Effect of concurrent administration of apoptotic inhibitors and hypothermia on post hypoxic cerebral injury in the newborn

Maria Delivoria-Papadopoulou, Shadi Malaeb

Department of Pediatrics, Drexel University College of Medicine, Philadelphia PA, USA

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Abstract

Neonatal hypoxic-ischemic encephalopathy is known to cause long-term neurodevelopmental impairment. Experimental studies and clinical trials demonstrated that treatment with hypothermia after hypoxic-ischemic insults reduced brain injury. As a result of these data, hypothermia has emerged as the standard of care for treatment of neonatal hypoxic-ischemic encephalopathy. However up to 40% of newborns with hypoxic-ischemic encephalopathy who are treated with hypothermia have significant neurocognitive deficits on follow-up. Obviously, there remains a need to further optimize cooling strategies and to identify adjuvant therapies that could potentially augment the neuroprotective effects and accentuate neuroprotection by hypothermia. As the occurrence of hypoxia in the newborn brain can not be predicted beforehand, the only opportunity we have to improve outcomes after hypoxic-ischemic encephalopathy is to pursue neuroprotective strategies that can be used as an adjunct to therapeutic hypothermia in the post-hypoxic-ischemia period, with special emphasis on mechanism mediating the early stages of hypoxic injury. Previously, we have demonstrated in the newborn piglet that within one hour of exposure to hypoxia, there is increased activation of the enzyme Ca++/calmodulin kinase (CaM Kinase) IV localized in the nucleus, a key regulator of transcription of apoptotic genes. We have also demonstrated that the hypoxia-induced enzyme CaM kinase IV activation is mediated by activation of two protein tyrosine kinases, Src kinase and EGFR kinase and by increased Ca++ influx into the nucleus. Inhibition of Src kinase by the selective inhibitor PP2 and of EGFR kinase by the selective inhibitor PD168393 at the onset of hypoxia prevented CaM kinase IV activation and decreased subsequent hypoxia-induced neuronal death. The aim of this study was to test the hypothesis that the combined treatment with hypothermia and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), a highly selective inhibitor of Src kinase, immediately after the hypoxic insult may augment the beneficial effect of hypothermia on hypoxia/ischemia-induced neuronal necrosis. To this aim we assessed the levels of CaM Kinase IV activity as well as the levels of Na+K+-ATPase in the Cerebral Cortex of Newborn Piglets exposed experimental hypoxia that were treated with hypothermia with or without concomitant PP2 administration. 2-3 day old piglets were anesthetized and ventilated. In conclusion, our preliminary data show that concurrent administration of Src kinase inhibitor in combination with induction of whole body hypothermia results in augmented neuroprotection as indicated by further attenuation of hypoxic-ischemic induced CaM kinase IV activation and improvement in neuronal membrane integrity compared to hypothermia alone.

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**Experimental protocol**

2-3 day old piglets were anesthetized and ventilated. Hypoxia-ischemia (PaO2 20-22 cm H2O and a 40-50% decrease in blood pressure from baseline) were induced by decreasing FiO2 from 21% to 7% for 1h. Following hypoxia-ischemia, all animals were wrapped in a circulating water blanket. Piglets assigned to hypothermia were cooled to a core temperature of 33-34°C over a 30min period, and kept at this temperature for 4h. Then, hypothermic piglets were rewarmed to normal core temperature and allowed to recover in room air. Controls were kept at a normal body temperature of 38-39°C. At the end of the recovery period, cerebral cortex from piglets in each group were frozen in liquid nitrogen for isolation of subcellular fractions (neuronal nuclei, mitochondria, cell membranes, and cytosol) and biochemical analyses.

**Results**

**Levels of CaMK IV activity in the cerebral cortex of newborn piglets**

Levels of CaMK IV activity in the cerebral cortex of newborn piglets that were exposed to hypoxia/ischemia were significantly higher than in the controls.

- Treatment of hypoxemic piglets with PP2 resulted in lower levels of CaMK IV activation that did not differ significantly from those in the controls.
- Effect of hypothermia, CaMK IV activity in hypoxic animals that were kept in normal environmental temperature were higher than in hypoxemic piglets that were treated with hypothermia.
- Combined treatment of hypoxic piglets with hypothermia and PP2 further decreased the levels of CaMK IV activity.

**Levels of Na+-K+-ATPase in the cerebral cortex of newborn piglets**

Levels of Na+-K+-ATPase decreased following hypoxia and they were restored by hypothermia. Administration of PP2 in hypoxic piglets treated with hypothermia further increased the Na+-K+-ATPase levels, suggesting a synergistic effect between hypothermia and PP2.

**Summary and conclusions**

Our preliminary data show that concurrent administration of Src kinase inhibitor in combination with induction of whole body hypothermia results in augmented neuroprotection as indicated by further attenuation of hypoxic-ischemic induced CaM kinase IV activation and improvement in neuronal membrane integrity compared to hypothermia alone. This work will provide novel insight into the mechanisms of hypothermic neuroprotection that will increase our understanding of the protective effects of hypothermia in the newborn brain. Of particular interest is the observation that the early stage mechanisms of hypoxic-ischemic injury targeted in these studies are amenable to inhibition by medications which are already used clinically in other situations. Therefore, these studies pioneer a fundamental experimental frame work in the newborn piglet that mimics the clinical scenario reflecting the window of opportunity between the time when hypoxic-ischemic encephalopathy is diagnosed and when hypothermia is started, and provide a basis for future translational studies in human newborns that will investigate the use of medications that could act synergistically with hypothermia to ameliorate hypoxic-ischemic brain injury.

*The authors declare that they have no conflicts of interest.*