\Psi$, negative inside) but increase the activity of electron transport chain, and hence the chemical proton gradient (ΔpH, alkaline inside); thus the total the protonmotive force hardly changes (Garlid, 1996b; Halestrap, 1994; Szewcyk et al., 1996; Holmuhamedov et al., 1998). However, Ca^{2+} uptake depends selectively on $\Delta\Psi$. Mitochondria readily uptake Ca^{2+} when intracellular levels increase above a so-called mitochondrial "buffer" concentration. Ca^{2+} is transported through the mitochondrial inner membrane via the electrogenic Ca^{2+} uniporter down its electrochemical gradient, thus the rate of this transport is solely dependent upon $\Delta\Psi$ (Gunter et al., 1994). Opening of mitoK_{ATP}-s should decrease mitochondrial Ca^{2+} uptake by decreasing $\Delta\Psi$, and KCO-s do induce release of Ca^{2+} from Ca^{2+}-preloaded mitochondria *in vitro* (Holmuhamedov et al., 1998). Mitochondrial Ca^{2+} overload substantially influences the recovery of mitochondrial function following ischemic stress: for example increased mitochondrial Ca^{2+} sequestration has been demonstrated to increase production of ROS (Dykens, 1994; Dugan et al., 1995). In conclusion, we carefully hypothesize, that activation of mitoK_{ATP}-s by DIAZ may prevent mitochondrial overload and subsequent ROS production resulting in preserved NMDA-induced vasodilation after I/R (Figure).

Protein synthesis inhibitors preserve NMDA-induced vasodilation by rapid inhibition of cyclooxygenase expression

Attenuation of the NMDA-induced vascular sequence is thought to be elicited by ROS-induced alterations of the NMDA receptor positive neurons, or the NMDA receptor complex itself (Hoffman et al., 1994; Marro et al., 1998). Recovery of the NMDA-induced vascular sequence thus may involve repair and resynthesis of protein structures involved in this sequence that are damaged by ROS. Therefore our finding that protein synthesis inhibitors Act-D and CHX are preserving this sequence after I/R is somewhat surprising. However, we have several reasons to believe that this effect is indeed mediated by specific effects of these drugs (Act-D and CHX) on transcription and translation inhibition,

respectively. (1) The doses used were proved to be effective in other systems (Pavlik and Teisinger, 1980), and (2) the effect on preservation of NMDA-induced vasodilation was dose-dependent. Further, (3) Act-D and CHX are structurally different and also target different sites of protein synthesis. Finally, (4) neither Act-D nor CHX affected NMDA-induced vasodilation directly, thus the preservation of this response after I/R is not due to some non-specific facilitation.

Similarly to our results of short-term preservation of neuronal-vascular function, other studies concluded that pretreatment with protein synthesis inhibitors results in long-term protection such as inhibition of delayed cell death in the CA1 region of the hippocampus (Goto et al., 1990; Papas et al., 1992), and decreased infarction volume after transient focal ischemia (Aronowski et al., 1997; Du et al., 1996). Therefore, selected genes do get expressed following H/I that may exaggerate the neuronal damage. Several potential neuroprotective mechanisms have been proposed concerning the actions of CHX pretreatment in reducing neuronal death after H/I stress. These include (1) CHX-induced mild hypothermia, (2) inhibition of apoptosis, and (3) suppression of the postischemic induction of an unidentified "noxious/killer protein". The former two possibilities are not feasible in our model since (1) mild systemic hypothermia does not preserve NMDA-induced vasodilation after I/R (Perciaccante et al., 2000), and (2) the time frame of our experiments is too short to develop apoptosis. At present we cannot exclude the rapid postischemic appearance of noxious proteins. However, we propose a fourth, previously not considered mechanism, that CHX pretreatment would reduce activity of potentially harmful proteins (like COX) during the pretreatment period before the onset of ischemic stress.

Previous evidence strongly indicated the role of COX-derived ROS in the pathomechanism of attenuated NMDA-induced vasodilation. COX is thought to be rapidly inactivated by self-produced superoxide anions, such that in an active system COX has a half-life not more than 5-10 min (Egan et al., 1981). This suggests that maintaining active COX levels would require continuous *de novo* enzyme synthesis. These facts offered an attractive hypothesis to explain the protective effect of protein synthesis inhibitors on NMDA-induced vasodilation after I/R. Namely, protein synthesis inhibitors rapidly decrease bioavailability of COX during the pretreatment period resulting in less ROS production during I/R. *Ex vivo* experimental data are in support of this theory, since CHX does inhibit baseline and AA-stimulated PGE_2 and prostacyclin production in brain, spleen and muscle slices obtained from rats (Fagan and Goldberg, 1986). CHX also blocked $PGF_{2\alpha}$ production induced by interleukin-1α in cultured piglet and sheep astrocytes within 20 min (Nam et al., 1995a,

1995b). We wanted to test this hypothesis *in vivo* by assessing COX activity rather than quantitative determination of COX protein levels. For instance, immunoblotting of COX after CHX treatment would probably have not yielded additional support for this theory. Since COX is rapidly inactivated and degraded, functional and immunoreactive COX levels may not be equivalent.

Changes in cortical COX activity after CHX treatment were assessed via 2 independent techniques: (1) COX-dependent vascular reactivity, and (2) cortical AA metabolism. Previous evidence suggests that the prostaglandins required for arteriolar vasodilation to hypercapnia and arterial hypotension are synthesized in the vascular endothelium (Leffler and Busija, 1987; Leffler et al., 1994a). The presented data suggested, that CHX treatment likely affected vascular endothelial cells shown by the attenuation of vascular responses to hypercapnia and arterial hypotension. We also found, that 6-keto-$PGF1_{\alpha}$ levels were reduced in the aCSF after CHX treatment, indicating decreased prostacyclin synthesis, further confirming the effect of CHX on cerebrovascular endothelial cells. In contrast, the reduced baseline and AA-stimulated $PGF_{2\alpha}$ levels may represent a more general inhibitory effect of CHX on COX synthesis in both neural and vascular cells.

At least 2 distinct isoforms of COX exist (COX-1,-2) (DeWitt, 1991). Originally COX-1 was considered the constitutively expressed isoform, and COX-2 was designated as the inducible isoform. However, in the brain and cerebral blood vessels of newborn pigs, COX-2 but not COX-1 has been identified as the major constitutively expressed isoform (Peri et al., 1995; Dégi et al., 1998a). COX-2 is an immediate early gene, and is readily inducible by a wide variety of stimuli including ischemic stress in piglet cerebral cortex and blood vessels (Busija et al., 1996b; Dégi et al., 1998b), and in adult brains including gerbils, rats, and humans (Ohtsuki et al., 1996; Planas et al. 1995; Sairanen et al., 1998). There is a substantial increase in porcine cortical and vascular COX-2 mRNA levels as soon as 2 hrs following ischemic stress, and COX-2 immunoreactivity is also increased within 8 hrs of cerebral ischemia (Dégi et al., 1998b; Domoki et al., 1999). However, the short half-life and extremely rapid turnover rate of the COX enzyme may conceal an even more dramatic change in COX expression after ischemic stress. The increased expression of COX-2 may participate in the brain pathology after ischemic stress by increasing the production of oxygen radicals and inflammatory prostanoids. This is an interesting possibility, since overall protein synthesis is assumed to be inhibited even by short periods of cerebral ischemia and reperfusion (Krause and Tiffany, 1993). However, translation of some other immediate early gene mRNA-s including heat shock proteins and proto-oncogenes appear to be increased

rapidly following ischemic stress in contrast to the generally depressed protein synthesis (Koistinaho and Hökfelt, 1997). It is quite conceivable that after cerebral ischemia when protein synthesis is generally inhibited, 1mg/kg CHX has a greater inhibition on COX synthesis than we have shown with the approaches used in our present study under normoxic conditions.

The most likely mechanism of how CHX inhibits COX-activity in our experimental model is through its inhibitory effect on translation of proteins. The effect of CHX and other protein synthesis inhibitors were found to be proportional to their effect on general protein synthesis inhibition (Fagan et al., 1986). In the present study, however, we cannot exclude the remote possibility that CHX could affect other proteins as well that may modulate COX-activity. But the potent, rapid decrease in COX-activity after CHX treatment explains the preservation of NMDA-induced vasodilation after I/R. According to our results, preservation of the NMDA-induced vascular sequence occurs probably via inhibited ROS production of the piglet cerebral cortex by inhibition of COX expression. Our present data thus reveal that pretreatment with protein synthesis inhibitors can result in not only inhibiting the appearance of a "noxious/killer protein" after ischemia, but also in the rapid disappearance of a potentially harmful albeit continuously expressed protein: COX (Figure 5). At present we do not know whether this effect of CHX pretreatment may account for the neuroprotection observed in other studies. Nevertheless, early events like ROS-induced injury seem to determine the fate of brain tissue affected by I/R.

Summary

In conclusion, our studies yielded additional evidence that NMDA-induced vasodilation is vulnerable to hypoxic/ischemic stress. The mechanism of this injury appears to be principally inflicted by ROS, simple depolarization of cortical neurons in the absence of ischemia does not affect this unique neuronal-vascular sequence. Therefore, assessment of cerebrovascular reactivity to NMDA provides a sensitive assay to investigate ROS-induced acute neuronal damage that reflects rather a metabolic than a classical electrophysiological function. We were able to demonstrate that specific activators of ligand-gated potassium channels and protein synthesis inhibitors preserved NMDA-induced pial arteriolar vasodilation when administered prior to I/R. The mechanism of preservation by KCO-s is unknown. However, we speculate that the KCO-s act on both plasmolemmal and mitochondrial channels, and the principal effect may be the modulation of calcium ion flux between the extracellular space

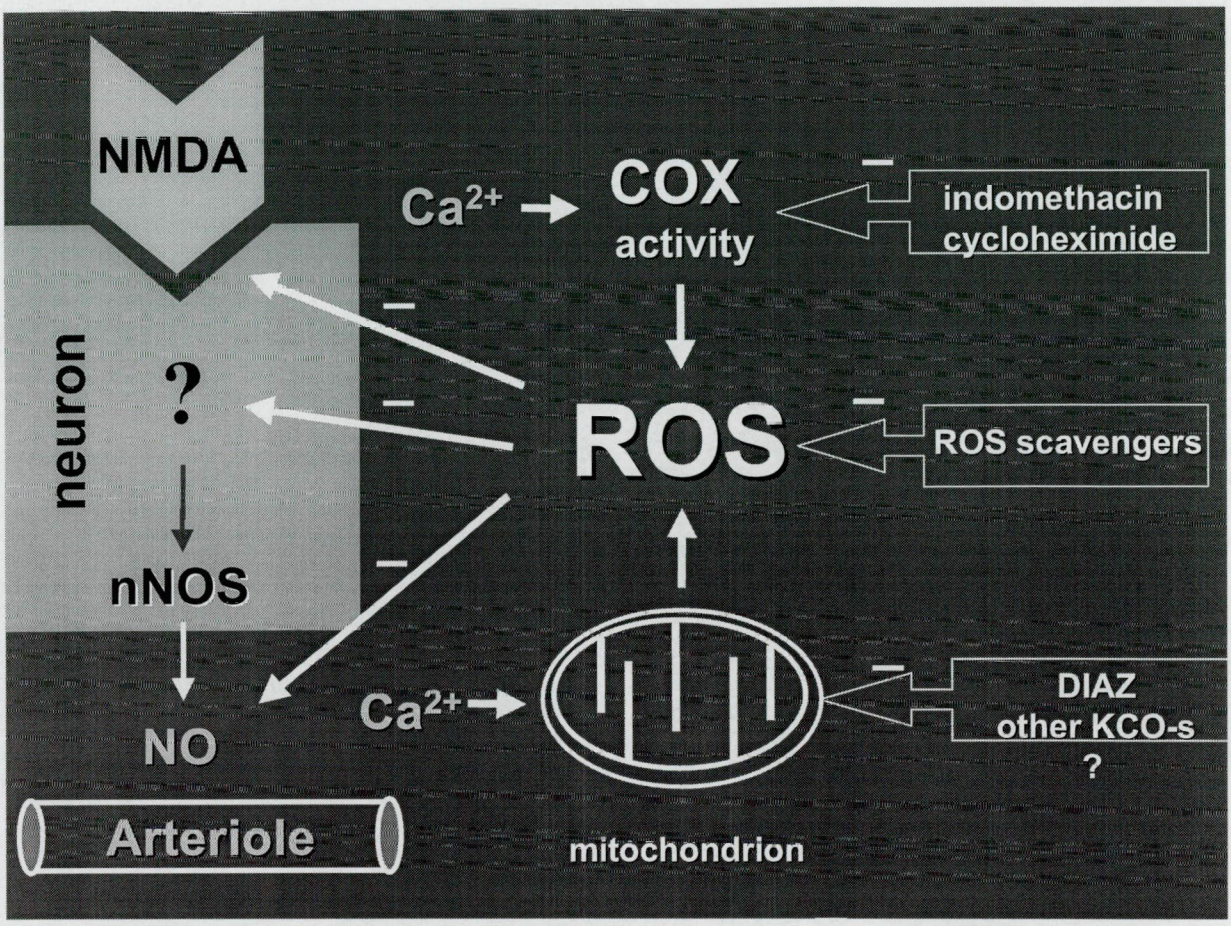


Figure 5. Preservation of N-methyl-D-aspartate (NMDA)-induced pial arteriolar vasodilation after ischemia/reperfusion (I/R). NMDA activates neuronal NMDA receptors, and results in activation of neuronal nitric oxide (NO) synthase (nNOS) and subsequent NO release, diffusion and action on pial arterioles in a multi-step process. Reactive oxygen species (ROS) produced by mitochondria and cyclooxygenase (COX) during reperfusion seem to play a pivotal role in attenuating the NMDA-induced vasodilation. Both sources of ROS are stimulated by increases in cytosolic and mitochondrial Ca^{2+} concentrations, respectively. ROS may target the NMDA receptor complex, yet unknown neuronal processes (?), or NO itself via peroxynitrate formation. In accordance with this hypothesis, increased ROS elimination by ROS scavengers, or decreased ROS production by cyclooxygenase (COX) inhibitor indomethacin, or protein (including COX) synthesis inhibitor cycloheximide shortly prior to I/R results in preserved vascular responsiveness to NMDA. NMDA-induced dilation is also preserved by pretreatment with diazoxide (DIAZ), a selective activator of mitochondrial ATP-sensitive potassium channels ($mitoK_{ATP}$), and other K^+ channel openers (KCO). Activation of $mitoK_{ATP}$ may reduce generation of ROS in the reperfusion phase by reducing mitochondrial Ca^{2+} sequestration. Other KCO-s may also activate mitochondrial K^+ channels or prevent increases in intracellular Ca^{2+} concentrations by hyperpolarization. Reduction in either COX-derived or mitochondrial ROS production appears to be sufficient to preserve the vascular response to NMDA after I/R

and the cytosol, as well as the cytosol and mitochondrial matrix. The mechanism how protein synthesis inhibition preserved neuronal-vascular responsiveness appears to occur via inhibition of COX-derived ROS production. Treatment with CHX that effectively preserved NMDA-induced vasodilation also was shown to inhibit COX-activity within the pretreatment period similarly to indomethacin. Indeed, according to our knowledge we demonstrated for the first time that CHX rapidly inhibits COX-activity *in vivo* in the cerebral cortex.

Our results with KCO-s and protein synthesis inhibitors eliciting pharmacological preservation of this sequence indicate mechanisms that reduce acute oxidative injury. These drugs may also reveal new targets of pharmacotherapy against neuronal injury in neonates.

Acknowledgments

I would like to devote this section to all the wonderful people I was fortunate to meet when working on the matter of this thesis.

I thank Dr. Ferenc Bari for introducing me to the subject of Physiology as my lab and seminar teacher ten years ago. The teacher-student relationship between us has gradually changed when I started to work first as a student in your laboratory. You taught me basic surgical skills of doing *in vivo* experiments, designing elegant and useful protocols. Your devotion captured my interest in the study of circulatory physiology. Your helpful comments were substantial for the preparation of manuscripts that are forming the basis of the present thesis. Finally, I would like to thank you for the professional and friendly atmosphere you created in your laboratory that makes this lab the most attractive place to work in.

I deeply thank Prof. David W. Busija for allowing me to work in your lab at the Department of Physiology and Pharmacology, Wake Forest University, Winston-Salem, North Carolina, USA. You introduced me to the study of neonatal cerebrovascular physiology, and showed me your quest to do excellent science. Our daily discussions provided guidance and encouragement for the continuation of experiments that emanated in the results discussed in the present thesis. I also thank you and your family for caring to make my and my family's stay in the Unites States as pleasant as possible.

I respectfully thank Prof. Gábor Jancsó for his support throughout my student years and scientific career. Prof. Jancsó steered my interest towards the study of the cerebral circulation, and kindly introduced me to various techniques to study the blood-brain barrier. His insightful comments were also helpful during the preparation of the present thesis.

I thank Prof. György Benedek, the chairman of the Department of Physiology, Faculty of Medicine, University of Szeged, for providing me support for my scientific activities both at my home institution and in the United States.

I want to say thanks to my friends and colleagues: Dr. Krisztina Boros, Nishadi Thrikawala, Dr. Janusz Osada, Dr. Péter Sántha, Dr. Roland Veltkamp, Dr. James V. Perciaccante, Gregory Robins, and Rick Parker. I thank all of you for your help and support all these years. You made me want to be a better scientist and a better person. Thanks!

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