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Tyrosine phosphorylation of neuronal nitric oxide synthase (nNOS) during hypoxia in the cerebral cortex of newborn piglets: The role of nitric oxide

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ABSTRACT

The present study aims to investigate the mechanism of activation of nNOS during hypoxia and tests the hypothesis that the hypoxia-induced increased tyrosine phosphorylation of nNOS in the cerebral cortical membranes of newborn piglets is mediated by nNOS-derived nitric oxide (NO). Fifteen newborn piglets were divided into normoxic (Nx, $n=5$), hypoxic (Hx, $n=5$) and hypoxic-pretreated with nNOS inhibitor I (Hx-nNOSi) groups. Hypoxia was induced by an FiO_2 of 0.07 for 60 min. nNOS inhibitor I (selectivity > 2500 vs endothelial NOS and >500 vs inducible NOS) was administered (0.4 mg/kg, i.v.) 30 min prior to hypoxia. Cortical membranes were isolated and tyrosine phosphorylation of nNOS determined by Western blot. Membrane protein was immunoprecipitated with nNOS antibody, separated on 12% SDS-PAGE and blotted with anti-phosphotyrosine antibody. Protein bands were detected by enhanced chemiluminescence, analyzed by densitometry and expressed as absorbance ($OD \times mm^2$). Density ($OD \times mm^2$) of tyrosine phosphorylated nNOS was 51.66 ± 14.11 in Nx, 118.39 ± 14.17 in Hx ($p < 0.05$ vs Nx) and 45.56 ± 10.34 in Hx-nNOSi ($p < 0.05$ vs Hx, p = NS vs Nx). The results demonstrate that pretreatment with nNOS inhibitor prevents the hypoxia-induced increased tyrosine phosphorylation of nNOS. We conclude that the mechanism of hypoxia-induced increased tyrosine phosphorylation of nNOS is mediated by nNOS-derived NO.

Hypoxia results in increased activation of neuronal nitric oxide synthase (nNOS) and generation of nitric oxide (NO) free radicals in the cerebral cortex of newborn piglets. Nitric oxide synthase generates NO, a signal transducer as well as a cytotoxic molecule, from L-arginine. In mammalian systems, three isoforms of NOS have been identified. NOS I (nNOS) is present in neurons and is a constitutively expressed enzyme whose activity is regulated by Ca^{++} and calmodulin (CaM) [4,16]. NOS II (iNOS) is an inducible enzyme whose activity is independent of Ca^{++}. NOS III (eNOS) is constitutively expressed in endothelial cells and is also regulated by Ca^{++} and calmodulin. Increasing evidence indicates that NOS expression is altered during various physiological and pathological conditions. nNOS mRNA up-regulation represents a general response of neuronal cells to stress conditions including hypoxia [5] and ischemia [20]. Neuronal nitric oxide synthase is only active in the dimeric form. nNOS exhibits a bidomain structure in which an N-terminal oxygenase domain containing binding sites for heme, BH_4 and L-arginine is linked by a calmodulin-recognition site to a C-terminal reductase domain that contains binding sites for FAD, FMN, and NADPH [1,8,25]. The calmodulin binding site is located between the oxygenase and reductase domain and the electron transfer reaction is regulated by calmodulin, a Ca^{++} binding protein. Conversion of L-arginine to NO by nNOS occurs in two steps that involve an initial formation of N-hydroxy-L-arginine (NOHA) as an enzyme bound intermediate, followed by its oxidation to NO and citrulline. Both steps require the transfer of NADPH-derived electrons from reductase domain flavins to the heme group in the oxygenase domain. In the reductase domain, NADPH first reduces FAD, which shuttles electrons to FMN. The FMN to heme electron transfer in neuronal NOS is triggered by calmodulin binding and it is imperative for the catalytic reaction to proceed because it enables the heme iron to bind and activate oxygen in both steps of the reaction sequence. During cerebral hypoxia, a post-translational modification (tyrosine phosphorylation) of nNOS may alter its activation leading to increased generation of NO that contributes to cell proliferation and cell death. In the proposed study, we focus on investigating the hypoxia-induced tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets. We propose that hypoxia-induced tyrosine phosphorylated nNOS binds its substrate with a higher affinity as compared to non-phosphorylated and results in increased activation of nNOS.

Previously, we have shown that oxygen free radical generation, lipid peroxidation and cell membrane dysfunction in the hypoxic brain can be reduced or prevented by using inhibitors of

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NOS such as N-nitro-L-arginine (NNLA) [17]. Hypoxia results in modification of the N-methyl-D-aspartate (NMDA) receptor ion-channel and its recognition and modulatory sites [13,6] and in NMDA receptor-mediated Ca^{++} entry into neurons. An increase in NMDA receptor-mediated Ca^{++} concentration in hypoxic synaptosomes [24] may activate several pathways of oxygen free radical generation including the NOS pathway. Cerebral hypoxia results in increased generation of NO free radicals [15]. Furthermore, hypoxia resulted in nitration of the NMDA receptor subunits NR1, NR2A, and NR2B [23]. Hypoxia also results in increased expression of cell death promoter protein Bax, whereas the expression of cell death repressor protein, Bcl-2, is not increased. NO increased neuronal nuclear Ca^{++}-influx and administration of a NOS inhibitor or a selective inhibitor of nNOS prior to hypoxia prevented the hypoxia-induced increase in intranuclear Ca^{++}-influx, increase in Ca^{++}/calmodulin-dependent protein kinase (CaM kinase IV) activity in neuronal nuclei, increase in cyclic AMP response element binding (CREB) protein phosphorylation, increased expression of cell death promoter protein Bax, activation of poly(ADP-ribose) polymerase, caspase-3 activation and damage to nuclear DNA [27,12,26,11,18,14]. nNOS mRNA up-regulation represents a response to stress conditions including hypoxia [4,5] and ischemia [20]. These studies provide compelling evidence for the role of NO generation and nNOS activation in hypoxic neuronal death.

The present study specifically focuses on investigating the effect of hypoxia on tyrosine phosphorylation of nNOS and the mechanism of hypoxia-induced tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets. In the present study we have tested the hypothesis that hypoxia results in increased tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets and that the increased tyrosine phosphorylation is mediated by nitric oxide (NO) derived from nNOS. Therefore, administration of a highly selective nNOS inhibitor, prior to hypoxia, will prevent the increased tyrosine phosphorylation of nNOS during hypoxia in the cerebral cortex of newborn piglets.

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 divided into three groups: normoxic ($n=5$), hypoxic ($n=5$), and hypoxic with nNOS inhibitor [(4s)-N(4-amino-5(aminoethyl)aminopentyl)-N′-nitroguanidine, hypoxic-nNOSi $n=5$]. nNOSi (0.4 mg/Kg) was administered i.v. 30 min prior to hypoxia. This nNOS inhibitor (Ki, 120 nM) is highly selective for nNOS (selectivity > 2500 fold vs eNOS and >500 fold vs iNOS).

The animals were anesthetized and ventilated for 1 h under either normoxic condition ($FiO_2 = 0.21$) or hypoxic condition; hypoxia was induced by lowering the fraction of inspired oxygen, FiO_2, to 0.06 for 60 min. The $PaCO_2$ was maintained between 35 and 45 mmHg. Blood pressure, heart rate, core temperature and end-tidal CO_2 were monitored continuously. 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 cortical cell membranes or in liquid nitrogen, and then stored at −80 °C for biochemical studies.

Preparation of cerebral cortical membrane fraction: Cerebral cortical tissue was homogenized in 10 vol of buffer (1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 158 mM NaCl, 10 mM Tris–HCl buffer, pH 7.0, 1 mM EGTA, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mM sodium orthovanadate (SOV), 0.1 mM phenylmethylsulfonyfluoride (PMSF) and 0.1% IGEPAL. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 40,000 × *g* for 60 min and the pellet homogenized in the homogenization buffer and used as membrane fraction. Protein was determined by the method of Lowry et al. [10].

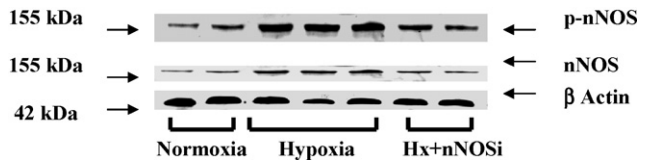

Fig. 1. Representative Western blots of tyrosine phosphorylated nNOS in cortical membranes of the cerebral cortex of normoxic, hypoxic and hypoxic-nNOS newborn piglets. Western blot analysis was performed using anti-phosphotyrosine (p-Tyr), anti-nNOS (Santa Cruz Biotechnology, CA) and anti-actin antibody (Chemicon). Protein bands were detected using enhanced chemiluminescence detection system and analyzed by imaging densitometry. Lanes 1 and 2 represent normoxic; lanes 3 and 4 represent hypoxic; lanes 5 and 6 represent hypoxic-nNOSi piglets.

Western blot analysis of tyrosine phosphorylated nNOS: The membrane protein was solubilized and brought to a final concentration of 1 μg/μl in a modified RIPA buffer (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 aprotinine, leupeptin and pepstatin). 200 μg protein was heated at 95 °C and immunoprecipitated with anti-phosphotyrosine (p-Tyr) antibody overnight at 4 °C. The immunoprecipitate was washed and suspended in 20 μl RIPA buffer. Then 5 μl of Laemmli buffer (100 mM Tris–HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added. The samples were heated for 5 min at 95 °C. Equal protein amounts of each sample was separated by using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were electrically transferred to nitrocellulose membranes and blocked with 10% non-fat dry milk in PBS buffer for 4–6 h at 4 °C. The proteins were then probed with primary antibodies directed against anti-nNOS antibody overnight at 4 °C on a rocking platform. Immunoreactivity was then detected with horseradish peroxidase conjugated secondary antibody (Rockland, Gilbertsville, PA). Specific complexes were detected by enhanced chemiluminescence using the ECL detection system and analyzed by imaging densitometry using Quantity One Software (Bio-Rad). The data are expressed as optical density (OD) × mm^2.

Determination of ATP and phosphocreatine: ATP and phosphocreatine concentrations were determined according to the method of Lamprecht et al. [9].

Statistical analysis: Data were analyzed using one-way analysis of variance ANOVA to compare normoxic, hypoxic, and hypoxic-nNOSi groups. A *p*-value of less than 0.05 was considered statistically significant. All values are presented as mean ± standard deviation (SD).

Cerebral cortical tissue hypoxia in newborn piglets was documented by determining the levels of ATP and PCr in the cerebral cortical tissue. The level of ATP (μmol/g brain) decreased from 4.35 ± 0.21 in normoxic (Nx), to 1.43 ± 0.28 in hypoxic (Hx) ($p < 0.05$ vs Nx), and 1.73 ± 0.33 in Hx-nNOSi ($p < 0.05$, vs Nx, p = NS vs Hx). PCr level (μmol/g brain) decreased from 3.80 ± 0.26 in Nx to 0.96 ± 0.20 in Hx ($p < 0.05$ vs Nx), and 1.09 ± 0.39 in Hx + nNOSi ($p < 0.05$ vs Nx, p = NS vs Hx). The level of high-energy phosphates decreased significantly in the hypoxic group as compared to normoxic and the data demonstrate that cerebral tissue hypoxia was achieved in the hypoxic group. In addition, these results demonstrate that the level of cerebral tissue high-energy phosphates, ATP and PCr, were comparable in the hypoxic and hypoxic-treated with nNOSi groups.

Representative Western blots of tyrosine phosphorylated (pTyr)-nNOS for normoxic, hypoxic and hypoxic-nNOSi groups are shown in Fig. 1 The results show an increased expression of total tyrosine phoshorylated nNOS in the Hx group indicating increased level of tyrosine phoshorylated nNOS in the cerebral cortex of newborn piglets during hypoxia. The results also demonstrate that nNOSi administration prevents the hypoxia-induced increased tyrosine phosphorylation of nNOS. The nNOS protein expression also increased during hypoxia. However, the increase in tyrosine

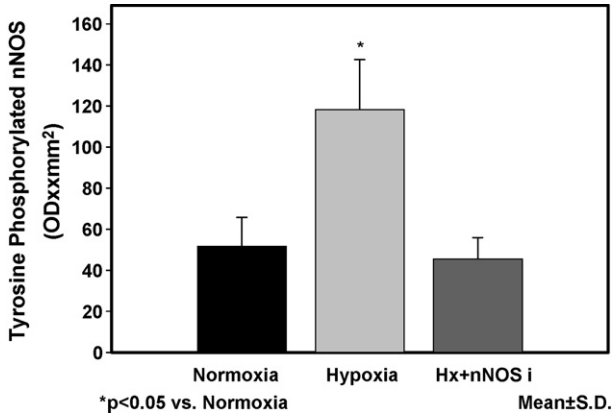

Fig. 2. Effect of hypoxia on the tyrosine phosphorylation of nNOS in cortical membranes of the cerebral cortex of normoxic, hypoxic and hypoxic-nNOS newborn piglets. The protein density (OD × mm^2) is presented on *Y*-axis. The data are expressed as mean ± SD.

phosphorylation is several fold higher as compared to increase in protein.

The data presented in Fig. 2 show that the density (expressed as optical density × mm^2) of tyrosine phosphorylated nNOS was 51.66 ± 14.11 in Nx, 118.39 ± 14.17 in Hx ($p < 0.05$ vs Nx and Hx-nNOSi) and 45.56 ± 10.34 in Hx-nNOSi ($p < 0.05$ vs Hx). The data show that hypoxia resulted in increased tyrosine phosphorylation of nNOS and the increased tyrosine phosphorylation of nNOS was prevented by nNOS inhibitor, indicating that the hypoxia-induced increased tyrosine phosphorylation of nNOS is mediated by nNOS-derived NO. The data for normoxic control with the inhibitor were not different from the normoxic alone.

We have shown that hypoxia results in increased nuclear Ca^{++} influx and increased activity of CaM kinase IV which is predominantly located in the nucleus [27]. Hypoxia resulted in increased phosphorylation of cyclic AMP response element binding (CREB) protein and increased expression of proapoptotic protein Bax [12,26]. Furthermore, hypoxia resulted in increased generation of NO [15]. The present study specifically investigated the effect of hypoxia on tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets and tested the hypothesis that cerebral hypoxia results in increased tyrosine phosphorylation of nNOS and the hypoxia-induced tyrosine phosphorylation of nNOS is mediated by nitric oxide derived from nNOS. Therefore, administration of a highly selective nNOS inhibitor prior to hypoxia will prevent the hypoxia-induced increase in tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets.

The results of the present study show that cerebral hypoxia results in increased tyrosine phosphorylation of nNOS in the cortical membrane fraction of the cerebral cortex of newborn piglets. Administration of a highly selective nNOS inhibitor prevented the hypoxia-induced increased tyrosine phosphorylation of nNOS. These results demonstrate that nitric oxide derived from nNOS mediates the tyrosine phosphorylation of nNOS.

The increased tyrosine phosphorylation of nNOS during hypoxia may lead to increased activation of nNOS. We propose that during hypoxia NO-mediated increased tyrosine phosphorylation of nNOS results in binding of the tyrosine phosphorylated nNOS with increased affinity to its substrate L-arginine. We propose that NO free radicals generated during hypoxia lead to EGFR and Src kinase-dependent increased phosphorylation of the tyrosine residue at the active site of nNOS. There is a conserved tyrosine residue (Tyr^{588}) at the active site of nNOS. Tyrosine 588 mutation resulted in markedly decreased substrate binding affinity for L-arginine and NO formation activity [21]. Studies have shown that in rat nNOS, the substrate L-arginine binds by hydrogen bonding with the Tyr^{588} and Glu^{592} [3]. The amino acid sequences of the active site domain (Tyr^{588} and Glu^{592}) are shown in bold and underline: ^{576}GLEFSACPFSGW**<u>Y</u>**MGT**<u>E</u>**IGV. We anticipate that during hypoxia, the phosphorylated Tyr^{588} (negatively charged) will bind the substrate, L-arginine (positively charged) with a much higher affinity as compared to the non-phosphorylated tyrosine at the active site.

In addition during hypoxia, NO-mediated EGFR and Src kinase-dependent tyrosine phosphorylated calmodulin may bind to the calmodulin binding domain of nNOS with a higher affinity leading to increased activation of nNOS. We propose that phosphorylated tyrosine at the substrate active site of nNOS binds the substrate, L-arginine, with a higher affinity and leads to nNOS activation. We have observed that hypoxia results in NO-mediated increased tyrosine (Tyr^{99}) phosphorylation of calmodulin. Therefore, we propose that NO-mediated Tyr^{99} phosphorylation of calmodulin and the NO-mediated tyrosine phosphorylation of the active site of nNOS lead to increased activation of nNOS. Thus, NO-mediated tyrosine phosphorylation of nNOS as well calmodulin is the novel mechanism of nNOS activation during hypoxia. We propose that it is the higher affinity binding between phosphorylated tyrosine (a phosphoryl group can make three H-bonds) and the L-arginine residue that takes place at both the substrate as well as the activator site results in increased nNOS activation.

During hypoxia, the tyrosine phosphorylated calmodulin (negatively charged) may bind with a higher affinity to the calmodulin domain of nNOS, a positively charged domain (725–756) rich in basic amino acid residues, Lys and Arg. The sequence of the calmodulin binding domain [22] of nNOS showing basic amino acid residues (in bold italics) is as follows: ^{725}***Lys*-*Arg*-*Arg***-Ala-Ile-Gly-Phe-***Lys*-*Lys***-Leu-Ala-Glu-Ala-Val-***Lys***-Phe-Ser-Ala-***Lys***-Leu-Met-Gly-Gln-Ala-Met-Ala-***Lys*-*Arg***-Val-***Lys***^{756}.

The increased activation of nNOS leading to increased generation of nitric oxide free radicals during hypoxia may result in activation of EGFR kinase and Src kinase by inactivating protein tyrosine phosphatases, SH-PTP-1 and SH-PTP-2. Since all protein tyrosine phosphatases contain a cysteine residue at their active site, enzyme activity can be affected by redox mechanisms. NO free radicals generated during hypoxia can combine with superoxide radicals to produce peroxynitrite. The reaction between NO free radical and superoxide to form peroxynitrite is favored over the reaction between superoxide and superoxide dismutase [7]. Hypoxia-induced nitration of NMDA receptor subunits indicates formation of peroxynitrite during hypoxia [23]. Therefore, during hypoxia the peroxynitrite anion ($ONOO^-$) can be easily attracted to the active site loop of PTP and result in oxidation of the essential cysteine residue and inactivation of the enzyme. We propose that during hypoxia NO-mediated peroxynitrite-dependent inactivation of SH-PTP-1 and SH-PTP-2 may lead to increased activation of EGFR and Src kinase. Furthermore, the increased activation of EGFR and Src kinase may result in increased tyrosine phosphorylation of nNOS and (p-Tyr^{99}) of calmodulin. Thus a cycle between nNOS activation and activation of EGFR and Src kinase can continue and perpetually ongoing in the hypoxic brain.

The results of the present study raise some very fundamental questions regarding the role of tyrosine phosphorylation of nNOS during hypoxia in cell proliferation and cell death. The increased tyrosine phosphorylation of nNOS to NO-mediated increased activation of EGFR kinase may be a potential mechanism for a variety of cancer conditions including breast cancer and glioblastoma [2]. As shown in this study, cerebral hypoxia leads to increased tyrosine phosphorylation of nNOS. Hypoxia also results in activation of EGFR kinase in the cerebral cortex of newborn piglets. Hypoxia is also known to result in cell death in the hypoxic brain. Therefore, the role of tyrosine phosphorylated nNOS via EGFR kinase activation in leading to both the cell proliferation and cell death needs serious consideration. In addition, nNOS may potentially be a unique tar-

get molecule that may mediate both the cell survival and cell death in the cancerous tissue and the hypoxic brain, respectively. The activated EGFR is able to recruit and activate several downstream signaling cascades including Ras/RAF/MEK/ERK, phosphoinositol-3-kinase (PI3K)-Akt, STAT and phospholipase C gamma pathways. The activation of these pathways ultimately promotes tumor-cell proliferation, survival, invasion and angiogenesis.

Overexpression of Bax or an increase in the ratio of Bax to Bcl-2, leads to programmed cell death. 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. 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. Neurons with elevated Bax levels had morphologic evidence of ischemic degeneration with apoptotic features including nuclear DNA fragmentation [19].

Increased nNOS activation can play a central role in hypoxia-induced 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 including nNOS pathway. The increased free radicals generated result in increased peroxidation of cellular and sub-cellular membranes leading to necrotic cell death. Second, the increased intracellular Ca^{++} may lead to increased intranuclear Ca^{++}. The increased intranuclear Ca^{++} may activate Ca^{++}-dependent endonucleases leading to DNA fragmentation. In addition, increased intranuclear Ca^{++} can activate CaM kinase IV in the nucleus leading to increased phosphorylation of cyclic AMP response element binding protein (CREB) resulting in increased transcription of apoptotic genes such as Bax and initiating the early events of DNA fragmentation and programmed cell death. Thus a central role for NO is proposed in regulating neuronal function and specifically in hypoxic neuronal death.

The results of the present study demonstrate that hypoxia results in increased tyrosine phosphorylation of nNOS. We propose that increased tyrosine phosphorylation of nNOS results in its increased activation due to increased affinity for binding its substrate arginine. We propose that increased activation of nNOS leading to increased generation of NO during hypoxia leads to increased activation of EGFR and Src kinases by inhibiting protein tyrosine phosphatases SH-PTP-1 and SH-PTP-2. Thus tyrosine phosphorylated calmodulin-dependent nNOS activation leads to EGFR and Src kinase activation that subsequently activates nNOS. Thus this perpetual cycle of nNOS activation → EGFR and Src kinase activation → nNOS activation goes on in the hypoxic brain resulting in hypoxic brain injury.

In summary: These results show that cerebral tissue hypoxic results in increased tyrosine phosphorylation of nNOS in the cerebral cortex of newborn piglets. In addition, administration of nNOSi, a highly selective inhibitor of nNOS, prior to hypoxia prevented the hypoxia-induced increased tyrosine phosphorylation of nNOS. We conclude that the mechanism of increased tyrosine phopshorylation of nNOS during hypoxia is mediated by nitric oxide derived from nNOS. We propose that nNOS-derived NO, by inhibiting protein tyrosine phosphatases (SH-PTP-1 and SH-PTP-2) mediates the increased activation of EGFR and Src kinases that leads to increased tyrosine phosphorylation of nNOS that may regulate cell proliferation and cell death.

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