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Mechanism of Tyrosine Phosphorylation of procaspase-9 and Apaf-1 in Cytosolic Fractions of the Cerebral Cortex of Newborn Piglets During Hypoxia

Om P Mishra and Maria Delivoria-Papadopoulos

Department of Pediatrics, Drexel University College of Medicine and St Christopher's Hospital for Children, Philadelphia, PA

Abstract

Previous studies have shown that cerebral hypoxia results in increased activity of caspase-9 in the cytosolic fraction of the cerebral cortex of newborn piglets. The present study tests the hypothesis that hypoxia-results in increased tyrosine phosphorylation of procaspase-9 and apoptotic protease activating factor-1 (Apaf-1) and the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 and Apaf-1 is mediated by nitric oxide. To test this hypothesis, 15 newborn piglets were divided into three groups: normoxic (Nx, n=5), hypoxic (Hx, n=5) and hypoxic treated with nNOS inhibitor I (Hx+nNOS I 0.4 mg/Kg, i.v., 30 min prior to hypoxia). The hypoxic piglets were exposed to an FiO_2 of 0.06 for 1 hour. Tissue hypoxia was documented by ATP and phosphocreatine (PCr) levels. Cytosolic fractions were isolated and tyrosine phosphorylated procaspase-9 and Apaf-1 were determined by immunoblotting using specific anti-procaspase-9, anti-Apaf-1 and anti-phosphotyrosine antibodies. ATP levels ($\mu\text{moles/g}$ brain) were 4.3 ± 0.2 in the Nx and 1.4 ± 0.3 in the Hx and 1.7 ± 0.3 in Hx +nNOS I group ($p < 0.05$ vs Nx) groups. PCr levels ($\mu\text{moles/g}$ brain) were 3.8 ± 0.3 in the Nx and 0.9 ± 0.2 in the Hx and 1.0 ± 0.4 in the Hx+nNOS I ($p < 0.05$ vs Nx) group. Density ($\text{OD} \times \text{mm}^2$) of tyrosine phosphorylated procaspase-9 was 412 ± 8 in the Nx, 1286 ± 12 in the Hx ($p < 0.05$ vs Nx) and 421 ± 10 in the Hx+nNOS I ($p < 0.05$ vs Hx) group. Density of tyrosine phosphorylated Apaf-1 was 11.72 ± 1.11 in Nx, 24.50 ± 2.33 in Hx ($p < 0.05$ vs Nx) and 16.63 ± 1.57 in Hx+ nNOS I ($p < 0.05$ vs Hx) group. We conclude that hypoxia results in increased tyrosine phosphorylation of procaspase-9 and Apaf-1 proteins in the cytosolic compartment and the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 and Apaf-1 is mediated by nNOS derived nitric oxide. We propose that increased interaction between the tyrosine phosphorylated procaspase-9 and Apaf-1 molecules lead to increased activation of procaspase-9 to caspase-9 in the hypoxic brain that initiates programmed neuronal death.

Keywords

Tyrosine phosphorylation; nNOS; NO; Procaspase-9; Apaf-1; Hypoxia; Newborn

The mechanism of activation of procaspase-9 to active caspase-9, a key step that initiates execution of programmed cell death in brain following hypoxia, is not well understood and might hold the promise for developing strategies for neuroprotection. Initial studies focused

Address for Correspondence: Om P. Mishra, Ph.D., Drexel University College of Medicine 245N 15th Street, Mail Stop 1029 New College Building, Rm 7410 Philadelphia, PA 19102 Phone: 215 762 7515 Fax: 215 762 7960 omprakash.mishra@drexelmed.edu.

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primarily on the expression of pro- and anti-apoptotic proteins and their ratios. However, recent studies indicate that post-translational modification may alter the function of these proteins including that of procaspase-9. Caspases are group of cysteine proteases that play an important role in the initiation and execution of programmed cell death [10,14,15,17,29,32]. Studies conducted on *Caenorhabditis elegans* (*C. elegans*) have demonstrated that an aspartate specific cysteine protease is essential for programmed cell death of all somatic cells during development [8,33]. In *C. elegans*, a group of genes including *egl-1* (*egl*, egg-laying defective), *ced-3* (cell death abnormal), *ced-4* and *ced-9* are at the core of programmed cell death. Three protein components (*Ced-3*, *Ced-4* and *Egl-1*) are required for cell death. These code for a caspase (*Ced-3*), an adaptor protein (*Ced-4*) and a proapoptotic member of the Bcl-2 family of proteins (*Egl-1*). The Bcl-2 homolog *Ced-9* is needed for cell survival. Protein-protein interactions between *Ced-3*, *Ced-4*, *Ced-9* and *Egl-1* provide a direct link between caspases as the effector arm of the programmed cell death pathway and Bcl-2 family proteins [3,4,28].

In mammalian cells, the adaptor protein comparable to *Ced-4* is apoptotic protease activating factor-1 (*Apaf-1*). [34,36]. Antiapoptotic proteins Bcl-2 and Bcl-x_L bind to *Apaf-1* and this binding is essential for the antiapoptotic function of Bcl-2 family member proteins [34,36]. *Apaf-1* acts upstream of caspases, and that *Ced-9* or the antiapoptotic proteins Bcl-2 or Bcl-x_L act as inhibitors of *Apaf-1*. *Ced-4* or *Apaf-1* can simultaneously bind to procaspase-9 (*Ced-3* homolog), as well as the apoptotic proteins (*Ced-9* homologs) [5,10]. In brief, the genetic components of programmed cell death have been identified, with a possible activation sequence of these components as follows: In *C. elegans*: *Egl-1* → *Ced-9* → *Ced-4* → apoptosis; In mammals: Bax → Bcl-2/Bcl-x_L → *Apaf-1* → procaspase-9 → caspase-9 → procaspase-3 → caspase-3 → apoptosis.

The mechanism of hypoxia-induced activation of procaspase-9 that initiates programmed cell death in mammalian brain tissue is not known. In vitro studies indicated that the apoptotic caspase cascade is activated by cytochrome c and ATP. In vitro studies using 100,000 g supernatant (S-100) extracts of HeLa 60 cells demonstrated that incubation with dATP or ATP (1–2 mM) and cytochrome c (10 μM) together for 1 hr at 37°C resulted in cleaved products of poly-(ADP-ribose)polymerase (PARP) indicating activation of caspase-3 [18,19]. Cleaved active caspase-9 and caspase-3 were also demonstrated. Using caspase-9 deficient mice, it was shown that caspase-9 is needed for caspase-3 activation [14]. On the basis of these studies it was generally accepted that ATP and cytochrome c together activate caspase-9. There are several studies, however, not in agreement with this general idea regarding the role of cytochrome c in programmed cell death [9] and have raised questions regarding the appropriateness of the concentrations of ATP and cytochrome c and apoptosome formation, as well as caspase-9 activation [12,23].

Apaf-1 is a crucial part of the apoptosome that is assembled in response to several cellular stresses (i.e., hypoxia, DNA damage, oncogene activation, etc.). Studies on *Apaf-1* knockout mice demonstrated that *Apaf-1* is an essential component of the apoptotic response to hypoxia [31]. In stress-induced apoptosis, mitochondria releases apoptogenic factors such as cytochrome c. Pro-apoptotic members of the Bcl-2 family such as Bax and Bid induce the release of apoptogenic factors, whereas anti-apoptotic members such as Bcl-2 or Bcl-X_L prevent their releases. Cytochrome c binds to *Apaf-1* and, when dATP is added to the cytochrome c-bound *Apaf-1*, the oligomeric complex of *Apaf-1* forms and leads to the recruitment of caspase-9 to form apoptosome and activates procaspase-9 [30].

Apaf-1 and caspase-9 are essential for caspase-3 activation and seem to play important roles in normal development [2]. *Apaf-1*-deficient mice exhibit reduced apoptosis in the brain and

striking craniofacial abnormalities with hyperproliferation of neuronal cells. Apaf-1 and caspase-9 control tumor development by interacting with p53 [31].

We have shown that cerebral hypoxia results in increased activity of caspase-9 and nNOS inhibition prevented the hypoxia-induced increased activity of caspase-9 indicating that the hypoxia-induced increase in caspase-9 activity is mediated by NO [7,13,25]. The present study was designed to investigate the mechanism of post-translational modification of procaspase-9 and Apaf-1 during hypoxia and tested the hypothesis that hypoxia-results in increased tyrosine phosphorylation of procaspase-9 and Apaf-1 and the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 and Apaf-1 is mediated by nitric oxide. An extremely selective nNOS inhibitor nNOS I (selectivity for nNOS >2500 vs eNOS and > 500 vs iNOS) was administered prior to the hypoxic exposure. The results of the present study provide evidence that hypoxia results in increased tyrosine phosphorylation of procaspase-9 and Apaf-1 in the cytosolic compartment the cerebral cortex of newborn piglets and the hypoxia-induced increased tyrosine phosphorylation is mediated by nNOS derived NO. The increased tyrosine phosphorylation of procaspase-9 and Apaf-1 may lead to increased interaction between the two molecules leading to increased activation of procaspase-9 to caspase-9 that initiates cascade of programmed cell death in the hypoxic brain.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Drexel University. Studies were conducted on anesthetized, ventilated and instrumented 3-5 days old newborn piglets. Fifteen piglets were divided into three groups: normoxic (Nx, n=5), hypoxic (Hx, n=5) and hypoxic and hypoxic treated with nNOS inhibitor I (Hx+nNOS I 0.4 mg/Kg, i.v., 30 min prior to hypoxia). Anesthesia was induced with 4% halothane and lowered to 1% during surgery while allowing the animals to breathe spontaneously. Lidocaine 2% was injected locally before instrumentation for endotracheal tube insertion and femoral arterial and venous catheter insertion. After instrumentation, the use of halothane was discontinued, and anesthesia was maintained with nitrous oxide 79%, oxygen 21% and Fentanyl (50 µg/kg) throughout the experiment. Tubocurarine (0.3 mg/kg) was administered after placing the animal on a volume ventilator. Arterial blood gases, heart rate and blood pressure were monitored in all animals throughout the study. Core body temperature was maintained at 38.5–39°C with a warming blanket. Baseline measurements were obtained in both groups for one hour after surgery to ensure normal arterial pressures and blood gas values. After stabilization following surgery, the piglets assigned to the hypoxic group were exposed to hypoxia ($\text{FiO}_2 = 0.06$) for 1 hr, while the piglets assigned to the normoxic group were ventilated at FiO_2 of 0.21 for 1 hr. At the end of the experiment, the cortical brain tissue was removed and placed in buffer for the preparation of cytosolic fractions or frozen in liquid nitrogen within 5–7 sec for the analysis of high energy phosphates, ATP and phosphocreatine (PCr), to document cerebral tissue hypoxia.

Cerebral tissue ATP and phosphocreatine (PCr) concentrations were determined spectrophotometrically by the method of Lamprecht et al. [17] and calculated from the increase in the absorbance at 340 nm.

Cerebral cortical cytosolic fraction was isolated in a buffer containing 50 mM Tris-HCl, (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM Na_3VO_4 , 1 mM NaF and 1 µg/ml each of aprotinin, leupeptin and pepstatin. Equal amounts (150 µg) of each protein sample was incubated overnight with 5 µg of anti-phosphotyrosine antibody at 4°C and then incubated with 150 µl of protein A agarose beads (Pharmacia) for an additional 2 hr at 4°C. The beads were pelleted and washed three times with the buffer. Proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membrane and incubated

with polyclonal anti-procaspase-9 or anti-Apaf-1 antibody (Santa Cruz). Subsequently, the nitrocellulose membrane was washed 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 for 2–3 min. Protein bands were analyzed using imaging densitometry. The density of proteins was expressed as absorbance (OD mm²).

The statistical analysis of the data on ATP, PCr, tyrosine phosphorylated procaspase-9 and Apaf-1 proteins were performed using Student's t-test. A p value <0.05 was considered significant.

The mean blood pressure and PaCO₂ among the three groups were as follows: 89.50±11.99 mmHg and 35.23± 5.71 mm Hg in Nx; 61.8 ± 7.97 mmHg and 37.70±1.27 mmHg in Hx; and 62.00± 10.58 mmHg and 36.50 ± 6.25 mm Hg in Hx pretreated with nNOS I groups (For mean blood pressure: p< 0.05 Nx vs Hx and Hx +nNOS I, p=NS Hx vs Hx + nNOS I, p=NS among the three groups) . The mean blood pressure was not significantly different between the two hypoxic groups. The PaCO₂ levels were similar among the three groups.

The levels of tissue high energy phosphates in the cerebral cortex of normoxic, hypoxic and hypoxic treated with nNOS I piglets were determined. Cerebral tissue hypoxia was documented by decreases in the levels of high energy phosphates, ATP and phosphocreatine (PCr). ATP levels (μmoles/g brain) were 4.3 0.2 in the Nx and 1.4 0.3 in the Hx and 1.7± 0.3 in the Hx +nNOS I group (p<0.05 vs Nx) groups. PCr levels (μmoles/g brain) were 3.8 0.3 in the Nx and 0.9 0.2 in the Hx and 1.0± 0.4 in the Hx+nNOS I (p<0.05 vs Nx) group. These results demonstrate that cerebral tissue hypoxia was achieved in the hypoxic group and the degree of hypoxia in the cerebral cortex of hypoxic and hypoxic with the nNOS I groups was comparable.

The representative expression of tyrosine phosphorylated procaspase-9 protein in the cytosolic fraction of the cerebral cortex of normoxic, hypoxic and hypoxic with nNOS I groups of piglets is shown in Fig 1. The tyrosine phosphorylated protein density data from all the animals is shown as bar graph in Fig 1.

Tyrosine phosphorylated procaspase-9 density (ODx mm²) was 412± 8 in Nx, 1286± 12 in Hx (p<0.05 vs Nx) and 421 ±10 in the Hx+nNOS I (p<0.05 vs Hx, p=NS vs Nx) group. These results demonstrate that cerebral tissue hypoxia results in increased density of tyrosine phosphorylated of procaspase-9 protein in the cytosolic compartment indicating that hypoxia results in increased tyrosine phosphorylation of procaspase-9 protein in the cytosolic compartment of the cerebral cortical tissue of newborn piglets. Pretreatment with the nNOS inhibitor prevented the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 indicating that the increased tyrosine phosphorylation of procaspase-9 is mediated by nitric oxide.

The representative expression of tyrosine phosphorylated Apaf-1 protein in the cytosolic fraction of the cerebral cortex of normoxic, hypoxic and hypoxic with nNOS I groups of piglets is shown in Fig 2. The tyrosine phosphorylated protein density data from all the animals is shown as bar graph in Fig 2.

Tyrosine phosphorylated Apaf-1 density (ODx mm²) was 11.72 ±1.11 in Nx, 24.50± 2.33 in Hx (p<0.05 vs Nx) and 16.63 ± 1.57 in Hx+ nNOS I (p<0.05 vs Hx) group. Similar to tyrosine phosphorylated procaspase-9, these results demonstrate that cerebral tissue hypoxia results in increased levels of tyrosine phosphorylated Apaf-1 in the cytosolic fraction indicating that hypoxia results in increased tyrosine phosphorylation of Apaf-1 protein in the

cerebral tissue of newborn piglets. Pretreatment with the nNOS inhibitor prevented the hypoxia-induced increased tyrosine phosphorylation of Apaf-1 indicating that the increased tyrosine phosphorylation of Apaf-1 is mediated by nitric oxide derived from nNOS.

Previously we have demonstrated that hypoxia results in increased generation of nitric oxide free radicals in the cerebral cortical tissue. NO produced during hypoxia results in inactivation of protein tyrosine phosphatase (PTP, SH-PTP1 and SH-PTP2) activity that leads to increased tyrosine phosphorylation of a number of proteins. We showed that cerebral hypoxia results in increased tyrosine phosphorylation of Src at its active site Tyr416 (an index of Src kinase activation) and increased activity of Src kinase in the cerebral cortex of newborn piglets and the hypoxia-induced increased activation of Src is NO-mediated. We propose that NO-mediated increase in tyrosine phosphorylation of procaspase-9 results in its increased activation during hypoxia. We have shown that hypoxia results in inhibition of protein tyrosine phosphatase activity and increase in protein tyrosine kinase (PTK, Src kinase) activity. The administration of a nitric oxide synthase inhibitor prevented both the decrease in PTP (SH-PTP1 and SH-PTP2) activity and the increase in PTK (Src kinase) activity [1,24]. Therefore, NO produced during hypoxia in the cerebral cortex of newborn piglets mediates tyrosine phosphorylation of procaspase-9 and Apaf-1, which initiate cascade of neuronal death.

We propose that tyrosine phosphorylated procaspase-9 strongly binds the Apaf-1 molecule due to additional H-bondings between the phosphate group of tyrosine residue of procaspase-9 and the amino and imino groups of two arginine residues present in the CARD domain of Apaf-1, thus increasing interaction between the two molecules for activation of procaspase-9. Simultaneously, tyrosine phosphorylated Apaf-1 binds strongly with two arginine residues present in the CARD domain of procaspase-9 and results in further increasing the interaction between these two molecules, thereby leading to exceedingly strong interaction between procaspase-9 and Apaf-1, resulting in increased activation of procaspase-9 to active caspase-9. The increased H-bondings between the procaspase-9 and Apaf-1 are like additional rungs in a ladder providing increased pull on the two sides of the ladder. (**See our proposed ladder-and-rung model in Fig 3**) The proposed ladder-and-rung model depicts the interaction between the phosphate group of phosphorylated Tyr¹⁵³ of procaspase-9 and the two arginines of Apaf-1 as well as the interaction between the phosphate group of phosphorylated Tyr²⁴ of Apaf-1 and the two arginines of procaspase-9 illustrating exceedingly increased interaction between procaspase-9 and Apaf-1.

Studies showed that the recognition of procaspase-9 by Apaf-1 is essential for formation of apoptosome and activation of procaspase-9 [26,27,6]. Binding between the procaspase-9 and Apaf-1 is due to the basic surface from one molecule to the acidic surface of the other through electrostatic interactions that results in 1:1 binding stoichiometry between procaspase-9 and Apaf-1. Mutational studies have shown that a mutation at Tyr²⁴ with Ala decreased the ability of Apaf-1 CARD to precipitate procaspase-9 CARD indicating that mutation at Tyr²⁴ is essential for Apaf-1 and procaspase-9 interaction. Mutation at Tyr¹⁵³ decreased the activation of procaspase-9. These studies provide structural basis for increased electrostatic charge-charge interaction between tyrosine phosphorylated procaspase-9 and Apaf-1 molecules leading to procaspase-9 activation.

We have demonstrated that NO generated during hypoxia results in increased activation of Ca⁺⁺/calmodulin-dependent protein kinase IV (CaM kinase IV) [21,38]. CaM kinase IV is predominantly present in the nucleus and activates cyclic-AMP response element binding protein (CREB) by phosphorylating it at Ser133. CREB phosphorylation triggers transcription of a large number of apoptotic genes. We have demonstrated that the increased expression of apoptotic proteins during hypoxia is NO-mediated [21,22,37]. The increase in

proapoptotic proteins such as Bax and Bad during hypoxia may lead to Apaf-1 activation resulting in activation of procaspase-9.

In summary: we have shown that cerebral tissue hypoxia results in increased tyrosine phosphorylation of procaspase-9 and Apaf-1 in the cytosolic fraction of the cerebral cortex of newborn piglets and the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 and Apaf-1 is prevented by pretreatment with a highly selective nNOS inhibitor. We conclude that hypoxia results in increased tyrosine phosphorylation of procaspase-9 and Apaf-1 and the hypoxia-induced increased tyrosine phosphorylation of procaspase-9 and Apaf-1 proteins is mediated by nNOS derived NO. We propose that that tyrosine phosphorylated procaspase-9 strongly binds the Apaf-1 molecule due to additional H-bondings between the phosphate group of tyrosine residue of procaspase-9 and the amino and imino groups of two arginine residues of the CARD domain of Apaf-1, thus increasing interaction between the two molecules for activation of procaspase-9. Similarly and simultaneously, tyrosine phosphorylated Apaf-1 binds strongly with two arginine residues of the CARD domain of procaspase-9, thereby leading to exceedingly strong interaction between the procaspase-9 and Apaf-1, resulting in increased apoptosome formation and activation of procaspase-9 to active caspase-9.

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Figure 1.

Tyrosine phosphorylated procaspase-9 protein in the cytosolic fraction of the cerebral cortex of normoxic (n=5), hypoxic (n=5) and hypoxic pretreated with nNOS inhibitor (n=5) groups of newborn piglets. The protein density is presented as absorbance (OD x mm²). The data is presented as mean ± standard deviation. *p<05 vs Nx; **p<05 vs Hx and NS vs Nx.

Figure 2.

Tyrosine phosphorylated Apaf-1 protein in cytosolic fraction of the cerebral cortex of normoxic (n=5), hypoxic (n=5) and hypoxic pretreated with nNOS inhibitor (n=5) groups of newborn piglets. The protein density is presented as absorbance (OD x mm²). The data is presented as mean $\pm$ standard deviation. *p<0.05 vs Nx; **p<0.05 vs Hx

Figure 3.
The proposed Ladder-and Rung Model for interaction between tyrosine phosphorylated procaspase-9 and tyrosine phosphorylated Apaf-1.