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ORIGINAL PAPER

Differential Expression of Apoptotic Proteins Following Hypoxia-induced CREB Phosphorylation in the Cerebral Cortex of Newborn Piglets

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Abstract The present study investigates the correlation between the hypoxia-induced phosphorylation of cyclic AMP response element binding protein and the expression of apoptotic proteins (proapoptotic proteins Bax and Bad and antiapoptotic proteins Bcl-2 and Bcl-xl) during hypoxia in the cerebral cortex of newborn piglets. Piglets were divided into normoxic (Nx) and hypoxic (Hx, $FiO_2 = 0.06$ for 1 h) groups. Cerebral tissue hypoxia was documented by ATP and phosphocreatine (PCr) levels. Ser^{133} phosphorylation of cyclic AMP response element binding (CREB) protein was determined by Western blot analysis using a specific anti-phosphorylated Ser^{133}-CREB protein antibody. The expression of apoptotic proteins was determined by using specific anti-Bax, anti-Bad, anti-Bcl-2 and anti-Bcl-xl antibodies. ATP and PCr values (μmoles/g brain) in Hx were significantly different from Nx (ATP: 4.40 ± 0.39 in Nx vs. 1.19 ± 0.44 in Hx, $P < 0.05$ vs. Nx; PCr: 3.60 ± 0.40 in Nx vs. 0.70 ± 0.31 in Hx, $P < 0.05$ vs. Nx). Ser^{133} phosphorylated CREB protein ($OD \times mm^2$) was 74.55 ± 4.75 in Nx and 127.13 ± 19.36 in Hx ($P < 0.05$ vs. Nx). The expression of proapoptotic proteins Bax and Bad increased and strongly correlated with the increase in CREB protein phosphorylation (correlation coefficient $r = 0.82$ and $r = 0.85$, respectively). The expression of antiapoptotic proteins Bcl-2 and Bcl-xl did not show correlation with CREB protein phosphorylation. We conclude that cerebral hypoxia results in differential regulation of CREB protein-mediated expression of proapoptotic and antiapoptotic proteins in the cerebral cortex of newborn piglets. We propose that the increased expression of proapoptotic vs antiapoptotic genes will lead to an increased potential for apoptotic programmed cell death in the Hx newborn brain.

Keywords CREB protein · Phosphorylation · Bax · Bad · Bcl-2 · Bcl-xl · Hypoxia · Brain

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Introduction

Intranuclear Ca^{++} regulates a number of critical nuclear functions including regulation of transcription factors, cell cycle regulation, gene transcription, DNA replication and nuclear envelope breakdown [1, 2]. Furthermore, nuclear Ca^{++} signals potentially control a number of events leading to hypoxia-induced programmed cell death. Nuclear and cytosolic Ca^{++} signals are differentially regulated, and the extranuclear Ca^{++} concentration determines the mode of Ca^{++} entry into the nucleus.

The increased intracellular Ca^{++} is a primary mediator of activity-dependent gene transcription under a number of experimental conditions [3–6]. The patterns of neuronal impulse and the specific properties of the stimulus-induced calcium transients determine the nature and amplitude of the genomic response [5, 7]. Several factors including the site of calcium entry, the amplitude and the spatial properties of the calcium signals determine the calcium regulated gene expression

[8–13]. Furthermore, the duration of calcium signal also contributes to the specificity of the transcription induction. In cells of the immune system only a continuous rise in intracellular Ca^{++} concentration, but not a brief spike, induced translocation of transcription factors, NF-ATc [14]. It was demonstrated that gene expression in neurons is also determined by the duration of calcium transients and the activity-dependent transcription is regulated by the duration of calcium transients [6].

In previous studies we have shown that cerebral hypoxia results in increased nuclear Ca^{++}-influx in neuronal nuclei of the cerebral cortex of newborn piglets [15, 16]. The nuclear Ca^{++}-influx increased as a function of increase in cerebral tissue hypoxia, as measured by decrease in high energy phosphates, ATP and phosphocreatine (PCr). We have demonstrated that cerebral hypoxia results in increased Ca^{++}/calmodulin kinase (CaM kinase) IV activity and increased cyclic AMP response element binding (CREB) protein phosphorylation in neuronal nuclei of newborn piglets [17, 18].

Previously, we have shown that hypoxia results in increased expression of apoptotic proteins in the cerebral cortex of newborn piglets. Bcl-2 family of proteins (including Bcl-2 and Bax) control cell proliferation, differentiation and programmed cell death during normal brain development [19–21]. Bax and Bcl-2 are inducible genes found in the developing and adult central and peripheral nervous systems [22–24]. Bcl-2 prevents apoptosis by forming a heterodimer with the proapoptotic protein Bax and protects cells from programmed cell death following hypoxia [19–21].

The present study specifically focuses on investigating during hypoxia the relationship between phosphorylation of CREB protein at Ser133 and the expression of proapoptotic proteins Bax and Bad, and antiapoptotic proteins Bcl-2 and Bcl-xl in neuronal nuclei of the cerebral cortex of newborn piglets. In the present study we have tested the hypothesis that hypoxia-induced increase in phosphorylation of CREB protein at Ser^{133} has a strong correlation with the expression of proapoptotic proteins. On the basis of our previous studies we anticipate a relationship between CREB protein phosphorylation and proapoptotic protein expression.

Materials and methods

Animal experimentation and induction of hypoxia

Studies were performed on 3–5 day old Yorkshire piglets obtained from the Willow Glenn Farm, Strausburg, PA. The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of Drexel University. Newborn piglets were randomly assigned to one of two groups: normoxic (Nx) ($n = 6$) and hypoxic (Hx) ($n = 6$). The animals were ventilated for 1 h under either Nx condition ($FiO_2 = 0.21$) or Hx condition; hypoxia was induced by lowering the FiO_2 to 0.07 for 60 min. At the end of the experimental period, the animal was sacrificed; the cortical tissue was removed and placed either in homogenization buffer for isolation of neuronal nuclei or in liquid nitrogen, and then stored at –80°C for later biochemical studies.

Isolation of cerebral cortical neuronal nuclei

Cerebral neuronal nuclei were isolated and purified according to the methods of Giuffrida et al. 1975 [25] and purified as described by Austoker et al. 1972 [26]. Cortical tissue was homogenized by habd ib Dounce-type glass homogenizer (200 ± m clearance) in 22 strokes in 15 volumes of a medium containing 0.32 M sucrose, 10 mM Tris–HCl (pH 6.8) and 1 mM $MgCl_2$ to achieve a final concentration of 2.1 M sucrose, at which neuronal nuclei are settled. The nuclei were then purified by centrifuging at 53,000*g* for 60 min . All procedures were carried out at 4°C. The nuclear pellet was suspended in the medium (0.32 M sucrose, 10 mM Tris–HCl buffer, pH 6.8 and 1 mM $MgCl_2$) and the purity of neurona nuclei was assessed by phase contrast microscope (Olympus, Melville, NY, USA). The neuronal nuclei were characterized by the presence of a centrally located nucleolus (one nucleolus/nucleus) as compared with the presence of multinucleoli in the astrocytic and oligodendrocytic nuclei. The final nuclear preparation was devoid of any microsomal, mitochondrial or plasma membrane contaminant with a purity for neuronal nuclei of 90%. Protein content was determined by the method of Lowry et al. 1951 [27]. The nuclear membrane preparation was diluted to a final concentration of 100 μg protein/100 μl.

Immunoprecipitation and western blot analysis of Ser^{133}-CREB protein phosphorylation and pro-and antiapoptotic proteins Bax, Bad, Bcl-2 and Bcl-xl

Neuronal nuclear membranes were prepared as described above in the presence of protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 0.2 mM sodium orthovanadate]. Protein content was determined by the method of Lowry et al. 1951 [27], and the nuclear membrane preparation was diluted to a final concentration of

100 μg/100 μl. The membrane protein was solubilized in modified RIPA buffer [50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 1 μg/ml each of apoprotein, leupeptin and pepstatin]. Equal amounts of each neuronal nuclear protein were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes. Nitrocellulose membranes in duplicate were then blocked with 10% non-fat milk in phosphate-buffered saline. The membranes were subsequently incubated with anti-phosphorylated (serine-133) CREB protein antibody (Upstate Biotechnology, Lake Placid, NY, USA) or specific anti-Bax, anti-Bad, anti-Bcl-2 and anti-Bcl-xl antibodies (Upstate Biotechnology, Lake Placid, NY, USA). Immunoreactivity was then detected by incubation with horseradish peroxidase-conjugated secondary antibody (Rockland, Gilbertsville, PA, USA). Specific complexes were detected by enhanced chemiluminescence method using the ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and analyzed by imaging densitometry (GS-700 densitometer, Bio-Rad). The densitometric scanning data were expressed as autoradiographic values ($OD \times mm^2$) per immunoblot protein representing CREB protein phosphorylation. In another set, CREB protein expression was also determined by anti-CREB antibody using the above method.

Determination of ATP and phosphocreatine

ATP and PCr concentrations were determined according to the method of Lamprecht et al. 1974 [28].

Statistical analysis

Data was analyzed using one way analysis of variance ANOVA to compare Nx and Hx groups. A P value of less than 0.05 was considered statistically significant. All values are presented as mean ± standard deviation (SD).

Results

Brain tissue hypoxia in piglets was documented by determining the ATP and PCr levels in the cerebral cortical tissue. ATP values (μmoles/g brain) decreased from 4.40 ± 0.39 in Nx to 1.19 ± 0.44 in Hx ($P < 0.05$ vs. Nx). PCr values (μmoles/g brain) decreased from 3.60 ± 0.40 in Nx to 0.70 ± 0.31 in Hx ($P < 0.05$ vs. Nx). The level of high energy phosphates decreased significantly in the Hx group as compared to Nx and the data demonstrate that cerebral tissue hypoxia was achieved in the Hx group.

CREB protein phosphorylation at Ser^{133} in neuronal nuclei of Nx and Hx groups are shown in Fig. 1. The relationship of CREB protein phosphorylation with the cerebral high energy phosphates ATP and PCr are shown in Fig. 1. The results show an increased phosphorylation of CREB protein as afunction of decrease in high energy phosphates during hypoxia in the cerebral cortical neuronal nuclei of newborn piglets. These results confirm our previous findings that cerebral hypoxia results in increased CREB protein phosphorylation with decreased high energy phosphates.

The expression of proapoptotic proteins Bax and Bad were plotted against increasing concentrations of phosphorylated CREB protein. The relationship of CREB protein phosphorylation with the expression of Bax and Bad is shown in Fig. 2. The results show an increased expression of Bax and Bad with increase in

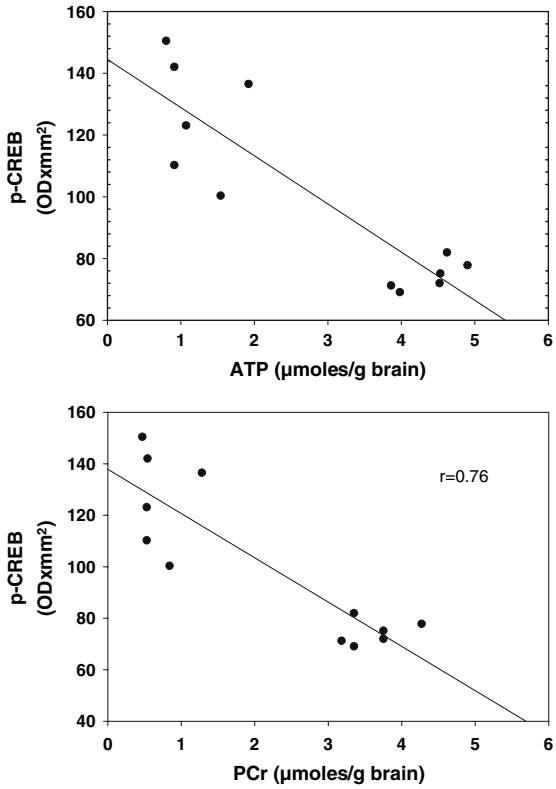

Fig. 1 The effect of hypoxia on CREB protein phosphorylation at Ser^{133} in neuronal nuclei of the cerebral cortex of newborn piglets. Densitometry are shown as a function of cerebral high energy phosphates, ATP, and phosphocreatine. Experiments were performed on six normoxic and six hypoxic newborn piglets. The CREB protein phosphorylation at Ser^{133} in neuronal nuclei is expressed as $OD \times mm^2$

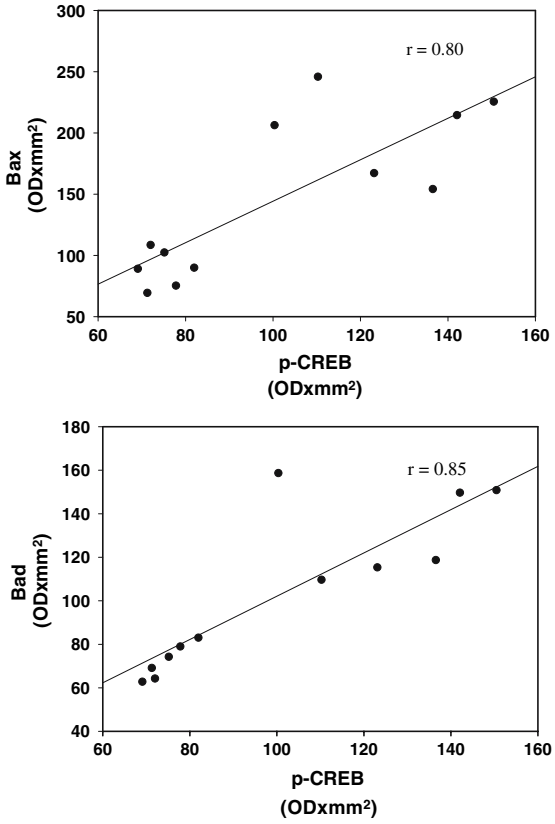

Fig. 2 Relationship between CREB protein phosphorylation at Ser133 and expression of proapoptotic proteins Bax and Bad in neuronal nuclei of the cerebral cortex of normoxic ($n = 6$) and hypoxic ($n = 6$) newborn piglets. Western blot analysis was performed using anti-Bax and anti-Bad antibodies (Santa Cruz biotechnology, CA) and anti-actin antibody (Chemicon). Protein Bands were detected using enhanced chemiluminescence detection system and analyzed by imaging densitometry. The densitometry data of Bax and Bad protein expression is presented as a function of CREB protein phosphorylation

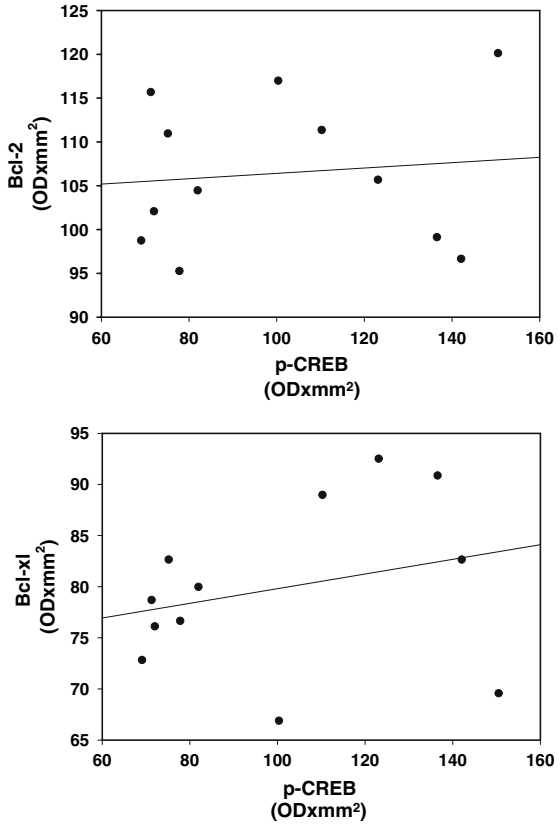

Fig. 3 Relationship between CREB protein phosphorylation at Ser133 and expression of antiapoptotic proteins Bcl-2 and Bcl-xl in neuronal nuclei of the cerebral cortex of normoxic ($n = 6$) and hypoxic ($n = 6$) newborn piglets. Western blot analysis was performed using anti-Bcl-2 and anti-Bcl-xl antibodies (Santa Cruz biotechnology, CA) and anti-actin antibody (Chemicon). Protein Bands were detected and analyzed by imaging densitometry. The densitometry data of Bcl-2 and Bcl-xl protein expression is presented as a function of CREB protein phosphorylation

phosphorylation of CREB protein at Ser133. The data show a strong correlation between CREB protein phosphorylation and the expression of proapoptotic proteins Bax and Bad (correlation coefficient: $r = 0.82$ and $r = 0.85$, respectively). These results demonstrate that expression of proapoptotic proteins increases as a function of increase in CREB protein phosphorylation on Ser133 during hypoxia in neuronal nuclei of the cerebral cortex of newborn piglets.

The expression of antiapoptotic proteins Bcl-2 and Bcl-xl were plotted against increasing concentrations of phosphorylated CREB protein. The relationship of CREB protein phosphorylation with the expression of Bcl-2 and Bcl-xl is shown in Fig. 3. The results show that hypoxia does not result in increased expression of Bcl-2 and Bcl-xl. The data do not show a correlation between CREB protein phosphorylation and the expression of antiapoptotic proteins Bcl-2 and Bcl-xl (correlation coefficient: $r = 0.82$ and $r = 0.85$, respectively). These results demonstrate that the expression of antiapoptotic proteins did not increase during hypoxia and had no correlation with the phosphorylation of CREB protein in neuronal nuclei of the cerebral cortex of newborn piglets. These results indicate differential expression of pro- and antiapoptotic proteins during hypoxia in the cerebral cortex of newborn piglets.

Discussion

Intracellular Ca^{++} is a critical mediator of hypoxic-ischemic neuronal excitotoxicity. The Ca^{++}-dependent neuronal damage is due to NMDA receptor-mediated excitotoxicity that is initiated as a result of hypoxia. During hypoxia an increase in intracellular Ca^{++} is a

result of the NMDA receptor ion-channel-mediated Ca^{++}-influx as well as the release of Ca^{++} from intracellular stores such as mitochondria and the endoplasmic reticulum [29]. Previously, we have shown that cerebral hypoxia results in increased nuclear Ca^{++}-influx, increased CaM kinase IV activity and increased phosphorylation of CREB protein at Ser^{133} in neuronal nuclei of the cerebral cortex of newborn piglets. The present study tests the hypothesis that cerebral hypoxia-induced increased CREB phosphorylation correlates with the expression of proapoptotic proteins in the cerebral cortex of newborn piglets.

The results of the present study show that cerebral hypoxia results in increased phosphorylation of CREB protein at Ser133 in neuronal nuclei of the cerebral cortex of newborn piglets. The data presented in the graphs show a strong correlation between CREB protein phosphorylation and the expression of proapoptotic proteins Bax and Bad. There is no correlation between CREB phosphorylation with the expression of antiapoptotic proteins Bcl-2 and Bcl-xl. These results show a differential effect of hypoxia on the CREB mediated transcription of pro- and anti-apoptotic genes of the same Bcl-2 gene family. This is quite interesting to note how cerebral hypoxia can have such a selective effect on transcription mediated by CREB.

CaM kinase IV is present in nucleus and activates transcription factors including CREB protein. The importance of CaM kinases in transcription was supported by the observation that Ca^{++}-dependent transcription of three immediate early genes (c-fos, NGFI-A, and NGFI-B) was blocked by the CaM kinase inhibitor KN-62 [30]. CaM kinase IV is a member of CaM kinase cascade and is predominantly located in the nucleus, and along with CaM kinase kinase is abundant in the brain. CaM kinase kinase and CaM kinase IV mediate Ca^{++}-regulated gene expression by activating CREB. CaM kinases are activated by a conformational change induced by binding to Ca^{++}/calmodulin complex, which in turn results in decreased interference of auto-inhibitory domain of CaM kinase with its active site. CaM kinase kinase activates CaM kinase IV, via phosphorylation of Thr^{200} or Thr^{196} in a process that requires Ca^{++}/calmodulin and ATP/Mg^{++}. Activated CaM kinase IV mediates Ca^{++}-regulated transcription by phosphorylating and activating CREB protein at Ser^{133} in a cyclic-AMP-independent process.

The CaM kinase cascade, leading to activation of transcription can be regulated at different points. The activity of CaM kinase IV is tightly regulated by protein kinases and protein phosphatases. First, CaM kinase kinase, the activator of CaM kianse IV, is inactivated when phosphorylated by CaM kinase I, CaM kinase IV or protein kinase A. Second, CaM kinase IV can be inactivated by protein phosphatase 2A. Third, protein serine/threonine phosphatases (protein phosphatase 1, protein phosphatase 2A and protein phosphatase 2B) can dephosphorylate CREB at Ser^{133} causing termination of transcriptional activity. In addition CREB activation can also be blocked by CaM kinase II dependent phosphorylation at Ser^{142} that blocks the activation of CREB. The differential response of hypoxia on the expression of proapoptotic and antiapoptotic proteins could be due to the differential effect on mRNA translation of these specific genes.

As observed in this study during hypoxia, overexpression of Bax or an increase in the ratio of Bax to Bcl-2, is associated with cells undergoing programmed cell death [21]. Up-regulation of Bax and/or down-regulation of Bcl-2 mRNA or protein levels have been observed in several experimental models including transient global ischemia [22, 31, 32]; the expression of Bax and Bcl-2 genes may be regulated by p53 [33]. Using a permanent middle cerebral artery occlusion model, DNA breaks occurred within 6 h and levels of Bax mRNA significantly increased within the infarcted hemisphere, indicating a shift in the gene expression ratio of Bcl-2 to Bax [31]. Using immunohistochemistry before and after 10 min of global ischemia, there were high levels of Bax, low levels of Bcl-2, and DNA-strand breaks in the same population of neurons found to be degenerating morphologically [34]. Neurons with elevated Bax levels almost uniformly had morphologic evidence of ischemic degeneration with apoptotic features including nuclear DNA fragmentation [24].

The increased Bax/Bcl-2 ratio favoring an increase in free Bax and promoting cell death observed during post-hypoxic reoxygenation can also be attributed to phosphorylation of Bcl-2 in neuronal nuclear membranes where phosphorylation of Bcl-2 compromises its anti-apoptotic potential [35]. Bax and Bcl-2 are located primarily in the membranes of the nuclear envelope, mitochondria, and parts of the endoplasmic reticulum [30, 36]. Bax and Bcl-2 within the nuclear membrane help regulate the intranuclear Ca^{2+} concentration to be independent of the cytosolic Ca^{2+} concentrations [37]. Mitochondrial membrane Bcl-2 does not prevent cell death by directly altering mitochondrial function [32], but instead by blocking release of cytochrome *c* from the mitochondria into the cytosol [38–40]. However, programmed cell death can occur in cells without mitochondrial DNA and these cells can be protected from apoptosis by the over expression of Bcl-2, suggesting that neither apoptosis nor the protective effect of Bcl-2 depends solely on mitochondria [32].

In a series of studies, we have demonstrated that administration of nitric oxide synthase (NOS) inhibitor, N-nitro-L-arginine (NNLA) prevented the hypoxia-induced increase in CaM kinase IV activity, increase in CREB phosphorylation, increase in the expression of pro-apoptotic protein Bax and increased fragmentation of nuclear DNA [17, 18, 41]. During hypoxia, NO and superoxide radicals are concurrently produced. NO produces peroxynitrite on combining with superoxide. The rate of reaction between NO and superoxide is several orders of magnitude faster than the reaction between superoxide and superoxide dismutase. Therefore, the production of peroxynitrite is favored over dismutation of superoxide. Thus NO produced during hypoxia may result in nitrosylation as well as peroxynitrite-mediated nitration of a number of proteins including proapoptotic and antiapoptotic proteins.

Nitric oxide is a critical mediator of neuronal injury as evidenced by the administration of pharmacological inhibitors of NOS that reduce neuronal injury from focal ischemia, NMDA dependent excitotoxicity and cerebral hypoxia [42–44]**.** Mice deficient in nNOS gene exhibit significant protection against cerebral ischemia and NMDA-mediated neurotoxicity [42, 43] Furthermore, cerebral hypoxia results in the generation of NO free radicals [45]. In addition, administration of NOS inhibitor prevented the hypoxia-induced generation of free radicals, nitration of the NMDA receptor subunits, CaM kinase IV activation, increased phosphorylation of cAMP response element binding (CREB) protein at Ser^{133} , increased expression of apoptotic protein Bax and fragmentation of nuclear DNA [18, 41, 44, 46].

We have shown that the activity of PP and PP2A are decreased during hypoxia in the cerebral cortex of newborn piglets [47]. The decrease in phosphatase activity could be due to NO-mediated modification of cysteine residue in the enzyme protein. We have also shown that cerebral hypoxia results in inhibition of mitogen activated protein (MAP) kinase phosphatase-1 and MAP kinase phosphatase-3 in the cerebral cortex of newborn piglets and the hypoxia-induced decrease in MAP kinase phosphtase-1 and -3 is mediated by NO. Therefore, NO produced during hypoxia may mediate modification of pro= and ant-apoptotic proteins by phosphorylation/dephosphorylation mechanisms.

In view of these observations, NO can play a central role in Hx neuronal death by both the necrotic as well as apoptotic or programmed cell death mechanisms. First, the NO-induced increase in NMDA receptor mediated intracellular Ca^{++} potentially initiates a number of reactions leading to increased free radical generation via a number of enzymatic pathways such as Ca^{++} activation of phospholipase A_2, causing release of arachidonic acid which then can be metabolized by cyclooxygenase and lipoxygenase, the conversion of xanthine dehydrogenase to xanthine oxidase by Ca^{++}-dependent activation of proteases and activation of nitric oxide synthase by Ca^{++} to further generate NO leading to formation of peroxynitrite and oxygen free radical species. The increased free radicals generated result in increased peroxidation of cellular and subcellular membranes leading to necrotic cell death. Second, the increased intracellular Ca^{++} may lead to increased intranuclear Ca^{++} by mechanisms of Ca^{++}-influx such as the IP3 receptors and the nuclear membrane high affinity Ca^{++} ATPase. Furthermore, we have demonstrated that NO increases nuclear Ca^{++} influx. Increased intranuclear Ca^{++} may activate Ca^{++}-dependent endonucleases leading to DNA fragmentation. In addition, increased intranuclear Ca^{++} by activating CaM kinase IV and increased phosphorylation of CREB protein resulting in increased transcription of proapoptotic genes such as Bax and Bad, and initiating the early events of DNA fragmentation and programmed cell death. Thus a central role for NO is proposed in regulating neuronal function in Hx neuronal death, by altering the nuclear membrane mechanisms of Ca^{++}-influx resulting in increased CaM kinase activity and subsequent increased phosphorylation of CREB protein at Ser^{133}.

In summary: These results show that hypoxia results in increased phosphorylation of CREB protein at Ser133 in neuronal nuclei of the cerebral cortex of newborn piglets. The results demonstrate a strong correlation between the CREB protein phosphorylation at Ser133 and the expression of proapoptotic proteins Bax and Bad. There was no correlation between CREB protein phosphorylation and the expression of antiapoptotic proteins Bcl-2 and Bcl-xl. The results of this study show a selective hypoxia-induced expression of proapoptotic proteins vs antiapoptotic proteins that would result in an increased ratio of pro-/antiapoptotic proteins leading to increased potential for the initiation of programmed cell death in the Hx brain. We conclude that hypoxia results in a differential expression of pro- and anti-apoptotic proteins in neuronal nuclei of the cerebral cortex of newborn piglets.

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