

Mechanism of CaM Kinase IV Activation During Hypoxia in Neuronal Nuclei of the Cerebral Cortex of Newborn Piglets: The Role of Src Kinase

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Abstract The present study aims to investigate the mechanism of CaM kinase IV activation during hypoxia and tests the hypothesis that hypoxia-induced increased activity of CaM kinase IV is due to Src kinase mediated increased tyrosine phosphorylation of calmodulin and CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets. Piglets were divided into normoxic (Nx, n = 5), hypoxic (Hx, F₁O₂ of 0.07 for 1 h, n = 5) and hypoxic-pretreated with Src kinase inhibitor PP2 (Hx-Srci, n = 5) groups. Src inhibitor was administered (1.0 mg/kg, I.V.) 30 min prior to hypoxia. Neuronal nuclei were isolated and purified, and tyrosine phosphorylation of calmodulin (Tyr⁹⁹) and CaM kinase IV determined by Western blot using anti-phospho-(pTyr⁹⁹)-calmodulin, anti-pTyrosine and anti-CaM kinase IV antibodies. The activity of CaM kinase IV and its consequence the phosphorylation of CREB protein at Ser¹³³ were determined. Hypoxia resulted in increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹, tyrosine phosphorylation of CaM kinase IV, activity of CaM kinase IV and phosphorylation of CREB protein at Ser¹³³. The data show that administration of Src kinase inhibitor PP2 prevented the hypoxia-induced increased tyrosine phosphorylation of calmodulin (Tyr⁹⁹) and tyrosine phosphorylation of CaM.kinase IV as

well as the activity of CaM kinase IV and CREB phosphorylation at Ser¹³³. We conclude that the mechanism of hypoxia-induced increased activation of CaM kinase IV is mediated by Src kinase-dependent tyrosine phosphorylation of the enzyme and its activator calmodulin. We propose that Tyr⁹⁹ phosphorylated calmodulin, as compared to non-phosphorylated, binds with a higher affinity at the calmodulin binding site (rich in basic amino acids) of CaM kinase IV leading to increased activation of CaM kinase IV. Similarly, tyrosine phosphorylated CaM kinase IV binds its substrate with a higher affinity and thus increased tyrosine phosphorylation leads to increased activation of CaM kinase IV resulting in increased CREB phosphorylation that triggers increased transcription of proapoptotic proteins that initiate hypoxic neuronal death.

Keywords Calmodulin · Tyr⁹⁹ · CaM kinase IV · Tyrosine phosphorylation · CREB phosphorylation · Src kinase · PP2 · Hypoxia

Introduction

Previously, we have shown that hypoxia results in increased activation of calcium/calmodulin-dependent protein kinase IV (CaM kinase IV) in neuronal nuclei of the cerebral cortex of newborn piglets [1]. Calmodulin (CaM), an activator of CaM kinase IV, is an ubiquitous highly conserved acidic protein that binds Ca⁺⁺ and is present in all eukaryotic organisms [2]. CaM has a dumb-bell shape formed by two globular clusters at its C- and N-terminals that are connected by a flexible α -helix [3]. The binding of CaM can be modified by its post-translational modification [4]. Studies have shown that calmodulin is phosphorylated by receptor protein tyrosine kinase

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(PTK) as well as non-receptor PTK. CaM possesses only two tyrosine residues, Tyr⁹⁹ and Tyr¹³⁸ both of which are known to be phosphorylated [5]. Evidence show that most of the phosphorylation (90%) occurs at Tyr⁹⁹ and only 10% at Tyr¹³⁸ [6–8]. In the present study, we focus on investigating the hypoxia-induced phosphorylation of CaM at Tyr⁹⁹ and tyrosine phosphorylation of CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets. We propose that hypoxia-induced Tyr⁹⁹ phosphorylated calmodulin binds the calmodulin binding domain of CaM kinase IV with a higher affinity as compared to non-phosphorylated CaM and results in CaM kinase IV activation. Similarly, the increased tyrosine phosphorylation of CaM kinase IV may lead to increased binding with its substrate and result in increased activation of CaM kinase IV. During hypoxia, the post-translational modification (tyrosine phosphorylation) of calmodulin, a component central to all the Ca⁺⁺/calmodulin-activated enzymes including CaM kinase IV may alter the activation of a number of intracellular and intranuclear signaling pathways that contribute to cell proliferation and cell death.

An increase in intranuclear Ca⁺⁺, acting through the Ca⁺⁺ receptor calmodulin regulates diverse cellular responses [9]. Ca⁺⁺/calmodulin dependent protein kinase IV (CaMK IV), is a key enzyme of the CaM kinase cascade and is enriched in the brain, predominantly localized in cell nuclei [1, 10]. Phosphorylation of Threonine¹⁹⁶ in CaMK IV by an upstream protein kinase results in induction of Ca⁺⁺/calmodulin-dependent activity [11]. This regulatory mechanism allows a transient elevation in intracellular Ca⁺⁺ levels to produce prolonged CaMK IV activation to regulate gene transcription, through cyclic AMP response element binding (CREB) protein. CREB protein is phosphorylated by CaMK IV at serine¹³³ which initiates transcription. CREB protein is a transcription factor that mediates responses to a number of physiological and pathological signals [12, 13].

The present study specifically focuses on investigating the mechanism of CaM kinase IV activation during hypoxia and determines the effect of hypoxia on tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets. In the present study we have tested the hypothesis that hypoxia results in increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and CaM kinase IV that results in increased activity of CaM kinase IV, and the hypoxia-induced activation of CaM kinase IV is mediated by Src kinase-dependent tyrosine phosphorylation. Therefore, administration of a highly selective Src kinase inhibitor, prior to hypoxia, will prevent the increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and CaM kinase IV and the increased activity of CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets.

Experimental Procedures

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 divided into 3 groups: normoxic (n = 5), hypoxic (n = 5), and hypoxic treated with Src kinase inhibitor PP2, (n = 5). PP2 was administered (1.0 mg/kg, i.v.) 30 min prior to hypoxia.

The animals were ventilated for 1 h under either normoxic condition (FiO₂ = 0.21) or hypoxic condition. Hypoxia was induced by lowering the FiO₂ to 0.06 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 biochemical studies.

Isolation of Cerebral Cortical Neuronal Nuclei

Cerebral cortical nuclei were isolated according to the method of Giuffrida et al. [14]. One gram of brain tissue was homogenized in 15 volumes of a medium containing 0.32 M sucrose, 10 mM Tris–HCl and 1 mM MgCl₂ (pH 6.8). The homogenate was filtered through a nylon bolting mesh (size 110 μm) and subsequently centrifuged at 850g for 10 min. The nuclei were recovered through a discontinuous gradient with a final sucrose concentration of 2.1 M, which increases the yield of large neuronal nuclei. The nuclei were purified by centrifugation for 60 min at 70,000g. The nuclear pellet was collected, re-homogenized and used as the nuclear preparation. Purity of neuronal nuclei was assessed by phase contrast microscope. Neuronal nuclei were characterized by the presence of one nucleolus per nucleus, whereas, others have multiple nucleoli per nucleus. The final nuclear preparation was devoid of any microsomal, mitochondrial or plasma membrane contaminant with a purity of neuronal nuclei of 90%. Protein content was determined by the method of Lowry et al. [15].

Determination of CaM Kinase IV Activity in Neuronal Nuclei

CaM kinase IV activity was determined as described by Park and Soderling [16], by ³³P incorporation (2 min at 37°C) into syntide-2 in a medium containing 50 mM HEPES (pH 7.5), 2 mM DTT, 40 μM syntide-2, 10 mM Mg acetate, 5 μM PKI 5–24 (protein kinase A inhibitor), 2 μM PKC 19–36 (protein kinase C inhibitor), 1 μM

microcystin-LR (protein phosphatase 2A inhibitor), 200 μM sodium orthovanadate (inhibitor of ATPase, alkaline phosphatase, protein tyrosine phosphatase), 0.2 mM ATP, 1 μCi ^{33}P -ATP and either 1 μM calmodulin and 1 mM CaCl_2 (for total activity) or 1 mM EGTA (for Ca^{2+} /CaM independent activity) and 10 μl neuronal nuclei. Phosphorylated peptide medium (20 μl) was placed on phosphocellulose P81 membranes, washed and dried. The filter was placed in 10 ml of scintillation fluid and ^{33}P radioactivity counted. The difference in the presence and absence of CaM was calculated and the enzyme activity was expressed as pmol/mg of protein/min.

Western Blot Analysis of Tyrosine Phosphorylated Calmodulin [Tyr⁹⁹, CaM kinase IV and CREB protein (Ser¹³³)]

The nuclear protein was solubilized and brought to a final concentration of 1 $\mu\text{g}/\mu\text{l}$ 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 $\mu\text{g}/\text{ml}$ each of aprotinin, leupeptin and pepstatin). 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 to each 20 μg of nuclear membrane protein mixture. 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. The nitrocellulose membranes were blocked with 10% non-fat dry milk in PBS buffer for 4–6 h at 4°C. The proteins on the nitrocellulose membranes were then probed with primary antibodies directed against anti-phospho (pTyr⁹⁹)-calmodulin or anti-phospho (pSer¹³³) CREB protein antibodies, in case of CaM kinase IV, the nuclear protein was immunoprecipitated with anti-phosphotyrosine (p-Tyr) antibody and then probed with CaM kinase IV antibody (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C on a rocking platform. Immunoreactivity was then detected by incubation with horseradish peroxidase conjugated secondary antibody (Rockland, Gilbertsville, PA). Specific complexes were detected by enhanced chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and analyzed by imaging densitometry (GS 700 Imaging Densitometer, Bio-Rad) using Quantity One Software (Bio-Rad). The data are expressed as optical density (OD) \times mm².

Determination of ATP and Phosphocreatine

Brain tissue concentrations of ATP and phosphocreatine (PCr) concentrations were determined according to the

method of Lamprecht et al. [17]. Cerebral tissue hypoxia was confirmed biochemically by determining the levels of high energy phosphates ATP and phosphocreatine (PCr). Frozen cortical tissue was powdered under liquid nitrogen, extracted in 6% weight by volume perchloric acid. The extract was thawed on ice and centrifuged at 2,000 $\times g$ for 15 min at 4°C. The supernatant was neutralized to a pH of 7.6 using 2.23 M K_2CO_3 /0.5 M triethanolamine/50 mM EDTA buffer and then centrifuged at 2,000 $\times g$ for 15 min at 4°C. Supernatant (300 μl) was added to 1 ml of buffer (50 mM triethanolamine, 5 mM MgCl_2 , 2 mM EDTA, 2 mM glucose, pH 7.6) and 20 μl NADP. Glucose-6-phosphate dehydrogenase (10 μl) was added and the samples were incubated and read after 8 min. Hexokinase (10 μl) was then added, absorbance readings were taken until steady state was reached and ATP concentration was calculated from the increase in absorbance at 340 nm. Next, ADP (20 μl) and creatine kinase (20 μl) were added to the solution. The samples were read every 5 min for 60 min and phosphocreatine concentrations were calculated from the increase in absorbance at 340 nm.

Statistical Analysis

The data were analyzed using one way analysis of variance (ANOVA) to compare normoxic, hypoxic, and hypoxic-Srci groups. A *P* value of less than 0.05 was considered statistically significant. All values are presented as mean \pm standard deviation (SD).

Results

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 ($\mu\text{moles}/\text{g}$ brain) decreased from 4.41 ± 0.83 in Nx to 2.94 ± 0.67 in Hx ($P < 0.05$ vs. Nx), and 2.81 ± 0.80 in Hx-Srci ($P < 0.05$, vs. Nx, $P = \text{NS}$ vs. Hx). PCr level ($\mu\text{moles}/\text{g}$ brain) decreased from 3.39 ± 0.55 in Nx to 1.12 ± 0.27 in Hx ($P < 0.05$ vs. Nx), and 1.70 ± 0.84 in Hx-Srci ($P < 0.05$ vs. Nx, $P = \text{NS}$ 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 Srci groups.

Representative Western blots of phospho (pTyr⁹⁹)-calmodulin for normoxic, hypoxic and hypoxic-Srci groups are shown in Fig. 1. The results show an increased expression of phosphorylated (p-Tyr⁹⁹) calmodulin in the

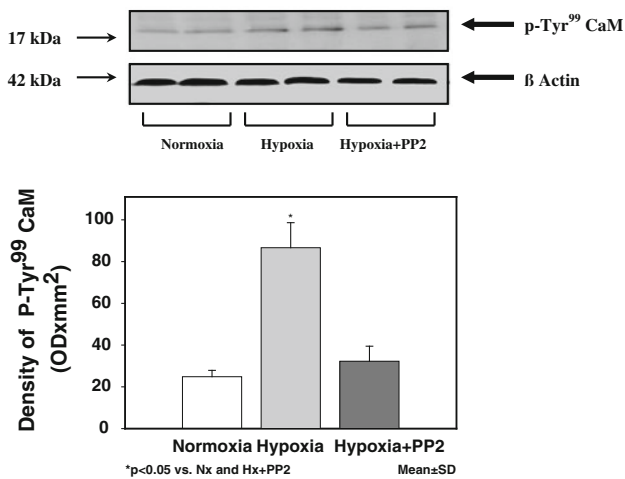


Fig. 1 a Representative western blots of phospho-(pTyr⁹⁹)-calmodulin: Representative western blots of phospho-(pTyr⁹⁹)-calmodulin neuronal nuclei of the cerebral cortex of normoxic, hypoxic and hypoxic-Srci newborn piglets. Western blot analysis was performed using anti-phospho tyrosine (p-Tyr⁹⁹)-calmodulin (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-Srci piglets. **b** Effect of hypoxia on tyrosine phosphorylation of calmodulin at Tyr⁹⁹: Effect of hypoxia on tyrosine phosphorylation of calmodulin at Tyr⁹⁹ in neuronal nuclei of the cerebral cortex of normoxic (n = 5), hypoxic (n = 5) and hypoxic-Srci (n = 5) of newborn piglets. The protein density (OD × mm²) is presented on Y-axis. The data are expressed as mean ± SD. P value is <0.05 by ANOVA

Hx group indicating increased level of phosphorylated (p-Tyr⁹⁹) calmodulin in neuronal nuclei during hypoxia. Administration of Src kinase inhibitor, PP2, prior to hypoxia prevented the hypoxia-induced increase in phospho (pTyr⁹⁹)-calmodulin.

The results (Fig. 1) show that the density (expressed as optical density × mm²) of the phosphorylated (pTyr⁹⁹) calmodulin was 25 ± 3 in Nx, 87 ± 12 in Hx (P < 0.05 vs. Nx) and 32 ± 12 in Hx-Srci (P < 0.05 vs. Hx, P = NS vs. Nx). The data show that hypoxia resulted in increased (pTyr⁹⁹)-phosphorylation of calmodulin and the increased (pTyr⁹⁹)-phosphorylation of calmodulin is prevented by prior administration of a selective Src kinase inhibitor, indicating that the hypoxia-induced increased (pTyr⁹⁹)-phosphorylation of calmodulin is mediated by Src kinase.

Representative Western blots of tyrosine phosphorylated CaM kinase IV for normoxic, hypoxic and hypoxic-Srci groups are shown in Fig. 2 The results show an increased expression of tyrosine phosphorylated CaM kinase IV in the Hx group indicating increased level of Tyrosine phosphorylated CaM kinase IV in the cerebral cortex during hypoxia. Administration of Src kinase inhibitor, PP2, prior to hypoxia prevented the hypoxia-induced increase in tyrosine phosphorylated CaM kinase IV.

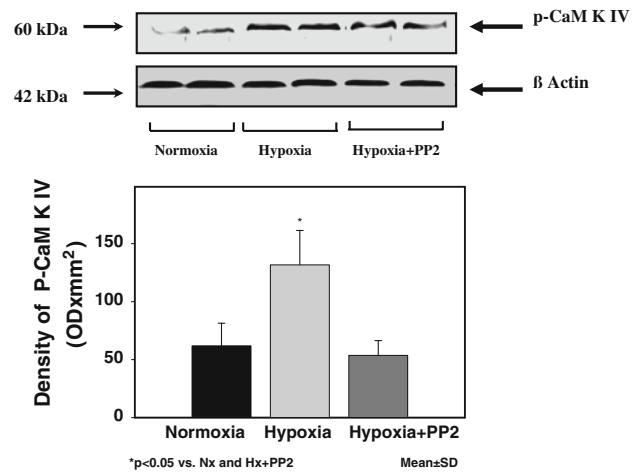


Fig. 2 a Representative western blots of tyrosine phosphorylated CaM kinase IV: Representative western blots of tyrosine phosphorylated CaM kinase IV in neuronal nuclei of the cerebral cortex of normoxic, hypoxic and hypoxic-Srci newborn piglets. Western blot analysis was performed using anti-phospho tyrosine and anti-CaM kinase IV antibodies (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-Srci piglets. **b** Effect of hypoxia on tyrosine phosphorylation of CaM kinase IV: Effect of hypoxia on tyrosine phosphorylation of CaM kinase IV in neuronal nuclei of the cerebral cortex of normoxic (n = 5), hypoxic (n = 5) and hypoxic-Srci (n = 5) of newborn piglets. The protein density (OD × mm²) is presented on Y-axis. The data are expressed as mean ± SD. P value is <0.05 by ANOVA

The results (Fig. 2) show that the density (expressed as optical density × mm²) of tyrosine phosphorylated CaM kinase IV was 62 ± 20 in Nx, 132 ± 30 in Hx (P, 0.05 vs. Nx) and 54 ± 13 in Hx-Srci (P < 0.05 vs. Hx, P = NS vs. Nx). The data show that hypoxia resulted in increased tyrosine phosphorylation of CaM kinase IV and the increased tyrosine phosphorylation of CaM kinase IV was prevented by prior administration of a selective Src kinase inhibitor, indicating that the hypoxia-induced increased tyrosine phosphorylation of CaM kinase IV is mediated by Src kinase.

The results (Fig. 3) show that the activity of CaM kinase IV (expressed as pmoles/mg protein/min) was 2605 ± 5956 in Nx, 5244 ± 802 in Hx (P < 0.05 vs. Nx) and 3700 ± 532 in Hx-Srci (P < 0.05 vs. Hx, P = NS vs. Nx). The data show that hypoxia resulted in increased CaM kinase IV activity in the hypoxic group (as shown previously by us) and the hypoxia-induced increase in CaM kinase IV activity in neuronal nuclei of newborn piglets is prevented by prior administration of a selective Src kinase inhibitor, indicating that the hypoxia-induced increase in CaM kinase IV is mediated by Src kinase.

Representative Western blots of phospho (p-Ser¹³³)-CREB protein for normoxic, hypoxic and hypoxic-Srci

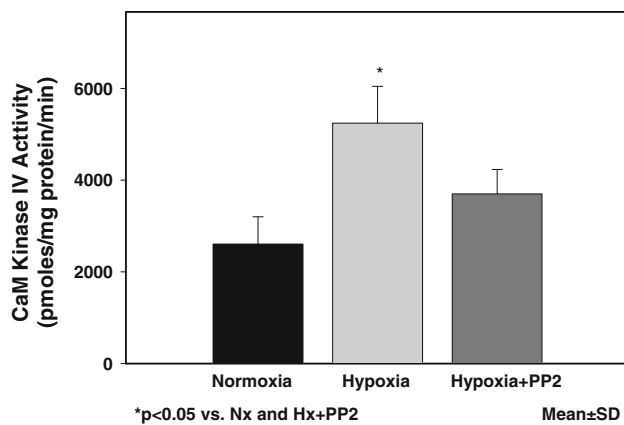


Fig. 3 Effect of hypoxia on CaM kinase IV activity: Effect of hypoxia on CaM kinase IV activity in neuronal nuclei of the cerebral cortex of normoxic ($n = 5$), hypoxic ($n = 5$) and hypoxic-Srci ($n = 5$) of newborn piglets. The CaM kinase IV activity (pmoles/mg protein/min) is presented on Y-axis. The data are expressed as mean \pm SD. P value is <0.05

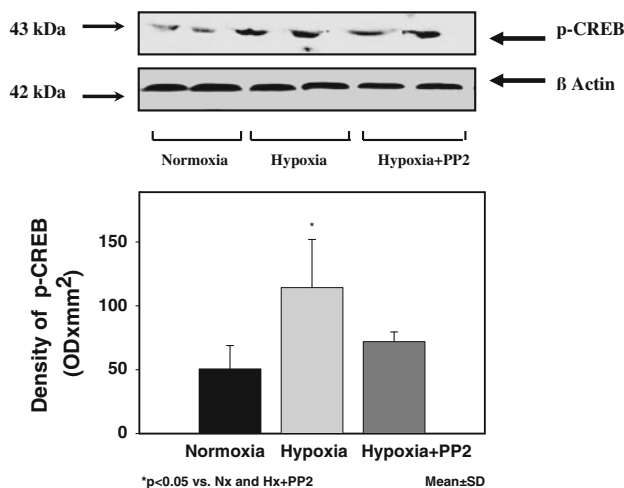


Fig. 4 a Representative western blots of phospho-(p-Ser¹³³)-CREB protein in neuronal nuclei of the cerebral cortex of normoxic, hypoxic and hypoxic-Srci newborn piglets. Western blot analysis was performed using anti-phospho serine (p-Ser¹³³)-CREB protein (Santa Cruz biotechnology, CA) and anti-actin 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-Srci piglets. **b** Effect of hypoxia on tyrosine phosphorylation of CREB protein at Ser¹³³: Effect of hypoxia on tyrosine phosphorylation of CREB protein at Ser¹³³ in neuronal nuclei of the cerebral cortex of normoxic ($n = 5$), hypoxic ($n = 5$) and hypoxic-Srci ($n = 5$) of newborn piglets. The protein density (OD \times mm²) is presented on Y-axis. The data are expressed as mean \pm SD. P value is <0.05 By ANOVA

groups are shown in Fig. 4. The results show an increased expression of phosphorylated (p-Ser¹³³)-CREB protein in the Hx group indicating increased level of phosphorylated (p-Ser¹³³) CREB protein in neuronal nuclei during

hypoxia. Administration of Src kinase inhibitor, PP2, prior to hypoxia, prevented the hypoxia-induced increase in phospho (p-Ser¹³³)-CREB protein.

The results (Fig. 4) show that the density (expressed as optical density \times mm²) of the phosphorylated (p-Ser¹³³)-CREB protein was 51 ± 18 in Nx, 114 ± 38 in Hx ($P < 0.05$ vs. Nx) and 72 ± 18 in Hx-Srci ($P < 0.05$ vs. Hx, $P = NS$ vs. Nx). The data show that hypoxia resulted in increased (p-Ser¹³³) phosphorylation of CREB protein and the increased (p-Ser¹³³) phosphorylation of CREB protein was prevented by prior administration of a selective Src kinase inhibitor, indicating that the hypoxia-induced increased (p-Ser¹³³) phosphorylation of CREB protein is mediated by Src kinase.

Discussion

Previously 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 [1, 18, 19]. We have also shown that hypoxia results in increased phosphorylation of cyclic AMP response element binding (CREB) protein and increased expression of proapoptotic protein Bax [13, 20]. The present study aims to investigate the mechanism of CaM kinase IV activation during hypoxia and tests the hypothesis that hypoxia-induced increased activity of CaM kinase IV is due to Src kinase mediated increased Tyr phosphorylation of calmodulin and CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets. Therefore, administration of a selective Src kinase inhibitor prior to hypoxia will prevent the hypoxia-induced increase in tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and CaM kinase IV as well as the increased activity of CaM kinase IV and phosphorylation of CREB protein at Ser¹³³ in neuronal nuclei of the cerebral cortex of newborn piglets.

The results of the present study show that cerebral hypoxia results in increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and CaM kinase IV as well as CaM kinase IV activity and CREB phosphorylation (Ser¹³³) in neuronal nuclei of the cortical membrane fraction of the cerebral cortex of newborn piglets. Administration of a selective Src kinase inhibitor prevented the hypoxia-induced increased Tyr⁹⁹ phosphorylation of calmodulin and CaM kinase IV as well as the increased CaM kinase IV activity and CREB protein phosphorylation at Ser¹³³ in neuronal nuclei of newborn piglets. These results demonstrate that Src kinase-mediated tyrosine phosphorylation results in increased activation of CaM kinase IV in the hypoxic brain.

The increased tyrosine phosphorylation of calmodulin during hypoxia may lead to increased activation of CaM

kinase IV, as an activator. We propose that during hypoxia Src kinase-mediated increased phosphorylation of calmodulin at Tyr⁹⁹ results in binding of the tyrosine phosphorylated calmodulin with increased affinity to the calmodulin binding domain of CaM kinase IV and leads to increased activation of CaM kinase IV in the hypoxic brain. We propose that during hypoxia, the phosphorylated calmodulin (negatively charged) will bind with a higher affinity to the calmodulin binding domain of CaM kinase IV, a positively charged domain (725–756) rich in basic amino acid residues, Lys and Arg. The sequence of the calmodulin binding domain of CaM kinase IV showing basic amino acid residues (in bold italics) is as follows: **-Arg-Arg-Lys**-Leu-**Lys**-Ala-Ala-Val-**Lys**-Ala-Val-Val-Ala-Ser-Ser-**Arg**-Leu-Ser-. Note the high presence of Arg and Lys residues in this domain. Similarly, the increased tyrosine phosphorylation of CaM kinase IV will facilitate binding of its substrate which is rich in basic amino acids. The amino acid sequence of the phosphorylated kinase-inducible-domain of CREB protein is as follows: **-Lys-Arg-Arg**-Glu-Ile-Leu-Ser-**Arg-Arg**-Pro-Ser¹³³-Tyr-**Arg-Lys**-Ilu-Leu-Asn-Asp-. Note the high presence of Arg and Lys residues in the domain.

The increased tyrosine phosphorylation of calmodulin and CaM kinase IV may lead to increased activation of Ca⁺⁺/calmodulin-dependent protein kinase IV and result in increased activation of Ca⁺⁺-dependent nuclear mechanisms and activates cascades of post-hypoxic programmed cell death. We have observed that hypoxia results in increased activation of CaM kinase IV in neuronal nuclei of newborn piglets. We have also demonstrated that hypoxia results in increased CaM kinase IV activity, increase in CREB phosphorylation, increase in the expression of pro-apoptotic protein Bax and increased fragmentation of nuclear DNA [1, 13, 20]. The expression of Bcl-2 did not increase in nuclei, mitochondria or cytosol. The increased ratio of Bax/Bcl-2 will increase the permeability of the mitochondrial membrane. In addition, the increased Bax may also increase the activation of caspase-9 within the mitochondria and may lead to proteolysis of mitochondrial proteins. Increased permeability and increased activation of caspase-9 may result in activation of mitochondrial endonuclease leading to fragmentation of mitochondrial DNA. Thus increased tyrosine phosphorylation of calmodulin can have consequences through increased CaM kinase IV activation and proapoptotic protein expression.

Previously we have shown that hypoxia results in increased generation of nitric oxide (NO) free radicals [21]. The NO free radicals generated during hypoxia may result in activation of 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 [22–24]. 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 [25, 26]. Hypoxia-induced nitration of NMDA receptor subunits indicates formation of peroxynitrite during hypoxia [27]. Therefore, during hypoxia peroxynitrite-dependent inactivation of SH-PTP-1 and SH-PTP-2 may lead to increased activation of Src tyrosine kinase. Furthermore, the increased activation of Src kinase may further result in increased tyrosine phosphorylation (p-Tyr⁹⁹) of calmodulin and CaM kinase IV, as demonstrated in the present study. Thus a cycle between nNOS activation and activation of Src kinase can continue and perpetually ongoing in the hypoxic brain.

Tyrosine phosphorylation of calmodulin may regulate the activation of protein tyrosine kinases (PTKs). PTKs mediate signal transduction and control many critical processes, such as transcription, cell death progression, differentiation, immune response, intercellular communication and programmed cell death [28–32]. Based on the human genome, potentially 90 genes encode protein tyrosine kinases whose functions are controlled by 107 genes that encode protein tyrosine phosphatases [33, 34]. Protein tyrosine phosphatases regulate the activation of PTK by dephosphorylating these tyrosine residues. Cytoplasmic protein tyrosine phosphatases SH-PTP-1 and SH-PTP-2 are known to dephosphorylate EGFR kinase. Therefore, nitric oxide generated during hypoxia may result in inactivation of cytoplasmic SH-PTP-1 and SH-PTP-2 leading to increased activation of EGFR and Src kinase.

The results of the present study raise some very fundamental questions regarding the role of tyrosine phosphorylation of calmodulin and CaM kinase IV during hypoxia in cell proliferation and cell death. The increased phosphorylation of calmodulin leading to increased activation of Src kinase, via nNOS activation, may be a potential mechanism for a variety of cancer conditions including breast cancer and glioblastoma [35–39]. As shown in this study, cerebral hypoxia leads to increased tyrosine phosphorylation of calmodulin and CaM kinase IV. Hypoxia also results in activation of Src kinase and EGFR kinase in the cerebral cortex of newborn piglets [40, 41]. Hypoxia is also known to result in cell death in the hypoxic brain. Therefore, the role of tyrosine phosphorylated calmodulin via Src kinase/EGFR kinase activation in leading to both the cell proliferation and cell death needs serious consideration. In addition, CaM kinase IV kinase, which activates CaM kinase IV, may potentially be a unique target molecule that may mediate both the cell survival and cell death in the cancerous tissue and the hypoxic brain, respectively.

In recent years, the role of CaM kinase IV cascade is increasing in health and diseases including the immune and inflammatory responses [42, 43].

The results of the present study demonstrate that hypoxia results in increased tyrosine phosphorylation of calmodulin and CaM kinase IV as well as increased activity of CaM kinase IV and CREB protein phosphorylation at Ser¹³³. We propose that increased tyrosine phosphorylation of calmodulin results in increased activation of CaM kinase IV. The Tyr⁹⁹ phosphorylated calmodulin may bind with increased affinity with the enzyme and lead to increased CaM kinase IV activity. In addition, increased tyrosine phosphorylation of CaM kinase IV during hypoxia may facilitate increased binding of its substrate (rich in basic amino acids). We propose that Src kinase mediated increased tyrosine phosphorylation of calmodulin and increased tyrosine phosphorylation of CaM kinase IV is the mechanism of CaM kinase IV activation during hypoxia.

In summary: These results show that cerebral tissue hypoxic results in increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹ and increased tyrosine phosphorylation of CaM as well as increased CaM kinase IV activity and CREB protein phosphorylation at Ser¹³³ in neuronal nuclei of newborn piglets. These studies demonstrated that administration of Src kinase inhibitor PP2 prior to hypoxia prevented the hypoxia-induced increased tyrosine phosphorylation of calmodulin at Tyr⁹⁹, tyrosine phosphorylation of CaM kinase IV and CaM kinase IV activity and consequent CREB protein phosphorylation. We conclude that the mechanism of increased activation of CaM kinase IV during hypoxia is mediated by Src kinase-dependent tyrosine phosphorylation of CaM and CaM kinase IV.

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