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Active forms of Akt and ERK are dominant in the cerebral cortex of newborn pigs that are unaffected by asphyxia

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Chemical compounds studied in this article:
Akt1/2 kinase inhibitor A-6730 (PubChem CID: 16218954) and the MAPK/ERK kinase (MEK) inhibitor U0126 monoethanolate (PubChem CID: 16218944) both from Sigma Aldrich (St. Louis, MO, USA).

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ABSTRACT

Aims: Perinatal asphyxia (PA) often results in hypoxic–ischemic encephalopathy (HIE) in term neonates. Introduction of therapeutic hypothermia improved HIE outcome, but further neuroprotective therapies are still warranted. The present study sought to determine the feasibility of the activation of the cytoprotective PI-3-K/Akt and the MAPK/ERK signaling pathways in the subacute phase of HIE development in a translational newborn pig PA/HIE model.

Main methods: Phosphorylated and total levels of Akt and ERK were determined by Western blotting in brain samples obtained from untreated naive, time control, and PA/HIE animals at 24–48 h survival (n = 3–3–6,respectively). PA (20 min) was induced in anesthetized piglets by ventilation with a hypoxic/hypercapnic (6% $O_2$20%CO_2) gas mixture. Furthermore, we studied the effect of topically administered specific Akt1/2 and MAPK/ERK kinase inhibitors on Akt and ERK phosphorylation (n = 4–4) in the cerebral cortex under normoxic conditions.

Key findings: PA resulted in significant neuronal injury shown by neuropathology assessment of haematoxylin/eosin stained sections. However, there were no significant differences among the groups in the high phosphorylation levels of both ERK and Akt in the cerebral cortex, hippocampus and subcortical structures. However, the Akt1/2 and MAPK/ERK kinase inhibitors significantly reduced cerebrocortical Akt and ERK phosphorylation within 30 min.

Significance: The major finding of the present study is that the PI-3-K/Akt and the MAPK/ERK signaling pathways appear to be constitutively active in the piglet brain, and this activation remains unaltered during HIE development. Thus, neuroprotective strategies aiming to activate these pathways to limit apoptotic neuronal death may offer limited efficacy in this translational model.

1. Introduction

According to the World Health Organization's estimates, perinatal asphyxia (PA) affects 4 neonates out of 1000 live births [1]. The neuronal injury triggered by PA may lead to the so-called hypoxic-ischemic encephalopathy (HIE) in the survivors leading to lifelong neurodevelopmental disorders. The neuronal injury eliciting HIE is no longer considered as a single “event” during PA but rather an evolving process that lasts for days after the resolution of the asphyxia. Correspondingly, mild whole body hypothermia applied in the first 2–3 days after PA is currently the only effective therapy to mitigate HIE severity [2]. However, hypothermia cannot cure HIE alone, and thus clearly further therapies are needed that can augment the neuroprotection afforded by cooling.

The newborn piglet is an established large animal model of the term neonate because of its gyrencephalic brain, developmental stage, body size, cerebrovascular regulation and its cerebral glucose metabolic rate are similar to the term human neonate [3]. We have recently created and characterized a piglet PA/HIE model that resulted in moderate/severe brain damage in most of the animals [4]. This model faithfully reproduces all major hallmarks of human PA/HIE, and can be used to assess the molecular mechanisms of HIE development, and to identify appealing targets for neuroprotective interventions. One of these may be the brain-derived neurotrophic factor (BDNF) signaling pathway. BDNF is a member of the neurotrophic factor family that plays an important role in regulating neural proliferation, differentiation and survival [5,6]. The neuroprotective actions of BDNF are mediated through the phosphatidylinositide 3-kinases (PI-3-K) and the mitogen-activated

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protein kinase (MAPK) pathways [7]. The PI-3-K pathway activates the kinase Akt, one of its direct downstream protein kinase effectors, which regulates multiple biological processes including cell survival, proliferation, growth, and glycogen metabolism [8,9]. Similarly, MAPK-s also regulates widespread cellular processes including cell survival, and apoptosis. Extracellular signal-regulated kinase (ERK) is an anti-apoptotic MAPK, as its downstream pathways are usually linked to growth factor action: cellular differentiation and proliferation. Thus, ERK usually promotes cell survival [10]. Maintaining the delicate balance between the simultaneously active and inactive kinases is critical in determining cell fate [11]. There are both *in vitro* and *in vivo* studies providing evidence that BDNF-supported neuronal survival is mediated *via* the activation of the ERK pathway [12,13], while others have shown that rather the PI-3-K pathway is involved [14]. Therefore, it appears that growth factor-mediated protection of neurons can occur *via* different pathways depending on various factors such as cell types, environmental conditions, and cellular stimuli. However, the potential *in vivo* role of these pathways in the neuronal injury/survival during HIE has not yet been previously studied in a PA/HIE large animal model.

Therefore, the major objective of the present study was to determine the *in vivo* activation of the PI-3-K/Akt and the MAPK/ERK signaling pathways under normoxic conditions and over the course of HIE development (24–48 h). The results emanating from these studies urged us to test also if cortical kinase activities can be *in vivo* modified by specific inhibitors.

2. Materials and methods

2.1. Experimental groups

All necessary permits to perform the *in vivo* animal experiments including the approval from the Institutional Animal Care and Use Committee have been obtained preceding the experiments. Animal care and handling were in accordance with the National Institutes of Health guidelines. The levels of activated ERK and Akt kinases have been determined in the following experimental groups (Fig. 1): (1) untreated animals without any interventions (n = 3); (2) normoxic time controls (n = 3) with 24 or 48 h of survival (n = 1–2, respectively); (3) animals undergoing 20 min PA (n = 6) also with 24 or 48 h of survival (n = 3–3, respectively); (4) non-asphyxiated animals treated topically with the MAPK/ERK kinase (MEK) inhibitor U0126 monoethanolate (n = 4); and (5) non-asphyxiated animals treated topically with the Akt1/2 kinase inhibitor A-6730 (n = 4) onto the cerebral cortex. The detailed procedures applied to the different groups are given in the subsequent paragraphs.

2.2. Animals

Newborn (< 24 h old) male Large-White piglets (weighing between 1.5 and 2.5 kg, n = 20) were used in the present study, they were delivered to the laboratory on the morning of the experiments from a local company (Pigmark Ltd., Co., Szeged, Hungary). The animals were anesthetized with an intraperitoneal injection of sodium thiopental (45 mg/kg; Sandoz, Kundl, Austria). The animals were placed on a heating pad. The skin was disinfected, and the animals were intubated through a tracheotomy. Then the piglets were artificially ventilated with warmed, humidified medical air (21% O_2, balance N_2) at a frequency of 30–35 breaths/min, using a pressure controlled ventilator applying peak inspiratory pressure = 120–135 mmH_2O. Aseptic technique was followed during all aspects of the animal surgery. With the exception of the untreated animals, the right carotid artery and femoral vein were cannulated with catheters. To maintain anesthesia/analgesia the mechanically ventilated animals were given a bolus injection of morphine (100 μg/kg; Teva, Petach Tikva, Israel) and midazolam (250 μg/kg; Torrex Pharma, Vienna, Austria), then were infused with morphine (10 μg/kg/h), midazolam (250 μg/kg/h) and fluids (5% glucose, 0.45% NaCl 3–5 ml/kg/h) throughout the whole experiment. The wounds were closed and covered with warm compress to minimize heat and fluid losses.

2.3. Experimental PA/HIE

The piglets between the time control and the asphyxia groups were randomized with coin flip. The animals were placed into a neonatal incubator (SPC 78-1; Narco Air-Shields, Inc., Hatboro, Pa., USA). Oxygen saturation, mean arterial blood pressure (MABP) through the arterial catheter, heart rate (HR), and ECG were continuously monitored using a Hewlett-Packard M1094 monitor (Palo Alto, California, USA) and recorded online (MecifView, Arlington, Mass., USA). Core temperature was maintained rigorously between 38.5 ± 0.5 °C with a servo-controlled heating pad. Prophylactic antibiotics were given intravenously (penicillin: 50 mg/kg/12 h, Teva, Petah Tikva, Israel and gentamicin: 2.5 mg/kg/12 h, Sanofi, Paris, France) and the urinary bladder was tapped by suprapubic puncture every 12 h of survival. Arterial blood gases, along with blood sugar and lactate levels were determined (~300 μl/sample; EPOC Blood Analysis, Epocal Inc., Ottawa, Canada) at defined intervals to monitor the effect of PA and to keep the values in their respective physiological ranges during the

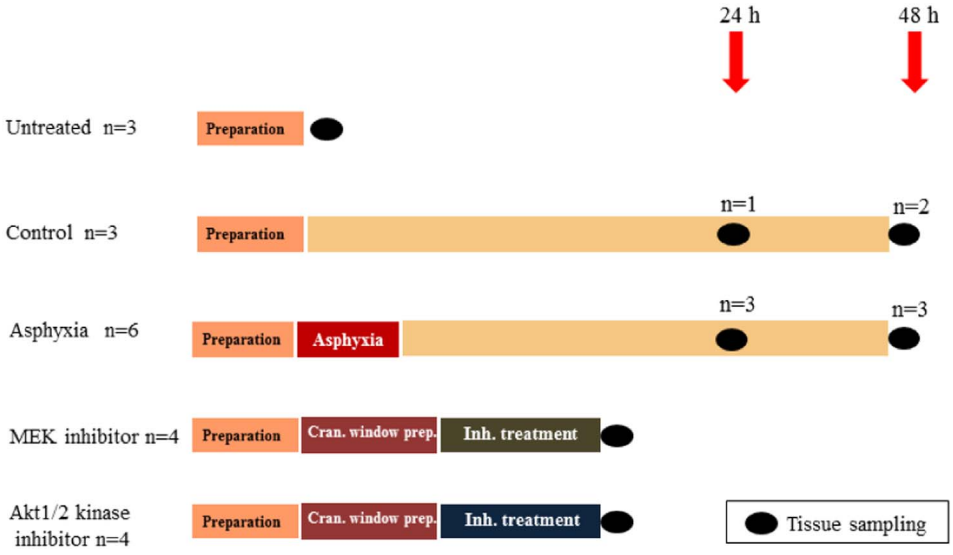

Fig. 1. Experimental protocol. Untreated animals served as naïve controls; Control animals were anesthetized, ventilated, and monitored, but not subjected to asphyxia. Asphyxia animals were like Controls but were exposed to 20 min asphyxia. The animals in the MEK and Akt1/2 kinase inhibitor groups were fitted with closed cranial windows and the respective drugs were applied locally onto the cerebral cortex.

survival period.

PA was induced by ventilation with a hypoxic/hypercapnic gas mixture containing 6% O_2 and 20% CO_2 for 20 min while respiratory rate was reduced from 30 to 15 breaths/min, and intravenous glucose administration was suspended. Arterial blood gases were also determined to check the severity of PA at the end of asphyxia (blood sample was taken at the 18th min of asphyxia). Reventilation was commenced with medical air throughout the survival period.

2.4. The study of ERK and Akt kinase inhibitors

In these groups, the head of the anesthetized, mechanically ventilated animals were fixed in a stereotactic frame. Monitoring of physiological parameters was similar to the previous groups. After retraction of the scalp, a circular craniotomy was made in the left frontoparietal region, where after careful removal of the dura mater, a stainless steel closed cranial window with three needle ports was inserted. The cranial window was sealed with bone wax, cemented in place with dental acrylic, and was filled with artificial cerebrospinal fluid (aCSF; containing KCl 220 mg/l, $MgCl_2$ 132 mg/l, $CaCl_2$ 221 mg/l, NaCl 7710 mg/l, urea 402 mg/l, dextrose 665 mg/l, and $NaHCO_2$ 2066 mg/l, warmed to 37 °C and equilibrated with a gas mixture containing 6% O_2, 6.5% CO_2 and 87.5% N_2). There was a 45 min stabilization period allowed after the implantation of the cranial window before commencing the experiments.

Both the MEK inhibitor U0126 and the Akt1/2 kinase inhibitor A-6730 drugs were prepared similarly: first a 10 mM stock solution was produced in dimethyl-sulfoxide (DMSO) that was further diluted in aCSF to obtain 10 μM drug solutions (final DMSO concentration was 0.01%). In the respective experimental groups, the drugs were applied topically onto the cerebral cortex with continuous superfusion through one of the injectable ports at a rate of 0.5 ml/min for 40 min. The other two ports allowed continuous efflux of the aCSF. Immediately after the completion of the drug treatment, the animals were euthanized with 300 mg Na-pentobarbital (Release®, WDT, Garbsen, Germany). The cortex below the cranial window as well as the corresponding contralateral untreated cortex were collected and processed as described in the following section.

2.5. Western blot analysis

At the end of the respective experimental periods (Fig. 1), both carotid arteries were catheterized in the distal direction, and the brains were perfused through them with cold (4 °C) physiological saline. The brains were gently removed from the skull, and tissue samples were snap frozen in liquid N_2, and stored at − 80 °C before Western-blot analysis.

Thawed samples were homogenized and harvested in ice-cold lysis buffer (50 mM Tris-base, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100). The homogenate was centrifuged at 40,000 × *g* at 4 °C for 30 min and the protein concentration of the supernatant was determined (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, Hercules, CA, USA).

Samples prepared from equal amounts (50 μg) of protein were mixed with Laemmli buffer (1 M Tris-HCl, pH 6.8, glycerol, SDS, 100 mM EDTA, 100 mM EGTA and 1% bromophenol blue) and denatured by boiling. Subsequently, they were loaded for 12% SDS-containing polyacrylamide gel and separated based on molecular size. The gels were electroblotted for polyvinylidene difluoride (PVDF) membranes (Hybond-P, GE Healthcare, United Kingdom).

Detection of the protein of interest was carried out by first blocking the membrane in 3% nonfat dry milk in Tris Buffered Saline -Tween (10 mM Tris-base, 150 mM NaCl, 0.2% Tween-20, pH 8.0; TBS-Tween). Membranes were probed overnight at 4 °C with antibodies recognizing the following antigens: phospho-p44/42 MAP kinase to detect phospho-ERK1/2, total p44/42 MAP kinase, anti-Akt and phospho-Akt (Ser473), (Cell Signaling Technology, Danvers, MA, USA), diluted 1:1000 in the blocking solution. Excess antibody was removed by five washes of TBS-Tween. Membranes were incubated with a horseradish-peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000 in blocking solution at room temperature for 2 h. Five washes in TBS-Tween were followed by detection of the enhanced chemiluminescent signal (WesternBright ECL, Advansta, USA) on X-ray films. The relative protein expressions were determined using densitometry (ImageJ software, National Institutes of Health, USA). Each experiment has been performed at least three times. Values are expressed as mean ± S.E.M.

2.6. Histology

Brain samples collected for histology were immersion-fixed in 4 °C, 4% paraformaldehyde solution for two weeks then processed further. From the cerebral cortex, paraffin embedded 4 μm sections were stained with haematoxylin-eosin. The slides were evaluated by two independent researcher blinded to the experimental protocol with light microscopy (Leica Microsystems, Wetzlar, Germany). The degree of cerebrocortical neuronal damage in the frontoparietal region was graded on a 10-step scale from 0 to 9 using our previously established scoring system [15,4]. Briefly, four pattern of neuronal injury (none; scattered; grouped; panlaminar) was determined in 20–20 non-overlapping fields of vision in the frontal region. Then, final score (0–9) were given to the region based on the most severe level of damage seen for a particular region.

2.7. Statistical analysis

Results were plotted using SigmaPlot (v12.0, Systat Software Inc., San Jose, CA., USA). Core temperature, saturation, HR and MABP as well as arterial blood gas and metabolic parameters were expressed as mean ± S.E.M.

Statistical comparisons include one-way analysis of variance (ANOVA) as well as two-way repeated measures ANOVA followed by the Holm-Sidak *post-hoc* test for pairwise comparisons. Neuropathology scores were expressed as median, 25–75 percentiles. For non-parametric data Mann-Whitney *U* test was applied. p values < 0.05 were considered to be significant.

3. Results

3.1. In vivo observations

At the beginning of the experiments, piglets in both the control and the asphyxia group had similar, physiological values of core temperature (38.6 ± 0.1 and 38.6 ± 0.1 °C), oxygen saturation (91 ± 3 and 97 ± 3%), heart rate (133 ± 10 and 150 ± 14 1/min), and MABP (68 ± 7 and 65 ± 9 mmHg), respectively. Also, arterial blood pH, blood gases and metabolites were similar and within the normal range for these and the untreated group as well (Table 1).

Asphyxia elicited marked increases in HR and MABP (peak values were 200 ± 60 1/min and 94 ± 18 mmHg, respectively) and large drop in oxygen saturation (to 27 ± 1%). Correspondingly, the blood gas analysis performed at the end of the asphyxia period revealed severe hypoxemia, acidosis due to both hypercapnia and elevated blood lactate levels, and hyperglycemia (Table 1). Reventilation restored oxygenation quickly, and the assessed physiological parameters all returned toward baseline levels and they were not then statistically different from the corresponding values of the control group with the exception of blood glucose and lactate levels. These remained significantly elevated at 1 h of reventilation but then returned to baseline values by 4 h of reventilation. (Fig. 2, Table 1). Then, the assessed

Table 1
Arterial blood gas and metabolite analysis in the different groups. Arterial blood pH, partial pressures of carbon dioxide, partial pressures of oxygen, as well as blood glucose- and lactate levels were in the normal ranges at baseline conditions in all three groups. Asphyxia resulted in severe hypoxemia, hypercapnia, acidosis, hyperglycemia and elevations in lactate indicating severe mixed respiratory and metabolic acidosis. Reventilation quickly restored blood gases, however, and blood sugar and lactate levels were still elevated at 1 h compared to controls, and normalized only by 4 h after reventilation. The values then remained in the physiological range and there was no difference between the experimental groups at any later time points (data beyond 20 h are not shown). Data are shown as mean ± S.E.M. Level of significance (p) was set at 0.05. *vs. baseline; †vs. asphyxia at given time point.

Arterial pH	Base	Asph.	1 h	4 h	8 h	12 h	16 h	20 h
Untreated	7.55 ± 0.09							
Control	7.51 ± 0.04	NA	7.51 ± 0.04	7.49 ± 0.02	7.5 ± 0.1	7.52 ± 0.07	7.54 ± 0.09	7.53 ± 0.08
Asphyxia	7.55 ± 0.05	6.87 ± 0.01*	7.47 ± 0.06	7.51 ± 0.06	7.31 ± 0.42	7.49 ± 0.08	7.48 ± 0.11	7.53 ± 0.04

Arterial pCO_2 (mmHg)	Base	Asph.	1 h	4 h	8 h	12 h	16 h	20 h
Untreated	35.3 ± 8.9							
Control	37.1 ± 6	NA	39.7 ± 7.3	43.3 ± 4.4	41.7 ± 14.2	39.2 ± 13.6	37.7 ± 8.2	38.6 ± 7.7
Asphyxia	36.5 ± 4.6	164.3 ± 31*	35.1 ± 5.4	41.4 ± 6.3	37.4 ± 17.6	38.8 ± 7.9	43.1 ± 12.5	40.8 ± 16.2

Arterial pO_2 (mmHg)	Base	Asph.	1 h	4 h	8 h	12 h	16 h	20 h
Untreated	76.7 ± 17.4							
Control	52.9 ± 4	NA	52.5 ± 5.9	58.4 ± 7.9	52.5 ± 2.4	51.3 ± 2.8	53.1 ± 4	53.8 ± 2.2
Asphyxia	63.5 ± 9.6	20 ± 6.2*	60.4 ± 7.7	60.9 ± 10.5	58.6 ± 9.4	60 ± 7.2	65.8 ± 5.3	70.3 ± 7.8

Blood glucose (mmol/l)	Base	Asph.	1 h	4 h	8 h	12 h	16 h	20 h
Untreated	5.5 ± 1.9							
Control	5.3 ± 0.2	NA	4.8 ± 0.9	5.7 ± 1.7	5.5 ± 2.4	4.3 ± 2.1	4.5 ± 0.7	5.1 ± 0.8
Asphyxia	5.8 ± 0.9	9 ± 1.2*	7.3 ± 1.1*	5.6 ± 0.6	4.1 ± 0.8*	4.6 ± 0.9*	4.7 ± 0.9*	4.6 ± 0.8*

Blood lactate (mmol/l)	Base	Asph.	1 h	4 h	8 h	12 h	16 h	20 h
Untreated	2 ± 1.3							
Control	2.2 ± 0.8	NA	1.2 ± 0.1	1.1 ± 0.4	1.3 ± 0.7	1.3 ± 0.7	1.5 ± 1	1.5 ± 0.6
Asphyxia	1.6 ± 0.7	7.4 ± 0.7*	7.7 ± 2.2*,†	1.5 ± 0.6	1.2 ± 0.5	1.2 ± 0.4	1.1 ± 0.4	1.1 ± 0.3

physiological parameters remained in the respective physiological ranges and were statistically not different from the time control group during the rest of the survival period.

3.2. Neuropathology

Asphyxia resulted in severe neuronal damage similar to previously reported values confirming HIE development. In the frontoparietal cortex, the neuropathology scores were significantly higher as compared to the values of the normoxic (combined untreated/time control animals) groups: the scores were 7; 6; 8 *versus* 1; 1; 4 *(median; 25th;75th percentiles; asphyxiated *versus* normoxic animals, respectively; n = 5–5, *p = 0,008, Mann-Whitney U test).

3.3. Activation of ERK/Akt signaling pathways

Total ERK levels in the frontoparietal cortex were remarkably similar in all experimental groups. In addition, the levels of phosphorylated ERK (P-ERK) were also similar to the total levels; therefore the ratio of the P-ERK to total ERK was typically between 80 and 100% indicating high degree of activation of the pathway (Fig. 3.) The results concerning total and phosphorylated Akt (P-Akt) levels, the ratio of P-Akt to total Akt levels were remarkably high and there were no statistical differences among the groups, despite a slight tendency of activation by asphyxia (Fig. 3.)

High degree of ERK and Akt phosphorylation in the frontoparietal cortex was also demonstrated in the samples taken from the control side in our short-term experiments (Fig. 4). However, under normoxic conditions, local, topical *in vivo* treatment with the MEK inhibitor U0126 and the Akt1/2 kinase inhibitor A-6730 resulted in significant inhibition of ERK and Akt phosphorylation, respectively (Fig. 4).

The ratios of P-ERK/total ERK and the P-Akt/total Akt in the other assessed brain regions were remarkably similar to the values determined in the neocortex (Fig. 5). In the untreated as well as in the 48 h asphyxia animals, the phosphorylation ratios were remarkably high and there were no statistical differences among the groups, indicating no effect of asphyxia.

4. Discussion

The most important novel findings of the present study are the following: (1) we could assess activation levels of two signaling pathways important for neuronal survival in a large animal model of HIE; (2) we found that 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 anesthesia and/or exposure to asphyxia; (3) we proved that the high cerebrocortical ERK and Akt phosphorylation levels under normoxic conditions can be *in vivo* modulated by specific inhibitors.

The pathophysiology of HIE is not yet fully understood, however, in recent years the neuronal injury during HIE is increasingly being viewed as a multi-step process consisting of distinct pathomechanisms characteristic of the successive phases of HIE development. These phases include (1) the primary energy failure during the PA stress, (2) the reoxygenation/reperfusion injury associated with the reventilation/resuscitation efforts, and (3) the so-called “secondary energy failure” triggered perhaps by accumulated mitochondrial damage and associated clinically with bouts of apnoe periods and seizures [16]. During the primary energy failure of PA, the lack of oxygen and the reduction of blood flow shifts cellular metabolism from an aerobic to a less

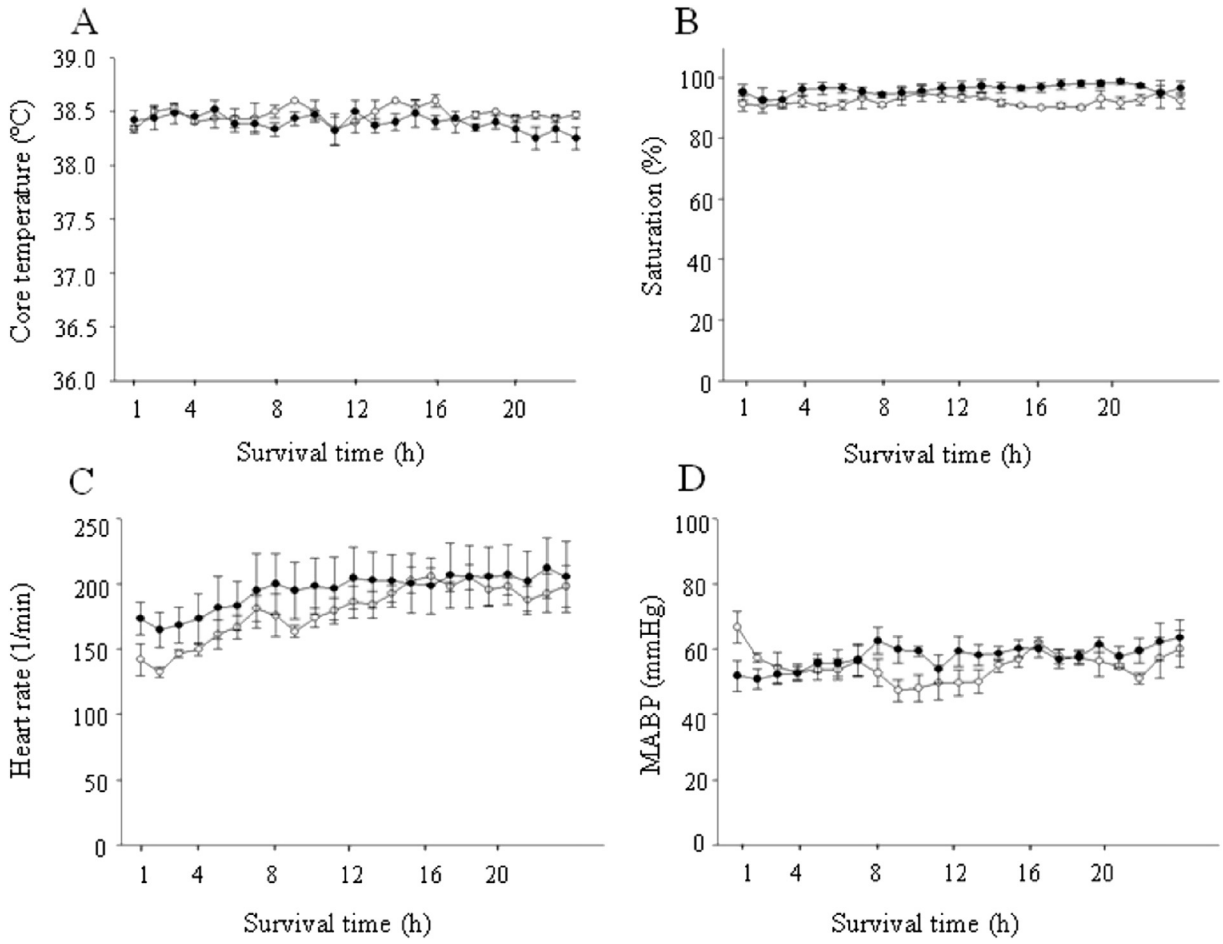

Fig. 2. Core temperature (Panel A), oxygen saturation (Panel B), heart rate (Panel C), and mean arterial blood pressure (MABP) (Panel D) data in the control and in the asphyxia groups during the first 24 h survival period. There were no significant differences between the groups in the monitored parameters either in the first or the second (data not shown) 24 h period. Data are shown as mean ± S.E.M.

efficient anaerobic pathway, resulting in the depletion of NAD^+ stores, decrease in ATP levels, and lactate accumulation [17]. ATP depletion will soon lead to the depression of neuronal activity soon followed by anoxic depolarization. Anoxic depolarization is characterized by the loss of transmembrane ionic gradients eliciting the accumulation of intracellular Na^+, H^+, and Ca^{2+} ions causing edema and worsening tissue acidosis [18,19], the unregulated release of excitatory amino acids along with the inhibition of astrocytic uptake can also contribute to excitotoxic damage *via* the *N*-methyl-D-aspartate (NMDA) receptor [20,21]. Upon reventilation/reoxygenation, the high energy phosphate levels and the transmembrane ionic gradients are gradually restored, but the initially still increased intracellular and intramitochondrial Ca^{2+} levels will activate a host of intracellular proteases and nucleases, as well as enzymatic and non-enzymatic synthesis of reactive oxygen species (ROS). These alterations will trigger mitochondrial dysfunction [17] and DNA fragmentation [22] setting the stage for further delayed and programmed neuronal cell death during the secondary energy failure stage. Based on the above delineated sequence of events, rational neuroprotective therapies aimed to mitigate neuronal injury of HIE should focus on (1) shortening the duration of asphyxia, (2) administration of cytoprotective/antioxidant agents simultaneously with the resuscitation to limit reoxygenation/reperfusion injury, and (3) to implement neuroprotective interventions before the neuronal injury related to secondary energy failure occurs. These options can and should be combined to maximize their neuroprotective potential, but perhaps the third option has the largest translational feasibility as there is usually a latent phase between the restoration of brain energy metabolism upon reoxygenation and the onset of secondary energy failure that offers at least a 5–6 h long therapeutic window for interventions.

In the current experiments, we used a large animal (piglet) PA/HIE model that was previously characterized [4] and now confirmed to present all the major metabolic hallmarks of PA also observed in humans at birth (hypoxemia, hypercapnia and lactic acidosis), and to develop HIE shown by neuropathology scores. This model has been successfully implemented to show neuroprotection afforded by reventilation with gas mixture containing 2.1% molecular hydrogen, likely mitigating the oxidative stress-induced neuronal damage associated with reoxygenation/reperfusion [4]. Another attractive neuroprotective target would be the prevention of neuronal cell death through the activation of antiapoptotic signaling pathways. This approach could be used in the latent phase after reoxygenation but before the onset of secondary energy failure as although necrosis can be observed within minutes, apoptosis takes more time to develop [23]. For instance, exogenous or endogenous BDNF could exert neuroprotection through stimulation of neuronal survival and inhibition of apoptosis in this period. BDNF might be involved in the endogenous neuroprotective response after PA, as 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 [24]. Furthermore, exogenous BDNF was found neuroprotective in a rat neonatal hypoxic-ischemic brain injury model [13]. Neuroprotection elicited by intracerebroventrical 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 lasting up to at least 12 h. Furthermore, ERK activation was specifically shown to reduce apoptotic neuronal death assessed with cleaved caspase 3 immunohistochemistry [13].

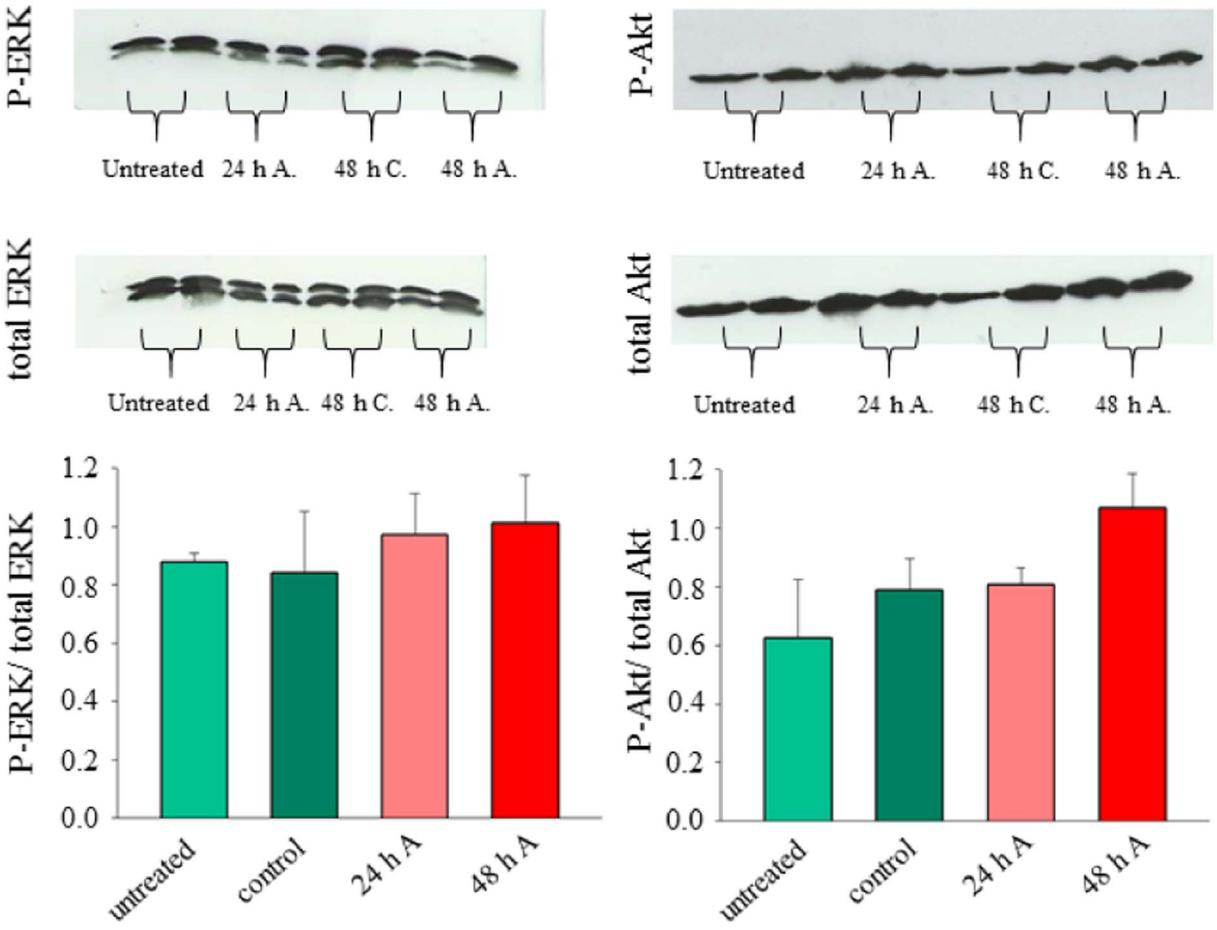

Fig. 3. Ratio of phosphorylated (P-ERK, P-Akt) and levels of total ERK and Akt in the cerebral cortex of the different experimental groups (bar graphs) with representative blots. There were no significant differences among the different groups. *Untreated* – untreated group; *Control*, *C* – time control animals without asphyxia; *A* – asphyxia group, 24 h–48 h – length of survival. Data are shown as mean ± S.E.M.

In addition to BDNF, other growth factors such as basic fibroblast growth factor have been identified as a factor promoting cell survival and neurogenesis, through activation of the ERK pathway [25,26]. In addition, Cohen-Armon et al. demonstrated that ERK2 phosphorylation can be modulated by PolyADP-ribose polymerase-1 (PARP-1) which catalyze a posttranslational modification of nuclear proteins by polyADP-ribosylation [27]. PARP-1 activation could play a role in our model as it has been found to be induced by hypoxia in newborn piglets [28]. The importance of the activation of the PI3K/Akt signaling pathway has been demonstrated in an adult rat stroke model, where cerebral infarct was elicited with permanent middle cerebral artery occlusion (pMCAO). Again, BDNF could reduce infarct size that was associated with the phosphorylation of Akt. Wortmannin, a selective PI3K inhibitor, could reverse the increment in p-Akt level and the afforded neuroprotection [29]. The transient elevation in p-Akt levels was also noted in adult mice within hours of MCAO, and this endogenous activation was regarded as neuroprotective [30].

Our current study yielded markedly different results concerning the phosphorylation levels of both ERK and Akt. Unlike in the previously cited adult or P7 neonatal rodent studies [13,29,30], ERK and Akt

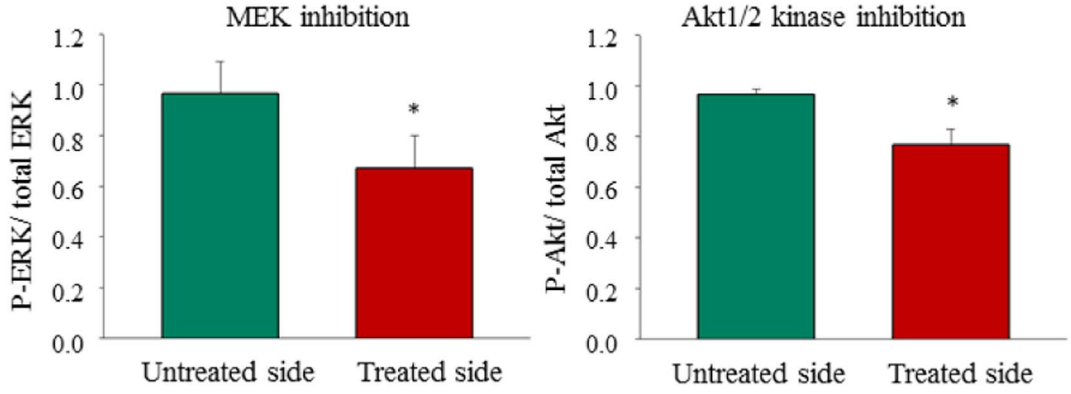

Fig. 4. Ratio of phosphorylated (P-ERK, P-Akt) and levels of total ERK and Akt in the normoxic, non-asphyxiated cerebral cortex after inhibition with the MEK inhibitor U0126 and the Akt1/2 kinase inhibitor A-6730, respectively. Both inhibitor significantly reduced phosphorylation compared to the contralateral untreated side. *$p < 0.05$ compared to the untreated side. Data are shown as mean ± S.E.M.

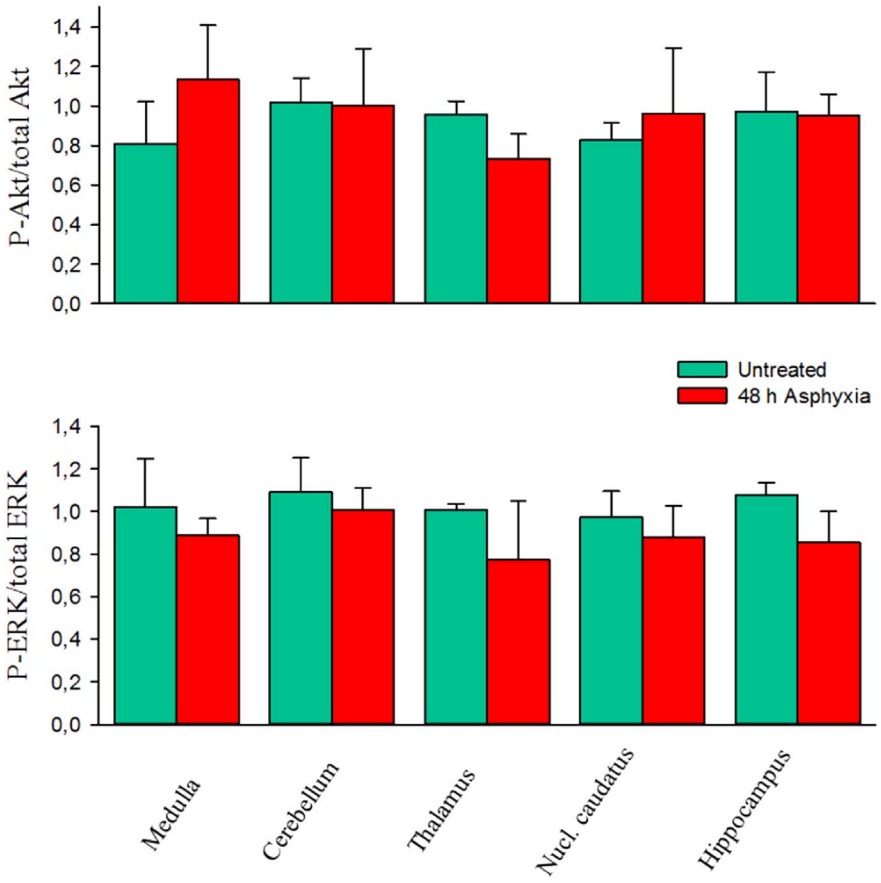

Fig. 5. Ratio of phosphorylated (P-ERK, P-Akt) and of total ERK and Akt levels in the assessed brain regions from the untreated and 48 h asphyxia groups (n = 3–3). Assessment of the different brain regions yielded essentially similar data: both ERK and Akt displayed high degree of phosphorylation that was unaffected by asphyxia, there were no significant differences among the different groups. *Untreated* – untreated group; *48 h Asphyxia* – asphyxia group with 48 h period of survival. Data are shown as mean ± S.E.M.

phosphorylation was virtually complete also in the cortex of untreated naïve animals, and it was not changed significantly either in the normoxic time controls or the animals subjected to PA for at least 48 h. This high degree of baseline phosphorylation/activation of untreated animals was also observed in virtually all of the assessed brain regions, not only in the neocortex, but also in the hippocampus, the caudate nucleus, the thalamus, cerebellum, and the medulla oblongata suggesting a general phenomenon. Similarly, high phosphorylation levels were maintained also at 48 h after asphyxia. Our pharmacological experiments proved that these findings 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. 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 [31]. However, our results suggest that in these models the effects of delivery on cerebral function may have already likely faded, and the activation levels of ERK and Akt are significantly different from the immediate perinatal period. These results question the efficacy of therapeutic interventions aiming to activate these antiapoptotic signaling pathways to obtain neuroprotection. This suggestion is in line with the recent findings of an elegant study. Robertson et al. 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 using cleaved caspase-3 immunohistochemistry could not be shown in the cerebral cortex [32].

5. Conclusion

We demonstrated in a translational newborn piglet model of PA/HIE that the endogenous activations levels of ERK and Akt kinases critical in antiapoptotic signaling are already high in the cerebral cortex and all other assessed brain regions under baseline conditions and would not significantly change up to 48 h following asphyxia capable to induce significant neuronal injury. We propose that further activation of these signaling pathways is difficult, and this limitation should be taken into consideration when designing rational neuroprotective treatments to mitigate the long term adverse effects of HIE.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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