Regional Differences in the Neuronal Expression of Cyclooxygenase-2 (COX-2) in the Newborn Pig Brain

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Cyclooxygenase (COX)-2 is the major constitutively expressed COX isoform in the newborn brain. COX-2 derived prostanoids and reactive oxygen species appear to play a major role in the mechanism of perinatal hypoxic-ischemic injury in the newborn piglet, an accepted animal model of the human term neonate. The study aimed to quantitatively determine COX-2 immunopositive neurons in different brain regions in piglets under normoxic conditions (n=15), and 4 hours after 10 min asphyxia (n=11). Asphyxia did not induce significant changes in neuronal COX-2 expression of any studied brain areas. In contrast, there was a marked regional difference in all experimental groups. Thus, significant difference was observed between fronto-parietal and temporo-occipital regions: 59±4% and 67±3% versus 41±2%* and 31±3%* respectively (mean±SEM, data are pooled from all subjects, n=26, *p<0.05, vs. fronto-parietal region). In the hippocampus, COX-2 immunopositivity was rare (highest expression in CA1 region: 14±2%). The studied subcortical areas showed negligible COX-2 staining. Our findings suggest that asphyxia does not significantly alter the pattern of neuronal COX-2 expression in the early reventilation period. Furthermore, based on the striking differences observed in cortical neuronal COX-2 distribution, the contribution of COX-2 mediated neuronal injury after asphyxia may also show region-specific differences.

Key words: neonatal pig, prostaglandin H₂ synthase, cerebral cortex, hippocampus, immunohistochemistry

I. Introduction

Phospholipase A₂ (PLA₂) releases arachidonic acid (AA) from membrane phospholipids initiating the production cascade of eicosanoid signalling molecules. Among the many AA metabolic pathways, the rate-limiting activity of cyclooxygenase (COX), also known as prostaglandin H₂ synthase is responsible for the formation of prostanoids, comprising the different prostaglandins, prostacyclins, and thromboxanes. In the central nervous system (CNS), COX-derived prostanoids are involved in the modulation of synaptic transmission including neurotransmitter release [23, 28]. Furthermore, prostanoids are also important mediators of local cerebrovascular control. The cellular elements of the neurovascular unit responsible for determining local blood flow: neurons, astrocytes, microvascular endothelial cells all express PLA₂, COX and other enzymes necessary to produce vasoactive prostanoids [2]. These prostanoids can directly affect the tone of cerebral resistance vessels or can exert a permissive effect on other vasoactive mediator systems [5, 6, 24, 25, 33].

Two COX isoforms have been described in the CNS: COX-1 and COX-2. The isoenzymes have similar catalytic activity and bear 63% amino acid sequence identity, but they differ in molecular weight and pharmacological properties, as well as tissue and cellular distribution [12, 23].
COX-1 and, in contrast to most other organs, COX-2 are both constitutively expressed in the CNS, and are involved in diverse neuronal functions as well as in the regulation of cerebral blood flow (CBF) [29]. Constitutive COX-2 expression is more widespread and predominant in the newborn brain [32], where COX-2 is responsible for 80% of the total COX activity unlike the 10% contribution found in adults [34].

Brain COX-2 expression is upregulated under pathological conditions such as hypoxic/ischemic stress [9, 10, 13, 15], seizures and cortical spreading depression [7]. After hypoxia/ischemia, COX-2-derived prostanoids and superoxide anions have been considered to enhance the neuronal and vascular damage in the CNS, but several studies showed that enhanced COX-2 expression could also be protective in the CNS [21]. Similar to the Janus-faced actions of COX-2 in the CNS, this enzyme also plays diverse role in various physiological and pathophysiological roles spanning from normal development to tumor invasion in virtually all organs [30, 35].

The newborn pig is perhaps the most important large animal model for study of the cerebrovascular physiology of the term neonate, and to test neuroprotective therapies after hypoxic/ischemic insults. Therefore, it is of great importance to describe neuronal COX-2 expression in this species. To our knowledge, few studies have yielded so far the percentage of COX-2 immunopositive neurons in all major cortical and subcortical areas of the piglet brain under normoxic conditions and after asphyxia/reventilation in the presence or the absence of neuroprotective hydrogen ventilation [17].

### II. Materials and Methods

The brain samples used for the present study were obtained from animals (Large-White piglets, less than 1-day old, body weight: 1–2 kg, n=26) used in a previous study [17]. The use of animals was approved by the Animal Care and Use Committee of the University of Szeged. The animals belonged to 4 experimental groups: normoxic (time) controls ventilated with room air or with 2.1% hydrogen-containing room air, and asphyxiated animals (10 min asphyxia followed by 4 hours reventilation either with room air or with hydrogen-containing room air (for details see Table 1).

Upon collection, the brains were immersion fixed in 4% paraformaldehyde. For COX-2 immunohistochemistry, brain samples from the frontal, parietal, temporal and occipital lobes of the cerebral cortex, the hippocampus, the caudate nucleus, the cerebellum, the pons, and the medulla oblongata were dissected. The samples were dehydrated, paraffin embedded, and 4 µm thin coronal sections were cut from the paraffin blocks. The sections were mounted on silanized slides, were incubated at 56°C overnight, then dewaxed in xylene and rehydrated in descending alcohol gradient. Slides were incubated in 3% H$_2$O$_2$ inmethanol to block endogenous tissue peroxidase activity. Antigen retrieval was performed by boiling the slides for 3 min using citrate buffer (pH=6.0) solution. After cooling at

| Table 1. | The percentage of COX-2 immunopositive neurons in the piglet cerebral cortex and hippocampus |
| --- | --- |
|  | Cortex | Hippocampus |
|  | Frontal | Parietal | Temporal | Occipital | CA1 | CA2 | CA3 | Granular layer |
| TC+RA (n=8) | 1+ | 28±6 | 34±4 | 17±3 | 16±3 |
|  | 2+ | 16±3 | 21±3 | 8±1 | 7±1 |
|  | 3+ | 5±1 | 7±1 | 7±3 | 4±1 |
|  | Σ | 48±7 | 62±6 | 31±4 | 27±4 | 14±3 | 4±1 | 3±1 | 3±1 |
| TC+H$_2$ (n=7) | 1+ | 34±4 | 34±5 | 21±2 | 15±3 |
|  | 2+ | 16±3 | 19±4 | 13±2 | 9±2 |
|  | 3+ | 10±4 | 9±2 | 7±2 | 5±1 |
|  | Σ | 60±5 | 63±8 | 40±2 | 29±4 | 10±3 | 3±1 | 2±1 | 2±1 |
| AR+RA (n=6) | 1+ | 27±8 | 28±4 | 26±6 | 17±4 |
|  | 2+ | 25±7 | 26±4 | 14±1 | 12±3 |
|  | 3+ | 13±5 | 12±3 | 8±2 | 7±2 |
|  | Σ | 65±12 | 66±6 | 48±9 | 36±8 | 12±2 | 3±0 | 1±0 | 2±1 |
| AR+H$_2$ (n=5) | 1+ | 33±5 | 32±8 | 25±3 | 18±4 |
|  | 2+ | 23±2 | 37±4 | 16±3 | 12±3 |
|  | 3+ | 10±2 | 13±3 | 9±4 | 6±1 |
|  | Σ | 66±5 | 81±5 | 49±4 | 36±7 | 24±7 | 8±5 | 2±1 | 5±3 |

Abbreviations: TC, time control; AR, asphyxia/4h reventilation; RA, room air ventilation; H$_2$, 2.1% hydrogen-supplemented room air ventilation; 1+–3+, staining intensity grading.
room temperature, slides were incubated with a 1:200 dilution rabbit monoclonal primary antibody against COX-2 (clone SP21, Labvision, Fremont, California, USA) for 30 min. The secondary anti-rabbit antibody was conjugated with high affinity horseradish peroxidase-polymer system (EnVision®; Dako, Glostrup, Denmark). For visualization, 3,3′-diaminobenzidine (DAB) tetrahydrochloride solution was used. Slides were counterstained with hematoxylin then dehydrated in increasing concentrations of alcohol, cleared in xylene and covered with a coverslip.

COX-2 immunoreactive neurons were manually counted by two independent observers. In each examined brain region, the percentage of stained neurons was determined by dividing the number of immunopositive neurons by the total number of neurons observed in 5 randomly selected fields of view at 20× magnification using light microscopy. In addition, reflecting staining intensity the immunoreactive neurons were ranked: 1+, 2+, or 3+ scores were given corresponding with weak, perinuclear staining (1+), moderate cytoplasmic staining (2+) and strong cytoplasmic and dendritic staining (3+) respectively.

Data are expressed as mean±SEM. For statistical analysis, one-way ANOVA followed by the Student-Newman-Keuls post hoc test was performed using a statistical software (SigmaPlot 11.0, Systat Software Inc., Chicago, Illinois, USA). p values of <0.05 were considered statistically significant.

III. Results

In the present study, we determined the ratio of COX-2 immunopositive neurons in various brain regions in 4 groups of piglets undergoing asphyxia/reventilation and/or the neuroprotective hydrogen ventilation treatment (Table 1).

In the cerebral cortex, COX-2 immunopositivity was observed in neurons and in microvessels. We observed COX-2 immunopositive neurons in all cortical samples from all cerebral lobes, in all cortical layers (Fig. 1A–D). In two distinct layers of the cerebral cortex, marked neuronal COX-2 positivity was found: in the oval-shaped cells in cortical layer III and pyramidal cells in layer V. There was no statistically significant difference among the 4 experimental groups concerning the ratio of immunopositive neurons or their staining intensity (Table 1). In contrast, we observed striking regional differences in the ratio of COX-2 immunopositive neurons among the four cerebral lobes. COX-2 immunoreactive neurons were almost twice as numerous in the frontal and the parietal cortices, than in the temporal and the occipital lobes (Fig. 2A). Interestingly, this regional difference was not reflected in the staining intensity of the immunopositive neurons; instead, the proportions of 1+, 2+, 3+ neurons were remarkably similar in all 4 regions: ~50%, 35%, 15%, respectively (Fig. 2B).

Fig. 1. Representative photomicrographs showing COX-2 immunostaining in different regions of the newborn pig brain. In the frontal (A) and the parietal cortex (B) strong cytoplasmic and dendritic expression can be observed in large percentage of the neurons, unlike in the temporal (C) and occipital cortex (D). All subregions of the hippocampus, such as the granular layer (E), the CA1 (F), the CA2 (G), and the CA3 (H) show generally weak neuronal staining. Bar=100 μm.
In the hippocampus, COX-2 immunostaining was considerably less pronounced than in the cortex with only few immunopositive neurons (Fig. 1E–H). There was also no significant difference among the 4 experimental groups (Table 1). The highest expression was found in the pyramidal cells of the CA 1 field, while there was minimal immunopositivity in the CA2–CA3 regions and the granular layer of the hippocampus (Fig. 2A). The staining intensity could not be reliably evaluated due to the scarcity of immunopositive neurons.

In the assessed subcortical regions, there was no remarkable COX-2 immunostaining. The numbers of stained neurons were less than 1% showing patchy staining of neurons in the pons, medulla, cerebellum, and the caudate nucleus (data not shown). In the cerebellum, COX-2 immunopositivity was observed in a small fraction of the Purkinje cells. Similar to the cortical regions, asphyxia or hydrogen ventilation did not cause significant alteration in the minimal COX-2 immunostaining.

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**IV. Discussion**

The major new findings of the study are the following: (1) in newborn pigs COX-2 expression is widespread in cortical neurons, however, its frequency is low in the hippocampus and virtually negligible in the subcortical brain regions and the cerebellum; (2) cortical COX-2 immunopositive neurons were approximately two times more numerous in the fronto-parietal cortex as compared to the temporo-occipital region; (3) despite the large regional difference among cortical regions in the number of COX-2 positive neurons, the pattern of staining intensity was remarkably similar in all regions; (4) asphyxia and hydrogen ventilation did not alter neuronal COX-2 expression in 4 hours.

In the piglet CNS, COX-2 is the major COX isof orm expressed both in the neurons, cerebral arteries, and the microvasculature [13, 32]. Accordingly, COX-2 inhibition resulted in more than 90% decrease in brain prostaglandin levels [32]. Basal expression of COX-2 appears to be regulated by natural synaptic activity in the developing and the adult rat brain and can have a profound role in NMDA receptor-dependent synaptic plasticity [22, 36]. COX-2 activity appears to be involved in the cognitive development and memory formation since the selective COX-2 inhibitor (SC58125) prevented memory formation in 1 day old chicks [19, 20]. These data highlight the importance of COX-2 derived prostanoids in learning. Basal neuronal COX-2 expression may have a similar role in the piglet: we showed previously that local cortical depolarisation by KCl or
activation ionotropic glutamate receptors with kainate or NMDA caused rapid induction of cortical neuronal COX-2 levels [14].

In contrast to the lack of studies on the physiological function of neuronal COX-2 in piglets, COX-2 has been extensively studied in conjunction with the mechanism of hypoxia/ischemia-induced damage of the neonatal cerebral cortex in this species. COX-2 produces superoxide anions in a 1:1 ratio with PGH₂, and COX-derived reactive oxygen species (ROS) have been shown to be important for the generation of oxidative stress following ischemic stress [1, 31]. Furthermore, COX-2 activity has been demonstrated to contribute to the neurovascular dysfunction after hypoxia/global cerebral ischemia in piglets. Neurovascular function was studied using NMDA-induced pial arteriolar vasodilation that has been repeatedly shown to be a ROS-sensitive neuronal-vascular response, for a recent review see [3]. Specifically, pretreatment with the selective COX-2 antagonist NS-398 prevented the posts ischemic attenuation of NMDA-induced vasodilation, indicating the role of COX-2 produced ROS in the pathomechanism [15]. This beneficial effect of COX-2 inhibition is likely contributing to the neuroprotection elicited by inhibiting the COX-2 pathway in various experimental models [4, 37].

COX-2 is known to be induced by anoxic stress, parenchymal and cerebrovascular COX-2 mRNA levels were shown to increase within 0.5–2 hours after global cerebral ischemia in the piglet [10, 11, 13]. In our previous study, asphyxia was shown to significantly increase the number of damaged neurons after 4 hours, moreover, hydrogen ventilation was shown to partially alleviate the hypoxic/ischemic neuronal damage [17]. However, we could not show statistically significant increases of COX-2 immunopositive neurons in the present study, perhaps there was a slight tendency for increased number of positive neurons after asphyxia in the parietal and occipital cortices. This perhaps unexpected result is likely due to the increased turnover rate of COX-2, for instance in cerebral arteries COX-2 mRNA levels increased within 30 min, but increased protein levels were detected only 8 hours after ischemic stress [13]. According to the results of our present study, the baseline pattern of cerebral COX-2 expression does not significantly change during the first few hours of reoxygenation—in the period when most oxidative stress occurs.

The role of COX-2 in the mechanism of hypoxic-ischemic injury in the piglet has been virtually exclusively derived from studies on the parietal cortex [1, 3, 15]. In these studies, a closed cranial window was implanted over the parietal cortex enabling visualisation of the pial microcirculation and sampling of the artificial cerebrospinal fluid for the determination of prostanoids. Our present findings suggest that coincidentally the parietal cortex might be one of the most sensitive regions of the piglet brain to COX-2 inflicted neuronal injury, since COX-2 expression appears to be the most widespread in the neurons of this cortical region. First Dégi et al. [8] examined qualitatively the regional distribution of COX-2 immunoreactivity in the piglet brain, however, the cerebral cortex was only represented by samples from the parietal cortex, thus the regional difference discovered in the present study could not be noted. Thus, our present study is the first in which this regional difference between the fronto-parietal and the temporo-occipital cortices has been described. Our findings indicate that COX-2 dependent oxidative stress upon reoxygenation/reventilation after hypoxic/ischemic stress can be more severe in those cortical areas where basal COX-2 expression is significantly higher. Further studies are clearly needed to address this possibility.

Compared to the cortex, the hippocampus and the subcortical areas studied had low neuronal COX-2 expressions. Dégi et al. [8] reported qualitatively strong COX-2 immunoreactivity in the stained hippocampal neurons, but in our study we found that the percentage of such stained neurons was in fact low. Interestingly, neuronal COX-2 expression appears to have an inverse relationship with regional cerebral blood flow. Indeed, regional blood flow in the midbrain, pons, medulla and cerebellum (expressing low levels of COX-2) is significantly higher as compared to the cortical regions (displaying high COX-2 expression) under normal conditions in piglets [18]. The cortical prostanoids appear to limit cortical blood flow by constricting cerebral arterioles, furthermore the high cortical COX-2 expression appears to be necessary for the expression of prostanoid receptors and coupled signal transduction proteins required for the age-dependent vascular regulation [26, 27]. We previously found that reactive hyperemia after asphyxia was significantly greater in the cerebellum than in the parietal cortex, and these hemodynamic differences were associated with increased vulnerability of the cerebellum but not the cortex to 100% O₂ versus room air ventilation after asphyxia [16]. Our present findings suggest that regional hemodynamic difference might exist within the cortex itself that need to be looked at.

In summary, the present study is the first to yield quantitative data on the pattern of neuronal COX-2 expression in the piglet brain describing high region-dependent cortical COX-2 expression, and low expression in the hippocampus and subcortical structures. The results from this study open new avenues for understanding the regional differences in the pathomechanism of perinatal hypoxic-ischemic injury, and the function of the neurovascular unit.

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