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Mitochondrial mechanisms of cell death and neuroprotection in pediatric ischemic and traumatic brain injury

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Abstract

There are several forms of acute pediatric brain injury, including neonatal asphyxia, pediatric cardiac arrest with global ischemia, and head trauma, that result in devastating, lifelong neurologic impairment. The only clinical intervention that appears neuroprotective is hypothermia initiated soon after the initial injury. Evidence indicates that oxidative stress, mitochondrial dysfunction, and impaired cerebral energy metabolism contribute to the brain cell death that is responsible for much of the poor neurologic outcome from these events. Recent results obtained from both *in vitro* and animal models of neuronal death in the immature brain point toward several molecular mechanisms that are either induced or promoted by oxidative modification of macromolecules, including consumption of cytosolic and mitochondrial NAD^+ by poly-ADP ribose polymerase, opening of the mitochondrial inner membrane permeability transition pore, and inactivation of key, rate-limiting metabolic enzymes, e.g., the pyruvate dehydrogenase complex. In addition, the relative abundance of pro-apoptotic proteins in immature brains and neurons, and particularly within their mitochondria, predisposes these cells to the intrinsic, mitochondrial pathway of apoptosis, mediated by Bax- or Bak-triggered release of proteins into the cytosol through the mitochondrial outer membrane. Based on these pathways of cell dysfunction and death, several approaches toward neuroprotection are being investigated that show promise toward clinical translation. These strategies include minimizing oxidative stress by avoiding unnecessary hyperoxia, promoting aerobic energy metabolism by repletion of NAD^+ and by providing alternative oxidative fuels, e.g., ketone bodies, directly interfering with apoptotic pathways at the mitochondrial level, and pharmacologic induction of antioxidant and anti-inflammatory gene expression.

Keywords

Metabolism; Calcium; Permeability transition pore; poly(ADP-ribose) polymerase; Reactive oxygen species; Pyruvate dehydrogenase; Cytochrome *c*; Nicotinamide adenine dinucleotide

Pediatric hypoxic and traumatic brain injury

Brain injury in the pediatric population can have a great economic and emotional impact on both the child's family and society. It is important to remember that in addition to regaining

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lost skills, developing children are expected to rapidly be developing new skills, making the burden of brain injury even greater. Children with severe injury can require an extended amount of chronic care throughout their lifetime. One of the most common causes of permanent neurologic injury in children is perinatal hypoxia– ischemia. Infants sustain asphyxial injury around the time of birth at a rate of 0.2 to 0.4% of all full-term births. The incidence is much greater in the preterm and low birth weight infant population (MacDonald et al., 1980). Up to 50% of the most severely affected infants will die in the newborn period, and the less severely injured infants can survive with significant, permanent neurologic deficits. These can include cerebral palsy, mental retardation, learning disabilities, epilepsy, deafness and blindness. Importantly, long-term outcome studies have shown that 20% of infants demonstrating moderate encephalopathy secondary to perinatal hypoxia– ischemia have marked neurologic deficits at 3.5 and 8 years of age (Robertson et al., 1989). Furthermore, in the subset of children that did not have marked deficits, up to 1/3 had delays in their academic progress in school.

Another cause of neurologic injury in children is cardiac arrest. There are many etiologies of cardiac arrest in children, but the majority (>60%) involve a period of asphyxia preceding loss of circulation (Manole et al., 2008; Berg et al., 2008). Outcome is relatively poor, as only ~30% of patients have return of spontaneous circulation, and only 12% of these patients survive to hospital discharge (Donoghue et al., 2005). The burden of neurologic impairment is high in survivors, with only ~4% having intact neurologic outcomes (no or mild impairment) (Donoghue et al., 2005).

Trauma is the leading cause of death in children ages 1–18, and traumatic brain injury (TBI) is the major determinant of outcome in these injured children (NIH 1998). Every year in the United States alone, almost 1/2 million children sustain traumatic brain injury, and ~3000 children per year die from these injuries (CDC). Despite recent advances in neurointensive care and reduction in the overall mortality rate (Tilford et al., 2005), the long-term morbidity of severe TBI in childhood remains high. Survivors of pediatric TBI suffer from many long-term physical, cognitive, psychological, and emotional impairments (Yeates et al., 2004; Max et al., 2004; Wechsler et al., 2005; Keenan and Bratton, 2006). For example, one study found that 40% of children with severe TBI had a persistent personality change after injury(Max et al., 2000), and other studies have shown profound declines in long-term academic performance (Ewing-Cobbs et al., 2004a). Importantly, children injured at younger ages (<4–6 years) may be especially vulnerable to declines in intelligence and executive functioning (Ewing-Cobbs et al., 2006), (Ewing-Cobbs et al., 2004b; Anderson et al., 2005). Despite these daunting facts, there are currently no effective neuroprotective therapies available for children with severe head injury. Additional studies elucidating the age-specific mechanisms of secondary injury and recovery are essential, in order to target these mechanisms for effective neuroprotective treatments in pediatric acute brain injury.

Neuroprotection for pediatric brain injury

Current therapeutic options for pediatric brain injury are limited and essentially focus on supportive care of the injured infant and avoidance of any additional systemic insults such as further hypoxia, hypotension, and secondary ischemia due to cerebral edema. Of note, most of these parameters, with the exception of hypoxia, have age-dependent definitions. For instance, normal blood pressure ranges increase steadily with age, and normal cerebral blood flow (CBF) is higher in children than in infants or teenagers. Peak global CBF is seen at ~3–4 years of age (108 ml/100 g/min), with a slow decline into the teenage years (~70 ml/100 g/min) (Suzuki, 1990). Most normal values for children remain above the normal CBF range for adults (~50 ml/100 g/min), with the exception of infants <6 months of age (~40 ml/100 g/min) (Suzuki, 1990) (Zwienenberg and Muizelaar, 1999).

No pharmacologic or therapeutic interventions, either alone or in combination, have proven effective in improving neurologic outcome of infants and children after asphyxial or traumatic insults. In fact, most medical therapies used in the past to treat hypoxic–ischemic brain injury, such as steroids, barbiturates and osmotic agents, have proven ineffective (Vannucci, 1990). Clinical trials in pediatric TBI are similarly limited (Natale et al., 2006), and the majority of clinical management guidelines are extrapolated from adult TBI guidelines (Adelson et al., 2003; Jankowitz and Adelson, 2006). Two main therapeutic interventions that have been tested prospectively in head-injured children recently are decompressive craniectomy (Taylor et al., 2001) and hypothermia (Biswas et al., 2002; Hutchison et al., 2008). Early decompressive craniectomy in children reduced intracranial hypertension and showed a trend toward improved long-term outcome, but the study was limited by small numbers (n = 27) (Taylor et al., 2001). Importantly, the recently completed multi-center randomized control trial on hypothermia for severe pediatric TBI (32.5 °C for 24 h) did not significantly improve neurologic outcome (Hutchison et al., 2008) (see discussion below). Discovery of successful treatment strategies will require continued study of the cellular and subcellular pathophysiological changes that occur in the brain subsequent to hypoxia–ischemia or trauma. The use of animal models of perinatal hypoxia–ischemia or head trauma that shows injury patterns similar to those seen in human newborns will facilitate this process (Vannucci et al. 1999, Prins and Hovda, 2003).

It is now well known that injury to the brain initiated during hypoxia or ischemia continues, and is magnified during the post-resuscitative period. This “reperfusion illness” is multifactorial in etiology and is caused in part by such processes as excitotoxicity, oxidative stress, metabolic failure, inflammation, apoptosis, and others. While many drugs provide neuroprotection in animal models of global cerebral ischemia, prevention of post-ischemic human brain injury through pharmacologic intervention has not yet been achieved (Harukuni and Bhardwaj, 2006). For example, no increased survival or neuroprotection among cardiac arrest (CA) survivors was demonstrated in adults through the use of glucocorticoids (Jastremski et al., 1989) (Jastremski et al., 1989), thiopental (Brain Resuscitation Clinical Trial I Study Group, 1986), or calcium channel antagonists (Brain Resuscitation Clinical Trial II Study Group, 1991). Similarly, several large clinical trials failed to demonstrate a role for high dose epinephrine in human victims of CA. (Goetting and Paradis 1991; Barton and Callaham 1991). Great excitement was generated in 2002, however, when two large clinical trials demonstrated significant improvement in neurologic outcome and reduced mortality in adults treated with moderate hypothermia following resuscitation from CA. (Bernard et al., 2002; Hypothermia Cardiac Arrest Study Group, 2002). This breakthrough helped renew the conviction that lessons learned in the laboratory can be successfully translated into improved long-term resuscitation outcomes following human CA. Pediatric studies of hypothermia to date have focused primarily on neonatal asphyxia and TBI. In neonates, both whole-body cooling (esophageal T=33.5 °C for 72 h) or selective head cooling (rectal T=34–35 °C, head cooling unit with water T=8–12 °C for 72 h) have shown promise in reducing the risk of death or severe disability (Shankaran et al., 2005; Gluckman et al., 2005). Preliminary evidence from clinical trials testing head cooling for neuroprotection after neonatal hypoxic ischemic encephalopathy suggests efficacy (Wyatt et al., 2007; Gunn et al., 2008), and a large multi-center clinical trial testing whole-body cooling on survival and neurologic outcome is in progress (Azzopardi et al., 2008). Initial studies of hypothermia in pediatric TBI (brain T=32–33 °C) have demonstrated that the treatment is relatively safe and improves control of ICP (Biswas et al., 2002; Adelson et al., 2005). In contrast, Hutchison et al. (2008) did not show efficacy of hypothermia (brain T=32.5 °C for 24 h) initiated within 8 h and continued for 24 h after pediatric TBI. In contrast to adult stroke victims, who often do not receive medical attention for many hours after the stroke onset, the vast majority of TBI victims reach a hospital within an hour or two after the injury and are therefore available for treatment. Together with successfully

resuscitated cardiac arrest survivors, TBI victims can receive potentially neuroprotective interventions, e.g., hypothermia, relatively early after the injury, thereby increasing the likelihood of success.

In order for any neuroprotective strategy (including hypothermia) to make an impact, however, the technique must be safe, easy to implement, and widely accepted. Despite successful hypothermia clinical trials, the emergency medicine/critical care community has not yet embraced the use of hypothermia in the clinical arena. Even if widely accepted, one recent editorial suggests that hypothermia should be only one part of a strategy designed to attack different components of the pathophysiologic cascade (Broccard, 2006). We have established that by avoiding unnecessary hyperoxia during the first hour following resuscitation after global cerebral ischemia (cardiac arrest) in adult animals, we significantly reduce oxidative stress, metabolic dysfunction, delayed neuronal death, and both short-term and long-term neurobehavioral impairment (Liu et al., 1998; Richards et al., 2006; Balan et al., 2006; Vereczki et al., 2006; Richards et al., 2007). Comparisons of the effects of hyperoxic and normoxic resuscitation in neonatal rat models of hypoxic ischemia are equivocal. One study found no overall difference in long-term histologic outcome after reperfusion under 100%, 40%, or 21% inspired O_2; however, there was a trend toward better outcome specifically for male rats using 40% O_2 (Grafe et al., 2008). Another study demonstrated that 100% O_2 after neonatal HI reduces cerebral blood flow (CBF) and, importantly, increases brain superoxide production (Fabian et al., 2008). Importantly, clinical studies comparing room air (21% O_2) to hyperoxic (100% O_2) resuscitation in depressed newborns have generally shown equivalent long-term neurologic outcomes between groups, and a recent meta-analysis found a lower mortality rate in room air resuscitated infants (Rabi et al., 2007). In pediatric cardiac arrest (CA), preclinical studies of asphyxial CA in immature rats found increased markers of tissue oxidative injury in rats resuscitated with hyperoxic conditions (Walson et al., 2008). To our knowledge, the effect of hyperoxic versus normoxic resuscitation on outcome after pediatric TBI has not been studied. In adult TBI, hyperoxic resuscitation has produced mixed results (Diringer, 2008). Some studies have shown that hyperoxia improves cerebral metabolism (Tolias et al., 2004; Tisdall et al., 2008), while others suggest it can produce oxidative injury to cellular constituents (Ahn et al., 2008). The role of oxygen following injury to the developing brain is clearly a controversial but clinically very important topic that requires more research using both animal models and clinical trials. While better management of oxygenation to avoid both hypoxia and unnecessary hyperoxia will likely improve clinical outcome, it is unlikely that this intervention alone will provide optimal long-term result. Additional approaches include those that target mitochondrial pathways of necrotic and apoptotic cell death.

Oxidative stress, mitochondrial dysfunction, and metabolic failure

A large body of evidence indicates that mitochondrial dysfunction plays a critical role in the pathophysiology of ischemic and traumatic brain injury (Blomgren and Hagberg, 2006; Chang et al.,1992; Fiskum et al., 1999; Kuroda et al., 1996; Rizzuto et al., 1992; Starkov et al., 2004). Consequences of mitochondrial dysfunction are numerous and include oxidative stress, loss of cellular Ca^{2+} homeostasis, promotion of apoptosis, and metabolic failure.

There are many possible causes of mitochondrial metabolic impairment and most involve oxidative modifications to proteins, lipids, or DNA. Identification of the sites at which oxidative stress impairs respiration can guide the development of counteractive interventions with neuroprotective potential. Complex I of the electron transport chain (ETC), which catalyzes the oxidation of NADH and the reduction of ubiquinone, is particularly sensitive to inhibition by both oxidative stress and ischemia/reperfusion and is generally considered to

be the rate-limiting component of the ETC (Hillered and Ernster, 1983; Rosenthal and Fiskum, 1990; Rosenthal et al., 1987; Sims, 1991). Another cause of impaired ETC activity is the release of cytochrome *c* through the outer mitochondrial membrane into the cytosol, an event that is also often followed by caspase-dependent apoptosis (Polster et al. 2001). Oxidative stress promotes cytochrome *c* release by several mechanisms, including those promoting translocation of Bax and Bak to the mitochondrial outer membrane (Fig. 1) (Castino et al. 2007; Perier et al. 2005). These proteins form megapores within the outer membrane when prompted to oligomerize by the binding of BH3 domain only proteins, e.g., tBid, to these proteins or to anti-apoptotic proteins, e.g., Bcl2 or $Bclx_L$, that normally block megapore formation by heterodimerizing with Bax and Bak. Although not previously measured, the loss of cytochrome *c* through such megapores could be responsible for the secondary brain mitochondrial respiratory impairment observed 24 h after neonatal H/I, when caspase 3 activation is robust (Puka-Sundvall et al., 2000d).

In addition to impaired ETC activities, oxidative phosphorylation can also be obstructed by inhibition of other mitochondrial enzymes and membrane transporters (Fig. 1). Thus, oxidative inactivation of mitochondrial matrix enzymes, e.g., pyruvate and α-ketoglutarate dehydrogenases and aconitase, are implicated in metabolic failure (Bogaert et al., 1994;Richards et al., 2006;Vereczki et al., 2006). Evidence also suggests that mitochondrial oxidative stress impairs the adenine nucleotide translocase, necessary for influx of ADP and efflux of ATP (Vesce et al., 2005). While their is little if any information about the effects of acute brain injury on other mitochondrial membrane transporters, one recent study suggests that even transient elevation of intramitochondrial Ca^{2+} can stimulate glycolytic lactate production through impairment of net efflux of mitochondrial α-ketoglutarate and subsequent inhibition of the malate/aspartate shuttle system (Contreras and Satrustegui, 2009). Stimulation of α-ketoglutarate dehydrogenase by Ca^{2+} can reduce the level of α-ketoglutarate within both brain and heart mitochondria to a level that limits its efflux through the oxoglutarate transporter. The lack of cytosolic α-ketoglutarate then limits the aspartate transaminase reaction that is necessary for the shuttling of reducing power from glycolysis-generated NADH to the mitochondria. Consequently, lactate dehydrogenase utilizes this NADH to form lactate from pyruvate, thus shunting pyruvate away from mitochondrial aerobic metabolism. While this scenario is likely to be very transient under physiological conditions, it may persist for some time after cerebral ischemia, head trauma, and other events associated with excitotoxicity, where mitochondria are exposed to and sequester elevated cytosolic Ca^{2+} for prolonged periods.

Much interest is also currently focused on the availability of the metabolic cofactor NAD^+, necessary for the numerous dehydrogenases present within the mitochondrial matrix (Ying, 2008). Cellular NAD^+ can be rapidly catabolized by nuclear and possibly mitochondrial poly-ADP-ribose polymerase in response to activation of this enzyme by oxidative DNA modifications and ERK kinase-mediated enzyme phosphorylation (Du et al., 2003; Eliasson et al., 1997; Kauppinen and Swanson, 2007). NAD^+ in its oxidized or reduced form (NADH) can be lost from the mitochondrial matrix following opening of the inner membrane permeability transition pore (PTP), which results in transmembrane equilibration of small ions and molecules of up to approximately 1500 Da (Fig. 1) (Crompton et al., 2002; Halestrap et al., 2002). The PTP is activated by abnormally high concentrations of Ca^{2+} and by oxidative stress (Bernardi et al., 1999). Little is known regarding the effects of brain development on mitochondrial permeability transition. One study provided evidence that the PTP is relatively sensitive to Ca^{2+}-induced opening and cyclosporin A-mediated inhibition in mitochondria from immature rats (Eliseev et al., 2007). We found that the relative sensitivity of PTP opening in immature rat brain mitochondria is dependent on the environment, e.g., the presence of ATP and the pH. In fact, the PTP is comparatively very

active in immature brain mitochondria at low pH and ATP, conditions that are present during and immediately following hypoxic ischemia (Robertson et al., 2004).

Contribution of PTP opening to ischemic and traumatic brain injury is supported by the neuroprotection observed with PTP inhibitors, e.g., cyclosporins (Alessandri et al., 2002; Hansson et al., 2004; Sullivan et al., 2002; Uchino et al., 2002), that bind to cyclophilin D, the one well-established protein associated with pore opening. Cyclophilin D knock-out mice are resistant to ischemic brain injury (Schinzel et al., 2005), and to spinal cord axonal damage associated with experimental autoimmune encephalomyelitis, an animal model characterized by extensive inflammation (Forte et al., 2007). These findings taken together with observations that post-ischemic inflammation is accompanied by considerable ROS formation via iNOS and NADPH oxidase (Wang et al., 2007), strongly suggest that the PTP participates in inflammatory neurodegeneration after global cerebral ischemia. Support for involvement of the PTP in neonatal hypoxic ischemia comes from the observations of mitochondrial Ca^{2+} accumulation, morphologic signs of mitochondrial swelling (Puka-Sundvall et al., 2000a), and an increase in mitochondrial entrapment of ^{14}C-labeled 2-deoxyglucose shortly after hypoxic ischemia (Puka-Sundvall et al., 2001). Although significant evidence suggests involvement of the PTP in adult animal models of TBI (Sullivan et al., 2005), this has not been directly investigated in pediatric models. Our finding of early mitochondrial release of cytochrome *c* after TBI in immature rats could be secondary to mitochondrial swelling via PTP opening, but this could also occur from other mechanisms. Future studies examining mitochondrial swelling and potential neuroprotection with CsA in immature animal models of TBI would be informative.

Mitochondria and the developing brain

Developmental differences in brain mitochondria of normal rats have been well documented (Table 1). In general, through the first 3–4 weeks of life in the rat, there is a 3-fold increase in mitochondrial protein per cell, with corresponding increases in respiratory enzyme activity and increasing oxygen consumption (Tyler and Harreveld, 1942;Murthy and Rappoport, 1963;Milstein et al., 1968). There are also potential differences in mitochondrial membrane composition (Sitkiewicz et al., 1982), and relative ratios of synaptosomal to non-synaptosomal brain mitochondria (Dienel et al., 1977). A series of studies by the laboratory of Holtzman and others have detailed developmental differences in brain mitochondrial activity in immature (<4 weeks old) versus mature rats (adult, >2 months). ADP/O ratios with NAD-lined substrates were lower in rats <2 weeks of age, increased between the 3rd and 4th week, and reached adult levels by the 4th week of life (Holtzman and Moore, 1973;Holtzman and Moore, 1975). There are also differences in State III and State IV respiratory rates at different ages, reaching adult levels at >5 weeks of age (Holtzman and Moore,1975). Additional studies of mitochondrial function demonstrate increased sensitivity of cortical slices from immature animals to altered pH, with a lower pH (6.7) inhibiting immature tissue respiration but not affecting adult tissue respiration (Holtzman et al., 1987).

Developmental aspects of mitochondrial susceptibility to brain insults have been shown using models of hypoxia–ischemia in immature rats (7 day old). A comparative *in vitro* study of isolated nerve terminals after hypoxic ischemia demonstrated differences in mitochondrial complex activity in the neonatal versus adult tissues (Keelan et al., 1999). In vivo studies of hypoxic ischemia in immature rats (7 day old) have shown significant abnormalities in mitochondrial respiration patterns (Yager et al., 1991a; Yager et al., 1991b; Yager et al., 1996; Gilland et al., 1998). The apoptotic pathway also has a role in neurodegeneration following hypoxic ischemia in the developing rat brain. In 21 day old rats, evidence of apoptosis was seen after both mild and severe unilateral hypoxic ischemic injuries (Beilharz et al., 1995). Younger rats (7 day old) had evidence of apoptotic changes throughout the brain, with prolonged increases in caspase-3 activity (Nakajima et al., 2000;

Puka-Sundvall et al., 2000b; Puka-Sundvall et al., 2000c; Puka-Sundvall et al., 2000d). This unique pattern of activated caspase-3 was also seen after hypoxic ischemia in rats of various ages, with the youngest rats (7 day old) having the highest levels of active caspase-3, that declined steadily during maturation (Hu et al., 2000). Perinatal models have shown alteration in mitochondrial mRNA and DNA levels associated with uteroplacental insufficiency in rats (Lane et al., 2000), and histologic evidence of apoptotic cell morphology on brain autopsy tissue from infants suffering intrauterine cerebral injury (Edwards et al., 1997). This apparent propensity toward apoptosis could be explained at least in part by a greater general expression of pro-apoptotic compared to anti-apoptotic proteins at the level of brain mitochondria and the greater sensitivity to release of mitochondrial apoptotic mediators, e.g., cytochrome *c*, in response to apoptotic triggers, including BH3 only proteins and elevated intracellular Ca^{2+}, as demonstrated by our lab and the Hagberg group (Polster et al., 2003c; Zhu et al., 2005; Soane et al., 2008).

Studies in pediatric TBI models have also demonstrated mitochondrial dysfunction and altered cerebral metabolism (Robertson et al., 2006). Investigators have found early hyperglycolysis followed by a 1–3 day period of metabolic depression in fluid percussion TBI in immature rats (17 day old) (Thomas et al., 2000). This group has also shown that the ability of alternative substrates, such as ketone bodies, to rescue brain metabolism may be developmentally regulated (Prins et al., 2005). Using the controlled cortical impact model, we studied isolated brain mitochondria early (1–4 h) after TBI in immature rats (17 day old) (Robertson et al., 2007). Mitochondria had significant alterations in respiratory capacity, with increases in State 4 respiration (1 h) and decreases in State 3 respiration (4 h). Mitochondria also had reduced cytochrome *c* content and decreased activity of the important metabolic enzyme, pyruvate dehydrogenase. Other investigators used gel-based proteomics to show significant reductions in pyruvate dehydrogenase subunit expression that extended to 2 weeks after TBI in the developing brain (Kochanek et al., 2006). Loss of pyruvate dehydrogenase activity in pediatric TBI may be especially important, as it is the critical enzymatic link between glycolysis and the TCA cycle. Evidence for metabolic derangements in clinical studies of pediatric TBI comes primarily from imaging investigations (Ashwal et al., 2006). Using magnetic resonance spectroscopy (MRS), children have marked elevations in brain lactate and reductions in N-acetyl aspartate (NAA), a marker of neuronal and/or mitochondrial integrigy, and these metabolic alterations correlate with long-term neurologic outcome (Ashwal et al., 2000). However, few of these imaging studies were obtained early after pediatric TBI (<48 h), so information is not available about very early metabolic alterations. Recently, we used proton (^{1}H) spectroscopy after CCI in immature rats (16–17 day old) to evaluate the time course of metabolic alterations (Casey et al., 2008). We showed that metabolic derangements begin early (<4 h) and are sustained for at least 7 days after TBI in the developing brain. Another study used ^{13}C-NMR spectroscopy to evaluate glucose metabolism 5–6 h after CCI in immature rats (21–22 day old) (Scafidi et al., 2009). This study showed that neuronal oxidative metabolism of glucose is delayed in both the ipsilateral and contralateral hemispheres, compared to uninjured sham controls. Specifically, findings suggested impairment in the TCA cycle, at the level of pyruvate dehydrogenase and possibly α-ketoglutarate dehydrogenase, as well as impairment of the malate–aspartate shuttle. Future studies using clinically relevant tools, such as MR spectroscopy, could allow development and testing of mitochondrially targeted neuroprotective strategies for the developing brain after injury. This study demonstrates the value of utilizing clinically relevant tools, such as MRS, to investigate metabolic alterations following acute pediatric brain injury.

Mitochondrial pathways for neuroprotection in the immature brain

Based on increasing knowledge of mitochondrial pathways toward cell death in the immature brain after hypoxia, ischemia, and trauma, several mitochondria-based neuroprotective strategies have been developed (Fig. 2). One approach is pharmacologic inhibition of mitochondrial Ca^{2+} overload, which together with oxidative stress, induces opening of the inner membrane permeability transition pore. While cyclosporin-based drugs appear effective at inhibiting PTP opening in mature brain mitochondria, the relatively low expression of cyclophilin D in immature brain mitochondria may be responsible for the ineffectiveness of cyclosporin A at improving outcome after neonatal brain injury (Puka-Sundvall et al., 2001;Eliseev et al., 2007). Other attempts at PTP inhibition should be made, however, including the use of agents, e.g., 2-aminoethoxydiphenyl borate, that are capable of inhibiting cyclosporin A-insensitive PTP (Chinopoulos et al., 2003). Another approach toward PTP inhibition includes the use of agonists for mitochondrial ATP-sensitive potassium channels, that indirectly inhibit PTP opening in adult brain mitochondria (Wu et al., 2006). These agents, e.g., diazoxide, also inhibit mitochondrial ROS production (Fornazari et al., 2008) and have exhibited neuroprotection in a neonatal hypoxic ischemia model (Rajapakse et al., 2002).

One consequence of PTP opening is loss of mitochondrial pyridine nucleotides, that are required in numerous reactions involved in normal energy metabolism and in ROS detoxification. Therapies directed at replenishing cellular and mitochondrial cofactors, e.g., NAD (H), have exhibited neuroprotection both *in vitro* and with adult animal models of brain injury and should be tested with pediatric brain injury models. This strategy includes administration of NAD^+ or nicotinamide (Ayoub et al., 1999; Sadanaga-Akiyoshi et al., 2003; Ying et al., 2007). One report indicated that when nicotinamide was administered to neonatal rats as long as 24 h after perinatal asphyxia, dopamine levels present within the striatum 3 months later were relatively preserved (Bustamante et al., 2007). Moreover, since a futile cycle of NAD^+ catabolism is generated by the activation of both PARP and PARG, inhibitors of these enzymes should preserve both cytosolic and mitochondrial energy metabolism. Poly-ADP ribosylation of proteins occurs both early (<1 h) and at delayed times (12 h) after stroke in neonates (Martin et al., 2005). The PARP inhibitor 3-aminobenzamide both inhibits this product formation and subsequent, delayed cell death (Joly et al., 2003). Although not yet reported for immature animals, TBI in adult rats results in mitochondrial protein poly-ADP-ribosylation that may contribute to inhibition of respiration and oxidative phosphorylation (Lai et al., 2008). Additional investigation of this form of metabolic impairment in the immature brain is clearly needed.

Another mitochondria-based approach to neuroprotection for acute brain injury is the administration of agents that in one way or another act as enhancers of aerobic energy metabolism. The administration of exogenous pyridine nucleotides and their precursors, as mentioned above, is one example. Other examples include agents that can directly serve as fuel for cerebral energy metabolism, e.g., ketone bodies, acetyl-L-carnitine, and pyruvate. Of these, β-hydroxybutyrate may be the most straight forward approach for the immature brain since the vascular monocarboxylic acid transporters that carry ketone bodies and the brain enzymes that metabolize them are relatively high during the suckling period (Vannucci and Simpson, 2003). Thus, the immature brain is genomically primed to utilize ketones as fuel, particularly if the activity of enzymes, e.g., pyruvate dehydrogenase, become inhibited, limiting aerobic glucose metabolism. β-hydroxybutyrate, a ketogenic diet, or fasting has been shown to be neuroprotective in various rat models of acute brain injury (Yamada et al., 2005; Puchowicz et al., 2008; Tai et al., 2008; Davis et al., 2008), with at least one report for young rats (Prins et al., 2005). While a ketogenic diet is used to treat intractable epilepsy in children, there is some concern that it can also impair normal brain development (Zhao et al., 2004). An alternative approach, based on the same principle of compensating for

impaired glucose metabolism, is administration of acetyl-L-carnitine or free carnitine. These natural agents normally involved in acetyl and acyl carbon metabolism are neuroprotective and promote the production of acetyl-CoA, the same product generated from glucose derived pyruvate via pyruvate dehydrogenase. Large doses of L-carnitine administered prior to, but not after neonatal hypoxic ischemia in rats result in neuroprotection (Wainwright et al., 2003). While carnitine appears to improve cerebral energy metabolism after hypoxic ischemia (Macri et al., 1995), it may exert mechanisms of action independent of directly promoting mitochondrial energy metabolism (Winter et al., 1995; Akisu et al., 1998; Tastekin et al., 2005; Wainwright et al., 2006). Acetyl-L-carnitine has not been tested for neuroprotection with immature animals; however, it is highly effective against brain injury in adult animals (Rosenthal et al., 1992; Shuaib et al., 1995; Lolic et al., 1997; Zanelli et al., 2005).

Pyruvate is a third metabolic substrate that has exhibited neuroprotection in different acute brain injury models; however, it has not yet been reported using immature animals. The brain mitochondrial redox state is relatively oxidized soon after neonatal hypoxic ischemia (Yager et al., 1991a), which could be explained by either a limited concentration of pyruvate generated by glycolysis or by limited influx of carbon into the tricarboxylic acid cycle through impaired pyruvate dehydrogenase. If the first scenario is true, exogenous pyruvate should promote aerobic energy metabolism, possibly resulting in neuroprotection. The same result might even be reached by adding lactate, as endogenous lactate is a preferred substrate for immature brain metabolism, at least during anoxia and reoxygenation (Vannucci and Duffy, 1976). Stimulation of metabolism through pyruvate dehydrogenase can also occur by addition of dichloroacetate, which inhibits pyruvate dehydrogenase kinase, thereby de-inhibiting pyruvate dehydrogenase (Stacpoole, 1989). Dichloroacetate has exhibited protection for both ischemia and trauma in the adult brain (Chang et al., 1992; Biros and Nordness, 1996). In addition, one study reported that early post-treatment with dichloroacetate improved post-ischemic clearance of cerebral acidosis and elevated phosphocreatine in newborn pigs better than it did for 1 month old swine (Corbett et al., 1998); however, effects on neurologic or histologic outcome were not reported.

A host of other interventions could prove therapeutic for acute injury to the immature brain that indirectly compensate for mitochondrial dysfunction or that protect against mitochondrial damage from conditions, e.g., elevated intracellular Ca^{2+} and high levels of ROS, that exist during and after ischemia and trauma. For instance, pretreatment of rats with creatine was reported to improve the cerebral phosphocreatine/phosphate ratio and reduce brain edema after neonatal hypoxic ischemia (Adcock et al., 2002). Elevating total brain creatine may provide neuroprotection by elevating the high energy phosphate buffering capacity through accelerating the mitochondrial creatine kinase reaction. N-acetylcysteine, an antioxidant, was also shown to reduce cortical H_2O_2 and brain lactate and improve cerebral perfusion in piglets after hypoxia, suggesting an improvement in cerebral aerobic energy metabolism (Lee et al., 2008). In addition to avoiding brain tissue hyperoxia and adding antioxidants to reduce oxidative stress, a new approach has been reported that is based on activation of endogenous antioxidant and anti-inflammatory gene expression. Low levels of ROS and specific chemicals, e.g. sulforaphane, an isothiocyanate found in cruciferous vegetables, promote the translocation of Nrf2, a transcriptional activating factor, from the cytosol to the nucleus, where it binds to antioxidant response elements (ARE), thereby stimulating the expression of scores of genes (Jaiswal, 2004; Dinkova-Kostova and Talalay,2008). Some of the elevated gene products appear in mitochondria, including Mn-superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase (Zhu et al., 2008). Considering that the immature brain has relatively low expression levels for antioxidant genes (Cruz et al., 1998; Bayir et al., 2006), an antioxidant genomic post-

conditioning strategy could be particularly effective toward neuroprotection in infants and children.

Since neuronal apoptosis appears even more prominent following acute injury to the immature brain than it does in adults, interventions that target the mitochondrial (intrinsic) pathway of apoptosis are very promising. For instance, the release of apoptosis-inducing factor (AIF) and its role in DNA damage is important in neonatal hypoxic ischemic brain injury (Zhu et al., 2003). The Ca^{2+} activated protease calpain plays a critical role in mitochondrial AIF release (Polster et al., 2005; Cao et al., 2007), and one report indicates that calpain inhibitors are neuroprotective in neonatal rat cerebral hypoxic ischemia (Chen et al., 2008). Other anti-apoptotic approaches that could be applied to the immature brain include the use of agents, e.g., estrogen, that stimulate the expression of Bcl-2 and other apoptotic proteins (Zhang et al., 2004), or agents that impair the ability of Bax or Bad to create pores in the mitochondrial inner membrane resulting in the release of cytochrome *c* and other pro-apoptotic proteins into the cytosol (Polster et al., 2003a; Guan et al., 2006; Iriyama et al., 2008).

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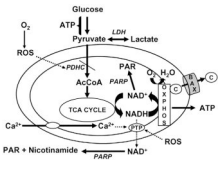

Fig. 1.

Oxidative stress and mitochondrial metabolic dysfunction. Mechanisms of mitochondrial and metabolic dysfunction following acute brain injury include direct inhibition of the pyruvate dehydrogenase complex (PDHC) by reactive O2 species (ROS), loss of mitochondrial NAD(H) through the permeability transition pore (PTP), consumption of NAD^+ by poly-ADP ribose polymerase 1 (PARP), and release of cytochrome *c* (c) through Bax or Bak megapores present in the outer membrane. Respiratory inhibition stimulates lactate dehydrogenase (LDH), resulting in lactic acidosis, which further inhibits oxidative phosphorylation and promotes oxidative stress.

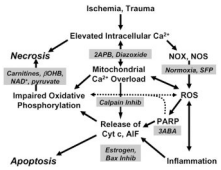

Fig. 2.

Mitochondria-targeted neuroprotective interventions for acute injury to the immature brain. One common early trigger of neuronal injury following either cerebral ischemia or head trauma is an abnormal elevation of intracellular Ca^{2+}, which if unabated, will directly lead to cellular necrosis through activation of degradative enzymes, e.g., phospholipases and proteases. Elevated Ca^{2+} can also induce delayed cell death by inducing mitochondrial metabolic dysfunction, e.g., through activation of the permeability transition pore (PTP), and by stimulation of activating enzymes that produce reactive O2 species (ROS), e.g., NADPH oxidase (NOX) and nitric oxide synthase (NOS). The ROS contribute to mitochondrial metabolic dysfunction, stimulate cellular inflammatory responses, e.g., microglial activation, and modify many different molecules throughout the cell including lipids, proteins, and DNA. DNA damage activates poly-ADP-ribose polymerase, which consumes NAD^+, thus impairing energy metabolism. Pro-apoptotic molecular pathways, including those activated by inflammatory cytokines, lead to the release of mitochondrial apoptotic proteins, e.g., cytochrome *c* (Cyt c) and apoptosis-inducing factor (AIF). Calpain cleavage of molecules, e.g., AIF and Bid, also promotes the mitochondrial intrinsic pathway of apoptosis. Potentially neuroprotective interventions that target these pathways include the PTP inhibitor 2-aminoethoxydiphenyl borate (2-APB), the ATP-sensitive K^+ channel opener diazoxide, normoxic resuscitation, the Nrf2 activator sulforaphane (SFP), calpain inhibitors, the PARP inhibitor 3-amino benzoamide (3ABA), estrogen, which induces Bcl2 expression, Bax inhibitors, and several agents that stimulate aerobic energy metabolism, including free carnitine and acetyl-L-carnitine, β-hydroxybutyrate (βOHB), NAD^+ or its precursor nicotinamide, and pyruvate.

Table 1

Unique age-dependent features of cellular response to acute brain injury from immature animal studies.

Mechanism	Unique response of developing brain	References
Energy metabolism	• Improved potential for use of alternative fuels (ketones)	(Thomas et al., 2000; Prins et al., 2005)
	• Shortened length of metabolic depression after TBI	
Excitotoxicity	• Regional expression of NMDA, AMPA and kainate receptors is age-dependent	(McDonald et al., 1988; McDonald et al., 1992; Monyer et al., 1994; Ben Ari et al., 1997; Pohl et al., 1999; Anderson et al., 1999; Sharma et al., 2000; Robertson et al., 2004)
	• Heightened sensitivity of young brain to excitotoxicity after HI insult	
	• Potential for NMDA inhibition to promote delayed (apoptotic) cell death	
	• Potential for GABA receptor excitatory transmission at young ages	
	• Age-dependent differences in mitochondrial calcium uptake capacity	
Mitochondrial permeability transition	• Lack of effect of cyclosporin A (CsA) on mitochondrial Ca^{2+} uptake or HI injury	(Puka-Sundvall et al., 2001; Robertson et al., 2004; Nakai et al., 2004; Setkowicz et al., 2004)
	• CsA protection with intrauterine ischemia	
	• CsA worsened mortality (PND 6) and seizure length (PND 30) in immature rats	
Oxidative stress	• Brain antioxidant enzymes have an age-dependent profile of activity and expression	(Mavelli et al., 1982; Ditelberg et al., 1996; Fullerton et al., 1998; Khan and Black, 2003; Fan et al., 2003; Robertson et al., 2006; Bayir et al., 2006)
	• Perinatal surges in antioxidant enzymes but lower levels of enzymes during other developmental times (versus adult)	
	• Worse outcome after HI insult in Cu,Zn-SOD overexpressing immature mice (PND 7) due to accumulation of H_2O_2	
	• Limited upregulation of GPX after TBI in immature mouse (PND 21) compared to adult mouse	
Cell death	• Immature brain has normal, basal programmed cell death mechanisms active in early development	(Raff et al., 1993; Alonso et al., 1997; Shimohama et al., 1998; Bittigau et al., 1999; Krajewska et al., 2002; Johnston et al., 2002; Polster et al., 2003b; Vannucci and Hagberg, 2004; Zhu et al., 2005; Northington et al., 2005; Soane et al., 2008)
	• Age-dependent expression of bcl-2 family proteins in tissue and mitochondria	
	• Susceptibility of immature brain to apoptotic cell death after hypoxic–ischemic insult	
	• Vulnerability of young brain to mitochondrial cytochrome *c* release upon exposure to pro-apoptotic stimuli	
	• Trauma-induced apoptosis is greater in young rats	

TBI – traumatic brain injury; HI – hypoxic–ischemic; PND – postnatal day; SOD – superoxide dismutase; GPX – glutathione peroxidase.