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Brain tissue energy dependence of CaM kinase IV cascade activation during hypoxia in the cerebral cortex of newborn piglets

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ABSTRACT

The present study aims to investigate the dependence of CaM kinase IV cascade activation during hypoxia and tests the hypothesis that hypoxia-induced tyrosine phosphorylation of CaM and CaM kinase IV, activation of CaM kinase IV and phosphorylation of CREB protein during hypoxia increases as a function of increase in cerebral tissue hypoxia as measured by decrease in tissue ATP and phosphocreatine (PCr). 3–5 days old newborn piglets were divided into normoxic (Nx, FiO_2 of 0.21 for 1 h) and hypoxic (Hx, FiO_2 of 0.07 for 1 h) groups. Cerebral tissue hypoxia was documented by determining the levels of high energy phosphates ATP and phosphocreatine (PCr). Cerebral cortical neuronal nuclei were isolated and purified, and tyrosine phosphorylation of calmodulin (Tyr^{99}), the activator of CaM kinase IV, and CaM kinase IV determined by Western blot using anti-phospho-($pTyr^{99}$)-calmodulin, anti-pTyrosine and anti-CaM kinase IV antibodies. The activity of CaM kinase IV and its consequence the phosphorylation of CREB protein at Ser^{133} were determined. The levels of ATP (μmole/g brain) ranged from 3.48 to 5.28 in Nx, and 0.41 to 2.26 in Hx. The levels of PCr (μmole/g brain) ranged from 2.46 to 3.91 in Nx and 0.72 to 1.20 in Hx. The $pTyr^{99}$ calmodulin (OD × mm^2) ranged from 20.35 to 54.47.60 in Nx, and 84.52 to 181.42 in Hx (r^2 = 0.5309 vs ATP and r^2 = 0.6899 vs PCr). Expression of tyrosine phosphorylated CaM kinase IV ranged from 32.86 to 82.46 in Nx and 96.70 to 131.62 in Hx (r^2 = 0.5132 vs ATP and r^2 = 0.4335 vs PCr). The activity of CaM kinase IV (pmole/mg protein/min) ranged from 1263 to 3448 in Nx and 3767 to 6633 in Hx (r^2 = 0.7113 vs ATP and r^2 = 0.6182 vs PCr). The expression of p-CREB at Ser^{133} ranged from 44.26 to 70.28 in Nx and 82.70 to 182.86 in Hx (r^2 = 0.6621 vs ATP and r^2 = 0.5485 vs PCr). The data show that hypoxia results in increased tyrosine phosphorylation of calmodulin (Tyr^{99}), increased tyrosine phosphorylation of CaM kinase IV , increased activity of CaM kinase IV and increased phosphorylation of CREB at Ser^{133} as an inverse function of cerebral concentration of high energy phosphates, ATP and PCr. We conclude that the hypoxia-induced increased activation of CaM kinase IV cascade increases with the increase in the degree of cerebral tissue hypoxia as measured by cerebral tissue high energy phosphates in a curvilinear manner. The tyrosine kinases (Src kinase and EGFR kinase) mediated activation of CaM kinase IV cascade potentially results in increased CREB phosphorylation that triggers transcription of proapoptotic proteins during hypoxia.

Cerebral hypoxia in the newborn occurs due to antepartum or perinatal hypoxia/asphyxia with an incidence ranging from 1% to 5% of all live births. Intrauterine hypoxia and birth asphyxia are associated with increased neonatal morbidity and mortality, as well as the long term sequelae of mental retardation, seizure disorders and cerebral palsy. Previously, we have shown that hypoxia results in increased expression and phosphorylation of apoptotic proteins and increased fragmentation of nuclear DNA. Studies also demonstrated that these indices increase as an inverse function of cerebral tissue high energy phosphates, an index of tissue hypoxia. Furthermore, hypoxia resulted in increased activation of calcium/calmodulin-dependent protein kinase IV (CaM kinase IV) in neuronal nuclei of the cerebral cortex of newborn piglets [23]. In the present study, we focus on investigating the relationship between the level of high energy phosphates in the cerebral tissue and activation of CaM kinase IV cascade.

Ca^{2+}/calmodulin dependent protein kinase IV (CaMK IV), the key enzyme of the CaM kinase cascade, is enriched in the brain and predominantly localized in cell nuclei [11,19]. Cyclic AMP response element binding (CREB) protein is phosphorylated by CaMK IV at $serine^{133}$ which initiates transcription. CREB protein is a transcrip-

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tion factor that mediates responses to a number of physiological and pathological signals [8,12].

The present study focuses on investigating the relationship between the levels of cerebral tissue high energy phosphates, ATP and PCr, with the activation of CaM kinase cascade and specifically examines tyrosine phosphorylation of CaM, CaM kinase IV, CaM kinase IV activity and phosphorylation of CREB protein at serine^{133} in neuronal nuclei of the cerebral cortex of newborn piglets. The study also aims to determine the degree of cerebral tissue hypoxia, as determined by the level of cerebral energy metabolism, at which the activation of CaM kinase IV cascade is triggered.

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 two groups: normoxic ($n = 5$) and hypoxic ($n = 5$). The animals were ventilated for one hour under either normoxic condition ($FiO_2 = 0.21$) or hypoxic condition. Hypoxia was induced by lowering the FiO_2 to 0.06–0.08 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.

Cerebral cortical nuclei were isolated according to the method of Giuffrida et al. [7] as described before [5]. 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. [10].Brain tissue concentrations of ATP and phosphocreatine (PCr) concentrations were determined according to the method of Lamprecht et al. [9] as described [5]. Cerebral tissue hypoxia was documented by determining the levels of high energy phosphates ATP and phosphocreatine (PCr).

CaM kinase IV activity was determined as described by Park and Soderling [16], by ^{33}P 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 orthovandate (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. The activity was expressed as pmol/mg of protein/min.

Western blot analysis of tyrosine phosphorylated calmodulin (Tyr^{99}, CaM kinase IV and CREB protein (Ser^{133}): The nuclear 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 aprotinin, leupeptin and pepstatin). Then 5 μl of Laemmli buffer 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 and probed with primary antibodies directed against anti-phospho (pTyr^{99})-calmodulin or anti-phospho (pSer^{133}) CREB protein. Specific complexes were detected by enhanced chemiluminescence using the ECL system (Amersham, Buckinghamshire, UK) and analyzed by imaging densitometry (GS 800 Densitometer, Bio-Rad) using Quantity One Software (Bio-Rad). The data are expressed as optical density (OD) × mm^2.

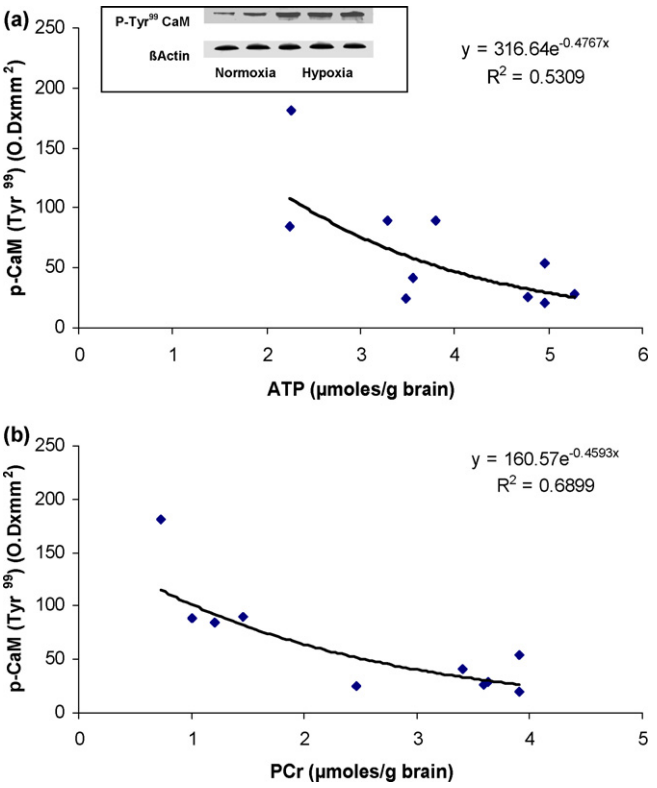

Fig. 1. (a) Representative western blots of phospho-(pTyr^{99})-calmodulin in neuronal nuclei of the cerebral cortex of normoxic and hypoxic piglets. Lanes 1 and 2 represent normoxic and lanes 3–5 represent hypoxic piglets. (b) Relationship between the level of cerebral high energy phosphates and phosphorylation of calmodulin at Tyr^{99}.

Data analysis: The phosphorylation data was plotted against the levels of high energy phosphates. The curves were analyzed and r^2 value (correlation coefficient) >0.5 was considered as a significant correlation.

Cerebral cortical tissue hypoxia in newborn piglets was documented by the levels of ATP and PCr in the cerebral cortical tissue. The levels of ATP (μmole/g brain) ranged from 3.48 to 5.28 in Nx, and 0.41 to 2.26 in Hx. The levels of PCr (μmole/g brain) ranged from 2.46 to 3.91 in Nx and 0.72 to 1.20 in Hx. The results show that cerebral tissue high energy phosphates ATP and PCr, indices of cerebral tissue hypoxia, decreased indicating that varying degrees of tissue hypoxia was achieved in the experimental animals.

Representative Western blots of phospho (pTyr^{99})-calmodulin for normoxic and hypoxic groups are shown in Fig. 1. The results show an increased expression of phoshorylated (p-Tyr^{99}) calmodulin in the Hx group indicating increased level of phosphorylated (p-Tyr^{99}) calmodulin in neuronal nuclei during hypoxia.

The results (Fig. 1) show that the density (expressed as optical density × mm^2) of the phosphorylated (pTyr^{99}) calmodulin (OD × mm^2) ranged from 20.35 to 54.47.60 in Nx, and 84.52 to 181.42 in Hx ($r^2 = 0.5309$ vs ATP and $r^2 = 0.6899$ vs PCr). The data show that hypoxia resulted in increased (pTyr^{99})-phosphorylation of calmodulin. A significant correlation was observed between the levels of (pTyr^{99})-phosphorylation of calmodulin with the levels of ATP and PCr. The results show that (pTyr^{99})-phosphorylation of calmodulin increases with an increase in degree of cerebral tissue hypoxia as an inverse function of ATP and PCr concentrations in the cerebral tissue.

Representative Western blots of tyrosine phosphorylated CaM kinase IV for normoxic and hypoxic groups are shown in Fig. 2 The results show an increased expression of tyrosine phosphorylated