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Effect of Concurrent Src Kinase Inhibition with Short Duration Hypothermia on Ca²⁺/Calmodulin kinase IV (CaM Kinase IV) Activity and Neuropathology after Hypoxia-Ischemia in the Newborn Swine Brain

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Abstract

BACKGROUND—Hypoxia-Ischemia (HI) results in increased activation of Ca²⁺/Calmodulin kinase IV (CaM Kinase IV) mediated by Src kinase. Therapeutic Hypothermia (HT) ameliorates neuronal injury in the newborn.

HYPOTHESIS—Inhibition of Src kinase concurrently with hypothermia further attenuates the hypoxia-induced increased activation of CaM kinase IV compared with hypothermia alone.

DESIGN/METHODS—Ventilated piglets were exposed to HI, received saline or a selective Src kinase inhibitor (PP2) and were cooled to 33°C. Neuropathology, adenosine triphosphate (ATP), Phosphocreatine (PCr) concentration and CaM Kinase IV activity were determined.

RESULTS—The neuropathology mean score (M±SD) was 0.4±0.43 in Normoxia-Normothermia (p<0.05 vs hypoxia-normothermia), 3.5±0.89 in hypoxia-normothermia (p<0.05 vs normoxia-normothermia), 0.7±0.73 in hypoxia-hypothermia (p<0.05 vs normoxia-normothermia) and 0.5±0.70 in normoxia-hypothermia (p<0.05 vs hypoxia-normothermia). CaM Kinase IV activity in cerebral tissue (pmol Pi/mg protein/min; M±SD) was 2002±729 in normoxia-normothermia,

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Author Contributions Statement:

MDP conceived and designed the experiment and edited the manuscript. PK performed the experiments and wrote the main manuscript text. SM and MDP supervised the experiments. PK and IK reviewed the cytopathology and edited the manuscript. IK and AJ did the statistics, prepared the Figures and wrote the statistic section of the manuscript. All authors reviewed the manuscript.

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1704±18 in normoxia-hypothermia, 6017±2510 in hypoxia-normothermia, 4104±542 in hypoxia-hypothermia ($p<0.05$ vs normoxia-hypothermia), and 2165±415 in hypoxia-hypothermia with PP2 ($p<0.05$ vs hypoxia-hypothermia). The hypoxic groups with and without hypothermia or Src kinase inhibitor were comparable in the levels of ATP and PCr indicating that they were similar in their degree of energy failure prior to treatments. Hypothermia or Src kinase inhibitor (PP2) did not restore the ATP and PCr levels.

CONCLUSIONS—Hypothermia and Src kinase inhibition attenuated apoptotic cell death and improved neuropathology after hypoxia. Combination of short duration hypothermia with Src kinase inhibition following hypoxia further attenuates the increased activation of CaM Kinase IV compared to hypothermia alone in the newborn swine brain.

Keywords

Cerebral hypoxia; Hypothermia; Src kinase; CaM Kinase IV

Introduction

Current estimates of the incidence of neonatal encephalopathy range from 1–8/1000 live births.[1–3] Approximately 15% to 20% of affected newborns die in the postnatal period, and an additional 25% may manifest substantial long-term disabilities. Neonates with severe encephalopathy have a high risk of death (85%), cerebral palsy (CP) and developmental delay among survivors. [1,4] Neonates with moderate encephalopathy may have significant motor deficits, memory impairment, visual dysfunction, increased hyperactivity and learning difficulties.[2]

Decreased oxygen and glucose delivery to the newborn brain during hypoxia-ischemia leads to primary energy failure, depletion of high energy compounds and activation of a downstream cascade of free radical injury. Excitotoxicity, and induction of a neuro-inflammatory response, exacerbated by reperfusion/re-oxygenation lead to secondary energy failure and delayed cell death.[5] Although the initial wave of cell death may occur too rapidly for effective intervention, treatments administered before the onset of secondary energy failure have the potential to attenuate or prevent the later wave of cell death.[5,6]

Hypothermia (HT) has emerged as the standard of care to treat infants with HIE.[7] Several systematic reviews and meta-analysis of term and late preterm infants with moderate/severe encephalopathy and evidence of intrapartum asphyxia concluded that therapeutic hypothermia reduced the combined outcome of mortality or major neurodevelopmental disability. However, the overall rate of death or disability following hypothermia reported in published trials was still 31% to 55%. [8] There remains a need to further optimize cooling strategies and to identify adjuvant therapies that could potentially augment the neuroprotective effects of hypothermia.[7] Several experimental and pre-clinical studies have documented the effects of hypothermia on the pathogenesis of brain injury in the acute, subacute and chronic stages of hypoxia. Exposure to hypothermia reduces excitotoxicity, free radical production, apoptosis, and inflammation. Hypothermia attenuates the influx of intracellular calcium during HIE, and reduces apoptotic cell death. [4] The mechanism of

apoptotic cell death following cerebral hypoxia and the point of action of therapeutic hypothermia and the Src kinase inhibitor is illustrated in Diagram 1.

Our laboratory has focused on molecular mechanisms of hypoxia induced brain injury and particularly the enzymes of the apoptotic cascade such as Src kinase and Ca^{++} /calmodulin kinase IV (CaM kinase IV).[9] We have previously shown that cerebral hypoxia results in increased activity of Src kinase and CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets.[10,11]

Ca^{++} /calmodulin-dependent kinase IV (CaM Kinase IV), located in the nucleus, phosphorylates the cyclic-AMP response element binding (CREB) protein, a regulator of the transcription of genes of cell survival or cell death pathways.[11,12]

We hypothesize that short duration of HT will attenuate the hypoxia induced overactivation of CaM kinase IV, and that inhibition of Src kinase in combination with HT will further attenuate the activation of CaM kinase IV compared with HT alone.

Materials and Methods

Experimental Procedures

Studies were performed on 2–3 day old male Yorkshire piglets obtained from the Willow Glenn Farm in Strasburg, PA. The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of Drexel University (IACUC-20401). All methods were performed in accordance with the relevant guidelines and regulations. Appropriate use of drugs was followed during experiments to eliminate pain and suffering, according to the NIH Guidelines for the Use of Laboratory Animals. Five piglets were included in each group for statistical comparison. Anesthesia was induced with 4% Isoflurane and maintained with 0.8% Isoflurane. The piglets were orally intubated and ventilated within 10 min of induction of anesthesia. A peripheral IV catheter was inserted for administration of fluids and medications. Intravenous fentanyl (25 $\mu\text{g/kg}$) and pancuronium bromide (0.1 mg/kg) were administered following placement on a pressure ventilator using a mixture of 75% nitrous oxide and 25% oxygen. Arterial blood gases and pH were taken every 15 min through a percutaneously placed peripheral arterial line.

Following 1 hr of baseline ventilation with normal blood gases, the piglets were randomly assigned to either the normoxic control group, for an additional 60 min of mechanical ventilation in room air, or the hypoxia group. Hypoxia was induced by decreasing the FiO_2 to 0.06 within 5 minutes, maintained for the 60 min period and titrated to achieve a 40 % reduction in systolic BP from baseline. In terms of temperature, the piglets were either maintained at 37–38°C (normothermic-NT) or cooled to 33–34°C for 4 hrs, then rewarmed over 6 hours (hypothermic-HT). HT to a target T_{rectal} of 33–34°C was achieved by placing the piglet on a neonatal cooling blanket.

The hypoxic piglets that were cooled were subdivided into a group that received Src kinase inhibitor PP2 (HI-HT-PP2) and to another group that underwent hypothermia only (HI-HT) without the inhibitor. PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-d]

pyrimidine) is a selective and potent inhibitor of Src-kinase (IC₅₀= 5 nM) (EMD Bioscience, CA). The inhibitor was administered one hour after HI to mimic the clinical scenario of HIE. After the re-oxygenation period, the animals were either transcardially perfused with phosphate buffer saline (PBS) and paraformaldehyde for neuropathology studies or the brain was rapidly harvested and placed in liquid nitrogen and stored at -80 °C for biochemical studies. For neuropathology studies, areas of motor and sensory cortex, hippocampus, putamen and caudate nucleus were prepared and fixed.

Neuropathology

Hypoxic injury was confirmed histologically, by Hematoxylin and Eosin (H&E) stain. Upon brain removal, mid-sagittal sections comprising of cerebrum, brainstem and cerebellum en block were fixed immediately by immersion in 10% neutral formalin, processed according to standard neurohistological procedures, and embedded in paraffin. Parasagittal sections corresponding to hippocampus were stained with H&E. Sections from motor cortex, somatosensory cortex, putamen, hippocampus, caudate nucleus and thalamus were utilized to evaluate the number of viable neurons, apoptotic profiles, pericellular edema, pyknotic nuclei and vascular thickening. Neuronal cell injury was demonstrated by neuropathology and scored by 2 independent observers. The scores ranged from 0 (normal) to 4 (injured neurons >75% of high power field) according to the method of Hoque et al. [13]

Isolation of Cerebral Cortical Neuronal Nuclei

Cerebral cortical nuclei were isolated according to the method of Giuffrida et al [14] and described in our previous studies[11]. 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 ATP and Phosphocreatine

Brain tissue concentrations of Adenosine Triphosphate (ATP) and phosphocreatine (PCr) concentrations were determined according to the method of Lamprecht et al.[16] Cerebral tissue hypoxia was confirmed biochemically by determining the levels of high energy phosphates ATP and PCr as described in our previous studies[11].

Determination of CaM Kinase IV Activity in Neuronal Nuclei

CaM kinase IV activity was determined as described by Park[17] and Soderling[18], 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 ³³P-ATP and either 1 μM calmodulin and 1 mM CaCl₂ (for total activity) or 1 mM EGTA (for Ca²⁺/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.

Statistical Analysis

The statistical analysis of the data on ATP, PCr and CaM kinase IV activity was performed using one way analysis of variance (ANOVA). A p value <0.05 was considered significant. Pair-wise comparison between the groups was done by Tukey-test.

Results

High Energy Phosphates

Cerebral hypoxia was confirmed by determining the levels of ATP and PCr ($\mu\text{moles/gr Brain Tissue}$) to assess whether the hypoxic groups with and without the inhibitor were comparably hypoxic. The ATP and PCr levels in the different groups are shown in Table 1. The hypoxic groups with and without hypothermia or Src kinase inhibitor were comparable in the levels of ATP and PCr indicating that they were similar in their degree of energy failure prior to treatments. Administration of HT or Src kinase inhibitor (PP2) did not restore the ATP and PCr levels.

Neuropathology

The neuropathology mean score ($M\pm SD$) was 0.4 ± 0.43 in normoxia-normothermia ($p<0.05$ vs hypoxia-normothermia), 3.5 ± 0.89 in hypoxia-normothermia ($p<0.05$ vs normoxia-normothermia), and 0.7 ± 0.73 in hypoxia-hypothermia ($p<0.05$ vs hypoxia-normothermia) and 0.5 ± 0.70 in normoxia-hypothermia ($p<0.05$ vs hypoxia-normothermia). Morphologically, neurons were damaged, with pyknotic nuclei, ruptured or irregular bordered nuclear membranes and marked presence of apoptotic profiles [Figure 1]. However, HT resulted in fewer damaged neurons, less karyorrhexis and pericellular edema, and thus attenuated neuronal cell injury compared to the group exposed to hypoxia without HT or Src kinase inhibition.

CaM kinase IV activity

CaM Kinase IV activity in cerebral tissue (pmol Pi/mg protein/min; $M\pm SD$) was 2002 ± 729 in normoxia-normothermia, 1704 ± 18 in normoxia-hypothermia, 6017 ± 2510 in hypoxia-normothermia, 4104 ± 542 in hypoxia-hypothermia ($p<0.05$ vs normoxia-hypothermia), and 2165 ± 415 in hypoxia-hypothermia with PP2 ($p<0.05$ vs hypoxia-hypothermia). CaM Kinase IV activation increased following hypoxia. However, treatment with short duration hypothermia attenuated the CaM kinase IV activation and Src kinase inhibition, when used concurrently with hypothermia, further reduced the activation of CaM kinase IV similar to the normoxia levels. [Figure 2]

Discussion

The present study showed that using small molecule inhibitors in combination with hypothermia (HT) acts synergistically and further attenuates the overactivation of apoptotic enzymes when compared to HT alone. Studies have shown that HT may attenuate the

neuronal injury caused by hypoxia-ischemia and reperfusion by decreasing the metabolism, limiting the cell and mitochondrial membrane leakage, reducing the calcium influx into the cell, decreasing excitotoxicity and the production of free radicals and by decreasing the activation of apoptotic enzymes such as caspase 3 and 9, Src kinase and CaM kinase IV.[19–22] However, following inhibition of Src, the activation of other enzymes, such as CaM kinase IV and Caspase 3 and 9 have been shown to be decreased. Growing evidence indicates that non-receptor tyrosine kinases have been implicated in the hypoxia induced neonatal brain injury in pre-clinical studies.[22] Single protein inhibition with small molecules resulted in satisfactory in vivo results in the HI porcine model.[10,22] In order to ensure adequate energy failure after hypoxia we measured the high energy compounds in the groups and our hypoxic groups were very comparable in the ATP and PCr levels. HT or Src inhibition did not restore the energy failure but they were applied one hour following the hypoxia.

A significant advantage of the combination treatment of hypothermia with the inhibitors is their already clinical establishment in other medical fields such oncology or rheumatology. [23] The newborn porcine shares comparative developmental ontogeny with the term newborn human brain and the pattern of hypoxic brain injury in piglets is similar to that in hypoxic human infants.[24] We and other investigators have used the porcine animal model, in contrast to a rodent model of HIE, as it provides the advantage of being able to make real time measurements of physiologic parameters such as arterial blood gases and continuous blood pressure monitoring which allows titration of the FiO₂ to achieve a precise and reproducible degree of hypoxia-ischemia.[24]

CaM kinase IV is a multifunctional CaM-dependent kinase protein with limited tissue distribution that has been implicated in transcriptional regulation in neurons. [11,25] In our previous studies we showed that Src kinase inhibition without hypothermia, attenuated the activity of CaM kinase IV but only to a certain level (3700 ± 532 in HI-PP2 ($P < 0.05$ vs. HI, $P = NS$ vs. Nx). [11] We now showed that HT has an additive role and further decreased the CaM kinase IV activity when combined with small molecules.

CaM Kinase IV phosphorylates and activates cyclic AMP response element binding protein (CREB), a key transcription factor. We have previously shown increased activation of apoptotic pathway mediators such as cytosolic non-receptor protein tyrosine kinase, Src kinase and nuclear CaM Kinase IV in the porcine brain acutely after HI. [11,18] Inhibition of Src kinase prevented HI-induced activation of CaM Kinase IV and reduced HI cerebral injury in normothermic (NT) piglets.[5]

Inhibition of Src kinase prevented the hypoxia-induced increase in tyrosine phosphorylation of calmodulin at Tyr99 and CaM kinase IV showing that it is the Src kinase that mediates the increased Tyr phosphorylation of calmodulin and CaM kinase IV in neuronal nuclei of the cerebral cortex of newborn piglets. [12]

Our data suggest that blockade of the apoptotic pathway works synergistically with HT and further attenuates the overactivation of the apoptotic enzymes. Since hypoxia ischemia (HI) cannot be predicted beforehand, agents such as PP2 that target mechanisms mediating early

stages of injury may provide a novel adjunct therapy to HT in the post-HI period to further improve outcomes of HIE.

The present study demonstrated that hypoxia resulted in increased activation of CaM kinase IV which was attenuated by short duration therapeutic HT and was further reduced by combined HT and Src inhibition in the newborn porcine HI model. We showed that the hypoxia-induced increased activation of apoptotic proteins is attenuated by the inhibition of Src kinase which acted synergistically with HT. Together with our previous studies, these data further elucidate the effects of Src kinase on the apoptotic cascade, and identify Src kinase as a potential target to interrupt cell death pathways.

In the course of perinatal hypoxic-ischemic-reperfusion insult, oxidative stress may be reduced by concurrent administration of a selective inhibitor of Src kinase (PP2) with hypothermia, even if this reduction serves as one of the mechanisms of action in therapeutic hypothermia.

Limitations

It was not deemed feasible to perform all studies (biochemical assays and histopathology) on the same animals. Biochemical studies such as high energy compounds and enzyme assays require deep freezing of brain tissue to -80°C within 5–10 seconds from harvesting. Conversely, transcardial perfusion and tissue fixation is required for neuropathology. Ultra-short duration of hypothermia was performed, however changes in enzymatic activity occur very rapidly.

Data Availability

The datasets generated during and/or analysed during the current study are available from Dr. Maria Delivoria-Papadopoulos' Laboratory at Drexel University College of Medicine in Philadelphia, PA, USA.

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Abbreviations

ATP	Adenosine Triphosphate
PCr	Phosphocreatine
HI	Hypoxia-Ischemia
NT	Normothermia
HT	Hypothermia
HI-NT	Hypoxic Normothermic
HI-HT	Hypoxic Hypothermic

NX-NT	Normoxic Normothermic
NX-HT	Normoxic Hypothermic
HIE	Hypoxic Ischemic Encephalopathy
PP2	(4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d] pyrimidine)
PBS	Phosphate Buffer Saline
CREB	cyclic-AMP response element binding
CaM Kinase IV	Ca ⁺⁺ /calmodulin kinase IV
H&E	Hematoxylin and Eosin
ANOVA	analysis of variance
NMDA	N-methyl-D-aspartate
Apaf-1	apoptotic protease activating factor-1
NO	Nitric Oxide
FAs	Focal Adhesions
HI-PP2	Hypoxia and treatment with Src Inhibitor, PP2
CP	Cerebral Palsy

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Figure 1.

Representative H&E-stained hippocampal sections illustrating a. a neuronal apoptotic profile (arrow) and b. normal neuronal cells (arrow heads). The photos were taken at × 600 magnification with oil immersion.

Figure 2.

CaM kinase IV activation during normoxia and hypoxia and treatment with hypothermia and with or without Src kinase inhibition. The hypoxia induced increase in CaM kinase IV activation was reduced by hypothermia and reduced further when hypothermia was combined with Src kinase inhibition (PP2).

Diagram 1.

Mechanism of apoptotic cell death following cerebral hypoxia. Note the point of action (marked with X) of hypothermia and the Src kinase inhibitor (PP2). Following inhibition of Src kinase, the activation of other enzymes, such as CaM kinase IV and Caspase 9 and 3 have been shown to be decreased.

Table 1

Levels of high energy compounds in the cerebral cortex of newborn piglets. The hypoxic groups with and without hypothermia or Src kinase inhibitor were comparable in the levels of ATP and PCr indicating that they were similar in their degree of energy failure prior to treatments. Administration of HT or Src kinase inhibitor (PP2) did not restore the ATP and PCr levels.

Study Groups	ATP (μ moles/g brain)	Phosphocreatine (μ moles/g brain)
Normoxia (n=5)	4.92 $\pm$ 0.83	3.59 $\pm$ 0.53
Hypoxia (n=5)	2.53 $\pm$ 0.67	1.23 $\pm$ 0.17
Hypoxia + HT (n=5)	2.32 $\pm$ 0.74	1.45 $\pm$ 0.84
Hypoxia+HT+PP2 (n=5)	2.68 $\pm$ 0.23	1.23 $\pm$ 0.43