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Mitochondrial Dysfunction Contributes to Cell Death Following Traumatic Brain Injury in Adult and Immature Animals

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Secondary injury following traumatic brain injury (TBI) is characterized by a variety of pathophysiologic cascades. Many of these cascades can have significant detrimental effects on cerebral mitochondria. These include exposure of neurons to excitotoxic levels of excitatory neurotransmitters with intracellular calcium influx, generation of reactive oxygen species, and production of peptides that participate in apoptotic cell death. Both experimental and clinical TBI studies have documented mitochondrial dysfunction, and animal studies suggest this dysfunction begins early and may persist for days following injury. Furthermore, interventions targeting mitochondrial mechanisms have shown neuroprotection after TBI. Continued evaluation and understanding of mitochondrial mechanisms contributing to neuronal cell death and survival after TBI is indicated. In addition, important underlying factors, such as brain maturation, that influence mitochondrial function should be studied. The ability to identify, target, and manipulate mitochondrial dysfunction may lead to the development of novel therapies for the treatment of adult and pediatric TBI.

KEY WORDS: Brain mitochondria; development; pediatric; cytochrome *c*; bcl-2; membrane permeability transition; apoptosis.

Approximately 1.5 million people sustain traumatic brain injury (TBI) in the United States each year (CDC, 1999). Of these, over 50,000 patients die annually, accounting for greater than one-third of all injury-related deaths (Sosin *et al*., 1995). Among survivors, almost one-fourth million people sustain a degree of injury that warrants hospitalization. In 1995, it was estimated that the total direct and indirect financial costs of TBI-related injuries were greater than $50 billion (Thurman, 2001). The long-term social and emotional burden may be even greater, with an estimated 5.3 million men, women, and children living with a TBI-related disability (CDC, 1999). Despite the significant public health impact of TBI, limited neuroprotective interventions exist for those suffering severe TBI.

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MITOCHONDRIA AND TBI – PRECLINICAL STUDIES

Growing evidence suggests an important role for mitochondria as subcellular targets for neuroprotection after TBI. Factors that both inhibit and promote neuronal apoptosis appear to work by influencing mitochondrial cytochrome *c* release, and pathways that promote necrotic cell death, such as excitotoxicity and oxidative stress, have profound influences on mitochondrial function (Fig. 1). Importantly, both preclinical and clinical studies have documented apoptotic and necrotic neural cell death occurring after TBI, and more recent studies have begun to define the significant influence of mitochondrial dysfunction on these cell death pathways.

Although the importance of mitochondria following brain injury has been suggested and studied for many years (reviewed in Fiskum *et al*., 1999), the majority of studies describing mitochondrial roles specifically in TBI have been conducted in the last decade. Early studies evaluated changes in mitochondrial respiration in the first few hours

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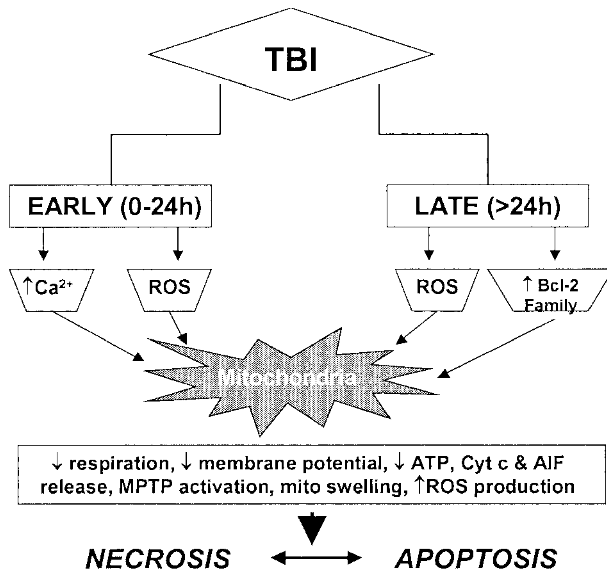

Fig. 1. Mechanisms of mitochondrial injury after TBI. TBI results in both early and delayed neural injury, both of which can have a profound influence on mitochondrial function and ultimately lead to necrotic and apoptotic cell death.

after injury and found slight reductions in the active, phosphorylating rate of mitochondrial respiration 4 h after fluid percussion TBI (Vink *et al.*, 1990). Subsequent studies using the controlled cortical impact model of TBI defined more dramatic alterations in mitochondrial respiration that began within 1 h of injury and persisted for at least 14 days (Xiong *et al.*, 1997a). In addition, mitochondria isolated from the hemisphere ipsilateral to injury demonstrated reduced ability to sequester Ca^{2+} (Xiong *et al.*, 1997a). These alterations in mitochondrial respiration and Ca^{2+} transport were reversible by postinjury treatment with the calcium channel blocker, SNX-111 (Verweij *et al.*, 1997) and the antioxidant, U-101033E (Xiong *et al.*, 1997b), both alone and in combination (Xiong *et al.*, 1998). These studies emphasize the potential for neuroprotection after TBI through pharmacologic intervention that directly targets mitochondrial dysfunction.

In addition to perturbations in mitochondrial respiration and Ca^{2+} homeostasis, TBI has recently been shown to have effects on mitochondrial membrane potential. Isolated mitochondria and synaptosomes from injured cortex show reduced membrane potential and evidence of mitochondrial inner membrane permeability changes (Sullivan *et al.*, 1999). Membrane potential was restored by the administration of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (MPTP). Similar types of mitochondrial abnormalities involving alterations in membrane potential with resultant mitochondrial swelling have also been seen in models of TBI examining axonal injury (Pettus and Povlishock, 1996). This series of investigations has described a key role for loss of mitochondrial integrity in the secondary axotomy that occurs following TBI (Buki *et al.*, 1999; Okondkwo *et al.*, 1999; Okonkwo and Povlishock, 1999).

Another important pathway with great significance in TBI is mitochondrial cytochrome *c* release and resultant neuronal apoptosis (reviewed in Raghupathi *et al.*, 2000). Experimental studies of TBI have demonstrated mitochondrial cytochrome *c* release in many models of TBI, including cold injury-induced brain trauma (Morita-Fujimura *et al.*, 1999), traumatic axonal injury (Buki *et al.*, 2000), and controlled cortical impact (Lewen *et al.*, 2001; Sullivan *et al.*, 2002). Downstream events, such as caspase activation, have also been well documented in animal models of TBI (Clark *et al.*, 2000; Keane *et al.*, 2001; Knoblach *et al.*, 2002; Sullivan *et al.*, 2002; Yakovlev *et al.*, 1997).

MITOCHONDRIA AND TBI – CLINICAL STUDIES

Although the number of studies directly evaluating mitochondrial function after TBI in humans is limited, they have generally supported findings seen in animal models of TBI. Brain mitochondria isolated from human victims of TBI have shown impaired rates of respiration and ATP synthesis (Verweij *et al.*, 1997, 2000). One interesting study of human autopsy tissue compared brain mitochondrial DNA deletions in short-term survivors of cardiac arrest, long-term survivors of TBI and age-matched controls, evaluating the role of mitochondrial gene expression in brain injury (McDonald *et al.*, 1999). They discovered a significantly lower incidence of mitochondrial DNA deletions in long-term survivors of TBI and hypothesized that chronic free radical-induced mitochondrial DNA damage may ultimately influence the survival of head-injury victims.

A few clinical studies have demonstrated evidence for apoptotic cell death involving mitochondrial pathways after TBI in adults and children. For example, caspase activation was documented in human TBI tissue (Clark *et al.*, 1999), and bcl-2 protein was increased in brain tissue from adult patients and in cerebrospinal fluid (CSF) from pediatric patients after TBI (Clark *et al.*, 1999, 2000). Importantly, bcl-2 CSF concentration correlated with patient survival, suggesting a neuroprotective role for bcl-2 in pediatric TBI victims. Very recent clinical studies in pediatric TBI have discovered elevations in two mitochondrial proteins, heat shock protein 60 (Hsp60) and

cytochrome *c*, in the CSF of head-injured children compared to noninjured pediatric controls (Lai *et al.*, 2003; Strange *et al.*, 2003). In these studies Hsp60 correlated with injury severity and cytochrome *c* levels correlated with child abuse victims and female gender. The presence of these integral mitochondrial proteins in the CSF suggests the presence of mitochondrial damage in these patients, and the correlation with specific injury and demographic features may prove to be helpful in defining subpopulations likely to respond to specific neuroprotective interventions. Ongoing investigation into the degree and features of mitochondrial dysfunction after TBI in humans is warranted, and could lead to the development of novel, subcellular, neuroprotective strategies aimed at early and sustained mitochondrial impairment.

MITOCHONDRIA AND THE DEVELOPING BRAIN

Developmental differences in brain mitochondria of normal rats have been well documented. In general, through the first 3–4 weeks of life in the rat, there is a threefold increase in mitochondrial protein per cell, with corresponding increases in respiratory enzyme activity and increasing oxygen consumption (Milstein *et al.*, 1968; Murthy and Rappoport, 1963). There are also potential differences in mitochondrial membrane composition (Sitkiewicz *et al.*, 1982) and relative ratios of synaptosomal to nonsynaptosomal 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 (adult) rats. 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, 1975).

To understand the role of mitochondrial dysfunction after injury to the developing brain, one must understand developmental aspects of mitochondrial function in normal (uninjured) brain. Initial studies in our lab have compared brain mitochondria isolated from immature rats to those isolated from adult rats. We evaluated Ca^{2+} uptake capacity of isolated mitochondria in both physiologic conditions and in those conditions potentially present after TBI, such as acidosis (pH = 6.5) and ATP depletion. The Ca^{2+} uptake capacity represents resistance to Ca^{2+}-induced mitochondrial injury. In a physiologic environment (pH = 7.0 with ATP), mitochondria isolated from adult rats had a higher Ca^{2+} uptake capacity than mitochondria from immature rats (Fig. 2(A)). Acidosis (pH = 6.5) caused a significant reduction in maximal Ca^{2+} uptake in both immature and adult rat brain mitochondria. As brain tissue acidosis contributes to poor outcome following TBI in both animals and humans, these observations suggest a subcellular mechanism of action that could be particularly important in immature animals and children. In contrast to the differences seen with pH modification, immature rats appear to tolerate the absence of ATP much better than adult rats. At both a pH of 7.0 and 6.5, immature rat brain mitochondria had a greater Ca^{2+}-uptake capacity than adult rat brain mitochondria (Fig. 2(B)). These results suggest that brain mitochondria from immature animals are more resistant than those of mature animals to Ca^{2+}-induced injury under extreme conditions (no ATP) that can occur within some brain cells following TBI.

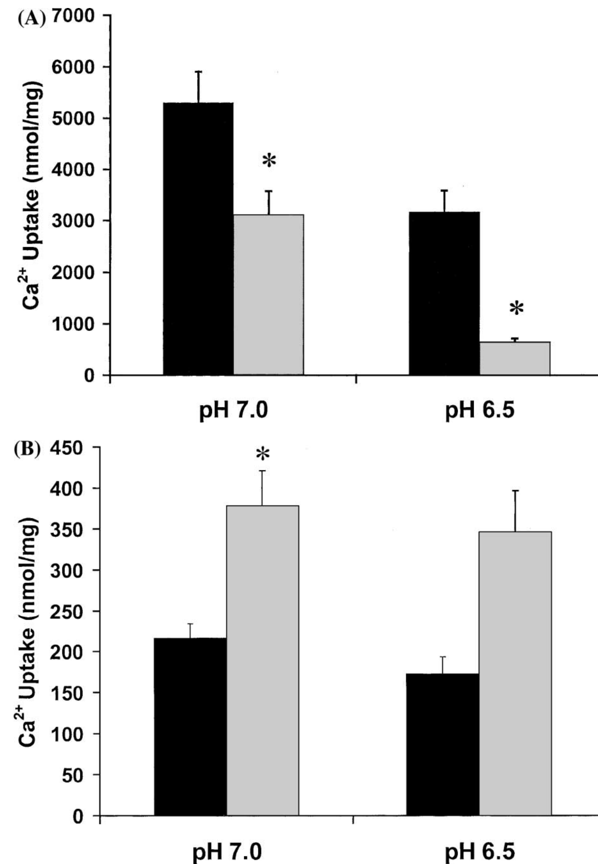

Fig. 2. Calcium uptake capacity of mature and immature brain mitochondria. In the presence of 3 mM ATP (Fig. 2(A)), maximal Ca^{2+} uptake capacity is greater in adult (black bars) versus immature (gray bars) rat brain mitochondria at both pH of 7.0 and 6.5. In the absence of ATP (Fig. 2(B)), maximal Ca^{2+} uptake capacity is greater in immature rat brain mitochondria.

Animal models of hypoxia-ischemia and traumatic brain injury have shown developmental differences in

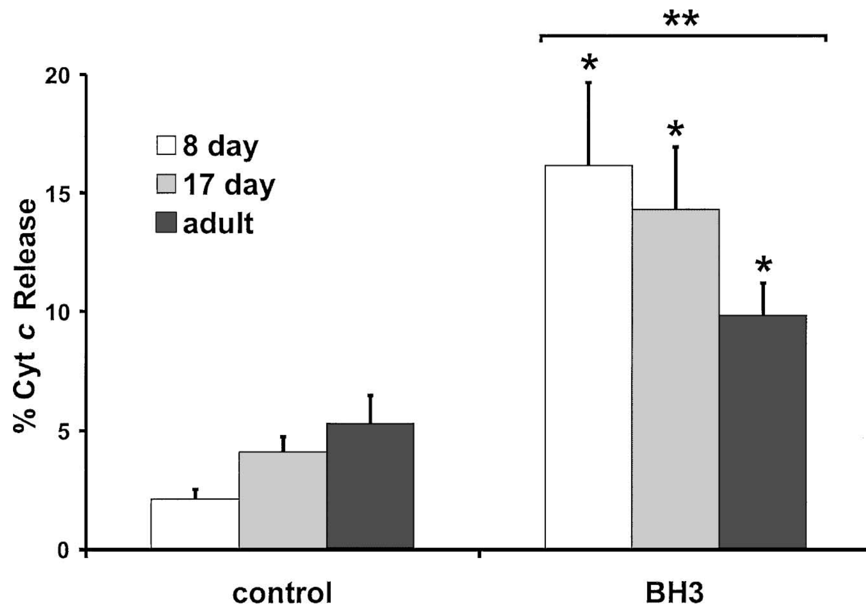

Fig. 3. Release of cytochrome *c* from brain mitochondria isolated from 8-day old, 17-day old, and adult rats in response to BH3 peptide. There is a significant difference between control and BH3 peptide treatment across groups and a significant affect of age on this difference (two-way ANOVA, $p < 0.05$).

apoptotic neuronal death. The exact mechanisms to explain these differences are unknown, but are likely multifaceted and related to mitochondrial response to injury. To begin to evaluate this, we studied the in vitro response of isolated brain mitochondria to proapoptotic peptides (BH3 cell death domain-containing peptide). Analysis by ELISA revealed greater cytochrome *c* release from mitochondria of immature rats exposed to BH3 peptide compared to adult rats, with the youngest rats (8do) showing the greatest release (Fig. 3). Previous studies in other laboratories have suggested that the protein Bax may be required for "BH3 only" proteins to promote cytochrome *c* release (Desagher *et al.*, 1999; Wei *et al.*, 2001), and brain levels may decline during maturation. Using immunoblot analysis, we found significant amounts of detectable Bax in 8do isolated rat forebrain mitochondria and moderate amounts of detectable Bax in 17do rats, but none detectable in adult rat brain mitochondria (Fig. 4).

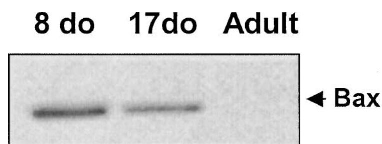

Fig. 4. Immunoblot for Bax in isolated rat brain mitochondria from 8-day- old, 17-day- old, and adult rats.

The presence of endogenous Bax in association with brain mitochondria may represent a potential explanation for the differences observed in sensitivity to BH3-induced cytochrome *c* release. The potentially very important conclusion we have reached from these results is that immature brain mitochondria are "primed" to release cytochrome *c* in response to BH3 domain proteins (e.g., tBid) due to the presence of endogenous mitochondrial Bax (Polster *et al.*, 2003). These characteristics of immature brain mitochondria could help explain the apparently greater contribution of apoptosis to brain cell death following TBI in immature animals.

MITOCHONDRIAL DYSFUNCTION AFTER EXPERIMENTAL TBI IN IMMATURE RATS

Given the important role that mitochondria likely play after TBI, and the unique aspects of mitochondrial development in the immature brain, it stands to reason that mitochondrial dysfunction following TBI in the immature brain would have profound effects. Very few studies have addressed this, although several centers have demonstrated unique patterns after TBI in immature rats, and explanations have discussed mechanisms with importance to mitochondrial function, such as alterations in CBF (Biagas *et al.*, 1996; Grundl *et al.*, 1994) and metabolism

(Thomas *et al.*, 2000). The most comprehensive investigation to date reveals two unique patterns of cell death after TBI in 7do rats, involving excitotoxic and apoptotic mechanisms (Pohl *et al.*, 1999). Neurons adjacent to the site of impact showed changes identical to those induced by glutamate, which peaked at 4 h and was not evident by 24 h. A delayed pattern of apoptotic cell death peaked at 24 h, and accounted for a much greater number of dying cells (2.2 million) than excitotoxicity (16,000). Interestingly, NMDA receptor antagonists protected against the primary excitotoxicity, but increased the severity of secondary apoptotic damage. Administration of SPBN, a free radical scavenger mitigated apoptotic damage. This study clearly demonstrates the importance of independent evaluation of pathologic pathways in the developing brain and supports the potential importance of mitochondrial dysfunction in this unique environment.

MITOCHONDRIA AND NEUROPROTECTION AFTER TBI

With the growing evidence for mitochondrial participation in traumatic neuronal injury, neuroprotective approaches must include strategies aimed to limit and reverse mitochondrial dysfunction. Interventions that have directly targeted mitochondria, such as calcium channel blockade (Verweij *et al.*, 1997, 2000; Xiong *et al.*, 1998) and antioxidant administration (Xiong *et al.*, 1997b, 1998, 1999) have documented reversibility of this mitochondrial dysfunction. Most importantly, animal studies have demonstrated that "mitoprotective" strategies have translated into neuroprotective strategies in models of TBI. Studies by Verweij *et al.* (2000) and Berman *et al.* (2000) initially examined time-window profiles and dose–response curves of the calcium channel blocker Ziconotide after TBI using mitochondrial outcome measures as endpoints. When the optimal mitochondrial dose was administered, rat showed improvements in motor and cognitive testing from 1 to 42 days after TBI. A number of studies involving the mitochondrial PTP inhibitor, cyclosporin A have shown improvement in both mitochondrial function, cerebral metabolism, and tissue damage after TBI (Alessandri *et al.*, 2002; Scheff and Sullivan *et al.*, 1999; Sullivan *et al.*, 1999). These studies suggest an important role for the mitochondrial PTP after TBI, especially with the lack of efficacy of the immunophilin ligand FK506 in one study (Scheff and Sullivan, 1999). However, the calcineurin interaction properties cannot be disregarded, as FK506 does protect against traumatic axonal injury (Singleton *et al.*, 2001). Finally, studies have begun to evaluate the role of uncoupling proteins after brain injury through proposed mechanisms of mild mitochondrial depolarization with resultant reduction in ROS generation. Specifically, overexpression of uncoupling protein-2 has been shown to reduce cortical damage and improve neurologic outcome after TBI in mice (Mattiasson *et al.*, 2003).

CONCLUSION

The importance of mitochondrial dysfunction following TBI in both preclinical and clinical studies is evident. Also evident, from clinical studies, is the extreme heterogeneity of injury following TBI, which can be influenced by age, gender, injury severity, injury mechanism, brain region, and number and degree of secondary insults. From the preclinical studies, cellular and even *subcellular* heterogeneity of alterations in metabolism and bioenergetics after TBI has been seen. Development of neuroprotective treatments must take into consideration this variability, and studies must continue to make attempts to understand the molecular mechanisms responsible for neuronal injury in different settings after TBI. This should include rigorous evaluation of important clinica, variables, such as patient age, as interventions that are protective in adult models may be ineffective or even detrimental in pediatric TBI (Pohl *et al.*, 1999). Continued study of mitochondrial participation in TBI may ultimately lead to translation into effective neuroprotective interventions targeted at specific patient profiles.

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