Molecular Mechanisms of Hypoxic-Ischemic Encephalopathy in Newborn Piglets

Ph.D. Thesis

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INTRODUCTION

Perinatal asphyxia (PA) is the medical condition elicited by the impairment of exchange of the respiratory gases – resulting hypoxemia and hypercapnia – during birth to a newborn infant that lasts long enough to cause damage to the infant's organs, including the heart, lungs, liver, guts, kidneys and the brain. It affects 1 to 6 newborns per 1000 full-term live births and represents one of the most common causes of neonatal death.

The hypoxic-ischemic encephalopathy (HIE) is a sub-set of cases of neonatal encephalopathy with a good evidence of a recent hypoxic-ischemic cause (including but not limited to PA), characterized as a clinically defined syndrome of disturbed neurological function in the earliest days of life in the term infant, manifested by difficulty with initiating and maintaining respiration, alterations in muscle of tone and reflexes, sleep-wakefulness cycle disorders including coma, and often by epileptic seizures. The prognosis depends on the severity of the PA. HIE cases can be graded into 3 clinical stages: mild, moderate and severe encephalopathy according to the Sarnat scoring system. Whereas a few infants survive even severe HIE without handicap, many of them develop life-long complications: 80% of dyskinetic cerebral palsy can be associated to PA at term, furthermore, hearing and visual loss, learning difficulties, memory impairments, attention deficits, hyperactivity, increased risk of schizophrenia are reported in children following PA/HIE.

Although most of the detailed epidemiological studies are based on data of high-income developed countries, unquestionably the burden of this disease is the most severe in the developing countries. Lack of modern healthcare results in 8 times higher mortality in low income countries than in the developed regions.

We need to strive for an effective, reliable and inexpensive therapeutic intervention to alleviate the threats of PA/HIE.

Currently, therapeutic whole-body hypothermia is the accepted treatment of PA/HIE patients, however, hypothermia cannot offer full neuroprotection and may result in unwanted effects such as bradycardia, hypotension, and thrombogenesis. The treatment requires a quite expensive cooling/heating equipment, as the cooling-rewarming procedure should be tightly controlled and therefore it is unavailable in many underprivileged medical institutions. Therefore, further studies aim to establish an alternative/ completive therapy, such as ventilation with neuroprotective gases.

We chose molecular hydrogen (H_2) as a putative neuroprotective agent to combat PA/HIE based on the Nature report of Ohsawa et al., they found H_2 an *ex vivo* antioxidant and

an *in vivo* neuroprotectant in an adult stroke rat model. H_2 is a small membrane-soluble gas molecule, therefore inhaled H_2 can easily penetrate the blood-brain barrier. Indeed, H_2 inhalation has been successfully used for measuring cerebral blood flow (the hydrogen clearance technique) since the 1960's. The optimally neuroprotective 2% H_2 concentration also used in our studies is expected to result in approximately 10-20 μmol/l brain H_2 levels in rats and humans alike. The neuroprotective effect of H_2 may be partly based on its antioxidant nature: it has been reported that H_2 selectively eliminates free radicals such as $OH^{\cdot}$ and ONOO, furthermore, H_2 is reported to also have a "mitohormetic" effect, increasing the expression of anti-oxidative enzymes underlying the Nrf2-pathway, but there can be further yet unknown pharmacological targets. H_2 is readily available, inexpensive and easy to apply, in neuroprotective concentrations not flammable, and human tolerance studies also prove its applicability.

To study the pathophysiology of PA/HIE, and to test neuroprotective strategies, our Experimental Neonatology Research Group developed newborn pig PA/HIE models. The newborn piglet is an accepted large animal model of the term human neonates as the brain of the newborn piglet has similarly gyrencephalic structure, its developmental state and metabolic rate are matching around birth in the two species.

Our group performed previously two sets of subacute (24 h survival) experiments to study the neuroprotective potential of H_2 against PA/HIE in term newborn piglets: in the first study PA was induced with 8-minute long trachea-occlusion, in the second study with 20-minute long ventilation of a hypoxic-hypercapnic gas mixture, completed with respective time control and H_2 treated asphyxia groups.

During the whole experiment the physiological parameters (heart rate, arterial blood pressure, O_2 saturation, core temperature), blood gas and blood glucose levels at baseline were always in the physiological range in all animals, except the duration of asphyxia. Our meta-analysis showed that by the end of the asphyxia, acidosis, hypercapnia, and hyperglycemia was more severe in the 20-min long PA model. Hematoxylin-eosin stained sections revealed significantly more severe cerebrocortical lesion in the 20-min long asphyxiated animals. In a similar fashion, more severe neuronal injury was observed in the hippocampus and in the basal ganglia as well. In summary, we can state that 8 min trachea-occlusion elicited mild-moderate whereas 20 min asphyxia resulted moderate-severe HIE. Thus, the 20-min long PA was more appropriate to demonstrate the neuroprotective potential of H_2.

Cyclooxygenases (COXs) are the rate-limiting enzymes catalysing the first committing step of prostaglandin secretion. There have been at least two COX isoforms described in the

central nervous system, COX-1 and COX-2. They are both membrane-associated enzymes and they maintain 63% homology in their amino acid sequence. Although they have similar catalytic activity, they differ in their pharmacological properties, as well as in cellular and tissue distribution. Both COX-1 and COX-2 are constitutively expressed in the central nervous system, furthermore, COX-2 proved to be the dominant isoform of COXs in the neonatal brain, providing up to 80% of the total brain COX activity. Whereas COX-1 is constitutively expressed in most brain regions, COX-2 is especially enriched in the hippocampus and the cortex. COX-2 plays a role in the modulation of neuronal activity and the neurovascular coupling. Additionally, expression of COX-2 is well-known to be upregulated by various brain disorders including brain trauma, cerebral ischemia, and proinflammatory insults. Several studies have shown the efficacy of pharmacological COX-2 inhibition to limit neuronal injury, for instance the decrease of stroke volume after experimental ischemia.

During prostaglandin production, reactive oxygen species (ROS) are co-released by COX-2, which are eliminated by endogenous antioxidants under physiological conditions. Neonates are particularly sensitive for oxidative stress by ROS due their organs' structural and functional immaturity, the overloading of aerobic metabolism with rapidly growing energy demand, the reduced ability to induce efficient homeostatic mechanisms, as well as the lack of effective antioxidant systems that mature during the first year of life in human neonates.

COX-2 has an undeniable role in the pathogenesis of HIE. A study showed that the administration of COX-2 inhibitors provided neuroprotection in a rat HIE model. Additionally, human neonatal brain ischemia has been also linked to high level of neuronal COX-2 expression by a human autopsy study. Therefore, the role of COX-2 in the pathomechanism of HIE could be best investigated in a translational large animal model that could also be used to establish neuroprotective treatments.

COX-2 has been extensively monitored in newborn piglets as well. It has been shown that anoxic stress increases COX-2 but not COX-1 mRNA and protein levels both in the cerebral vascular endothelium as well as in the cortical and hippocampal neurons, meanwhile this elevation was not successfully evoked by asphyxia within 2-8 hours of survival. The regional distribution of COX-2 immunopositive neurons in piglet brain was already described by our group, however within 4 hours following trachea-occlusion no change in COX-2 immunopositivity occurred. This study raised the question about whether a more effective asphyxia induction or longer time window of observation was needed.

Neuroinflammation likely plays an important role in the pathomechanism of neuronal injury during PA/HIE, and the role of microglial cells appears to be crucial, However, microglia have dual, contrasting roles in neuroinflammation. The microglial activation and effect is influenced by the mechanism, location, and severity of the injury and the age at which the injury occurs, Previous reports about microglial activation in the piglet model of PA/HIE were inconsistent, therefore it is necessary to describe the microglial activation in our recent PA/HIE model.

While PA/HIE causes severe neuronal damage, many, sometimes the majority of the neurons still survive the insult. As the antiapoptotic mechanisms work in the favour of preserving the neurons, therefore their stimulation can be valid potential targets of neuroprotective strategies.

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophic factor family and plays an important role in neuronal development and maturation in the central nervous system. The neuroprotective effects of BDNF are mediated by the mitogen-activated protein kinase (MAPK) or phosphatidylinositide-3-kinase (PI-3-K) pathway. The activation of these pathways can be described with the activation – phosphorylation – level of protein kinase-B (Akt) and signal related kinase (ERK). Intracerebroventricular administration of BDNF to postnatal day 7 rats elevated the phosphorylation level of ERK and Akt kinases. Pharmacological inhibition of ERK increased the tissue loss after the hypoxic-ischemic insult in rodents, however little is known about the activation of these pathways in a newborn large animal model after hypoxic-ischemic injury.

To better describe our model, and also to tackle some of the cellular events of the developing HIE, our aims were to answer the following questions:

1.) Regional differences in neuronal COX-2 expression have been previously reported. We therefore asked if the 24 h observation of the anesthetized animals would *per se* influence the percentage of COX-2 immunopositive neurons?
2.) Does PA induce neuronal COX-2 after 24 hours of survival? Are there any regional differences in the response to PA?
3.) Is there any correlation between the percentage of COX-2 immunopositive neurons and the severity of the neuronal lesion?
4.) Is there any correlation between the percentage of COX-2 immunopositive neurons and the oxidative damage of neurons?

5.) Can microglial activation be demonstrated in our PA/HIE model? Is there any correlation between the percentage of COX-2 immunopositive neurons and the degree of microglial activation?

6.) Can the neuroprotective H_2 treatment interfere with the effect of PA on the percentage of COX-2 immunopositive neurons or affect the microglial response?

7.) Can the activation of the Akt and ERK play an important role in neuronal survival in 24-48 hours after PA?

MATERIALS AND METHODS

All procedures were approved by the Animal Care and Use Committee of the University of Szeged. Animal care and handling were in accordance with the National Institutes of Health guidelines.

1. Analysis of neuronal damage and neuroinflammation with immunohistochemistry

For immunohistochemistry, we used the brain samples of newborn male Large-White piglets (age < 24 h at beginning of experiments, body weight: 1.5-2.5 kg, n=47) obtained mainly from the previous studies. The major difference between the two studies was the induction method and the duration of PA: asphyxia was elicited by 8 minutes of trachea-occlusion and suspended ventilation in the first, and by 20-minute-long ventilation with hypoxic-hypercapnic gas mixture (6% O_2, 20% CO_2) in the second study. In both studies, the asphyxia group was complimented with time control and H_2-treated asphyxia groups (n=7-7). A naïve group of animals was (n=5) also added: animals were sedated with sodium thiopental (45 mg/kg, ip.) and their brain were perfused with physiological saline through the catheterized common carotid arteries, then harvested for further experiments. Before histological experiments, the brains were immersion-fixed with 4%, 4°C paraformaldehyde for 2 weeks. The tissues were paraffin-embedded and 4 μm slices were prepared. To determine neuronal lesion, we performed hematoxylin-eosin staining followed by cell counting or neuropathological scoring.

Immunohistochemistry was performed with automatized immunostainer. Slides were digitalized. We determined the ratio of COX-2 immunopositive neurons with cell counting in the frontal, parietal, temporal and occipital cortices, as well as in the hippocampal CA1, CA3 regions and dentate gyrus, in the thalamus, basal ganglia and among cerebellar Purkinje-cells. We also counted the number of 8-hydroxi-2'-deoxyguanozine (8-OHdG) immunopositive neuronal nuclei in the parietal cortex.

We also investigated the microglial activation in the parietal cortex, characterized with the so-called ramification index, which considers the number of Iba-1 positive microglial cell bodies within a grid and the microglial processes crossing the gridlines. As the activated microglia can be identified with reduced processes and amoeboid-like shape, the lower the ramification index is, the more activated the microglia are.

2. Investigation of neuroprotective signalling pathways with Western blot analysis

We examined the activation of Akt and ERK signalling pathways at 24 and 48 hours following 20-min asphyxia in cortical and subcortical brain regions as well, then compared the values to time control and naïve animals (n=12). The total and phosphorylated protein levels were determined with Western blot analysis.

To double-check our results, we topically administered specific ERK and Akt kinase inhibitors: U0126 and A-6730, respectively (n=4-4).

RESULTS

1. The effect of anesthesia on neuronal COX-2 expression

Neuronal COX-2 expression showed marked regional differences: in the naïve animals the highest percentage of COX-2 positive neurons could be observed in the frontal and parietal cortices similar to our previous results in time controls at 4 hours of survival, as described previously. However, this regional expression pattern appeared markedly changed in the 24h time controls, as the COX-2 expression was significantly reduced in all neocortical regions compared to naïve or 4h survival animals. This reduction was found limited to the neocortex: the ratio of COX-2 immunopositive neurons remained unchanged in the hippocampus.

2. Effect of asphyxia and H_2 ventilation on neuronal COX-2 expression

In the brain regions obtained from the study of 8-minute-long asphyxia, there was no significant alteration in neuronal COX-2 expression as no difference was noted between the time control and asphyxiated groups in any of the observed regions. In contrast, 20-minute-long asphyxia elicited significant increases in neuronal COX-2 immunopositivity in the parietal and occipital cortices, as well as in the hippocampal CA3 region. Furthermore, a tendency of asphyxia-induced elevation in COX-2 immunopositive neurons could also be observed in the frontal and temporal cortices and in the basal ganglia too, albeit these changes did not reach statistical significance in these regions. In the H_2-treated group, despite exposure to the same level of asphyxia, the ratio of COX-2 immunopositive cells was similar to the time control group. In contrast to the CA3 subfield, the hippocampal CA1 region and the dentate gyrus displayed low percentage of COX-2 immunopositive neurons in time controls, and the ratio of immunopositive neurons were unchanged by asphyxia, they were similar in all three groups. In a similar fashion, COX-2 was present in about 30% of the cerebellar Purkinje cells in all experimental groups.

Notably, strong COX-2 immunopositive areas were only recorded in the asphyxia group of animals.

3. Assessment of correlation between neuronal COX-2 expression and neuronal damage

As neuronal COX-2 expression was affected by 20-minute-long asphyxia only, we performed all subsequent studies on these animals. We first investigated whether the ratio of COX-2 immunopositive neurons correlated with the previously determined neuropathology scores, higher scores reflecting more severe neuronal damage in the cortex. We found no correlation between the neuropathology scores and neuronal COX-2 expression; however, we could clearly identify 3 expression patterns: low neuropathology scores were always associated with low-moderate levels of neuronal COX-2 expressions; high neuropathology scores were associated either with high or with low COX-2 expression levels. Notably, low neuropathology score never coincided with high COX-2 expression level.

4. Correlation between neuronal COX-2 expression and oxidative DNA damage

In the parietal cortex, 20-minute-long asphyxia significantly increased the ratio of 8-OHdG immunopositive neuronal nuclei compared to both the time control and the H_2-treated asphyxia group indicating oxidative stress induced by asphyxia and the mitigating effect of molecular H_2. By assessing the correlation between the ratios of COX-2 and 8-OHdG immunopositive neurons, we found a marked tendency, either considering data from all groups or from the 20 min asphyxia group alone.

5. Microglial activation and its correlation with COX-2 expression

The Iba-1 immunohistochemistry visualized the distribution and morphology of microglia in the parietal cortex. Microglial activation is associated with reduced branching and assuming an amoeboid shape, and this change was quantified by ramification index determination. We found that ramification index was significantly lower – indicating microglial activation - in the group subjected to 20 min asphyxia, than in the time control group. However, there was no statistical difference between the time controls and the H_2-treated asphyxiated animals. When considering all data points, RI showed no correlation with neuronal COX-2 expression, but when regarding exclusively the 20 min asphyxia group significant negative correlation was observed.

6. Activation of Akt and ERK signalling pathways

The Akt and ERK kisases showed high activational state in the frontoparietal cortex considering the naïve, the time control and asphyxia grops as well, following the asphyxia with 24 or 48 hours too.

We successfully decreased the Akt and ERK phosphorylation with topical administration of specific inhibitors, compared to the contralateral untreated side of the cortex.

The Akt and ERK activation was similarly high in hippocampal and subcortical brain samples, as in the frontoparietal cortex in all experimental groups.

DISCUSSION

The major findings of the present study are as follows:

1) region-specific neuronal COX-2 expression in the neocortical areas is maintained at 4 hours of survival, but greatly reduced in 24-h anaesthetized time controls;

2) 20-min, but not 8-min long PA can significantly elevate the number of COX-2 positive neurons in the neocortex and the hippocampal CA3 subfield but not in the assessed subcortical areas or other hippocampal structures;

3) high ratios of COX-2 immunopositive neurons are always associated with severe neuronal lesion;

4) the ratio of COX-2 expressing neurons correlates with the oxidative damage of the neurons in the parietal cortex;

5) microglial activation can be detected in our HIE model already after 24 hours of survival in the parietal cortex, and the ratio of COX-2 immunopositive neurons correlates with microglial activation;

6) the neuroprotective H_2 administration prevents the upregulation of neuronal COX-2 expression in all sensitive brain regions, reduces oxidative damage, and alleviates microglial activation after PA;

7) both ERK and Akt are constitutively phosphorylated/ active in the cerebral cortex and all other assessed brain regions of newborn pigs irrespective of the length of anaesthesia and/or exposure to asphyxia; meanwhile we proved that the high cerebrocortical ERK and Akt phosphorylation levels under normoxic conditions can be *in vivo* modulated by specific inhibitors.

We are the first to describe the induction of neuronal COX-2 expression following asphyxia in a translational subacute piglet PA/HIE model. Previously, we extensively characterized the effect of 20-min asphyxia elicited in newborn piglets by ventilation with a hypoxic-hypercapnic gas mixture on hemodynamics, blood gases, metabolites, electroencephalogram and neuropathology. The applied insult resulted in alterations that matched both human pathology and corresponded to moderate to severe HIE. Our present results elucidated the confounding results from previous studies in which elevations in COX-2 levels were reported after 10 min of global cerebral ischemia but not 10 min of asphyxia. Our current results suggest that this reported difference was due to the more severe hypoxic/ischemic insult elicited by global cerebral ischemia, and in the present study, the longer

asphyxia duration rather than the shorter treatment elicited conditions similar to those observed with global ischemia, which resulted in the upregulation of COX-2.

In the present study, neuronal COX-2 abundance was conspicuously reduced in all neocortical areas of the 24 h time control animals compared to the values previously reported in our 4 h survival study or compared to naïve animals. The decreased number of COX-2-expressing neurons may be in part explained by the inactivation of cortex due to anaesthesia, as COX-2 expression is stimulated by neuronal activity. The applied anaesthetic/analgesic drugs could exert an inhibitory effect on COX-2 expression by interacting with the nuclear transcription factor NF-κB signalling pathway, which is a well-known transcriptional regulator of COX-2. Although morphine has been reported to have ambiguous stimulatory and inhibitory effects on NF-κB activation, midazolam is unequivocally known to inhibit the NF-κB pathway. The applied anaesthetic regimen was chosen to enhance the translational potential of our animal model, as morphine/midazolam analgesia/sedation is routinely used in the management of human neonates affected by PA/HIE. Furthermore, experimental data suggested that morphine analgesia may be an important permissive factor by allowing neuroprotective therapies such as therapeutic hypothermia to be effective; mild hypothermia failed to exhibit neuroprotection in the absence of anaesthesia and analgesia in newborn pigs.

Our findings suggested a time-dependent role of COX-2-derived ROS and prostanoids in the pathomechanism of HIE development in different brain regions, as COX-2 activity-dependent neuronal injury in the early reventilation/ reoxygenation phase will be most likely pronounced in regions with high baseline COX-2 expression (especially the frontoparietal neocortex). However, in the delayed secondary energy failure phase, COX-2 will likely remain a more important pathogenic factor for neuronal injury in those areas where the asphyxia-induced elevation dominates the anaesthesia-induced depression of COX-2 levels.

The present data thus suggested that at least in the neocortical areas, two factors affect neuronal COX-2 abundance. Long-term anaesthesia tends to decrease the enzyme levels, whereas asphyxia elevates them. Both the increase in COX-2 levels and the increase in the level of neuronal injury were variable in the piglets subjected to asphyxia, in accordance with the spectrum of human HIE severity. We found that very high percentages of COX-2-immunopositive neurons were inevitably accompanied by the most severe types of cortical neuronal damage. In some cases, however, similarly high neuropathology scores coincided with rather low COX-2 immunopositivity. These areas may perhaps represent those severely damaged areas where the hypoxia/ischemia-induced translational blockade might have prevented the expression of COX-2. Thus, the areas displaying very high neuronal COX-2

levels may represent those areas that were still able after asphyxia to translate new proteins, and the deleterious effects of COX-2 may have contributed most in these areas to the observed neuronal damage.

We assessed the effect of a neuroprotective (2.1%) concentration of H_2 on neuronal COX-2 expression. In addition to our previous studies, this concentration was found to be neuroprotective in a number of other disease models as well.

Our current results concerning the correlation of nuclear 8-OHdG immunoreactivity with COX-2 expression and the remarkable efficacy of molecular H_2 inhalation to attenuate elevations in both 8-OHdG and COX-2 levels after asphyxia suggest a role for ROS in the mechanism of COX-2 expression. 8-OHdG is used as a biomarker of oxidative modifications to DNA: elevations in the number of 8-OHdG-positive nuclei indirectly indicate significant oxidative stress imposed by hydroxyl radicals. Importantly, as molecular H_2 was originally described as a selective hydroxyl radical scavenger, the efficacy of H_2 to attenuate elevations in 8-OHdG levels after asphyxia further confirms the presence of significant oxidative stress perhaps characterized by significant production of hydroxyl radicals in our present PA/HIE piglet model. The connection between this oxidative stress and the observed induction of COX-2 expression may be the activation of NF-κB, which is a transcription factor known to be induced by ROS and inhibited by antioxidants. The general physiological functions of NF-κB include the regulation of apoptosis, cell growth, cellular stress responses and intracellular signalling. NF-κB affects various brain functions as well as neuronal development, inflammation and neurodegeneration. Brain injury has been shown to increase NF-κB activity. The COX-2 gene is a neuronal target of NF-κB, and therefore the asphyxia-induced increase in neuronal COX-2 expression is very likely mediated via NF-κB. A recent report in neonatal rats demonstrated reduced NF-κB activation in H_2-treated rat pups, which lends experimental support to our hypothesis.

We found that microglia were activated by asphyxia only but not by asphyxia followed by H_2. As microglial activation has also been reported to be accompanied by NF-κB activation, hydroxyl radicals may have activated the microglia by promoting NF-κB activity in the present study.

Another possible neuroprotective therapy would be the prevention of neuronal cell death through the activation of antiapoptotic signalization. Both endogenous and exogenous BDNF could exert neuroprotection: BDNF mRNA levels detected at 48 h survival were significantly increased in all brain areas compared to naïve animals in a newborn piglet HIE model. Furthermore, exogenous BDNF was found neuroprotective in a rat HIE model; administration

of BDNF to postnatal day 7 rat pups was causally linked to pronounced and rapid increases in the phosphorylation/activation of ERK and Akt kinases. Pharmacological modulation of Akt and ERK kinases also supported their neuroprotective role. In our current piglet PA/HIE model ERK and Akt phosphorylation was virtually complete also in the cortex of untreated naïve animals, and it was not changed significantly for at least 48 h either in the normoxic time controls or the animals subjected to PA. This high degree of baseline phosphorylation/activation of untreated animals was also observed in virtually all of the assessed brain regions. Similarly, high phosphorylation levels were maintained also at 48 h after asphyxia. Given the high basic activation of these neuroprotective kinases, H_2 was ineffective to accomplish neuroprotection via Akt or ERK pathway. Our pharmacological experiments proved that our results cannot be simply attributed to technical limitations. We could show in the neocortex, where local pharmacological treatment through the closed cranial window was possible that the MEK and Akt1/2 kinase inhibitors could reduce rapidly and statistically significantly the ratio of the phosphorylated forms of ERK and Akt, respectively. The background of this substantial difference is unknown, but one can assume to be associated with the fact that the piglets in the present study were indeed in the perinatal period: they were all born < 24 h before the experiments. Vaginal delivery even under physiological conditions is associated with mild asphyxia and perhaps this physiological amount of cerebral hypoxia is enough to trigger the observed activation of ERK and Akt. BDNF acts as a participant of neuroendocrine cascade of delivery, thus its level is elevated – measured in umbilical cord samples – and may interact through Akt and ERK signalisation. This situation may be in sharp contrast with most of the neonatal rodent HIE models. In these models P7–P8 pups are used because brain maturity is closest to the term human baby at this postnatal age. However, our results suggest that in these models the effects of delivery on cerebral function may have already likely faded. These results question the efficacy of therapeutic interventions aiming to activate these antiapoptotic signalling pathways to obtain neuroprotection. This suggestion is supported by the findings of Robertson et al., who studied melatonin-induced neuroprotection in a very similar newborn piglet 48 h survival HIE model. Although melatonin was found neuroprotective, however, significant anti-apoptotic effect of melatonin could not be shown in the cerebral cortex. These results support the findings of Yue et al. describing that the cell death following hypoxia-ischemia is predominantly caused by necrosis.

CONCLUSION

In newborn pigs, PA induces neuronal COX-2 expression that depends on the severity of PA and shows marked region-dependence as well. We also showed that neuronal COX-2 expression is also dependent on the duration of anaesthesia in neocortical areas.

Neuronal COX-2 expression correlates with the oxidative damage of the neurons as well as the microglial activation.

The H_2-treatment – likely through its antioxidant activity – alleviates the PA-induced elevation of neuronal COX-2 expression, the oxidative stress and the microglial activation.

The antiapoptotic signalling pathways appear to be constitutively active in our model, in accordance with the suggestion that the PA-induced neuronal loss is mostly caused by necrosis in the observed period of HIE.

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PUBLICATIONS RELATED TO THE PHD THESIS

I. Viktória Varga, János Németh, Orsolya Oláh, Valéria Tóth-Szűki, Viktória Kovács, Gábor Remzső, and Ferenc Domoki: Asphyxia-induced neuronal cyclooxygenase-2 expression is alleviated by molecular hydrogen in newborn pigs. *Acta Pharmacol Sin* [Epub ahead of print] doi: 10.1038/aps.2017.148 2018.
IF: 3.223

II. Viktória Kovács, Valéria Tóth-Szűki, János Németh, Viktória Varga, Gábor Remzső, and Ferenc Domoki: Active forms of Akt and ERK are dominant in the cerebral cortex of newborn pigs that are unaffected by asphyxia. *Life Sci* 192:1-8 2018.
IF: 2.936