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Differential Effects of the Mitochondrial Uncoupling Agent, 2,4-Dinitrophenol, or the Nitroxide Antioxidant, Tempol, on Synaptic or Nonsynaptic Mitochondria After Spinal Cord Injury

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Abstract

We recently documented the progressive nature of mitochondrial dysfunction over 24 hr after contusion spinal cord injury (SCI), but the underlying mechanism has not been elucidated. We investigated the effects of targeting two distinct possible mechanisms of mitochondrial dysfunction by using the mitochondrial uncoupler 2,4-dinitrophenol (2,4-DNP) or the nitroxide antioxidant Tempol after contusion SCI in rats. A novel aspect of this study was that all assessments were made in both synaptosomal (neuronal)- and nonsynaptosomal (glial and neuronal soma)-derived mitochondria 24 hr after injury. Mitochondrial uncouplers target Ca^{2+} cycling and subsequent reactive oxygen species production in mitochondria after injury. When 2,4-DNP was injected 15 and 30 min after injury, mitochondrial function was preserved in both populations compared with vehicle-treated rats, whereas 1 hr postinjury treatment was ineffective. Conversely, targeting peroxynitrite with Tempol failed to maintain normal bioenergetics in synaptic mitochondria, but was effective in nonsynaptic mitochondria when administered 15 min after injury. When administered at 15 and 30 min after injury, increased hydroxynonenal, 3-NT, and protein carbonyl levels were significantly reduced by 2,4-DNP, whereas Tempol only reduced 3-NT and protein carbonyls after SCI. Despite such antioxidant effects, only 2,4-DNP was effective in preventing mitochondrial dysfunction, indicating that mitochondrial Ca^{2+} overload may be the key mechanism involved in acute mitochondrial damage after SCI. Collectively, our observations demonstrate the significant role that mitochondrial dysfunction plays in SCI neuropathology. Moreover, they indicate that combinatorial therapeutic approaches targeting different populations of mitochondria holds great potential in fostering neuroprotection after acute SCI.

Keywords

electron transport system; excitotoxicity; mitochondrial bioenergetics; mitochondrial membrane potential (Ψ); mitochondrial permeability transition; oxidative damage

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Spinal cord injury (SCI) results in a cascade of secondary events such as oxidative stress and mitochondrial dysfunction (Luo et al., 2004; McEwen et al., 2007; Sullivan et al., 2005, 2007). Mitochondrial dysfunction, resulting from the disruption of Ca^{2+} homeostasis, release of excitatory amino acids, and the generation of toxic free radicals, is a central process that ultimately leads to neuronal death (Maragos and Korde, 2004; Sullivan et al., 2004b, 2005). We have recently demonstrated compromised mitochondrial bioenergetics in a time-dependent manner after acute contusion SCI (Sullivan et al., 2007). During excitotoxic insult, Ca^{2+} uptake in the mitochondria is known to increase reactive oxygen species (ROS) production, inhibit adenosine triphosphate (ATP) synthesis, and induce mitochondrial permeability transition (Dugan et al., 1995; Reynolds and Hastings, 1995; White and Reynolds, 1996; Sengpiel et al., 1998; Brustovetsky et al., 2002). It is known that by reducing mitochondrial membrane potential (Ψ) their uptake of Ca^{2+} can be inhibited (Nicholls and Budd, 1998a, 1998b; Stout et al., 1998). Therefore, pharmacological strategies that target mitochondrial dysfunction and oxidative stress may prove beneficial in treatment of SCI.

Mitochondrial uncoupling disconnects the flow of electrons through the mitochondrial electron transport system (ETS). Chemical uncouplers, such as 2,4-dinitrophenol (2,4-DNP), are protonophoric compounds that allow protons to reenter the mitochondrial matrix without taking part in ATP synthesis, which results in reduced Ψ (Skulachev, 1998; Jin et al., 2004). Chronic or severe chemical uncoupling of oxidative phosphorylation is deleterious to normal mitochondrial or cell function. However, in a SCI model with preinjury treatment with 2,4-DNP, as well as postinjury treatment after traumatic brain injury and ischemia reperfusion injury, it has been demonstrated that partial or mild mitochondrial uncoupling reduces mitochondrial Ca^{2+} uptake and ROS production (Jin et al., 2004; Ljubkovic et al., 2007; Pandya et al., 2007). In addition to chemical uncoupling agents, the nitroxide antioxidant Tempol has been shown to be a catalytic scavenger of the peroxynitrite (PON)-derived radicals NO_2 and CO_3 , which are mediators of PON-induced oxidative damage (Carroll et al., 2000; Bonini et al., 2002). PON is formed by the combination of $\cdot\text{NO}$ and superoxide radicals (Beckman, 1991). A recent report suggests that Tempol treatment improves neurological function after contusion SCI (Hillard et al., 2004). Importantly, we have established recently that synaptic (neuronal) and nonsynaptic (glia and neurons) mitochondria isolated from various central nervous system tissues respond differentially to insults and therefore most likely potential therapeutic interventions (Sullivan et al., 2004a; Brown et al., 2006; Naga et al., 2007).

In the current study we set out to assess the possible roles of two distinct mechanisms that could underlie SCI-induced mitochondrial dysfunction by targeting mitochondrial Ca^{2+} overload by using the mitochondrial uncoupler 2,4-DNP and PON damage using the nitroxide antioxidant Tempol. Additionally we designed the experiments to test the hypothesis that synaptic and nonsynaptic mitochondria would be differentially affected by our interventions after acute injury. Therefore, before designing involved experiments testing the effects on long-term behavioral improvements and tissue sparing after injury, the current study evaluated the effects of 2,4-DNP or Tempol administration at various time points after

injury on the integrity of synaptic and nonsynaptic mitochondrial populations after acute contusion SCI.

MATERIALS AND METHODS

SCI and Treatment

Female Sprague Dawley rats ($n = 144$) (Harlan Labs, IN) weighing 200–250 g were housed in the animal facility, Biomedical and Biological Sciences Research Building, University of Kentucky and allowed ad libitum access to water and food. All animal procedures were approved by the Institutional Animal Care and Use Committee. Before surgeries, animals were anesthetized with ketamine (80 mg/kg intraperitoneally) and xylazine (10 mg/kg intraperitoneally). A dorsal laminectomy was performed to expose the spinal cord at the tenth thoracic level (T10). Spinal cord contusions (200 kdyn) performed with the Infinite Horizon impactor (PSI, Lexington, KY), as previously described (Sullivan et al., 2007). Injured rats demonstrated total hind limb paralysis 24 hr after contusion injuries. Sham animals received a dorsal laminectomy only and had normal hind limb locomotion after surgery. Injured animals were treated with either vehicle, dimethyl sulf-oxide (DMSO), 5 mg/kg 2,4-DNP, or 300 mg/kg Tempol at either 15 min, 30 min, or 1 hr after contusion SCI.

Mitochondrial Preparation

Animals were euthanatized with CO₂ and decapitated at 24 hr after sham operation or drug treatment for isolation and characterization of synaptic and nonsynaptic mitochondria, as described previously (Brown et al., 2006; Naga et al., 2007). The spinal cords were rapidly removed and placed on an ice cold dissecting plate containing isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin (BSA), 20 mM HEPES, 1 mM EGTA; pH adjusted to 7.2 with KOH). The spinal cords were dissected into 2 cm segments centered on the injury site and homogenized in 2 mL of ice cold isolation buffer with EGTA. The homogenate was then centrifuged twice at 1,300g for 3 min at 4°C and the resulting supernatant removed and centrifuged at 13,000g for 10 min at 4°C. On the basis of pilot studies, mitochondria from two spinal cord segments were pooled in 500 µL of isolation buffer with EGTA to augment protein concentration for reliable mitochondrial respiration during experiments. The resulting crude mitochondrial/synaptosomal pellets were then placed atop a discontinuous Ficoll gradient (7.5%/10%) and centrifuged at 30,000g for 30 min at 4°C. The synaptosomal fractions were carefully removed from the interphase of Ficoll gradient and placed in a 2-mL centrifuge tube and the synaptic layer was washed with isolation buffer. The synaptosomal pellet was resuspended in 350 µL of isolation buffer with EGTA and burst in a nitrogen cell disruption chamber (1200 psi, 10 min) that was cooled to 4°C. The synaptosomal fractions were then applied for Ficoll purification again. The sedimented synaptosomal and nonsynaptosomal mitochondrial pellet was suspended in isolation buffer without EGTA and centrifuged for 10 min at 10,000g. The mitochondrial pellet was resuspended in EGTA-free isolation buffer at a concentration of ~10 mg/mL and stored on ice until further use. The protein concentration was determined with the BCA protein assay kit by measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, Vermont).

Measurement of Mitochondrial Function

Mitochondrial respiration was assessed as described previously (Sullivan et al., 2003) with a miniature Clark-type electrode (Hansatech Instruments, Norfolk, England) in a sealed, thermostatically controlled chamber at 37°C. Mitochondria were added to the chamber to yield a final protein concentration of ~200–300 µg/mL respiration buffer (125 mM KCl, 2 mM MgCl₂, 2.5 mM KH₂PO₄, 20 mM HEPES and 0.1% BSA, pH 7.2). Respiration was initiated by the addition of oxidative substrates pyruvate (5 mM) and malate (2.5 mM), which is designated as state II respiration. This was followed by the addition of 120 nmol of adenosine 5'-diphosphate (ADP; state III respiration) and the addition of oligomycin (1 µM) to induce state IV respiration. The mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (1 µM) was added to the chamber to assess complex I-driven maximum electron transport. The complex I inhibitor rotenone (0.8 µM) was then added to the chamber, followed by the addition of succinate (10 mM) to allow for quantification of complex II-driven maximum electron transport. The respiratory control ratio (RCR) was calculated by dividing the slope of the response of isolated mitochondria to state III respiration (presence of ADP) by slope of the response to state IV respiration (presence of 1 µM oligomycin and absence of ADP).

Mitochondrial Complex Activities

The mitochondrial ETS complex activities were measured with the Multi-detection Microplate Reader (Bio-Tek Instruments, INC, Winooski, VT). The mitochondria were freeze-thawed and sonicated three times before measuring the complexes activities.

The complex I (NADH dehydrogenase) assay was performed in 25 mM KPO₄ buffer (pH 7.2) containing mitochondrial protein (6 µg), 5 mM MgCl₂, 1 mM KCN, 1 mg/mL BSA, and 150 µM NADH at 30°C; the reaction was initiated by the addition of coenzyme Q-1 (50 µM). In this reaction ubiquinone 1 is the final electron acceptor. The decrease in NADH absorbance at 340 nm was monitored. The assay was also performed in the presence of rotenone (10 µM) to determine the rotenone-insensitive and the rotenone-sensitive complex I enzyme activity (Sullivan et al., 2004a).

The complex II (succinate dehydrogenase) activity: the rate of reduction of coenzyme Q by succinate was determined by measuring the rate of reduction of 2,6-dichloroindophenol, which is reduced rapidly by (coenzyme Q)H₂. The reaction mixture contained 100 mM KPO₄ buffer, 20 mM succinate, 10 µM EDTA, 0.01% Triton X-100, 1 µg/100 µL coenzyme Q₂ containing mitochondrial protein (6 µg) at 30°C and the reaction was initiated by the addition of 0.07% 2,6-dichloroindophenol. Decrease in absorbance was monitored at 600 nm (Ziengler, 1967).

Complex IV (cytochrome c oxidase) activity was measured in 10 mM KPO₄ buffer and 50 µM reduced cytochrome c. The reaction was initiated by addition of 6 µg mitochondrial protein. The rate of oxidation of cytochrome c was measured at 30°C by measuring decrease in absorbance of reduced cytochrome c at 550 nm (Smith, 1955; Wharton, 1967).

Markers of Mitochondrial Oxidative Damage

Measurements of the oxidative damage markers 3-nitrotyrosine (3-NT), 4-hydroxynonenal (4-HNE), and protein carbonyls were carried out as described (Sullivan et al., 2007).

Data Analysis

Data from mitochondrial preparations were analyzed by an analysis of variance (ANOVA). When appropriate, post hoc comparisons were made by Student-Newman-Keuls, with significance set at $P < 0.05$.

RESULTS

Effects of 2,4-DNP and Tempol on Synaptic Mitochondrial Bioenergetics

Contusion SCI was found to significantly alter synaptic mitochondrial bioenergetics over the first 24 hr after injury. Figure 1 shows the typical respiration traces for treatments at 15 min after injury with DMSO, 2,4-DNP and Tempol, as well as sham animals. Compared with sham-treated animals, compromised mitochondrial respiratory activity was evident in vehicle-treated injured animals. Treatment with 2,4-DNP after 15 min (Fig. 1) and 30 min (traces not shown) maintained near normal mitochondrial function vs. vehicle-treated injured animals, whereas 1 hr postinjury treatment with 2,4-DNP failed to preserve mitochondrial function (traces not shown). Conversely, Tempol treatment did not preserve mitochondrial integrity (Fig. 1). These results were confirmed by deriving the RCR (Fig. 2), which is an important measure of how well the ETS is coupled to ATP synthesis, indicative of mitochondrial integrity. Compared with sham-treated animals, there were significant reductions in the RCR for all injured animal groups at 15 min [$F(3,20) = 57.56$, $P < 0.0001$], 30 min [$F(3,18) = 65.78$, $P < 0.0001$], and 1 hr [$F(3,17) = 131.93$, $P < 0.0001$] after injury (Fig. 2). However, when 2,4-DNP was administered at 15 min and 30 min after injury, it significantly ($P < 0.05$) improved mitochondrial integrity compared with vehicle-treated animals, although the values remained significantly ($P < 0.05$) lower compared with sham-treated animals (Fig. 2). In contrast, treatment with Tempol had no effect on RCR compared with vehicle-treated injured animals (Fig. 2).

At 24 hr after injury, quantification of mitochondrial bioenergetics in terms of respiration rates showed a significant decrease in ADP phosphorylation (state III respiration) after 15 min [$F(3,20) = 29.997$, $P < 0.0001$], 30 min [$F(3,18) = 14.267$, $P < 0.0001$], and 1 hr [$F(3,17) = 54.897$, $P < 0.0001$] treatments in all injured animals compared with sham-treated animals (Fig. 3). NADH-linked (complex I driven) ETS capacity (state V–complex I) also decreased significantly at 15 min [$F(3,20) = 23.065$, $P < 0.0001$], 30 min [$F(3,18) = 16.308$, $P < 0.0001$], and 1 hr [$F(3,17) = 83.599$, $P < 0.0001$] in all injured animals compared with sham-treated animals (Fig. 3). Post hoc analysis showed a significant ($P < 0.05$) decrease in both state III and state V–complex I respiration in vehicle-treated injured spinal cord mitochondria compared with sham-treated animals. However, treatment with 2,4-DNP at either 15 or 30 min after injury significantly ($P < 0.05$) maintained state III and state V–complex I respiration compared with vehicle-treated injured animals, whereas treatment at 1 hr after injury was unable to preserve mitochondrial function (Fig. 3A–C). Notably, after 2,4-DNP treatment at 15 and 30 min after injury, both state III and state V–complex I

respiration were not statistically different compared with sham-treated animals. Conversely, Tempol administration at all time points was ineffective in maintaining mitochondrial bioenergetics (Fig. 3A–C).

In vehicle-treated animals, direct measurement of synaptic mitochondrial ETS complex enzymatic activity revealed that at 24 hr after injury, complex I activity decreased significantly after 15 min [$F(3,8) = 13.793$, $P < 0.005$], 30 min [$F(3,8) = 12.269$, $P < 0.005$], and 1 hr [$F(3,8) = 19.238$, $P < 0.0005$] treatments in all injured groups compared with sham-treated animals (Fig. 4). Also, in all vehicle-treated injured animals the activity of complex IV decreased significantly after 15 min [$F(3,8) = 26.733$, $P < 0.001$], 30 min [$F(3,8) = 6.682$, $P < 0.01$], and 1 hr [$F(3,8) = 43.339$, $P < 0.0001$] compared with sham-treated animals (Fig. 4). However, 2,4-DNP administered 15 or 30 min after injury, but not after 1 hr, significantly ($P < 0.05$) maintained both complex I and complex IV activities compared with vehicle-treated injured animals (Fig. 4A–C). Notably, after 2,4-DNP treatment at 15 and 30 min after injury, both complex I and complex IV activities were not statistically different compared with sham-treated animals. In contrast, Tempol administration was only effective for complex IV activity when administered at 15 min after injury (Fig. 4A). Despite trends, there were no significant changes in complex II activity for any experimental group (Fig. 4A–C).

Mitochondrial oxidative damage was assessed by using three unrelated markers of oxidative damage including lipid peroxidation (4-hydroxynonenal, HNE), PON-derived 3-nitrotyrosine (3-NT) and protein oxidation (protein carbonyls) from the same samples of mitochondria used for respiration rate assessments. In vehicle-treated injured animals, all three markers of oxidative stress we assessed were significantly increased compared with sham-treated animals: HNE at 15 min [$F(3,20) = 5.815$, $P < 0.005$], 30 min [$F(3,20) = 6.912$, $P < 0.005$], and 1 hr after injury [$F(3,20) = 15.846$, $P < 0.0001$]; 3-NT at 15 min [$F(3,20) = 9.544$, $P < 0.005$], 30 min [$F(3,20) = 8.333$, $P < 0.005$], and 1 hr after injury [$F(3,20) = 15.734$, $P < 0.0001$]; protein carbonyls at 15 min [$F(3,20) = 8.968$, $P < 0.005$], 30 min [$F(3,20) = 5.185$, $P < 0.01$], and 1 hr after injury [$F(3,20) = 5.287$, $P < 0.01$]. Post hoc analysis showed that compared with vehicle-treated injured animals, both 2,4-DNP and Tempol administered 15 or 30 min after injury significantly ($P < 0.05$) reduced 3-NT and protein oxidation to levels comparable to shams ($P > 0.05$) (Fig. 5A,B). Moreover, only 2,4-DNP, but not Tempol, significantly ($P < 0.05$) reduced the increased levels of HNE to sham values. (Fig. 5A,B). Notably, neither treatment significantly reduced mitochondrial oxidative damage when administration was delayed until 1 hr after injury.

Effects of 2,4-DNP and Tempol on Nonsynaptic Mitochondrial Bioenergetics

Typical respiration traces for nonsynaptic mitochondrial populations were comparable to those of synaptic mitochondria (traces not shown). Like synaptic mitochondria, there were significant reductions in RCR for all injured animal groups after 15 min [$F(3,20) = 10.311$, $P < 0.0001$], 30 min [$F(3,18) = 22.295$, $P < 0.0001$], and 1 hr [$F(3,18) = 27.384$, $P < 0.0001$] treatments compared with sham-treated animals (Fig. 6). Notably, 2,4-DNP treatment at all time points significantly ($P < 0.05$) maintained RCR compared with vehicle-treated injured animals, and the values for 15 and 30 min treatments were comparable to shams (Fig. 6).

However, the RCR was significantly less than shams after 1 hr 2,4-DNP treatment. In comparison, Tempol treatment was effective only when administered 15 min after injury ($P < 0.05$) compared with vehicle-treated injured rats, and maintained RCR levels near normal ($P > 0.05$ vs. shams) (Fig. 6).

Measurements revealed that the rates of respiration in nonsynaptic mitochondria from sham animals were 20%–30% higher than that of sham synaptic mitochondria (Figs. 3 and 7). Significant reductions in state III respiration were noted for all injured groups after 15 min [$F(3,20) = 9.893$, $P < 0.0005$], 30 min [$F(3,18) = 46.152$, $P < 0.0001$], and 1 hr [$F(3,18) = 62.112$, $P < 0.0001$] treatments compared with sham-treated animals (Fig. 7). State V–complex I–driven respiration was also decreased significantly for all injured animal groups after 15 min [$F(3,20) = 5.119$, $P < 0.005$], 30 min [$F(3,18) = 20.104$, $P < 0.0001$], and 1 hr [$F(3,18) = 11.356$, $P < 0.0005$] treatments compared with sham-treated animals (Fig. 7A–C). Post hoc analysis showed that 2,4-DNP treatment at all time points significantly ($P < 0.05$) increased state III and state V–complex I respiration compared with vehicle-treated animals. Notably, with 15 min 2,4-DNP treatment the state III and state V–complex I respiration rates were comparable to shams ($P > 0.05$), whereas the rates were significantly reduced ($P < 0.05$) with 2,4-DNP treatment at 30 min and 1 hr after injury compared with sham-treated animals (Fig. 7A–C). Conversely, treatment with Tempol only at 15 min after injury significantly ($P < 0.05$) preserved state III respiration compared with vehicle-treated injured animals in nonsynaptic mitochondria; however, respiration remained significantly ($P < 0.05$) lower than sham values (Fig. 7A).

Complex I activity decreased significantly compared with sham-treated animals after 15 min [$F(3,8) = 7.862$, $P < 0.01$], 30 min [$F(3,8) = 18.053$, $P < 0.001$], and 1 hr [$F(3,8) = 47.486$, $P < 0.0001$] treatments in all injured groups (Fig. 8). Significantly compromised complex I activity ($P < 0.05$) was observed at all the time points in vehicle-treated injured animals compared with sham-treated animals (Fig. 8A–C). Treatment with 2,4-DNP at 15 and 30 min after injury significantly ($P < 0.05$) maintained complex I activity near normal levels ($P > 0.05$ vs. shams) (Fig. 8A,B). Notably, complex I activity was significantly ($P < 0.05$) lower compared with sham-treated animal after 1 hr after injury treatment (Fig. 8C), although still significantly increased ($P < 0.05$) compared with vehicle-treated injured rats. Conversely, complex II and IV activities demonstrated insignificant trends for maintaining normal activity (Fig. 8).

Results of nonsynaptic mitochondrial oxidative damage are shown in Figure 9. In vehicle-treated injured animals, all three markers of oxidative stress we assessed were significantly increased compared shams: HNE at 15 min [$F(3,20) = 6.861$, $P < 0.001$], 30 min [$F(3,20) = 6.332$, $P < 0.005$], and 1 hr after injury [$F(3,20) = 11.835$, $P < 0.0001$]; 3-NT at 15 min [$F(3,20) = 10.200$, $P < 0.0005$]; 30 min [$F(3,20) = 163.652$, $P < 0.0001$], and 1 hr after injury [$F(3,20) = 6.237$, $P < 0.005$]; protein carbonyls at 15 min [$F(3,20) = 9.658$, $P < 0.001$]; 30 min [$F(3,20) = 11.242$, $P < 0.001$], and 1 hr after injury [$F(3,20) = 5.287$, $P < 0.01$]. Post hoc analysis showed that compared with vehicle-treated injured animals, both 2,4-DNP and Tempol administered 15 or 30 min after injury significantly ($P < 0.05$) reduced 3-NT and protein oxidation, whereas only 2,4-DNP, but not Tempol, reduced the increased

levels of HNE (Fig. 9A,B). Conversely, protein oxidation was reduced to sham levels by both the treatments at all time points of administration (Fig. 9).

DISCUSSION

The results from the present study demonstrate that “mild” mitochondrial uncoupling holds great promise as a potential therapy that is capable of maintaining both synaptic and nonsynaptic mitochondrial integrity after acute contusion SCI in rats. On the other hand, administration of a nitroxide antioxidant was ineffective in maintaining normal synaptic mitochondrial function. These results appear to indicate that in order to maintain mitochondrial function after SCI it is necessary to target more upstream targets. For example, “mild” mitochondrial uncoupling with 2,4-DNP reduces Ψ after injury, which in turn decreases ROS production, mitochondrial Ca^{2+} uptake, and delays mitochondrial transition pore formation. Conversely, PON scavengers target downstream events that occur as a result of increased production of mitochondrial free radicals. Collectively, this demonstrates that the pharmacological maintenance of mitochondrial integrity in the acute traumatized spinal cord is critical for limiting the cascade of molecular events leading to secondary cell death. Such a novel, potentially multifaceted neuroprotective strategy, used alone or in combination with other restorative therapeutics, has immense potential in minimizing neuropathology and optimizing the potential for functional recovery after contusion SCI.

Consistent with our recent characterization of total mitochondria (combined synaptic and nonsynaptic populations) from injured spinal cord (Sullivan et al., 2007), in the current study both synaptic and nonsynaptic mitochondria from vehicle-treated injured spinal cords demonstrated significantly compromised bioenergetics and increased oxidative damage at 24 hr after injury. Only treatment with 2,4-DNP at 15 and 30 min after injury, but not at 1 hr, significantly maintained normal synaptic mitochondrial bioenergetics, as well as reduced markers of oxidative stress. Alternatively, results from nonsynaptic mitochondria showed that 2,4-DNP was effective in maintaining normal mitochondrial bioenergetics at all the time-points of administration, whereas Tempol was effective in maintaining RCR and state III respiration only with treatment at 15 min after injury. This disparity may stem from the fact that synaptic mitochondria are more susceptible to excitotoxic insults, Ca^{2+} overload, and resultant mitochondrial transition pore than nonsynaptic mitochondria in the brain (Brown et al., 2006; Naga et al., 2007). Hence, targeting these upstream events that disrupt mitochondrial integrity and bioenergetics with 2,4-DNP promoted the greatest neuroprotection.

The synaptic mitochondrial fraction mainly contains mitochondria from neurons whereas the nonsynaptic mitochondrial fraction contains mitochondria from axonal bodies, glia, astrocytes etc. It is also known that rat cortical astrocytes have a greater Ca^{2+} uptake capacity than do cerebellar granule neurons (Bambrick et al., 2006). In addition to this, we also found higher respiration rates for nonsynaptic mitochondria compared with synaptic mitochondria in sham animals, which is suggestive of a higher ETS activity and capacity of nonsynaptic mitochondria compared with synaptic mitochondria under basal conditions. This difference was also evident in the enzymatic activities measured for complex I and IV

of the ETS in the nonsynaptic mitochondrial fraction. Although the precise mechanism underlying these differences is not clear, this may indicate that higher levels of 2,4-DNP are required to uncouple nonsynaptic mitochondria to the same extent as synaptic mitochondria, again illustrating the need to design therapies for specific mitochondrial populations. Regardless, these results show that mitochondrial dysfunction after contusion SCI is a rapid event and that “mild” mitochondrial uncoupling with 2,4-DNP injection after injury can be neuroprotective. It is important to note that “mild” mitochondrial chemical uncoupling has been clearly demonstrated to be neuroprotective after traumatic brain injury (Pandya et al., 2007).

Administration of 2,4-DNP at 15 and 30 min after injury significantly reduced mitochondrial levels of HNE, 3-NT and protein oxidation, whereas Tempol was only effective in reducing 3-NT and protein oxidation. This is most likely because Tempol is a catalytic scavenger of PON-derived radicals (3-NT) (Carroll et al., 2000; Bonini et al., 2002). Also, from our results it is clear that Tempol was ineffective in reducing the level of HNE, a marker for lipid peroxidation. It is well known that lipid peroxidation is a self-perpetuating form of free radical damage that is believed to be a major factor in the spread of spinal tissue degeneration after SCI (Hall, 1991; Anderson and Hall, 1993; Hall and Braughler, 1993). Therefore, these data indicate that, at least in the case of mitochondrial lipid peroxidation, reactive nitrogen species may not be playing a pivotal role or that Tempol cannot transverse the mitochondrial membranes. The functional decline after contusion SCI is contributed to both direct mechanical injury and secondary pathophysiology that are induced by the initial trauma (Profyris et al., 2004). Although the mechanisms of secondary neuronal injury are not very well understood, it is believed that excitotoxic amino acids (EAA) play an important role in the neuronal toxicity (Nicholls and Budd, 1998a, 1998b; White and Reynolds, 1996; Stout et al., 1998). after SCI, increased EAA levels lead to increased intracellular Ca^{2+} levels, which in turn results in increased mitochondrial Ca^{2+} uptake/cycling (Lifshitz et al., 2004; Sullivan et al., 2004b, 2005). Mitochondrial Ca^{2+} uptake is highly regulated by Ψ an increase in Ψ increases the uptake of Ca^{2+} into the mitochondria. Mitochondrial overload with Ca^{2+} results in increased ROS production, reduced ATP production, and induction of permeability transition that leads to neuronal cell death (Dugan et al., 1995; Reynolds and Hastings, 1995; White and Reynolds, 1996; Brustovetsky et al., 2002). In vitro studies suggest that reducing mitochondrial Ca^{2+} uptake by reducing Ψ (i.e., chemical uncoupling) after excitotoxic insults prevents neuronal cell death, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in EAA-mediated neuronal cell death (Nicholls and Budd, 1998a, 1998b; Stout et al., 1998).

A possible mechanism for the neuroprotection provided by mitochondrial uncouplers is their ability to decrease mitochondrial Ca^{2+} uptake, thereby maintaining normal mitochondrial function and decreasing neuronal cell death after excitotoxic insults. We found in the present study that 15 and 30 min postinjury treatment with 2,4-DNP significantly maintained normal mitochondrial function in terms of RCR, respiration rates and enzyme complexes of ETS compared with vehicle-treated injured animals. In addition to maintaining normal mitochondrial parameters, treatment with 2,4-DNP also decreased mitochondrial oxidative damage. The lack of effect when Tempol, a nitroxide scavenger, is used may be because it has a short half-life that varies from tissue to tissue (Chen and McLaughlin, 1985; Ueda et

al., 2003) and/or its inability to enter mitochondrial at concentrations sufficient to reduce reactive nitrogen species-induced mitochondrial damage. Additionally, “mild” mitochondrial uncoupling not only prevents mitochondrial Ca^{2+} overload by reducing Ψ but also decreases the ensuing ROS production, suggesting that this may be a pivotal neuroprotective therapy for acute SCI. Accordingly, we are currently assessing the Ca^{2+} concentrations in isolated mitochondria after acute contusion SCI with and without “mild” mitochondrial chemical uncoupling after injury. Our results imply that strategies that target multiple upstream events (e.g., mitochondrial uncoupling) are more efficacious than therapies focused on singular, particular downstream mechanisms (e.g., oxidative stress). On the basis of these findings, we are also assessing tissue sparing (i.e., gray matter and neuronal cell counts) and behavioral outcome measures after contusion SCI and “mild” mitochondrial chemical uncoupling.

In conclusion, these observations show that mitochondrial dysfunction after SCI is a rapid event that differentially affects mitochondria in different cellular populations. The results also demonstrate that mitochondrial-targeted therapeutic interventions affect synaptic (neuronal) and nonsynaptic (glial and neuronal soma) mitochondria to varying degrees after SCI, which may indicate the extent to which different mechanisms are involved in the neuropathology of SCI. This is of particular importance in designing optimal therapeutics because of the prominent role that nonneuronal cells, such as oligodendrocytes, play in behavioral improvement after SCI. Overall, these findings signify that therapeutic strategies that uncouple mitochondria may hold great potential as a pharmacological therapy for acute SCI.

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Fig. 1.

Contusion SCI leads to mitochondrial dysfunction as demonstrated by oxygram traces for synaptic mitochondria isolated from either sham spinal cords, 24 hr after injury alone or treatment with 2,4-DNP (DNP) or Tempol at 15 min after injury. Mild mitochondrial uncoupling after injury with DNP significantly maintained mitochondrial bioenergetics after injury, whereas Tempol showed little effect.

Fig. 2.

The RCR (calculated as the ratio of state III vs. state IV slopes) is a measure of mitochondrial integrity and coupling of the ETS to oxidative phosphorylation. The RCR for synaptic mitochondria was significantly decreased in vehicle-treated injured animals at 24 hr after injury, whereas 15- and 30-min postinjury treatment with 2,4-DNP (DNP) significantly maintained the RCR. Notably, the RCR for all the 2,4-DNP-treated groups remained significantly ($P < 0.05$) lower compared with sham-treated animals. Bars represent group mean $\pm$ SEM, $n = 5-6$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 3.

Respiration rates from synaptic mitochondria were calculated as nmol oxygen/min/mg protein in at 24 hr after SCI after treatment with either vehicle, 2,4-DNP (DNP) or Tempol at 15 min (A), 30 min (B), and 1 hr (C) after injury. Significantly impaired mitochondrial function was observed with vehicle treatment at all times points of administration. Notably, only mild mitochondrial uncoupling with DNP injections at 15 and 30 min after injury maintained normal mitochondrial function vs. vehicle-treated injured groups; notably, state III and state V–complex I respiration rates were comparable to shams ($P > 0.05$). Conversely, Tempol treatments were ineffective at all time points delivered. Bars represent group mean $\pm$ SEM, $n = 5-6$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 4.

Activities of complexes I, II and IV were assessed by using their specific substrates and calculated as nmol/min/mg protein. Activities are shown for synaptic mitochondria isolated from 15 min (A), 30 min (B), and 1 hr (C) postinjury treatment groups, respectively. With vehicle treatment, the activities of complexes I and IV were significantly decreased 24 hr after contusion SCI. Treatment with 2,4-DNP (DNP) at 15 and 30 min, but not 1 hr after injury, maintained normal levels of activities that were not significantly ($P > 0.05$) different than shams. At no time point of administration did Tempol demonstrate protective effects. Bars represent group mean $\pm$ SEM, $n = 3$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 5.

Synaptic mitochondrial oxidative stress was assessed by quantifying the oxidative markers 3-NT, 4-HNE and protein carbonyls by slot blot analysis. Data for synaptic mitochondria are expressed as intensity (AU). Data are shown at 24 hr after contusion SCI from the 15 min (A), 30 min (B), and 1 hr (C) postinjury treatment groups, respectively. Treatment with 2,4-DNP (DNP) at 15 and 30 min, but not 1 hr after injury, significantly decreased the increased levels of all the markers from vehicle-treated injured rats to levels comparable to shams ($P > 0.05$). Tempol treatment at 15 and 30 min after injury also significantly reduced 3-NT and protein carbonyls levels compared with vehicle-treated injured rats to levels comparable to shams ($P > 0.05$), but not when administered 1 hr after injury. Unlike with DNP treatment, HNE levels were not reduced by Tempol administration at any time point. Bars represent group mean $\pm$ SEM, $n = 6$ per group. $\star P < 0.05$ vs. vehicle-treated injured group.

Fig. 6.

The RCR (calculated as the ratio of state III vs. state IV slopes) is a measure of mitochondrial integrity and coupling of the ETS to oxidative phosphorylation. The RCR for nonsynaptic mitochondria was significantly decreased in vehicle-treated injured animals at 24 hr after injury. Treatment with 2,4-DNP (DNP) at all the time points significantly maintained the RCR compared with vehicle-treated injured animals. Although the values with 15- and 30-min treatments were comparable to shams ($P > 0.05$), the RCR remained significantly lower than shams after 1 hr DNP treatment. Conversely, Tempol treatment only at 15 min after injury was significantly effective in maintaining RCR, comparable to shams ($P > 0.05$). Bars represent group mean $\pm$ SEM, $n = 5-6$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 7.

Respiration rates from nonsynaptic mitochondria were calculated as nmol oxygen/min/mg protein in at 24 hr after SCI after treatment with either vehicle (A), 2,4-DNP (DNP) (B), or Tempol (C) at 15 min, 30 min, and 1 hr after injury. Significantly impaired mitochondrial function was observed with vehicle treatment compared with sham-treated animals. Notably, mild mitochondrial uncoupling with DNP at all time points, as well as treatment with Tempol only at 15 min after injury, significantly maintained normal mitochondrial function compared with vehicle-treated injured animals. However, with DNP treatments at 30 min and 1 hr after injury, state III and state V–complex I respiration rates remained significantly lower compared with sham-treated animals ($P < 0.05$). Also, state III respiration rates with Tempol treatment at 15 min in the postinjury groups remained significantly lower than shams ($P < 0.05$). Bars represent group mean $\pm$ SEM, $n = 5$ –6 per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 8.

Activities of complexes I, II, and IV were assessed by using their specific substrate and calculated as nmol/min/mg protein. Shown are activities for nonsynaptic mitochondria isolated from 15 min (A), 30 min (B), and 1 hr (C) postinjury treatment groups, respectively. Only complex I activity was significantly decreased after acute contusion SCI compared with sham-treated animals ($P < 0.05$). Treatment with 2,4-DNP (DNP) at all time points after injury significantly maintained complex I activity compared with vehicle-treated injured animals. With both 15 and 30 min treatments, complex I activity remained comparable to shams ($P > 0.05$), but not with treatment at 1 hr after injury. At no time point of administration did Tempol demonstrate protective effects. Bars represent group mean $\pm$ SEM, $n = 3$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.

Fig. 9.

Nonsynaptic mitochondrial oxidative stress was assessed by quantifying the oxidative markers 3-NT, 4-HNE and protein carbonyls by using slot blot analysis. Data for nonsynaptic mitochondria are presented as intensity (AU). Data are shown at 24 hr after contusion SCI from the 15 min (A), 30 min (B), and 1 hr (C) postinjury treatment groups, respectively. Compared with vehicle-treated injured animals, treatment with 2,4-DNP (DNP) at 15 and 30 min after injury significantly reduced the increased levels of HNE and 3-NT, comparable to shams ($P > 0.05$). Notably, Tempol administration at 15 and 30 min after injury was effective only in reducing increased 3-NT levels to sham values, but did not reduce HNE levels at all time points of administration. Both DNP and Tempol administration up to 1 hr after injury significantly decreased the increased levels of protein carbonyls vs. vehicle-treated injured animals, comparable to sham values ($P > 0.05$). Bars represent group mean $\pm$ SEM, $n = 6$ per group. ★ $P < 0.05$ vs. vehicle-treated injured group.