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Mitochondrial Uncoupling as a Therapeutic Target Following Neuronal Injury

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Mitochondrial dysfunction is a prominent feature of excitotoxic insult and mitochondria are known to play a pivotal role in neuronal cell survival and death following injury. Following neuronal injury there is a well-documented increase in cytosolic Ca^{2+}, reactive oxygen species (ROS) production and oxidative damage. In vitro studies have demonstrated these events are dependent on mitochondrial Ca^{2+} cycling and that a reduction in membrane potential is sufficient to reduce excitotoxic cell death. This concept has gained additional support from experiments demonstrating that the overexpression of endogenous mitochondrial uncoupling proteins (UCP), which decrease the mitochondrial membrane potential, decreases cell death following oxidative stress. Our group has demonstrated that upregulation of UCP activity can reduce excitotoxic-mediated ROS production and cell death whereas a reduction in UCP levels increases susceptibility to neuronal injury. These findings raise the possibility that mitochondrial uncoupling could be a potential novel treatment for acute CNS injuries.

KEY WORDS: Neuronal cell death; traumatic brain injury; excitotoxicity; spinal cord injury; reactive oxygen species.

Central Nervous System (CNS) trauma results in several pathophysiological events that contribute to neuronal damage and death, including glutamate-mediated excitotoxicity and the formation of reactive oxygen species (ROS) (Azbill *et al*., 1997; Braughler and Hall, 1992; Faden *et al*., 1989; Sullivan *et al*., 1998, 1999a). The ensuing loss of neuronal tissue is believed to evolve in a biphasic manner consisting of the primary mechanical insult and a progressive secondary necrosis (Cooper, 1985; Faden, 1993; Siesjo *et al*., 1995). Alterations in excitatory amino acids (EAA), increased oxidative stress (ROS), and the disruption of Ca^{2+} homeostasis are major factors contributing to the ensuing neuropathology (Braughler *et al*., 1985; Braughler and Hall, 1992; Choi *et al*., 1990; Faden *et al*., 1989). Compelling experimental data also demonstrates that mitochondria play a fundamental role in the death cascade, and mitochondria have been directly linked to EAA-mediated neurotoxicity (Brustovetsky *et al*., 2002; Jiang *et al*., 2001; Nicholls and Budd, 1998a; Stout *et al*., 1998; Sullivan *et al*., 2003). The present studies are based on the hypothesis that injury-induced glutamate release increases mitochondrial Ca^{2+} cycling/overload ultimately leading to mitochondrial dysfunction and that transient mitochondrial uncoupling can confer neuroprotection following traumatic brain injuries (TBI) and spinal cord injuries (SCI).

Extrinsic mitochondrial uncouplers are compounds that facilitate the movement of protons from the mitochondrial inner-membrane space into the mitochondrial matrix. Intrinsic uncoupling can be mediated via the activation of endogenous mitochondrial uncoupling proteins (UCP) which utilize free fatty acids to translocate protons. This short circuit "uncouples" the pumping of protons out of the matrix via the electron transport system (ETS) from the flow of protons through the ATP synthase and results in a coincidental reduction in the mitochondrial membrane potential ($\Delta\Psi$). While it is obvious that long-term, complete uncoupling of mitochondria would be detrimental, a transient or "mild uncoupling" could confer neuroprotection. Mild uncoupling during the acute phases

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of injured-induced excitotoxicity would be expected to reduce mitochondrial Ca^{2+} uptake (cycling) and ROS production, since both are $\Delta\Psi$-dependent.

Following TBI and SCI, there is a significant loss of mitochondrial homeostasis, resulting in increased mitochondrial ROS production and disruption of synaptic homeostasis (Azbill *et al*., 1997; Sullivan *et al*., 1998, 1999a,b; Xiong *et al*., 1997). This implicates an underlying pivotal role for mitochondria in the sequalae of injury-related neuropathology. Our laboratories and others have solidified this theory by demonstrating that therapeutic intervention with cyclosporin A following experimental TBI significantly reduces mitochondrial dysfunction (Sullivan *et al*., 1999a) and cortical damage (Scheff and Sullivan, 1999; Sullivan *et al*., 2000a,c), as well as cytoskeletal changes and axonal dysfunction (Okonkwo *et al*., 1999; Okonkwo and Povlishock, 1999). At least part of the mechanism by which CsA affords neuroprotection is via the maintenance of mitochondrial homeostasis by inhibiting the opening of the mitochondrial permeability transition pore (Buki *et al*., 1999; Okonkwo *et al*., 1999; Okonkwo and Povlishock, 1999; Scheff and Sullivan, 1999; Sullivan *et al*., 1999a). Furthermore, maintaining mitochondrial bioenergetics by dietary supplementation with creatine has also proved effective in ameliorating neuronal cell death and reduces mitochondrial ROS production and maintaining ATP levels following TBI (Sullivan *et al*., 2000b).

Although the complex mechanisms of secondary neuronal injury are poorly understood, it is clear that EAA neurotoxicity plays an important role (Rothman and Olney, 1995). This results in excessive entry of Ca^{2+}, leading to a loss of cellular homeostasis and subsequent neuronal Ca^{2+} overload. Ca^{2+} is the most common signal transduction element in cells, but unlike other second-messenger molecules, it is required for life. Paradoxically, prolonged high levels of $[Ca^{2+}]_i$ leads to cell death (Choi, 1992). Since Ca^{2+} cannot be metabolized like other second-messenger molecules, it must be tightly regulated by cells. Numerous intracellular proteins and some organelles have adapted to bind or sequester Ca^{2+} to ensure that homeostasis is maintained. *Mitochondria are one such organelle* (Ichas and Mazat, 1998; Rizzuto *et al*., 1999, 2000). During excitotoxic insult, Ca^{2+} uptake into mitochondria has been shown to increase ROS production, inhibit ATP synthesis and induce mitochondrial permeability transitions (Brustovetsky *et al*., 2002; Dugan *et al*., 1995; Reynolds and Hastings, 1995; Sengpiel *et al*., 1998; White and Reynolds, 1996). It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing $\Delta\Psi$ (chemical uncoupling) following excitotoxic insults is neuroprotective, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in EAA neuronal cell death (Nicholls and Budd, 1998a,b; Stout *et al*., 1998).

Free radical production is a byproduct of ATP generation in mitochondria via the electron transport chain. Electrons escape from the chain and reduce O_2 to $O_2^{-\cdot}$. Normally cells convert $O_2^{-\cdot}$ to H_2O_2 utilizing both manganese superoxide dismutase, which is localized to the mitochondria, and copper–zinc superoxide dismutase found in the cytosol. H_2O_2 is rapidly converted to H_2O via catalase and gluthione peroxidase, but has the potential to be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction, underlying ROS neurotoxicity. $OH^{\cdot}$ rapidly attacks unsatured fatty acids in membranes causing lipid peroxidation and the production of 4-hydroxynonenal (HNE) that conjugates to membrane proteins, impairing their function (Azbill *et al*., 1997; Keller *et al*., 1997a,b; Mark *et al*., 1997; Sullivan *et al*., 1998). In particular, ROS induction of lipid peroxidation and protein oxidation products may be particularly important in neurodegeneration (for review see Mattson, 1998) and TBI (Braughler *et al*., 1985; Braughler and Hall, 1989, 1992; Sullivan *et al*., 1998).

Mitochondrial ROS production is intimately linked to $\Delta\Psi$ such that hyperpolarization (high $\Delta\Psi$) increases and promotes ROS production (Liu *et al*., 2002; Skulachev, 1996, 1998; Starkov *et al*., 2002; Starkov and Fiskum, 2003; Votyakova and Reynolds, 2001). Since the magnitude of ROS production is largely dependent on—and correlates with—$\Delta\Psi$ even a modest reduction via increased proton conductance (decreases $\Delta\Psi$, the electrochemical proton gradient) across the mitochondrial inner membrane (uncoupling) reduces ROS formation (Kim-Han *et al*., 2001; Skulachev, 1996; Sullivan *et al*., 2003, in press-b; Votyakova and Reynolds, 2001).

Endogenous mitochondrial uncoupling is mediated by members of the UCP family, which function to dissociate ATP production from oxygen consumption in mitochondria of muscle and fat tissues (Nicholls and Ward, 2000), leading to heat generation. UCPs are activated by FFAs, superoxide and inhibited by purine nucleotides (Echtay *et al*., 2002) (also see Argiles *et al*., 2002; Harper *et al*., 2001; Zackova and Jezek, 2002 for review). Five mitochondrial UCPs exist in the human genome and among characterized uncoupling proteins, UCP2, UCP4, and UCP5/BMCP1 have recently been shown to be significantly expressed in the CNS (Arsenijevic *et al*., 2000; Diano *et al*., 2000; Horvath *et al*., 1999; Kim-Han *et al*., 2001). However, unlike UCP1 that is present only in brown adipose tissue and used to generate heat in cold environments (i.e., thermogenesis), their physiological role(s) are unclear.

Several hypotheses have been put forth concerning possible physiological roles of the UCPs including energy partitioning, energy balance, and control of metabolism which may be pivotal in obesity and diabetes (for review see Argiles *et al*., 2002; Jezek, 2002). Skulachev was the first to hypothesis that mild uncoupling could be beneficial since it causes a decrease in ROS production (Skulachev, 1996; and preceding section). Several studies have now demonstrated roles for UCPs in modulating ROS production. UCP2 (Arsenijevic *et al*., 2000) or UCP3 (Vidal-Puig *et al*., 2000) knockout mice exhibit increased ROS in macrophages and muscle, respectively. Leptin-deficient mice have decreased levels of UCP2 and increased ROS production in macrophages (Lee *et al*., 1999). Overexpression of UCP2 (Li *et al*., 2001) or UCP5/BMCP1 (Kim-Han *et al*., 2001) has also been shown to decrease cell death following H_2O_2 exposure and ROS production, respectively. UCP2 overexpression has also been demonstrated to reduce ROS production and increase tissue sparing in vivo following ischemia or TBI (Mattiasson *et al*., 2003).

On the basis of these initial reports, it is reasonable that increasing UCP activity by modulating dietary fat could directly modulate and reduce mitochondrial ROS production and subsequent oxidative damage. We have indeed shown that the converse (i.e., reducing dietary fat in immature animals) does rapidly reduce neuronal UCP expression/activity and increases mitochondrial ROS production. These changes in mitochondrial UCP activity and ROS production decrease the resistance of these immature animals to excitotoxic insult resulting in increased neuronal cell death following seizure activity, implicating a neuroprotective role for UCP2 and mitochondrial uncoupling in neuronal injury (Sullivan *et al*., 2003). These data also suggest that increasing dietary fat content would increase UCP activity and reduce ROS production, both of which we have recently demonstrated to occur in vivo (Sullivan *et al*., in press-a).

Several studies have demonstrated that mitochondrial uncoupling in vitro reduces neuronal mitochondrial Ca^{2+} loading and can inhibit excitotoxic cell death (Billups and Forsythe, 2002; Nicholls and Budd, 1998a,b; Pivovarova *et al*., 2002; Stout *et al*., 1998). To date only one study has assessed the potential for using mitochondrial uncouplers (2,4-DNP) as neuroprotective agents in an in vivo model of excitotoxicity (Maragos *et al*., 2003). Since it is well-established that excitotoxicity is a major player in TBI- and SCI-induced neuronal cell death and results in significant mitochondrial dysfunction, we designed several experiments to test the hypothesis that the mitochondrial uncouplers 2,4-DNP and FCCP would be neuroprotective following TBI and SCI.

The results demonstrate that rats administered a mitochondrial uncoupler have less tissue loss, improved behavioral outcomes and demonstrate a reduction in mitochondrial oxidative damage, Ca^{2+} loading, and dysfunction following SCI or TBI. The results also demonstrate that mitochondrial uncouplers significantly reduces mitochondrial dysfunction associated with injury whereas a 2,4-DNP analogue 2,4,6-trinitrophenol (TNP), which lacks the ability to uncouple intact mitochondria, did not provide any neuroprotection. Importantly, post-injury fasting of animals (24 h) following TBI yields similar results, perhaps by utilizing endogenous mitochondrial uncoupling proteins (UCP). Together these results implicate important mitochondrial events that could be potential novel interventions and novel targets for the treatment of TBI and SCI as well as other acute neuronal injuries.

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