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Phosphorylation of Caspase-9 in the Cytosolic Fraction of the Cerebral Cortex of Newborn Piglets Following Hypoxia

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Abstract

We have previously shown that hypoxia leads to increased expression and increased activity of caspase-9 in the cerebral cortex of newborn piglets. Previous studies have demonstrated the importance of caspase-9 in the initiation of the apoptotic cascade, however, the mechanism of caspase-9 activation is not well understood. Experiments were conducted on newborn piglets 2 – 3 days of age that were anesthetized and mechanically ventilated. Hypoxia was induced by lowering the FiO_2 to 0.05 – 0.07 $\times$ 1 hour, and was confirmed biochemically by demonstrating decreased levels of ATP and Pcr in the hypoxic groups in comparison with the normoxic group. The ATP level was 1.99 ± 0.66 in the hypoxic group versus 4.10 ± 0.19 in the normoxic group, $P < 0.05$, and the PCr value was 0.68 ± 0.14 in the hypoxic group, compared to 2.98 ± 0.39 in the normoxic group, $p < 0.05$. The cytosol of the neuronal nuclei from the cerebral cortex was probed with anti-phosphorylated Ser¹⁹⁶ caspase – 9 antibody, using Western blot analysis. Protein bands were analyzed using image densitometry. In both the hypoxic and normoxic samples, protein bands were demonstrated just above the 50 kD marker. Phosphorylated caspase – 9 expression in $OD \times mm^2$ was 43.85 ± 8.4 in the normoxic group and 67.6 ± 9.88 in the hypoxic group, $P < 0.05$. The results of this study demonstrate that caspase-9, a key protein in hypoxia induced apoptosis, is phosphorylated at the Ser¹⁹⁶ site during hypoxia. The results demonstrate that hypoxia results in a post-translational modification of caspase-9 at Ser¹⁹⁶, which may alter the activity of caspase-9 in the hypoxic newborn brain.

Keywords

Brain; Apoptosis; Caspase-9; Hypoxia; Newborn

1. Introduction

Apoptosis is a distinct form of controlled cell death which serves a number of physiological cell functions, including, control of cell numbers during the embryonic and fetal development, maintenance of tissue homeostasis, and the removal of abnormal cells [4]. This well organized process involves DNA fragmentation, membrane blebbing, cell shrinkage, and disassembly into membrane enclosed vesicles which are subsequently eliminated by phagocytosis, thereby preventing an inflammatory response to the intracellular components [6].

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Hypoxia is an apoptotic stimulus which has been implicated in neonatal morbidity and mortality. Our previous studies have shown that apoptosis occurs through a complex combination of processes including release of excitatory neurotransmitters, activation of excitatory amino acid receptors, increased intracellular calcium, production of oxygen free radicals, changes in cellular ion fluxes, alteration of cell membrane structure and function, changes in gene expression patterns, and programmed cell death which contributes to the cerebral dysfunction [17].

Caspases (cysteine aspartate proteases) are proteins which play an essential role at various stages of apoptosis. All caspases contain a cysteine residue at their active site, and specifically cleave substrate proteins at an aspartic acid residue. These proteases are found in the cytoplasmic compartment of animal cells as inactive zymogens and become activated by proteolytic cleavage at internal aspartate residues upon apoptotic stimulation [10].

The caspases are divided into two main classes. Those with the long prodomain are the class I caspases, and those with the short prodomain are the class II caspases. Class I caspases such as 8, 9, and 10 have the ability to auto-catalyze their own activation, and are activated in the early phases of apoptosis. These are known as the initiator caspases. Class II caspases include 3, 6, and 7. These are called the effector or the executioner caspases as these are responsible for the actual breakdown of the cells. These caspases require cleavage by another protease to become activated [5,10,18].

There are two major pathways of apoptosis in which caspases are involved. In the extrinsic pathway, the death signal is transmitted through the binding of an extracellular death ligand such as tumor necrosis factor (TNF) to its cognate receptor, the TNF receptor. The death receptor transmits the signals from outside the cell to the interior, where the apical proteases of the extrinsic pathway, caspase-8 and caspase-10 are recruited. The intrinsic pathway occurs as a consequence of cellular stress, and is mediated through the cytochrome c dependent activation of caspase-9 in the mitochondria of the cell [6]. Caspase-9 has been shown to be a major factor in apoptosis caused by tissue hypoxia, as it activates caspase-3 which is responsible for the breakdown of the cell structure observed in apoptosis.

In the proposed mitochondria-initiated pathway, caspase-9 activation occurs through the formation of a complex between procaspase-9, the apoptotic protease activating factor-1 (Apaf-1), and cytochrome c. This complex is known as the apoptosome. The apoptosome helps in the recruiting and activation of procaspase-9. The adapter protein Apaf-1 acts as a cofactor, increasing the proteolytic activity by orders of magnitudes, thereby greatly increasing the activation of caspase-9 [6,10].

Apaf-1 serves as a docking protein for caspase-9 and cytochrome c, and it interacts with caspase-9 at their homologous caspase recruitment domain (CARD). It has been demonstrated that both cytochrome c and either dATP or ATP are necessary for Apaf-1 to bind to caspase-9. It has been proposed that cytochrome c and dATP may induce a conformational change in Apaf-1 that exposes its CARD, allowing it to bind to caspase-9. Once caspase-9 has been activated, it cleaves and activates caspase-3 in as little as 15 minutes leading to apoptosis of the cell [12].

We have previously shown that hypoxia leads to increased expression and increased activity of caspase-9 in the cerebral cortex of newborn piglets, which in turn leads to activation of caspase-3 and cell death [10]. Previous studies have demonstrated the importance of caspase-9 in the initiation of the apoptotic cascade, however, the mechanism of caspase-9 activation is not well understood. It has been demonstrated that phosphorylation of caspase-9 both at the Ser¹⁹⁶ site and the Thr¹²⁵ site have important regulatory properties in the activity of caspase-9, and thus in the initiation or termination of apoptosis within a cell. This occurs because

phosphorylation at certain sites affects the processing and the proteolysis of caspase-9, which in turn controls its activation [1,2]. For example, it has been demonstrated that ERK2 mediated phosphorylation of caspase-9 at the Thr¹²⁵ site blocks post-translation processing of the protein, inhibiting its activation, and making apoptosis less likely to occur [1]. The present study tests the hypothesis that hypoxia leads to phosphorylation of caspase-9 at the Ser¹⁹⁶ site.

2. Material and Methods

Studies were performed in newborn piglets aged 2–5 days. The Institutional Animal Care and Use Committee of the Drexel University College of Medicine approved the animal protocol. Using 4% isoflurane for induction and 0.8% isoflurane for maintenance of anesthesia, a tracheostomy was performed and an endotracheal tube secured. Arterial and venous catheters were placed via the femoral vessels for blood sampling and administration of drugs. Lidocaine (1%) was used for local anesthesia at all incision sites. The animals were allowed to breathe spontaneously during instrumentation. Following surgery, isoflurane was withdrawn and the animals were placed on a volume ventilator with 72% nitrous oxide and 28% oxygen; tubocurarine (3 mg/kg) was administered, and fentanyl (30 µg/kg every 45 min) was used for analgesia. The ventilator settings and FiO₂ were adjusted in order to maintain a PaCO₂ of 35–45 mmHg and a PaO₂ of 80–110 mmHg. Blood pressure, heart rate, core temperature, and end-tidal CO₂ were monitored continuously. Core temperatures were maintained at 38.5–39°C using a servo-controlled warming blanket.

Piglets were randomly assigned to one of the two groups: a normoxic group, or a hypoxic group. Baseline measurements were obtained after 45 min in both normoxic and hypoxic groups while maintaining normal arterial blood gas values. Following baseline measurements, the five piglets assigned to the control group were maintained under normoxic conditions for 60 min. The six animals in the hypoxic group were ventilated with an FiO₂ of 0.06 for 1 h. At the end of the study the cerebral cortex was harvested within 5 sec of decapitation and placed in Tris–HCl buffer for isolation of cytosolic fractions; an additional section of brain was frozen in liquid nitrogen for determination of ATP and phosphocreatine levels, and stored at –80°C for biochemical analysis.

Brain tissue concentrations of ATP and PCr

Tissue hypoxia was confirmed biochemically by determining the levels of high energy phosphates ATP and phosphocreatine (PCr). The concentrations of ATP and PCr in the cerebral cortex were determined by an enzyme-coupled assay using the method of Lamprecht et al [11]. Deproteinized cortical homogenate from 500 mg of frozen cortex was ground to a powder in 7% (v/v) perchloric acid (1 ml/100 mg brain) under liquid nitrogen, allowed to thaw on ice, then centrifuged at 4000 g for 5 min. Aliquots of supernatant were neutralized with KOH–K₂CO₃ and centrifuged at 2000 g for 5 min. ATP and PCr concentrations were determined in a 1-ml volume containing buffer (50 mM triethanolamine, 5 mM MgCl₂, 1 mM EDTA, 2 mM glucose), 400 µl of the neutralized 2000 g supernatant, and 20 µl NADP. Readings were taken every 5 min after the addition of 10 µl hexokinase until a steady state was reached. The ATP concentration was calculated from the increase in absorbance at 340 nm during the 20 min after the addition of hexokinase. A 20 µl volume of ADP and 20 µl of creatine kinase were then added and readings taken at 5 min intervals until a second steady state was reached. PCr concentration was calculated from the increase in absorbance at 340 nm after the addition of creatine kinase.

Isolation of cerebral cortical cytosolic fraction

Cerebral tissue mitochondrial fraction was isolated by homogenizing one gram of cerebral cortical tissue by a Dounce-type glass homogenizer (seven strokes with pestle clearance 0.15

mm and seven strokes with pestle clearance 0.07 mm) in 30 ml of fresh isolation medium containing 0.32 M sucrose, 1 mM EDTA and 20 mM Tris-HCl buffer, pH 7.1. The 15,000 g supernatant was centrifuged at 100,000 g for 660 min to obtain the cytosolic fraction. The cytosolic preparations were diluted to a final concentration of 100 µg protein/100 µl.

Separation of cytosolic proteins

Proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose paper. The nitrocellulose paper was blocked with 10% phosphate-buffered saline (PBS)-milk at 4°C with constant agitation for 6–8 h. Incubation with anti-phosphorylated caspase-9 serine¹⁹⁶ antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) in 3% PBS-milk was done overnight at 4°C. Subsequently the nitrocellulose was washed with distilled water (dH₂O) and incubated with horseradish peroxidase conjugated secondary antibody (Rockland, Gilbertsville, PA, USA) in 3% milk for 1.5 h at room temperature with constant agitation. Following washings with dH₂O and PBS–0.05% Tween 20 the specific complexes were detected using ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2–3 min. Protein bands were analyzed using imaging densitometry (GS-700 imaging densitometer, Bio-Rad, Hercules, CA, USA). The density of proteins was expressed as absorbance ($OD \times mm^2$).

Statistical analysis

Statistical analysis of biochemical measurements was performed using a one-way analysis of variance (ANOVA) and Tukey-test for comparison among groups. A p value <0.05 was considered significant. All values shown are mean $\pm$ standard deviation (S.D.).

3. Results

Cerebral tissue hypoxia was documented by determining the levels of ATP and phosphocreatine (PCr). The data is presented in Figure 1. Cerebral cortical concentration of ATP was 4.10 ± 0.19 µmol/g brain in the normoxic group, as compared to 1.99 ± 0.66 µmol/g brain in the hypoxic group ($P < 0.05$). PCr level was 2.98 ± 0.39 µmol/g brain in the normoxic group and 0.68 ± 0.14 µmol/g brain in the hypoxic group ($p < 0.05$). The ATP and PCr levels significantly decreased in the hypoxic group demonstrating that significant cerebral tissue hypoxia was achieved in the hypoxic group.

Measurement of Ser¹⁹⁶ phosphorylated caspase-9 in the hypoxic and normoxic groups were obtained by measuring the density of the protein bands on the Western blots of the respective groups. The representative Western blots for the normoxic and the hypoxic groups are presented in figure 2. The data from all the animals are shown in figure 3. Phosphorylated caspase – 9 density expressed as in $OD \times mm^2$ was 43.85 ± 8.4 in the normoxic group and 67.6 ± 9.88 in the hypoxic group, $p < 0.05$. These results indicate that there was a statically significant increase in the expression of Ser¹⁹⁶ phosphorylated caspase-9 in the hypoxic sample verses the normoxic sample.

4. Discussion

Cerebral hypoxia in the perinatal period has been associated with damage to specific areas of the brain [16] and is associated with increased neonatal morbidity and mortality, as well as long term sequelae including seizure disorders, cerebral palsy, and mental retardation [17]. Over the last few years, it has been demonstrated that the damage to the brain which occurs from hypoxia in the neonatal period is not simply due to necrosis, but rather apoptosis, or programmed cell death. Apoptosis is a process where cell death is brought about through characteristic morphological changes that include shrinkage of the cytoplasm, membrane

blebbing, compaction of the nuclear chromatin, chromosomal DNA fragmentation, and the formation of small vesicles called apoptotic bodies, which are eventually phagocytosed by macrophages and other neighboring epithelial cells. These changes result from the activation of a set of apoptotic signaling pathways [3].

Caspase-3 is one of the main executioner caspases involved in hypoxic cell death. There are a number of ways which caspase-3, once activated, brings about apoptosis of a cell. Activated caspase-3 cleaves numerous intracellular proteins such as actin, fodrin, and lamins, which are important components in the maintenance of cell structure. Caspase-3 also cleaves and inactivates nuclear enzymes such as poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase activated Dnase (ICAD). Inactivation of PARP which is a DNA repair enzyme leads to cessation of cellular DNA repair, while inactivation of ICAD leads to activation of caspase activated Dnase (CAD), which then cleaves chromosomal DNA. This results in disruption of cell nuclei and the morphological changes that are characteristic of apoptosis [5,10].

Caspase-9 activates caspase-3. It has been demonstrated that caspase-9 can only become activated after forming a complex with apoptotic protease activating factor – 1 (Apaf-1). The Apaf-1 binds to procaspase-9 because the two proteins have homologous CARDs. It has also been demonstrated that Apaf-1 will only bind to procaspase-9 in the presence of dATP or ATP which undergoes hydrolysis of a high energy bond, as well as cytochrome c. It is likely that these two are integral to the binding of Apaf-1 to procaspase-9 because they induce a conformational change in the Apaf-1 that exposes its CARD domain, thereby allowing the binding of procaspase-9 to the Apaf-1 [12,15]. When the mitochondria are exposed to the apoptotic stimulus, cytochrome c is released from the mitochondria into the cytoplasm. Once in the cytoplasm, the cytochrome c associates with Apaf-1 in the presence of dATP or ATP, thereby forming the apoptosome, and activating procaspase-9 to active caspase-9 [3].

We have previously demonstrated that there is an increase in caspase-9 expression and activity as well as caspase-3 activity in the hypoxic brain [10]. We have also demonstrated that this increase in caspase-9 and caspase-3 activity is related to calcium influx into the hypoxic cell. Studies have shown that hypoxia, possibly through a nitric oxide mediated pathway, leads to modification of the N-methyl-D aspartate (NMDA) receptor, a subtype of the glutamate receptor located on the cell membrane. This receptor modification leads to an increased influx of calcium into the cell [5,8,17,19]. We have also demonstrated that hypoxia leads to modifications of high affinity Ca^{2+} - ATPase, IP_3 , and IP_4 , which are the nuclear envelope regulators of intranuclear Ca^{2+} responsible for transporting calcium from the cytoplasm into the nucleus. These receptor modifications lead to an increase in the level of intranuclear calcium. The hypoxia induced increase in intranuclear Ca^{2+} activates several calcium dependent pathways that result in the nuclear alterations observed in apoptosis, including gene expression via Ca^{2+} / calmodulin dependent kinases such as CaM kinase IV which is a critical enzyme for intranuclear Ca^{2+} mediated transcription [13,20]. Previously, we have demonstrated a hypoxia-induced increase in the activity of high affinity nuclear Ca^{2+} - ATPase, as well as an increase in the activity of Ca^{2+} / CaM kinase IV [13]. CaM kinase IV activation results in phosphorylation of cyclic-AMP-response element binding protein on Ser¹³³, resulting in increased gene transcription [14]. This may result in altered expression of the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax leading to an altered Bcl-2 to Bax protein ratio, which is a key determinant of the susceptibility of the cell to apoptosis [20]. Over expression of Bax has been demonstrated during hypoxia in the cerebral cortical neuronal nuclei of newborn piglets [17]. It is thought that Bax potentiates apoptosis through caspase activation, membrane pore formation, cytochrome c release from the mitochondria, and formation of heterodimers with Bcl-2. Bcl-2's anti-apoptotic properties include prevention of cytochrome c release, prevention of mitochondrial permeability transition pore opening during hypoxia, regulation of intracellular calcium, and formation of heterodimers with Bax. Bcl-2

also functions by acting as an antioxidant that prevents peroxidation of the nuclear membrane and protease inhibition [7]. In addition to enhancing cell death through increased transcription of pro-apoptotic genes, an elevated level of intranuclear Ca^{2+} may also participate in apoptosis through activation of Ca^{2+} / Mg^{2+} - dependent endonucleases leading to DNA cleavage at the internucleosomal region [9,20].

Although it has been demonstrated that procaspase-9 is cleaved and converted to caspase-9 through formation of the apoptosome, which requires binding to Apaf-1 in the presence of cytochrome c and dATP or ATP [6,12], the exact mechanism through which caspase-9 is activated during hypoxia has not been delineated. Numerous studies have demonstrated that phosphorylation of caspase-9, especially at the Ser¹⁹⁶ and the Thr¹²⁵ sites regulates its activity through various post-translational proteolytic modifications of the protein, making apoptosis more or less likely [1,2]. In the present study we have demonstrated a statistically significant greater concentration of phosphorylated caspase-9 at the Ser¹⁹⁶ site in the cytosol isolated from the cortical tissue of newborn piglets exposed to hypoxic conditions, compared to their normoxic counterparts. These results demonstrate a hypoxia induced post-translational modification of procaspase-9 at Ser¹⁹⁶. It has been demonstrated that modification at Ser¹⁹⁶ results in alterations of proteolytic cleavage of procaspase-9 and caspase-9 activity. The present data does not support previous studies [2] which have demonstrated that phosphorylation of caspase-9 at the Ser¹⁹⁶ site leads to a decrease in the activity of caspase-9.

In summary the results of the present study demonstrate that, in the cerebral cortex of newborn piglets, caspase-9 is phosphorylated at the Ser¹⁹⁶ site during hypoxia. This phosphorylation is believed to bring about a conformational change that alters its proteolytic activity within a hypoxic cell.

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Figure:1.

Figure:2.

Figure:3.