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Pharmacological evidence for a role of peroxynitrite in the pathophysiology of spinal cord injury

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Abstract

Evidence suggests that the reactive oxygen species peroxynitrite (PN) is an important player in the pathophysiology of acute spinal cord injury (SCI). In the present study, we examined the ability of tempol, a catalytic scavenger of PN-derived free radicals, to alleviate oxidative damage, mitochondrial dysfunction and cytoskeletal degradation following a severe contusion (200 kdyn force) SCI in female Sprague–Dawley rats. PN-mediated oxidative damage in spinal cord tissue, including protein nitration, protein oxidation and lipid peroxidation was significantly reduced by acute tempol treatment (300 mg/kg, i.p. within 5 min post-injury). Injury-induced mitochondrial respiratory dysfunction, measured after 24 h in isolated mitochondria, was partially reversed by tempol along with an attenuation of oxidative damage to mitochondrial proteins. Mitochondrial dysfunction disrupts intracellular Ca^{2+} homeostasis contributing to calpain-mediated axonal cytoskeletal protein (α -spectrin, 280 kD) degradation. Increased levels of α -spectrin breakdown proteins (SBDP 145 kD and 150 kD) were significantly decreased at 24 h in tempol-treated rats indicative of spinal axonal protection. However, a therapeutic window analysis showed that the axonal cytoskeletal protective effects require tempol dosing within the first hour after injury. Nevertheless, these findings are the first to support the concept that PN is an important neuroprotective target in early secondary SCI, and that there is a mechanistic link between PN-mediated oxidative compromise of spinal cord mitochondrial function, loss of intracellular Ca^{2+} homeostasis and calpain-mediated proteolytic axonal damage.

Keywords

Tempol; Peroxynitrite; Spinal cord injury; Oxidative damage; Protein nitration; Lipid peroxidation; Mitochondrial dysfunction; Cytoskeletal breakdown

Introduction

Reactive oxygen species-induced oxidative damage is a well documented secondary injury mechanism after CNS injury (Braugher and Hall, 1989, Hall, 2004, Hall and Braugher, 1989). Peroxynitrite (PN), formed by the diffusion rate-limited combination of nitric oxide (NO) and superoxide ($\text{O}_2^{\cdot-}$) free radicals (Alvarez et al., 2002, Beckman et al., 1990), has been proposed to be a key contributor to post-traumatic oxidative damage (Beckman, 1994, Crow

and Beckman, 1996, Hall, 2004), mainly because its highly reactive decomposition products nitrogen dioxide ($\cdot\text{NO}_2$), hydroxyl radical ($\cdot\text{OH}$) and carbonate radical ($\text{CO}_3^{\cdot-}$). These PN-derived radicals can oxidize proteins, nitrate tyrosine residues (Alvarez and Radi, 2003), induce cell membrane lipid peroxidation (Beckman et al., 1990, Hall, 2004, Kruman et al., 1997) and cause single-strand DNA breaks (Salgo et al., 1995, Szabo, 1996). Peroxynitrite can also inhibit mitochondrial respiration (Bolanos et al., 1995, Singh et al., 2007). In agreement with the importance of PN in acute CNS injury, recent studies have shown that certain PN-targeting compounds such as uric acid (Scott et al., 2005) and FeTSPP (Genovese et al., 2007) protect against secondary damage and improve motor functional recovery after spinal cord injury (SCI).

In the present study, we have examined the neuroprotective effects of tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a prototypical nitroxide antioxidant (Krishna and Samuni, 1994). Nitroxides have versatile antioxidant properties including, but not limited to, superoxide dismutase (SOD)-mimicry (Krishna et al., 1996), catalase and peroxidase-like activities (Krishna et al., 1996, Mehlhorn and Swanson, 1992), inhibition of $\cdot\text{OH}$ formation during Fenton-type reactions (Zeltzer et al., 1997) and inhibition of lipid peroxidation (Miura et al., 1993). Tempol is superior to many other antioxidants for neuroprotective use since it has a small molecule weight (MW:172) and can readily cross the blood brain barrier (Mitchell et al., 1991). More importantly, tempol can catalytically decompose the highly reactive PN-generated nitrogen dioxide radical ($\cdot\text{NO}_2$) and carbonate radical ($\text{CO}_3^{\cdot-}$) with a very rapid rate constant (Carroll et al., 2000). Consistent with this latter mechanism of action, tempol has been shown to decrease the levels of 3-nitrotyrosine (3-NT), a specific biomarker for PN oxidative damage (Carroll et al., 2000).

We have previously reported that PN-mediated oxidative damage in injured spinal cord tissue precedes two other critically important secondary injury events; mitochondrial dysfunction (Sullivan et al., 2007, Xiong et al., 2007) and calpain-mediated cytoskeletal protein breakdown (Xiong et al., 2007). Other studies in our group have demonstrated that PN-mediated oxidative damage is associated with mitochondrial dysfunction in traumatic brain injury (TBI) (Singh et al., 2006a) and SCI (Sullivan et al., 2007) models. Therefore, the current experiments were conducted to assess tempol's ability to reduce post-traumatic PN-induced oxidative damage, mitochondrial dysfunction and calpain-mediated cytoskeletal breakdown in a widely employed rat contusion SCI model. Using this pharmacological approach, we have established a mechanistic linkage between these pathophysiological events and validated a novel antioxidant approach for acute mitochondrial protection and ensuing neuroprotection after SCI.

Materials and methods

Subjects

This study employed a total of 126 young adult female Sprague–Dawley rats (Charles River, Portage, MI, USA) weighing between 200 and 225 g. The animals were randomly cycling and were not tested for stage of the estrus cycle. They were fed and watered ad libitum. All procedures described below have been approved by the University of Kentucky Institutional Animal Care and Use Committee and followed NIH guidelines.

Rat model of traumatic spinal cord contusion injury

Rats were anesthetized with ketamine (80 mg/kg i.p., Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (10 mg/kg i.p., Butler, Columbus, OH, USA) before a laminectomy at T10 vertebrae level was performed. Spinal cord injury was carried out using the Infinite Horizons contusion device as previously described (Scheff et al., 2003, Xiong et al., 2007).

The force applied to spinal cord was 200 kdyn, which produces a moderately severe contusion injury.

Tempol preparation and dosing

For the experiments described below, tempol was purchased from Sigma-Aldrich (Milwaukee, WI) and freshly prepared in 0.9% saline before intraperitoneal (i.p.) injection. In each of the experiments outlined below, separate cohorts of rats were randomly allocated into 3 groups ($n=6$ per group): (I) Sham: subjected to T10 laminectomy, but without SCI and no vehicle or drug treatment. (II) Vehicle: subjected to spinal cord injury and given 0.9% saline i.p. at 5 min after injury. (III) Tempol-treated: subjected to SCI and given tempol (300 mg/kg) i.p. at 5 min after injury. For initial experiments, a lower dose of tempol (150 mg/kg, i.p.) group was also included to determine the optimal dosing. For the immunohistochemical analysis of 3-NT and HNE, two sham, 2 vehicle-treated and 2 tempol (300 mg/kg I.P.) were also run. The doses chosen were based on previous studies of the effects of tempol on neurological recovery after contusion SCI in rats (Hillard et al., 2004) and on prior work in our laboratory concerning neuroprotective effects of tempol after experimental traumatic brain injury (Deng-Bryant et al., 2008).

Experiment 1: immunoblot and immunohistochemical analysis of the effects of tempol on post-traumatic oxidative damage

In experiment 1, the effects of tempol (300 mg/kg i.p. at 5 min post-injury) were examined on oxidative damage using either immunoblotting (slot and western) or immunohistochemical techniques. In the first part of this experiment, 18 rats were randomly allocated into 3 groups ($N=6$ /group): sham, injured plus vehicle and injured plus tempol. At 24 h after laminectomy or laminectomy plus SCI, a 1.5 cm section of the spinal cord centered on the T10 spinal cord segment and containing the immediately surrounding rostral and caudal tissue was rapidly removed at 24 h after laminectomy or SCI. From this larger piece, a 10 mm spinal cord (SC) section at the injury epicenter was dissected and homogenized in ice cold buffer (20 mmol/L Tris-HCL, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EGTA, 10 mmol/L EDTA, 20 mmol/L HEPES, 10% glycerol) containing protease inhibitor (Roche Inc., Nutley, NJ, USA). Following sonication, the dissolved proteins were centrifuged at $15,000 \times g$ for 30 min and the supernatant was collected and normalized to $1 \mu\text{g}/\mu\text{l}$ by protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). For slot blots, $2 \mu\text{g}$ of protein was loaded in each well of the slot blot apparatus and collected on the nitrocellulose membrane by gravity sedimentation. For western blot, $5 \mu\text{g}$ of protein was loaded on 3–8% SDS/PAGE gels and transferred to nitrocellulose membranes after electrophoresis. The membranes were blocked in Li-cor Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) for 1 h followed by incubation in primary antibody at 4°C overnight. Either rabbit polyclonal anti-hydroxynonenal (4-HNE) antibody (1:5000; Alpha Diagnostics International, Inc., San Antonio, TX, USA) or rabbit anti-nitrotyrosine (3-NT) antibody (1:2000; Upstate USA, Inc., Charlottesville, VA, USA) were applied to detect lipid peroxidation and protein nitration, respectively. The Oxy-blots detection kit (Chemicon International, Temecula, CA, USA) was employed to detect protein oxidation (protein carbonyl, PC). For α -spectrin breakdown products, mouse monoclonal anti- α -spectrin antibody (1:5000; Biomol International, LP Plymouth, PA, USA) was applied. The slot and western immunoblots were all analyzed using the Li-Cor odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE), which employs IRDye800 conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000, Rockland, Gilbertsville, PA, USA) as the secondary antibodies.

In a second part of the first experiment, 2 sham, 2 vehicle-treated and 2 tempol-treated (300 mg/kg i.p.), were overdosed with sodium pentobarbital (150 mg/kg) and perfused with 150 ml of 0.1 M PBS followed by 200 ml of 4% paraformaldehyde in 0.1 M PBS at 24 h after injury.

A 10 mm spinal cord segment containing the T10 impact epicenter was then harvested. After harvesting, the spinal cords were immersed in 4% paraformaldehyde in PBS for 4 h and then transferred into PBS overnight. The next morning, the spinal cords were cryopreserved in phosphate-buffered 20% sucrose for 2 days. A 5 mm portion of the injured spinal cords containing the epicenter from vehicle and tempol-treated injured and sham, uninjured animals were cryostat-sectioned in parallel at 20 μ m thickness in a transverse plane and every 5th section was transferred onto Superfrost plus slides (Fisher Scientific International, Inc., Hampton, NH, USA). After collection the sections onto the slides, the slides were placed on a tray and stored at 4 °C overnight to dehydrate after which they were stored at -20 °C until staining.

On the day of staining, the slides were removed from the freezer and thawed at room temperature for 30 min. After rinsing with 0.2 M PBS, the sections were incubated in 3% hydrogen peroxide in 0.2 M PBS for 30 min followed by incubation in blocking buffer (5% goat serum, 0.25% Triton-X, 1% powdered milk in 0.2 M PBS) for 1 h followed by exposure to the rabbit polyclonal anti-3-NT (1:2000) overnight. The following day, the sections were incubated for 2 h with biotinylated goat anti-rabbit secondary antibody (1:200; Vector ABC-AP Kit; Vector Labs, Burlingame, CA, USA). After rinsing, the sections were incubated in VECTASTAIN ABC reagent (Vector Labs) for 1 h followed by development of the staining using the Vector blue method (Vector Blue Alkaline Phosphatase Substrate Kit; Vector Labs) in the dark for 30 min. After reaction, spinal cord sections were counter-stained with nuclear fast red (Vector Labs), dehydrated and then photographed on an Olympus Provis A70 microscope with an Olympus Magnafire digital camera (Olympus America, Inc., Melville, NY USA).

Experiment 2: effects of tempol on post-traumatic mitochondrial dysfunction

In Experiment 2, the effects of tempol were examined on 24 h post-traumatic mitochondrial dysfunction. For this purpose, an additional 18 rats were randomly allocated into 3 groups ($N=6$ /group): sham, injured plus vehicle and injured plus tempol. At 24 h after laminectomy or laminectomy plus SCI, a 1.5 cm section of the spinal cord centered on the T10 spinal cord segment and containing the immediately surrounding rostral and caudal tissue was rapidly removed. The 24 h timepoint was chosen based upon it having been shown to be the time at which the peak of post-traumatic spinal cord mitochondrial dysfunction is seen in the currently employed SCI model (Sullivan et al., 2007).

Mitochondrial Ficoll gradient purification—Harvested spinal cord segment was homogenized in a Potter-Elvehjem homogenizer containing 2 ml of ice-cold isolation buffer (215 mmol/L mannitol, 75 mmol/L sucrose, 0.1% BSA, 20 mmol/L HEPES, adjusted to a pH of 7.2 with KOH) with 1 mmol/L EGTA. The homogenate was subjected to differential centrifugation at 4 °C. First, it was centrifuged twice at 1300 $\times$ g for 3 min in an Eppendorf microcentrifuge at 4 °C to remove cellular debris and nuclei. Then the pellet was discarded, and the supernatant was further centrifuged at 13,000 $\times$ g for 10 min. The crude mitochondrial pellet so obtained was then subjected to nitrogen decompression in a cell disruption bomb (Parr Instrument Company, Moline, IL) to release synaptic mitochondria and cooled to 4 °C under a pressure of 1200 psi for 10 min (Singh et al., 2006a,b). After nitrogen disruption, the mitochondria were placed atop a discontinuous Ficoll gradient (7.5%, 10%), and centrifuged at 100,000 $\times$ g for 30 min (Singh et al., 2006a,b, Sullivan et al., 2004). The mitochondrial pellets containing both synaptic and non-synaptic mitochondria at the bottom were then transferred to microcentrifuge tubes and topped off with isolation buffer without EGTA and centrifuged at 10,000 $\times$ g for 5 min at 4 °C to yield a tighter pellet (Singh et al., 2006a,b). The final mitochondrial pellet was re-suspended in 35 μ g isolation buffer without EGTA. The

mitochondrial protein concentration was determined using a BCA protein assay kit with a BioTek Synergy HT plate reader (Winooski, VT).

Mitochondrial respiration measurement—Mitochondrial respiratory rates were measured using a Clark-type electrode in a continuously stirred sealed and thermostatically controlled chamber (Oxytherm System, Hansatech Instruments Ltd., Norfolk, England, UK) maintained at 37 °C as described previously (Singh et al., 2006a, Sullivan et al., 2004). Thirty microliters of isolated mitochondrial protein was placed in a chamber containing 250 μ L of KCl-based respiration buffer (125 mmol/L KCl, 2 mmol/L $MgCl_2$, 2.5 mmol/L KH_2PO_4 , 0.1% BSA, and 20 mmol/L HEPES at pH 7.2) and allowed to equilibrate for 1 min. State II respiration was then fueled by addition of the Complex 1 substrates, 5 mmol/L pyruvate and 2.5 mmol/L malate and the resulting rate of oxygen utilization was followed for 2 min. Next, two boluses of 150 μ mol/L ADP were added to the mitochondria to initiate the State III respiratory rate for 2 min, followed by the addition of 2 μ mol/L oligomycin, an inhibitor of ATPase, to monitor the State IV respiration rate for another 2 min. For the measurement of the uncoupled respiratory rate (state V), 2 μ mol/L of the mitochondrial uncoupler FCCP was added to the mitochondria in the chamber, and oxygen consumption was monitored for 2 min, followed by the addition of rotenone (1 μ M) to shut down Complex 1-driven respiration completely. Complex 2-driven respiration is then initiated by the addition of 10 mmol/L of the Complex 2 substrate succinate (Singh et al., 2006a). Fresh mitochondria (mix of synaptic and non-synaptic) were prepared and run for respiration immediately for each experiment to assess the mitochondrial bioenergetics.

Experiment 3: effects of tempol on post-traumatic calpain-mediated cytoskeletal proteolysis

In a third experiment, the effects of tempol were investigated in regards to the degree of post-traumatic calpain-mediated degradation of the cytoskeletal protein α -spectrin as previously described (Xiong et al., 2007). Eighteen rats were randomly allocated into 3 groups ($N=6$ /group): sham, injured plus vehicle and injured plus tempol. At 24 h after laminectomy or laminectomy plus SCI, a 1.5 cm section of the spinal cord centered on the T10 spinal cord segment and containing the immediately surrounding rostral and caudal tissue was rapidly removed at 24 h after laminectomy or SCI. From this larger piece, a 10 mm spinal cord (SC) section at the injury epicenter was dissected and homogenized in ice cold buffer (20 mmol/L Tris-HCL, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EGTA, 10 mmol/L EDTA, 20 mmol/L HEPES, 10% glycerol) containing protease inhibitor (Roche Inc., Nutley, NJ, USA). Following sonication, the dissolved proteins were centrifuged at 15,000 $\times$ g for 30 min and the supernatant was collected and normalized to 1 μ g/ μ L by protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA).

Western blot analysis for α -spectrin breakdown—Fifteen micrograms of each sample was run on SDS/PAGE [3–8% (w/v) acrylamide, Bio-Rad Criterion XT precast gel] with a Tris-Acetate running buffer system and then transferred to nitrocellulose membranes using a semi-dry electrotransferring unit (Bio-Rad Laboratories, Hercules, CA) at 20 mA for 15 min. The blots were probed with mouse monoclonal anti- α -spectrin antibody (1:5000, Affiniti, Inc., Ft. Lauderdale, FL; now part of Biomol, International, LP) which recognizes an epitope that is common to the 280 kD parent α -spectrin as well as each of the 150 kD and 145 kD proteolytic fragments. Exposure to the primary antibody was followed by application of the secondary IRDye800 conjugated goat anti-mouse IgG (1:5000, Rockland, Gilbertsville, PA) for 1 h in the darkness. Imaging analysis of Western blots was done using the Li-Cor Odyssey Infrared Imaging System, to quantify the content of the 145 and 150 kD α -spectrin breakdown products (SBDP 145 and SBDP 150). Each western blot included a standardized protein loading control to allow for correction in regards to intensity differences from blot to blot.

Experiment 4: therapeutic window for the effect of tempol on post-traumatic calpain-mediated cytoskeletal proteolysis

In a final experiment, the therapeutic window for the neuroprotective effects of tempol in acute SCI was assessed using post-traumatic calpain-mediated α -spectrin degradation as a surrogate endpoint for axonal degeneration. A cohort of 36 animals was randomized to 6 groups of rats ($N=6$ /group): sham, injured plus vehicle treatment, injured plus 5 min post-injury tempol (300 mg/kg i.p.), injured plus 1 h tempol, injured plus 2 h tempol and injured plus 4 h tempol. At 24 h, after injury, spinal cord samples were harvested as described above and analyzed for levels of SBDP 145.

Statistical analysis

Statistical analyses were performed using the STATVIEW package (JMP Software, Cary, NC). All experimental values are expressed as mean $\pm$ standard error (SEM). In each experiment, a one-way analysis of variance (ANOVA) was first run. If the ANOVA revealed a significant effect, Student–Newman–Keuls (SNK) post-hoc testing was then carried out to compare individual pairings of the tempol-treated, vehicle (saline)-treated and sham groups. In all cases, a $p<0.05$ was considered significant.

Results

Tempol effects on peroxynitrite-mediated oxidative damage after SCI

Slot-immunoblot and western blot analysis—Three oxidative damage mechanisms were evaluated: protein nitration, lipid peroxidation and protein oxidation involved in PN-mediated oxidative damage following SCI (Xiong et al., 2007). The specific marker for PN formation 3-nitrotyrosine (3-NT), the lipid peroxidation product 4-hydroxynonenal (4-HNE) and protein oxidation-derived protein carbonyls (PC) were employed as markers for these oxidative damage events. Animals were sacrificed 24 h post-injury when the maximal oxidative damage had been previously shown to occur (Xiong et al., 2007). Fig. 1 shows the results obtained from quantitative immuno-slotblotting. For each oxidative marker, one-way ANOVA revealed that there was a significant effect associated with treatment (for 3-NT: $F(2,15)=30.677$; $p<0.0001$]; for 4-HNE: [$F(2,15)=14.02$; $p<0.001$]; for PC: [$F(2,15)=54.616$; $p<0.0001$]). Subsequent SNK analysis showed that injury resulted in significantly increased levels of 3-NT, 4-HNE and PC compared to the sham group, and that the 300 mg/kg tempol administration reduced the injury-induced elevations of 3-NT and 4-HNE significantly compared to the saline-treated vehicle group ($\#p<0.0001$, $n=6$). However, the beneficial effects of tempol were partial as 3-NT and 4-HNE levels remained significantly higher compared to the sham group ($*p<0.001$ vs. sham, $n=6$). A stronger protective effect of tempol was observed in terms of protein oxidation since tempol was able to bring the PC level back to normal ($\#p<0.0001$ vs. vehicle, $n=6$).

Western blot analysis of PN-mediated oxidative damage was also carried out to further confirm the effects of tempol. Representative blots of 3-NT, 4-HNE and PC shown in Fig. 2A provide a clear view of different staining patterns and side-by-side qualitative comparisons of protein nitration, lipid peroxidation and protein oxidation among the different groups. As demonstrated, injury (I) produced a rise in staining intensity for all markers compared to the sham groups (S) and tempol treatment (300 mg/kg i.p.) (T) reduced the degree of injury-induced staining. The results (Fig. 2B) are in agreement with what we obtained from slot blotting methods. One-way ANOVA indicated a significant effect associated with treatment: for 3-NT: [$F(2,15)=14.697$; $p<0.001$]; for 4-HNE: [$F(2,15)=12.550$; $p<0.001$]; and for PC: [$F(2,15)=32.515$; $p<0.0001$]). Post-hoc analysis showed injury produced significant increases in 3-NT, 4-HNE and PC levels compared to the sham group ($*p<0.05$, $n=6$). Tempol decreased injury-induced oxidative damage significantly ($\#p<0.05$, $n=6$). However, similar to the immuno-

slotblot measurements, the protective effects of tempol were partial since the tempol-treated groups still remained significant higher in regards to 3-NT, 4-HNE and PC when compared with the sham groups.

Immunohistochemical analysis—Fig. 3 displays the spinal cord cross sectional illustration of the intensity and spatial distribution of 3-NT immunostaining at 24 h post-injury. The 3-NT immunoreactivity was compared between a saline vehicle (Fig. 3A) and a tempol-treated (Fig. 3B) spinal cord, side by side. Intense 3-NT immunostaining is observed prominently in the gray matter, suggesting the gray matter is especially susceptible to PN-induced oxidative damage, which is not just limited to the injury epicenter but also spreads for some distance along the spinal cord. As displayed, extensive 3-NT immunoreactivity is still remarkable at the injured sections 2 mm rostral or caudal to the epicenter. In comparison, less 3-NT immunoreactivity in the gray and white matter were observed at the epicenter of tempol-treated spinal cord. Tempol administration clearly limited the 3-NT immunoreactivity observed in the sections 2 mm rostral and caudal to the epicenter.

Tempol effects on mitochondrial dysfunction after SCI

Previous studies have provided evidence that spinal cord mitochondrial respiratory functional impairment is at least partly attributable to PN-mediated oxidative damage (Sullivan et al., 2007, Xiong et al., 2007). In this study, we next determined whether tempol can protect mitochondria from PN toxicity following SCI. Mitochondrial bioenergetics were assessed by measuring oxygen consumption (respiration) of isolated mitochondria from four experimental groups including sham non-injured, injured plus saline-treated, injured plus 150 mg/kg tempol-treated and injured plus 300 mg/kg tempol-treated animals. Fig. 4 demonstrates the effects of tempol on mitochondrial respiration after SCI in terms of respiratory control ratio (RCR). A representative respiratory trace is shown in Fig. 4A for better understanding RCR and respiratory states (II–V). As illustrated, addition of pyruvate and malate (P/M) fuel electron transport system (ETS) Complex 1 initiates respiratory state II; 2 boluses of ADP activate ATP synthase and initiate State III; oligomycin is an inhibitor of ATP synthase, so little ADP or O₂ is consumed after addition of oligomycin, which is named State IV. FCCP is an uncoupler that dissipates the proton gradient across the mitochondrial inner membrane and ETS then works at its maximal extent to make up the loss of the gradient, thus forming Complex 1-driven State V. Addition of succinate fuels Complex 2-driven State V.

The respiratory control ratio (RCR) is a widely applied index in mitochondrial functional analysis since it is a sensitive measure of mitochondrial respiration and how well the ETS is coupled to oxidative phosphorylation. It is calculated as the ratio of the respiratory rate after the addition of the second bolus of ADP (State III) to the respiratory rate upon the addition of oligomycin (State IV). Mitochondria are considered healthy if their RCR is >5.0. A representative overlay of respiratory traces in Fig. 4B demonstrated the impaired oxygen consumption of SC mitochondria isolated from injured vehicle-treated rats and improved respiration of mitochondria isolated from injured tempol-treated (300 mg/kg) animals. Quantification of RCR shown in Fig. 4C indicated that mitochondria isolated from sham rats were metabolically intact and well coupled, with a RCR>7.0. A one-way ANOVA revealed a significant effect of treatments post-injury [$F(3,20)=31.501$, $p<0.0001$]. The RCR dropped significantly 24 h post-injury with the mean value decreased from 7.4 down to 2.9 ($*p<0.0001$ vs. sham, $n=6$). A single dose of tempol (150 mg/kg, i.p.) significantly improved mitochondrial bioenergetics by partially maintaining the mean RCR to 4.5 ($^{#}p<0.001$ vs. vehicle, $n=6$) (Fig. 4C). A higher dose of tempol (300 mg/kg) showed a slightly better effect by increasing the mean RCR to 4.7 ($^{#}p<0.001$ vs. vehicle, $n=6$). However, both tempol-treated groups attenuated injury-induced mitochondrial dysfunction only partially, with RCRs remaining significantly lower compared to the sham group ($*p<0.001$, $n=6$). A more detailed quantification of

mitochondrial bioenergetics (Fig. 5) shows significant injury effects in regards to each of the different mitochondrial respiratory states except Complex 2-driven state V, indicating that components of the ETS downstream of Complex 1 were still functioning (State II: [$F(3,20)=3.515, p<0.05$]; State III: [$F(3,20)=13.634, p<0.0001$]; State IV: [$F(3,20)=15.979, p<0.0001$]; Complex 1-driven state V: [$F(3,20)=4.887, p<0.0001$]). Post-hoc analysis with SNK indicated a significant decrease in state III respiration (RCR numerator) and increase in state IV (RCR denominator) 24 h post-injury. Both tempol doses increased state III and decreased state IV with a significant difference compared to the vehicle-treated group, indicating better capacity for ATP production and coupling ($^{\#}p<0.001, n=6$). However, state IV and Complex 1-driven state V in the tempol-treated groups were still significantly lower compared to the sham group, suggesting that the improved maintenance of mitochondrial function was significant, but incomplete. No significant differences in Complex 2-driven state V respiration could be detected across the experimental groups.

Tempol effects on calpain-mediated α -spectrin breakdown after SCI

Peroxyntirite-mediated oxidative damage and mitochondrial dysfunction results in intracellular Ca^{2+} overload, which activates the intracellular protease calpain leading to cytoskeletal protein (α -spectrin) degradation. Two fragments (145 kD and 150 kD) are formed due to the proteolysis of α -spectrin (Wang, 2000). Fragment 145 kD (SBDP 145) is generated specifically by calpain and therefore considered as an indicator of calpain activation. In contrast, SBDP 150 is generated by two enzymes: calpain and another cysteine protease caspase 3. Two groups of western blots are shown in Fig. 6A. The three bands from the top to the bottom are intact spectrin (280 kD), SBDP 150 and SBDP 145 respectively. As shown, an elevated level of calpain-specific SBDP 145 was observed following injury, and tempol administration (300 mg/kg, i.p.) reduced the SBDP 145 accumulation. Quantification of SBDP 145 (Fig. 6B) shows injury induced a ten-fold increase of this fragment ($^*p<0.0001$ vs. sham, $n=6$), whereas this Ca^{2+} -activated calpain-mediated spectrin proteolysis was significantly decreased in the tempol group ($^{\#}p<0.0001$ vs. vehicle, $n=6$). Quantification of the mixed calpain/caspase 3-generated SBDP 150 (Fig. 6C) indicated that tempol also reduced this breakdown fragment significantly ($^{\#}p<0.001$ vs. vehicle, $n=6$).

Therapeutic time window for tempol neuroprotection

Evidence from our studies has shown that a single dose of tempol acutely administered after SCI effectively protects spinal cord against PN-induced oxidative damage, mitochondrial dysfunction and spectrin breakdown, and improves fine hindlimb locomotor recovery. We then carried out experiments to determine the neuroprotective therapeutic time window using calpain-mediated α -spectrin degradation as a surrogate marker for spinal axonal neurodegeneration. Treatment was begun at the time of injury or after a 1, 2 or 4 h delay and the attenuation of calpain-activated cytoskeletal breakdown by a single dose of tempol (300 mg/kg, i.p.) was assessed at 24 h post-injury. A representative western blot is displayed in the upper panel of Fig. 7. As shown, injury induced a large accumulation of calpain-generated SBDP 145 that was attenuated in the acutely-treated tempol (<5 min post-injury) group and decreased to some extent in 1 h delay group. There was no such effect of tempol following a treatment delay of 2 or 4 h. Quantification of SBDP 145 is showed in the lower panel and a one-way ANOVA revealed a significant effect of treatments post-injury [$F(3,20)=8.659, p<0.0001$]. SBDP 145 fragments in all vehicle or tempol-treated groups were significantly higher compared to shams ($^*p<0.001, n=6$). Tempol treatment immediately post-injury significantly attenuated calpain-mediated proteolysis ($^{\#}p<0.001$ vs. vehicle, $n=6$), which is consistent with our earlier experiments (Fig. 6). Although there is a downward trend in the 1 h delay group observed in the blot, post-hoc SNK testing indicated no significant difference compared to the vehicle-treated injured group. Moreover, tempol treatment starting at either 2 or 4 h post-injury produced no reduction in α -spectrin breakdown when compared to the vehicle

group. This suggests that tempol by itself has a very short neuroprotective therapeutic window of ≤ 1 h.

Discussion

In the present study, a thorough analysis has been conducted to evaluate the therapeutic effects of tempol in regard to its ability to attenuate 1) PN-mediated oxidative damage; 2) mitochondrial dysfunction and 3) calpain-mediated proteolysis following spinal cord contusion injury in rats. The neuroprotective efficacy of tempol, a compound that possesses the ability to catalytically scavenge PN-derived free radicals (Carroll et al., 2000), strongly supports a pivotal role of PN-mediated oxidative damage in post-SCI pathophysiology, as we have previously hypothesized (Xiong et al., 2007). In addition, the current data helps to establish a mechanistic relationship between PN-induced oxidative damage, downstream spinal cord mitochondrial dysfunction and Ca^{2+} -activated, calpain-mediated cytoskeletal degradation after SCI. More importantly, the effectiveness of tempol strongly suggests that PN is an appropriate therapeutic target for preventing secondary injury after SCI. In the current study, we did not examine tempol effects on chronic locomotor recovery or spinal cord tissue sparing in the spinal cord-injured animals. However, we believe that our short term protective effects on oxidative damage, mitochondrial dysfunction and cytoskeletal degradation seen with a 300 mg/kg i.p. dose administered at 5 min post-injury, correlate well with the previous demonstration by another laboratory that single administration of a slightly lower dose (275 mg/kg i.p. at 20 min post-injury) significantly improves locomotor recovery together with an increase in spinal cord tissue sparing (Hillard et al., 2004).

Although a few other studies have also shown beneficial effects of tempol in various CNS injury models (Beit-Yannai et al., 1996, Cuzzocrea et al., 2000, Hillard et al., 2004), none of those explored the molecular mechanisms involved. In those earlier reports, it was suggested, but not demonstrated, that the protective effects of tempol were due to its ability to scavenge superoxide radicals ($\text{O}_2^{\cdot-}$) by acting as an SOD mimetic. However, with the recognition of the probable role of PN-mediated oxidative damage in secondary injury (Deng-Bryant et al., 2008, Deng et al., 2007, Genovese et al., 2007, Scott et al., 2005, Xiong et al., 2007), and tempol's potent catalytic scavenging of PN-derived radicals $\cdot\text{NO}_2$ and $\text{CO}_3^{\cdot-}$ (Carroll et al., 2000), this latter mechanism is more plausible. Consistent with this mechanistic viewpoint, tempol reduced the levels of 3-NT, a widely-accepted biomarker for PN-mediated cellular injury (Alvarez et al., 2002, Denicola and Radi, 2005, Radi et al., 2002a,b). Since PN-derived radicals can also lead to lipid peroxidative cellular damage (Radi et al., 1991) and direct protein oxidation, two other oxidative markers, 4-HNE and protein carbonyls, were also used to confirm tempol's antioxidant efficacy. Our results from quantitative slot and western blot analyses showed that acute treatment of animals with a single dose of tempol effectively reduced PN-mediated oxidative damage 24 h after SCI in terms of a decrease in 3-NT, 4-HNE and protein carbonyl levels. Immunohistochemical detection of reduced 3-NT formation confirmed that tempol protected against PN-induced oxidative damage following injury.

Mitochondria have been shown to be a key source of PN formation and a target of its free radical-mediated damaging effects (Bringold, 2000, Singh et al., 2006a, Stewart et al., 2002, Valdez et al., 2000). Peroxynitrite has been reported to increase mitochondrial proton leak and electron transport uncoupling (Echtay et al., 2003). In vitro studies from our laboratory with isolated brain mitochondria have shown that PN (generated by the PN-generating compound SIN-1) increased levels of 3-NT, along with an elevation in LP-induced 4-HNE and protein carbonyls in mitochondrial proteins coincident with an impairment of respiratory function (Singh et al., 2007). In the currently employed SCI model, a progressive increase in mitochondrial oxidative damage, including increased 3-NT, was observed prior to the loss of mitochondrial bioenergetics as previously shown (Sullivan et al., 2007). Collectively, these

studies strongly suggest that PN-derived free radical damage may be a major cause for post-traumatic mitochondrial dysfunction. Accordingly, we then evaluated the efficacy of tempol to antagonize mitochondrial dysfunction after SCI. Our results showed that tempol, administered immediately after injury, preserved mitochondrial function measured at 24 h following SCI. Quantification of mitochondrial respiration states showed that tempol improved mitochondrial bioenergetics indicative of better preserved ATP production capacity and coupling of ADP phosphorylation to electron transport, which is reflected by the increase in state III and decrease in state IV, respectively. In particular, the PN-directed damage and the protective effects of tempol are focused on complex I-driven respiratory function and ATP production since no differences in complex-II driven respiration were detected between mitochondrial samples from sham (non-injured) and injured animals. This is consistent with the fact that complex I components are more susceptible to oxidative damage than those in complex II (Martin et al., 2005, Starkov et al., 2002, Sullivan et al., 2007).

Secondary neuronal degeneration in the injured spinal cord begins with a depolarization-initiated excitotoxic insult involving massive release of glutamate and over-activation of the glutamatergic NMDA receptor that leads to intracellular Ca^{2+} overload (Stout et al., 1998). The early increase in intracellular Ca^{2+} levels is buffered by mitochondria, which act as a Ca^{2+} sink which plays a major role in maintaining intracellular homeostasis (Crompton, 1999, Kristal and Dubinsky, 1997). In the present study, we hypothesized that PN-oxidative damage and subsequent mitochondrial dysfunction contributes to the disruption of mitochondrial and other Ca^{2+} homeostatic mechanisms following SCI, resulting in excessive activation of the Ca^{2+} -activated proteolytic enzyme calpain and degradation of a large number of neuronal proteins (Bartus, 1997). We previously reported the temporal characteristics of the calpain-mediated breakdown of the neuronal cytoskeletal protein α -spectrin after spinal cord contusion injury (Xiong et al., 2007), indicating cytoskeletal degradation is indeed an important post-traumatic pathological event following SCI as has already been shown by others (Banik and Shields, 2000, Ray et al., 2003). In the current study, we investigated the effect of tempol on post-traumatic α -spectrin breakdown and observed that tempol significantly decreased the levels of the calpain-specific SBDP 145 and the mixed calpain/caspase SBDP 150 after injury, with greater effects observed in SBDP 145, suggesting that tempol's cytoskeletal-protective effect is mostly due to the attenuation of calpain activation (Wang, 2000). However, the present experiments do not rule out a contribution of caspase-3 activation in the degradation of the cytoskeleton, nor do they eliminate the possible reduction in caspase-3 activation as being part of the protective effect of tempol. Indeed, PN can activate caspases 3, 2, 8, 9 and lead to delayed, programmed apoptotic cell death (Vicente et al., 2006, Virag et al., 1998, Zhuang and Simon, 2000).

Despite the beneficial effects of early administration of tempol on oxidative damage, mitochondrial dysfunction and calpain-mediated α -spectrin proteolysis, our therapeutic window analysis using calpain-mediated α -spectrin degradation as a surrogate marker for axonal degeneration revealed that treatment with a single tempol dose must be undertaken within the first hour after injury which will be difficult to achieve in most acute SCI patients. Nevertheless, the protective efficacy of this compound strongly supports our hypothesis that PN-mediated oxidative damage is an early post-traumatic event that contributes to mitochondrial dysfunction, intracellular Ca^{2+} overload, cytoskeletal degradation and neurodegeneration and to improve neurological recovery and spinal cord tissue sparing as reported by others (Hillard et al., 2004). The relationship between these effects is schematically illustrated in Fig. 8. On the other hand, our finding of a short therapeutic window in terms of attenuation of cytoskeletal degradation is at odds with the results of Hillard et al. (2004) who found that even a delay in single dose tempol administration until 2 days post-injury was still able to significantly improve neurological recovery at least as well as early (20 min post-injury) treatment. That group, however, did not show that this beneficial effect on motor function was

associated with an increase in tissue sparing which they did not look at except in the group of animals that received tempol early. This claim that tempol, which is only known to possess free radical scavenging properties, can still have a benefit after a 2 day post-injury delay is incongruous with our previous demonstration in the rat contusion SCI model of the rapidly evolving time course of PN-mediated oxidative damage (Xiong et al., 2007), mitochondrial dysfunction (Sullivan et al., 2007) and calpain-mediated cytoskeletal degradation (Xiong et al., 2007), each of which peak or nearly peak by 24 h after SCI.

In any event, the present experiments do not rule out the possibility that more aggressive repeated dosing strategies (e.g. an i.v. bolus plus i.v. infusion) with tempol or other PN radical scavengers might display a more practical therapeutic window. Moreover, it is logical to contemplate the use of mechanistically-complimentary multi-drug strategies in which a tempol-like compound would be combined with compounds that prevent mitochondrial failure by inhibition of the mitochondrial permeability transition pore such as cyclosporine A (Rabchevsky et al., 2001) or its non-immunosuppressive analog NIM811 (McEwen et al., 2007) and/or compounds that directly inhibit calpain activation (Banik and Shields, 2000, Ray et al., 2003). Such a dual or triple mechanistic strategy may constitute a more pronounced and temporally practical neuroprotective approach.

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Fig. 1.

Quantitative slot immunoblotting showed tempol treatment reduced PN-induced oxidative damage. 3-nitrotyrosine (3-NT) is an indicator for PN-mediated nitration of protein tyrosine residues, 4-hydroxynonenal (4-HNE) is a marker for lipid peroxidation and protein carbonyl is a marker for protein oxidation. Injury induced significant increases in PN footprint 3-NT, lipid peroxidation byproduct 4-HNE and protein oxidation product carbonyls. Single dose of tempol (300 mg/kg, i.p.), a PN-derived free radical scavenger, was able to significantly decrease the accumulations of these oxidative markers 24 h post-injury (Values=mean $\pm$ standard error; * p <0.001, vs. sham, # p <0.0001, vs. vehicle, n =6/group).

Fig. 2. (A) Representative western blots showing staining patterns of 3-NT, 4-HNE and protein carbonyls. S, I, T, stand for the sham, vehicle and tempol-treated groups (300 mg/kg, i. p.), respectively. Compared to sham group, more intense staining patterns are observed in vehicle groups and tempol treatment attenuated the immunoreactivity. (B) Densitometric quantification of 3-NT, 4-HNE and protein carbonyls (across various molecular weight ranges included in the black box in each lane) showed significant injury-induced increases in PN formation, lipid peroxidation and protein oxidation which were each decreased by the PN-derived radical scavenger tempol compared to saline-treated injured group (values=mean $\pm$ standard error; * p <0.05, vs. sham, # p <0.05, vs. vehicle, n =6/group).

Fig. 3.

Tempol administration partially protected spinal cord from PN-induced oxidative damage following spinal cord injury. Representative cross sections of spinal cords subjected to contusion injury and treated with saline (vehicle, top) or tempol (bottom) are shown. In vehicle-treated injured group, intense 3-NT staining, mainly in the gray matter, can be observed throughout the spinal cord up to 2 mm rostral and caudal to the epicenter. Corresponding tempol-treated sections appeared to show better structural integrity and less 3-NT staining compared to saline-treated injured sections. Note: tissue disruption in three left hand vehicle-treated sections is due to fragility of the sections during sectioning and staining, not in vivo cavitation; tempol treated spinal cord shows less tissue fragility. Scale bar: 0.5 mm.

Fig. 4.

(A) A typical respiratory trace shows normal mitochondrial respiration. Mitochondrial oxygen consumption was measured using a Clark-type electrode in a continuously stirred sealed chamber (Oxygraph System; Hansatech Instruments Ltd.). Purified mitochondrial protein was suspended in respiration buffer in a final volume of 250 μ L. As indicated in the figure, RCR is the rate of respiration in the presence of ADP (state III) versus rate of respiration in the absence of ADP (state IV). Electron transport system (ETS) capacity is indicated by the rate of respiration in the presence of the uncoupler FCCP (state V). (B) Mitochondrial isolated 24 h after injury from the spinal cord show a reduction of RCR and a loss of ETS capacity. Tempol-treated group (300 mg/kg, i.p.) improved RCR by increasing the rate of state III and decreasing the rate of state IV, suggesting an attenuation of injury-induced mitochondrial dysfunction. (C) Quantification of RCR showed a significant drop after SCI and tempol administration increased RCR significantly at both dosages (150 mg/kg and 300 mg/kg), with a slightly better effect observed in 300 mg/kg treated group (Values=mean $\pm$ standard error; * p <0.001, vs. sham, # p <0.001, vs. vehicle, n =6/group).

Fig. 5.

Tempol partially preserved mitochondrial function following SCI. The X axis shows mitochondrial respiratory rates following SCI, expressed in nanomoles of oxygen per milligram of isolated mitochondrial protein. See text for detailed explanation. Quantification of state II, III, IV and complex 1-driven state V showed impaired mitochondrial respiration in injured groups. Tempol treatments prevented mitochondrial dysfunction by increasing state III (RCR numerator) and decreasing state IV (RCR denominator), indicating better coupling and respiration. Tempol was also able to increase complex 1-driven state V (Values=mean $\pm$ standard error; * p <0.001, vs. sham, # p <0.001, vs. vehicle, n =6/group). Note that complex 2-driven state V was not altered among any experimental groups.

Fig. 6.

Effect of tempol on calpain-mediated α -spectrin breakdown following SCI measured by quantitative western blot analysis. (A) Representative blot is showed and the bands from top to bottom demonstrated intact spectrin (280 kd), spectrin breakdown fragments SBDP 145 and SBDP 150, respectively. SBDP 145 is entirely attributable to calpain whereas SBDP 150 is produced by calpain and caspase-3. Quantification indicated that injured resulted in a significant increase of SBDP 145 (B) and SBDP 150 (C) 24 h post-injury. Tempol reduced the accumulations of both spectrin fragments, with a greater protective effect observed on SBDP 145 (values=mean $\pm$ standard error; * p <0.0001 vs. sham; # p <0.001 vs. vehicle, n =6/group).

Fig. 7.

Therapeutic time window for tempol's efficacy to decrease α -spectrin breakdown. (A) Representative western blots demonstrated different extent of α -spectrin breakdown from six experimental groups including sham, vehicle-treated injured and tempol treated at the onset of injury and with 1 h, 2 h and 4 h delays. (B) Quantification of calpain-generated SBDP 145 shows compared to vehicle group, only tempol treated without delay significantly reduced α -spectrin breakdown, suggesting the therapeutic time window of tempol is less than 1 h (* $p < 0.001$ vs. sham; # $p < 0.001$ vs. vehicle, $n = 6/\text{group}$).

Fig. 8.

Proposed interrelationships between PN-induced oxidative damage in neuronal mitochondria and the compromise of Ca^{2+} homeostasis, calpain-mediated proteolysis and neurological deficits based on the present studies. Tempol, by targeting the upstream PN-derived free radicals, decreases subsequent oxidative damage and respiratory dysfunction in spinal cord mitochondria which ameliorates Ca^{2+} overload, reduces calpain-mediated proteolysis as shown in the present study and decreases neurological deficits as reported by others (Hillard et al., 2004).