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Early Regional Response of Apoptotic Activity in Newborn Piglet Brain Following Hypoxia and Ischemia

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Abstract

Responses of selected neuroregulatory proteins that promote (Caspase 3 and Bax) or inhibit (Bcl-2, high Bcl-2/Bax ratio) apoptotic cell death were measured in the brain of piglets subjected to precisely controlled hypoxic and ischemic insults: 1 h hypoxia (decreasing FiO_2 from 21 to 6%) or ischemia (ligation of carotid arteries and hemorrhage), followed by 0, 2 and 4 h recovery with 21% FiO_2 . Protein expression was measured in cortex, hippo-campus and striatum by Western blot. There were no significant differences in expression of Caspase-3 between sham operated, hypoxic and ischemic groups. There were significant regional differences in expression of Bcl-2

and Bax in response to hypoxia and ischemia. The changes in Bcl-2/Bax ratio were similar for hypoxia and ischemia except for striatum at zero time recovery, with ischemia giving lower ratios than hypoxia. The Bcl-2/Bax ratio was also lower for the striatum than for the other regions of the brain, suggesting this region is the more susceptible to apoptotic injury.

Keywords

Brain; Apoptosis; Hypoxia; Ischemia; Newborn

Introduction

The severity and duration of hypoxic or ischemic insults are of critical importance for the outcome of brain function, though ischemic insults always seem to produce more severe brain injury than do hypoxic episodes of the same duration [1,2]. Numerous studies have indicated that some selectively vulnerable neurons in adults and neonates begin to die after hypoxic/ischemic insults, and that they die by at least two different mechanisms: apoptosis (programmed cell death) or necrosis [3–6]. The literature indicates that cell death by necrosis may be initiated during the hypoxic/ ischemic episode or in the period immediately following resuscitation. Apoptosis also plays a role in neuronal cell death after hypoxia–ischemia, brain trauma, and neurodegenerative diseases, although its role relative to necrosis remains unsettled [3,4,7–10]. Cheng et al. [4] showed that, in the neonate, apoptosis might be favored over necrosis as a cell death process following hypoxia–ischemia. Yue et al. [11] observed apoptosis and necrosis in neurons and glial cells following transient cerebral hypoxic-ischemic injury in newborn piglets. The authors suggested that immature neurons might be more prone to apoptotic death while terminally differentiated neurons die by necrosis. In adult and neonate models of global ischemia, apoptotic neuronal death began within hours of reperfusion and continued for several days [3,4]. The contribution of apoptosis to hypoxic-ischemic cell death varied with brain region and severity of the insult, being prominent in the neocortex, hippocampus and striatum [3,12,13]. Hypoxic-ischemic brain injury causes characteristic damage of these regions [3,12–15], with evidence for both apoptosis and necrosis detected in the brains of newborn piglets [12,16] and immature rats [17]. In rats, after transient occlusion of the middle cerebral artery followed by reperfusion, the number of apoptotic cells in the striatum and cortex was directly proportional to the duration of ischemia [18]. In newborn piglets, the severity of the initial ischemic insult, as judged by high-energy phosphate depletion in magnetic resonance spectroscopy, was correlated with the proportion of apoptotic cells in the cingulate sulcus studied at autopsy after 48 h [19]. In a deep hypothermic circulatory arrest model, all piglets displayed neurologic deficits and histological evidence of brain damage [20]. Injury was apparent by 6 h of reperfusion and was significantly worse in the neocortex and hippocampus than in striatum and cerebellum. This rapid cell death is consistent with cell suicide programs, which can kill a cell in 2–3 h [10,18].

A goal of present study was to determine the early regional responses of selected neuroregulatory proteins that promote (Caspase-3 and Bax) or inhibit (Bcl-2, high Bcl-2/Bax ratio) apoptotic cell death in piglet brain subjected to precisely controlled hypoxic or ischemic insults.

Experimental Procedure

Animal Preparation

Newborn piglets, age 3–5 days, were used for this study. Anesthesia was induced with 4% isoflurane (Novaplus, Hospira Inc., Lake Forest, IL). Pulse oximetry, ECG and temperature

measurements begun immediately after induction of anesthesia. A 1.0% lidocaine-HCl (Abbott Laboratories, North Chicago, IL) was used as a local anesthetic for tracheotomy and insertion of femoral arterial and venous lines. A bolus of fentanyl citrate (30 $\mu\text{g/kg}$) was administered intravenously (i.v.). Systemic blood pressure and heart rate were immediately monitored following insertion of the arterial line. Isoflurane was reduced to 2% after tracheotomy and pancuronium (Apothecon, Bristol-Meyers Squibb, Princeton, NJ) was injected (0.3 mg/kg, i.v.) to help maintain a balanced anesthesia and to induce respiratory paralysis. The piglets were placed on mechanical ventilation (Sechrist Infant Ventilator, model IV-100 B), isoflurane was withdrawn entirely and piglets were ventilated with a mixture of oxygen and nitrous oxide (at control condition with 21–22% oxygen and 79–78% nitrous oxide). Anesthesia was maintained during the experiments using nitrous oxide, fentanyl (10 $\mu\text{g/kg/h}$, i.v.) and pancuronium (0.1 mg/kg, i.v.). This model provides a balanced anesthesia using a potent inhalation agent (nitrous oxide) for amnesia and analgesia, a potent fast acting narcotic agent (fentanyl) for additional analgesia, and a neuromuscular blocking agent (pancuronium) to provide precise control of ventilation as well as surgical and experimental stillness. The piglets were monitored closely and any increase in heart rate or blood pressure of more than 10% was treated with supplemental fentanyl to increase anesthesia and return the values to control levels.

The head was placed in a Kopf stereotaxic holder and an incision was made along the midline of the scalp. The scalp was removed to expose the skull and a hole approximately 5 mm in diameter was made in the skull over one parietal hemisphere for measuring the oxygen pressure.

Blood pressure, body temperature, heart rate and end-tidal CO_2 were continuously monitored. Arterial blood samples were taken every 15 min and blood pH, arterial CO_2 pressure (PaCO_2) and arterial oxygen pressure (PaO_2) were measured using a Rapid Lab 850 Analyzer (Bayer). After the studies were completed, the anesthetized animals were euthanized by intravenous administration of saturated KCl solution.

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

Experimental Models

Hypoxia—Hypoxia was induced by changing the FiO_2 from 21 to 6%, lowering the mean cortical oxygen pressure to less than 10 mm Hg over 20 min and then holding the level of 8 mm Hg for 40 min followed by reoxygenation with 21% FiO_2 for 0, 2 and 4 h.

Ischemia: Ligation of Carotid Arteries and Hemorrhage—Ischemia was induced by ligation of both carotid arteries by pulling the snares snugly around them. Blood, a total of approximately 70 ml, was then withdrawn over a 20 min period from the arterial catheter into syringes to reduce the systemic arterial pressure to about 40 mm Hg and the mean cortical oxygen pressure to a level of 6 mm Hg and maintaining it for 40 min. After a total of 1 h of ischemia, the carotid ligatures were released and the withdrawn blood was reinfused. The animals were then maintained through a 0, 2 or 4 h period of recovery.

Measurements of Oxygen Pressure and Distribution Using the Oxygen-Dependent Quenching of Phosphorescence

Cortical oxygen pressure was measured using oxygen dependent quenching of phosphorescence. The technical basis for determining the distribution of oxygen in the microcirculation of tissue from the distribution of phosphorescence lifetimes in the serum of blood has been described in detail [21].

Briefly, a near infrared oxygen sensitive phosphor, Oxyphor G2 [22–24] was injected IV at approximately 1.5 mg/kg. The measurements were made through the dura using a multi-frequency phosphorescence lifetime instrument (PMOD 5000). The excitation light (635 nm) was carried to the tissue through a 3 mm optical fiber bundle. The phosphorescence ($\lambda_{\text{max}} = 790$ nm) emitted from the tissue was collected through a second optical fiber bundle forming a concentric circle around the excitation bundle and separated from it by approximately 6 mm. The end of the fiber bundles was placed within 1 mm of the surface of the dura. The separation of the excitation and emission collecting bundles results in effective sampling of brain microcirculation down to approximately 6 mm below the neocortical surface. Two types of measurements were made. (1) The excitation light was modulated at two frequencies [21] and the measured phosphorescence decay was fitted to a single exponential. This measurement method reports the oxygen levels as a single number representing the mean oxygen pressure in the microvasculature. Measurements were made at 15 s intervals through the period of hypoxia or ischemia. This provided real time data for adjusting the experimental parameters and ensuring that brain oxygenation was similar for all of the experimental insults. (2) The excitation light was modulated by the sum of 37 sinusoidal waves with frequencies spaced between 100 Hz and 40 kHz [21] and the frequency-dependence of the phase and amplitude of the phosphorescence was determined. The collected data was then analyzed to give the distribution of phosphorescence lifetimes by fitting using maximal entropy methods. The distribution of lifetimes and amplitudes was used to calculate the distribution of oxygen pressures and their relative volumes in the sampled tissue. Since there were substantial differences between animals with respect to exact placement of the light guide etc., the oxygen histograms were normalized to have a total signal (sum y values for all oxygen pressure values less than 120 mm Hg) equal to 1.0.

Western Blots

Samples of frozen tissue were homogenized in a buffer containing 2% SDS, 10 mM Tris–HCl (pH 7.4) freshly supplemented with NaF (10 mM), Na pyrophosphate (10 mM), Na_3VO_4 (1 mM), Na_2MoO_4 (1 mM), phenylarsine oxide (1 μM), aprotinin, leupetin and pepstatin (10 $\mu\text{g}/\text{ml}$) each. The homogenate was boiled for 5 min after addition of SDS–PAGE sample buffer. Protein concentration was determined in a homogenate aliquot using a BCA Protein Assay kit (Pierce, IL). An equal amount of protein from each sample (40 μg except for caspase where it was 80 μg) was separated by 12.5% SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech.). The membranes were then incubated in a blocking solution (phosphate buffered saline (PBS), pH 7.4, containing 5% non-fat milk) for 1 h at room temperature. This was followed by overnight incubation with specific antibodies for Bax (N-20) and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Beta-actin (ABCAM, Cambridge, MA, USA) which served as a loading control. After being washed in PBS containing 0.05% Tween 20 (PBS-T; Sigma–Aldrich), the membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Amersham Pharmacia Biotech.). For determination of Caspase 3 expression, the membranes were incubated with rabbit Cleaved Caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA) following with incubation in the HRP-conjugated secondary antibody solution.

The final reaction was visualized using enhanced chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech.), and the membranes were exposed to X-ray film.

Data Analysis

Oxygen histograms: Because the measurements were made on each piglet independent of the time of recovery (at which point the animals were euthanized) there are more

measurements for controls, during the ischemic or hypoxic period and at short recovery times than for longer recovery times. This is reflected in the *n* value for the reported data. All of the measurements are reported.

Autoradiographic films were analyzed using Scion Image software (NIH). The data are presented as the mean $\pm$ SD for the indicated number of independent experiments. The data were normalized by assigning a value of 100 to the control and presented the hypoxia and ischemia groups as a percent of the corresponding mean for the control. This is an exploratory study, we did not adjust for type 1 errors and statistical significance of differences were assessed using a two-tailed *t*-test with $p < 0.05$ considered significant. The two individual proteins are independent parameters and when testing for differences in the effects of hypoxia and ischemia on three brain regions this may be regarded as three comparisons. With Bonferroni's correction for multiple comparisons, significance at the 95% for a single difference would be equivalent to $p < 0.0167$ calculated by the two tailed *t*-test. Our hypothesis, that hypoxia would result in less injury than ischemia, predicts a direction for the changes in protein levels and a one tailed *t*-test would be appropriate. If readers wish to apply both corrections, significance for a single difference at the 95% level would be $p < 0.025$ calculated for the two tailed *t*-test.

Results

Effect of Ischemia and Hypoxia on Cortical Oxygen Pressure in Newborn Piglets

Histograms of the distribution of oxygen in the microcirculation of the brain cortex are shown in Fig. 1. Each histogram is the mean $\pm$ SE for the number of experiments given in the figure legend. The panels are the oxygen histograms measured before beginning the period of hypoxia or ischemia, at the end of the period of hypoxia or ischemia (60 min), and after recovery periods of 120 and 240 min. Since there were substantial differences among animals with respect to collected phosphorescence, the oxygen histograms were normalized to have a total signal (sum of *y* values for all oxygen pressure values less than 140 mm Hg) equal to 1.0.

The histograms for the two sets of animals (Control) were not significantly different, as expected for similarly treated animals. At the end of the 60 min period of hypoxia or ischemia the histograms were also very similar, with peak values near zero for both groups but with that for ischemia skewed toward higher oxygen pressures. This is consistent with the models used because in ischemia the arterial blood oxygen saturation is higher than for hypoxia and there is a small contribution to the histograms from blood in the small arterioles. The peak of the histogram for ischemia is slightly lower than for hypoxia, consistent with greater longitudinal gradients generated by the lower blood flow in ischemia. During recovery, the histograms were similar with the overall oxygen pressures lower than controls for both the hypoxic and ischemic animals, the histogram for ischemia being shifted slightly toward lower oxygen pressures compared to hypoxia. The histograms for 120 and 240 min of recovery are shown and these exemplify the differences observed. At both time points the ischemia histograms are slightly shifted to lower oxygen pressures compared to those for hypoxia. The greatest difference was observed for 240 min of recovery.

Time-Dependence of Bcl-2, Bax and Caspase-3 Levels in the Frontal Cortex of Newborn Piglets Following Ischemic and Hypoxic Episodes

Expression of Caspase-3 was not significantly different in the control, ischemic, or hypoxic group of animals. Significant increase in expression of Bcl-2 as compared to control was observed for both the ischemia and hypoxia groups only after 4 h of recovery ($157 \pm 29\%$ following ischemia, $p = 0.001$; $177 \pm 24\%$ following hypoxia, $p = 0.002$) (Fig. 2). The

immunoreactivities of Bax following ischemia and hypoxia are shown in Fig. 3. At 0 h of recovery, Bax was significantly higher as compared to control in both experimental groups ($159 \pm 26\%$ for the ischemic group, $p = 0.005$ and $134 \pm 17\%$ for the hypoxic group, $p = 0.008$). At 2 h of recovery significant increase in Bax was only in hypoxic group ($142.7 \pm 26.8\%$, $p = 0.009$). At 4 h of recovery, expression of Bax was similar in both groups and was not significant different from control. Since both Bax and Bcl-2 increased in both groups, the differences between them were significant only for Bcl-2 at 0 and 2 h recovery ($p = 0.007$ and 0.026 , respectively). The calculated ratios Bcl-2/Bax, relative to control, at 0, 2 and 4 h were 0.60 ± 0.15 , 0.97 ± 0.42 and 1.49 ± 0.27 , respectively, in the ischemia group and 0.97 ± 0.18 , 0.95 ± 0.27 and 1.53 ± 0.27 , respectively, in the hypoxia group (Fig. 4). At 0 h recovery the ratio for ischemia was significantly lower than control ($p = 0.007$) and at 4 h recovery they were significantly higher than control for both ischemia and hypoxia ($p = 0.019$ and 0.039 , respectively).

Time-Dependence of the Responses Caspase-3, Bcl-2 and Bax and in the Hippocampus of Newborn Piglets Following Ischemia and Hypoxia Insults

As was the case for the cortex, expression of Caspase-3 in hippocampus was not significantly different in the control, ischemic, or hypoxic groups of animals. Significant increase in expression of Bcl-2 compared to control was observed after every measured point of recovery for both the ischemia and hypoxia groups (Fig. 5). In the ischemia group, after 0, 2 and 4 h recovery the increase in Bcl-2 immunoreactivities were $177 \pm 41\%$ ($p = 0.004$), $152 \pm 27\%$ ($p = 0.008$) and $172 \pm 40\%$ ($p = 0.005$), respectively. In the hypoxia group the increases relative to control were $231 \pm 41\%$ ($p = 8 \times 10^{-4}$), $258 \pm 56\%$ ($p = 6 \times 10^{-4}$) and $271 \pm 26\%$ ($p = 3 \times 10^{-4}$), respectively. The hypoxic and ischemic groups were different from each other at 0, 2, and 4 h recovery with p values of 0.046, 0.002, and 0.001, respectively.

The immunoreactivity of Bax in the post-ischemia group was not different from control at the end of the ischemic period (0 h) but was significant lower at 2 h ($73 \pm 20\%$, $p = 0.034$) and 4 h ($65 \pm 15\%$, $p = 0.004$) (Fig. 6). In the post-hypoxia group, Bax was significantly increased compared to control at 0 h ($138 \pm 33\%$, $p = 0.041$) and 2 h of recovery ($163 \pm 12\%$, $p = 1 \times 10^{-4}$) with no significant difference from control at 4 h recovery (Fig. 6). The fact that Bax increased in the ischemia group and decreased in the hypoxia group lead to the p values for the differences between the groups being 3×10^{-6} and 0.002 after 2 and 4 h recovery, respectively.

The calculated ratios for Bcl-2/Bax, relative to controls, at 0, 2 and 4 h were similar in the ischemia (1.73 ± 0.27 , 2.07 ± 0.48 , and 2.63 ± 0.40 , respectively) and hypoxia (1.67 ± 0.16 , 1.58 ± 0.27 , and 2.62 ± 0.39 , respectively) groups, increasing with time of recovery in both groups (Fig. 7). The Bcl-2/Bax ratios for ischemia and hypoxia were different from control with p values at 0 h of 5×10^{-4} and 3.7×10^{-3} , respectively, at 2 h of 6.8×10^{-4} and 1.9×10^{-2} , respectively, and at 4 h of 8×10^{-6} and 3×10^{-5} , respectively.

The Levels of and Caspase-3, Bcl-2 and Bax in the Striatum of Newborn Piglets Following Ischemia and Hypoxia with Different Times of Recovery

Expression of Caspase-3 in striatum were not significantly different from that in the control and hypoxic groups of animals. There was a trend toward increase of Caspase-3 during the recovery time in ischemic group ($112 \pm 12\%$ compared to control at 0 time of recovery; $118 \pm 17\%$ compared to control at 2 h of recovery; $122 \pm 18\%$ as compared to control at 4 h of recovery). However, these changes were not statistically significant.

The Bcl-2 immunoreactivities in the striatum were not significantly different from controls for either the ischemic or the hypoxic groups or when the two were compared to each other at any point in the recovery (Fig. 8).

Immunoreactivities of Bax at 0 and 2 h recovery following ischemia were significantly increased to $170 \pm 31\%$ ($p = 0.001$) and $144 \pm 31\%$ ($p = 0.015$) compared to control, respectively (Fig. 9). At 4 h post-ischemia, the immunoradioactivity of Bax was not significantly different from control ($118 \pm 27\%$). In the hypoxia group, Bax immunoreactivities were not significantly different from control at any measured time of recovery. Direct comparison between the hypoxic and ischemic groups indicated significant differences at 0 and 4 h recovery, with p values of 0.0005 and 0.026, respectively.

The calculated ratios Bcl-2/Bax, relative to controls, at 0, 2 and 4 h were 0.45 ± 0.14 , 0.85 ± 0.21 and 1.00 ± 0.25 , respectively, in the ischemia group and 1.32 ± 0.39 , 0.72 ± 0.27 and 1.45 ± 0.30 , respectively, in the hypoxia group (Fig. 10). The Bcl-2/Bax ratio for ischemia was significantly different from control only at 0 h recovery ($p = 0.02$) and for hypoxia none of the values were different for control.

Discussion

In the present study, newborn piglets were subjected to controlled hypoxia and ischemia insults, the conditions determined by measuring the cortical oxygen pressure. During the one hour of hypoxia or ischemia, the cortical oxygen was decreased to the same value in order to be able to compare these experimental models and responses of selected proteins involve in anti- or pro-apoptotic activity. As shown in Fig. 1, during recovery, the histograms were similar with the overall oxygen pressures lower than controls for both the hypoxic and ischemic animals, the histogram for ischemia being shifted slightly toward lower oxygen pressures compared to hypoxia. Both the intensities (amplitudes) and lifetimes of phosphorescent signals decrease with increasing oxygen pressures. The decrease in signal with increasing oxygen pressure (decrease in signal to noise) results in asymmetric broadening of oxygen histograms as seen in the “tail” effect on the high oxygen end of the histograms. Oxygen pressures above the median should be used only for qualitative comparisons. Overall shift of the histograms to lower oxygen pressures despite the arterial blood oxygen saturation at control levels and blood pressures well above 45 mm Hg suggests the vas-culature of the brain has increased resistance consistent with widespread injury. This injury is not a blockade in individual vessels at the larger arteriolar level, which would cause increased contribution of values near zero to the histograms. Rather, the histograms show a general shift to lower oxygen pressures such as would be expected if there were widespread edema and/or increased adhesion of white cells to the endothelium lining the microcirculation. Either of these would increase the resistance to flow in the capillaries, producing the widespread regions of below normal oxygen pressures shown in the histograms. The oxygen histograms during recovery from ischemia are shifted to slightly lower oxygen pressures than those for hypoxia. Thus, although the histograms are consistent with substantial vascular injury following both ischemia and hypoxia, the injury due to ischemia appears to be worse than that for hypoxia.

A limitation of the present study was that the oxygen levels were determined in the frontal cortex only. We can only assume that similar changes in oxygenation occurred in other regions, such as the hippocampus and striatum.

As was described in the Introduction, exposure to hypoxia/ischemia triggers a variety of negative sequelae in the brain including, particularly in newborn brain, apoptotic activity. To determine the early apoptotic signaling in frontal cortex, hippocampus and striatum in

response to hypoxia or ischemia insults, we measured the expression of Caspase-3, Bcl-2 and Bax and, calculated the Bcl-2/Bax ratio. These proteins were chosen because they play important role in diminishing or activating apoptotic activity in newborn brain.

The proteins of Bcl-2 family are key regulatory factors, which can either promote cell survival (Bcl-2, Bcl-XL, A1, Mcl-1, and Bcl-W) or cell death (Bax, Bak, Bcl-XS, and Bok) by apoptosis [25–27]. Accumulating evidence indicates that increased level of Bcl-2 provides protection against apoptosis [28] and ischemic neuronal death [29,30]. The increased Bcl-2 enhances cell survival, possibly through regulating cytosolic and intranuclear Ca^{2+} concentration [31]. Similarly to Bcl-2, Bax protein is also a critical regulator of programmed cell death. However, Bax is apoptotic protein and acts by activating caspases [32]. Bax has been shown to form ion-conducting channels or pores in intracellular planar lipid bilayer membranes. These can lead to nuclear envelope breakdown and allow increase in intranuclear calcium [15,33]. The active form of Bcl-2 heterodimerizes with Bax and their ratio, rather than individual amount, determines the cellular susceptibility to apoptotic stimuli [32,34–37]. Increase in the ratio of Bax to Bcl-2 protein has been shown in piglets subjected to hypoxic and hypocapnic episodes, demonstrating an increased susceptibility to apoptosis in the brain of the newborn following hypoxia and hypocapnia [38,39].

Our study determined: (a) the early responses of above proteins and changes in Bcl-2/Bax ratio in three regions of piglet brain following hypoxia and ischemia and (b) regional differences and/or similarities in response of Bcl-2/Bax to hypoxia and ischemia. There were significant differences in the responses to hypoxia and ischemia. The insults were made as similar as possible, but the ischemic insult resulted in slightly lower oxygen pressures in the brain during recovery, suggesting greater injury to the vascular system. Perhaps most strikingly, in the hippocampus the levels of Bax was increased at zero and 2 h recovery in the ischemic group but was decreased at 2 and 4 h recovery in the hypoxic group. Bcl-2, in contrast increased in both groups with the increase in the hypoxic group greater than that in the ischemic group. The differences in response to hypoxia and ischemia could be due to difference in tissue pH, with lower tissue pH occurring during ischemia than during hypoxia. Because it is the Bcl-2/Bax ratio that is considered best correlated with apoptotic activity, however, and in the hippocampus this was not different between the hypoxic and ischemic groups, differences in apoptotic activity would not be expected.

In cortex and striatum, differences in Bcl-2/Bax were observed between hypoxia and ischemia at zero time of recovery. Following ischemic insult, in cortex and striatum the Bcl-2/Bax ratios were about 60 and 50%, respectively, of the ratios following hypoxic insult. The lower Bcl-2/Bax ratios in the cortex and striatum are consistent with ischemia causing greater activation of pro-apoptotic metabolism than hypoxia, and therefore greater possible brain injury through apoptotic cell death. The Bcl-2/Bax ratios were much higher in the hippocampus than in cortex and striatum at every time point in the recovery. As described above, the balance between these pro- and anti-apoptotic proteins is important in determining whether the cells undergo apoptotic death. The relatively large changes in Bcl-2 and Bax in the hippocampus suggests that this region of the brain may be more highly stressed by hypoxia/ischemia than other regions. The protective response is strong, however, as indicated by the higher Bcl-2/Bax ratio, and this may help to hold down the extent of apoptotic injury.

The lowest ratios of Bcl-2/Bax were observed in striatum. This is consistent with the cells in the striatum being more vulnerable to apoptotic injury than cells in the other regions of the brain. One possibility for the greater vulnerability may be differences in the response of pCREB (cAMP response element binding protein) to hypoxia and ischemia. CREB is a

transcription factor that is constitutively expressed in brain. Several studies involving over expression of dominant-negative CREB suggested a role for CREB as a survival factor in various cellular models [40–47], possibly acting downstream of the Akt/PKB survival pathway [48]. Mantamadiotis et al. [49] demonstrated that CREB family members are crucial to neuronal survival in vivo. Tanaka et al. [50] reported that neurons in the medial striatum showed persistently activated CREB phosphorylation with normally maintained morphology during the post-ischemic recovery.

In the earlier study, we measured the response of phosphorylation of CREB in striatum of newborn piglets to hypoxic and ischemic insult [51]. The experimental models were the same as for the present study and pCREB was measured at 2 h of recovery. The levels of pCREB were not altered following hypoxia but were decreased to about 50% of control after ischemia. We suggest that decreased pCREB in ischemia may be least partly responsible for observed differences in ratio of Bcl-2/Bax between hypoxia and ischemia. This suggestion is in agreement with data of Delivoria and et al. [52] showing that in the cortex of newborn piglets decrease in expression of pCREB is correlated with decrease of Bcl-2/Bax ratio. The present study shows that the Bcl-2/Bax ratios in the cortex of newborn piglets in hypoxia and ischemia are low and the values similar to that in the striatum following ischemia. It is therefore possible that pCREB plays a role in cortex similar to that we have suggested for striatum.

In this study we also measured expression of Caspase-3. Except for a small, not statistically significant, increase in striatum of ischemic animals, there were no differences in expression of Caspase-3 following hypoxia and ischemia. This was true for both the different times of recovery and the different regions of brain. Caspase-3 is one of the most important of the caspases involved in apoptotic brain injury. It has been shown by numerous studies to be activated by hypoxia or ischemia. Lack of significant increase in this enzyme in our study can be attributed to the relatively short time of recovery. Most of the studies in literature that report increase in this enzyme are for measurements made after much longer time of recovery than 4 h post ischemic-hypoxic insult.

Conclusion

Hypoxia and ischemia insults cause regional changes in expression of key proteins that regulate apoptosis in newborn piglet brain. In the hippocampus, the responses to the hypoxic/ischemic insults were greater than for the other regions of the brain. This is consistent with greater cellular stress in this region, but the Bcl-2/Bax ratios were also higher, and this would be expected to help keep down the numbers of cells lost to apoptosis.

Significant differences in the Bcl-2/Bax ratio between hypoxia and ischemia were observed only in striatum. In this region, ratio Bcl-2/Bax in ischemic group at zero time of recovery was significantly lower than for the hypoxic group. Studies are in progress to determine if these differences are correlated with differences in neuronal loss measured at longer times of recovery.

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Fig. 1.

Comparison of cortical oxygenation during ischemia and hypoxia and different times of post-ischemic and post-hypoxic recovery in newborn piglets. Histograms of oxygen distribution in the microcirculation of the cortex were determined by the distribution of phosphorescence lifetimes, as described in Methods. The presented histograms are for control conditions, at the end of the 60 min of ischemia or hypoxia, and after 120 and 240 min of recovery. The histograms for controls and for the end of the hypoxic/ischemic periods are $n = 19$ and 22 for hypoxia and ischemia, respectively. For 120 min of recovery the n values were 12 and 15 for hypoxia and ischemia, respectively, and at 240 min recovery they were 7 and 7 for hypoxia and ischemia, respectively. The values are presented as the mean $\pm$ SE for the indicated number of experiments

Fig. 2.

Bcl-2 expression in the frontal cortex of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of the corresponding values (100%) for the control group of piglets. *p* values for significant difference from control were determined by Student *t*-test. *p* < 0.05 was considered statistically significant

Fig. 3.

Bax expression in the frontal cortex of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of the corresponding values (100%) for the control group of piglets. p values for significant difference from control were determined by Student *t*-test. $p < 0.05$ was considered statistically significant

Fig. 4.
Bcl-2/Bax ratio in the frontal cortex measured at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery

Fig. 5. Bcl-2 expression in the hippocampus of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of corresponding values (100%) for the control group of piglets. p values for significant difference from control were determined by Student *t*-test. $p < 0.05$ was considered statistically significant

Fig. 6.

Bax expression in the hippocampus of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of the corresponding values (100%) for the control group of piglets. p values for significant difference from control were determined by Student t -test. $p < 0.05$ was considered statistically significant

Fig. 7.
Bcl-2/Bax ratio in the hippocampus measured at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery

Fig. 8.

Bcl-2 expression in the striatum of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of the corresponding values (100%) for the control group of piglets. *p* values for significant difference from control were determined by Student *t*-test. *p* < 0.05 was considered statistically significant

Fig. 9.

Bax expression in the striatum of newborn piglets at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery. The results are means from 6 experiments $\pm$ SD. The data are expressed in % of the corresponding values (100%) for the control group of piglets. *p* values for significant difference from control were determined by Student *t*-test. *p* < 0.05 was considered statistically significant

Fig. 10. Bcl-2/Bax ratio in the striatum measured at 0, 2 and 4 h of post-ischemic and post-hypoxic recovery