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Tempol protection of spinal cord mitochondria from peroxynitrite-induced oxidative damage

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Abstract
Peroxynitrite (PN)-mediated mitochondrial dysfunction has been implicated in the secondary injury process after traumatic spinal cord injury (SCI). This study investigated the detrimental effects of the PN donor SIN-1 (3-morpholinosydnonimine) on isolated healthy spinal cord mitochondria and the protective effects of tempol, a catalytic scavenger of PN-derived radicals. A 5 min exposure of the mitochondria to SIN-1 caused a dose-dependent decrease in the respiratory control ratio (RCR) that was accompanied by significant increases in complex I-driven states II and IV respiration rates and decreases in states III and V. These impairments occurred together with an increase in mitochondrial protein 3-nitrotyrosine (3-NT), but not in lipid peroxidation (LP)-related 4-hydroxynonenal (4-HNE). Tempol significantly antagonized the respiratory effects of SIN-1 in parallel with an attenuation of 3-NT levels. These results show that the exogenous PN donor, SIN-1, rapidly causes mitochondrial oxidative damage and complex I dysfunction identical to traumatic spinal cord mitochondrial impairment and that this is mainly due to tyrosine nitration. Consistent with that, the protection of mitochondrial respiratory function by tempol is associated with a decrease in 3-NT levels in mitochondrial proteins also similar to the previously reported antioxidant actions of tempol in traumatically-injured spinal cord mitochondria.

Keywords: *Tempol, spinal cord, mitochondria, nitrotyrosine, lipid peroxidation, oxidative damage*

Introduction

Over the past few years, there has been increasing appreciation of the role of reactive oxygen species (ROS)-induced mitochondrial dysfunction in the secondary injury cascade following central nervous system (CNS) trauma [1–4]. Among these reactive species, an important player is peroxynitrite (PN) formed by the diffusion-controlled combination of nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet -}$) [5,6]. Peroxynitrite, commonly referred to as a reactive nitrogen species (RNS), exerts its cytotoxic effects through its derived free radicals ($^{\bullet}OH$, $^{\bullet}NO_2$, $CO_3^{\bullet -}$), which lead to oxidative damage by nitrating and oxidizing proteins, lipids (lipid peroxidation, LP) and nucleic acids [7–10]. In particular, PN is a central contributor to protein nitration and its major product 3-nitrotyrosine (3-NT) is widely used as the footprint for PN formation [11]. Previous *in vitro* studies have demonstrated that PN impairs mitochondrial respiration through oxidative damage to multiple targets including: (a) cytochrome c, an important component of electron transport chain (ETC) [12]; (b) NADH: ubiquinone reductase (ETC Complex I) [13] and (3) mitochondrial anti-oxidant defense systems (i.e. manganese superoxide dismutase, glutathione) [14]. In agreement, *in vivo* studies using multiple brain injury models have supported a pivotal role of PN in mediating the progress of secondary injury cascades [15,16]. Additionally, spinal cord injury (SCI)-induced mitochondrial dysfunction is correlated with an increase in oxidative damage including 3-NT in mitochondrial proteins [4,17], strongly suggesting the

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direct linkage of PN and post-traumatic mitochondrial oxidative damage.

There were two purposes for the current study. First of all, we wanted to determine whether exogenous application of PN to normal spinal cord mitochondria could duplicate the characteristics of post-traumatic spinal cord mitochondrial dysfunction documented previously [17,18]. To test this, we applied the PN donor SIN-1 (3-morpholinosydnonimine) directly to healthy spinal cord mitochondria harvested from non-injured rats to evaluate PN's toxic effects on mitochondrial respiration. SIN-1 is capable of simultaneously generating the PN precursors, $^{\bullet}NO$ and $O_2^{\bullet -}$, which form PN at an ultra-rapid diffusion-limited rate [5,6] and it is therefore used as a PN donor for *in vitro* studies [19,20]. In previous work, we have shown that SIN-1 application to normal rat cerebral cortical mitochondria from uninjured brains is able to replicate mitochondrial dysfunction seen after traumatic brain injury (TBI) together with an increase in mitochondrial 3-NT levels [1,3]. However, in regards to the current work, we postulated that spinal cord mitochondria might be more sensitive to PN based upon the knowledge that rat spinal cord mitochondria have a higher baseline level of oxidative stress compared to brain mitochondria [21]. In addition, we wanted to more conclusively define the relative importance of protein nitration, assessed via 3-NT levels, vs LP-related modification of mitochondrial proteins, as measured by the content of the polyunsaturated fatty acid-derived aldehyde 4-hydroxynonenal (4-HNE). Our prior work with isolated brain mitochondria exposed to SIN-1 showed a significant increase in 3-NT, but only a non-significant trend towards an increase in 4-HNE [20], suggesting that protein nitration might be the more critical oxidative damage mechanism in this context.

A second purpose of this investigation was to examine the protective effects of tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a prototypical nitroxide antioxidant [22] which has been shown to possess neuroprotective effects in TBI [23,24] and SCI [18,25] models. It has been proposed that tempol's neuroprotective efficacy is largely due to it's ability to catalytically scavenge PN-derived free radicals [22]. Considering this, we hypothesized that tempol should be able to protect mitochondria from PN-induced oxidative damage. Although we have previously reported that tempol can protect rat brain mitochondria from *in vitro* SIN-1 damage [20], the current study with spinal cord mitochondria included a more complete tempol dose–response analysis in regards to mitochondrial respiratory protection and a more definitive assessment of it's relationship to attenuation of protein tyrosine nitration (3-NT) vs LP-mediated (4-HNE) protein modification.

The results described in this paper demonstrate that SIN-1 delivered PN is able to produce complex I-selective mitochondrial respiratory dysfunction essentially identical to that produced by SCI [18]. This dysfunction is associated with an increased modification of mitochondrial proteins by tyrosine nitration, but not by the LP-related 4-HNE. Furthermore, tempol is able to antagonize SIN-1-induced spinal cord mitochondrial functional impairment together with a complete suppression of the SIN-1 induced 3-NT levels in the mitochondrial proteins. These findings strongly support the hypothesis that PN contributes to post-traumatic mitochondrial failure in the injured spinal cord [4,17,18] and that the ability of tempol to attenuate mitochondrial dysfunction is largely due to protection against PN-mediated nitrative damage to mitochondrial proteins.

Materials and methods

Animals

Experiments were performed with isolated mitochondria from young adult female Sprague–Dawley rats (Charles River, Portage, MI) (200–225 g) that were fed and watered *ad libitum*. The animals were randomly cycling and were not tested for stage of the estrus cycle. The protocols for spinal cord removal and mitochondrial harvesting were approved by the University of Kentucky Institutional Animal Care and Use Committee and are consistent with the NIH Guidelines for the Care and Use of Animals.

Chemicals

Chemicals and mitochondrial respiration substrates including SIN-1 (3-morpholinosydnonimine), tempol (4-hydroxy-TEMPO, free radical), mannitol, sucrose, bovine serum albumin (BSA), ethylene glycol tetraacetate (EGTA), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) potassium salt, potassium phosphate monobasic anhydrous (KH_2PO_4), magnesium chloride ($MgCl_2$), malate, pyruvate, adenosine 5'-diphosphate (ADP), oligomycin A, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone and succinate were purchased from Sigma-Aldrich (St Louis, MO). BCA protein assay kit was obtained from Pierce (Rockford, IL).

Isolation of Ficoll-purified spinal cord mitochondria

Spinal cord mitochondria were extracted as described previously with some modifications [3,4,20,24,25]. Briefly, the rats were decapitated and the spinal cords were quickly squirted out by a 60 ml syringe filled with saline. By using the lumbar enlargement as a landmark, a 2 cm segment of spinal cord containing mid-thoracic region was dissected on a ice-cold glass plate and then homogenized in a Potter-Elvehjem homogenizer containing 2 ml isolation buffer with 1 mmol/L EGTA (215 mmol/L mannitol, 75 mmol/L

sucrose, 0.1% BSA, 20 mmol/L HEPES and 1 mmol/L EGTA at PH 7.2). The homogenate was first centrifuged twice at 1300 × g for 3 min to remove cellular debris and nuclei. Then the supernatant was collected and further centrifuged at 13 000 × g for 10 min. The resulting crude mitochondrial pellet was then subjected to nitrogen decompression to release synaptic mitochondria [4,26]. After nitrogen disruption, the mitochondria were placed on top of a discontinuous Ficoll gradient (7.5%, 10%) and centrifuged at 100 000 × g for 30 min. The mitochondrial pellets were then transferred and centrifuged again to yield a tighter pellet. Mitochondrial proteins were suspended with isolation buffer without EGTA and the concentration was determined using a BCA protein assay kit with a BioTek Synergy HT plate reader (Winooski, VT).

Mitochondrial respiration studies

Figure 1 shows an illustration of mitochondrial electron transport chain (ETC) and oxidative phosphorylation. The upper panel (Figure 1A) displays a typical respiratory trace of spinal cord mitochondrial respiration. The lower panel (Figure 1B) shows simplified pathways of electron transport through ETC. Briefly, electrons are fed into Complex I (NADH dehydrogenase) by pyruvate and malate or into Complex II (succinate dehydrogenase) by succinate, transported through Complex III (cytochrome reductase), cytochrome c and finally reach Complex IV (cytochrome oxidase). Each electron is accepted by O_2 reducing the latter to H_2O. As the electron flows, protons (H^+) are pumped from the matrix into the mitochondrial intermembrane (IMM) space, thus forming a proton gradient across the IMM. When H^+ flows back into the matrix through ATP synthase (Complex V), ADP is phosphorylated to ATP. Knowledge of this oxidative phosphorylation process is crucial to an understanding of the subsequent mitochondrial respiration studies we employed (Figure 1B).

Mitochondrial respiratory rates were measured using a Clark-type electrode as described previously [4,17,20,24,25]. Briefly, 30–35 μg of mitochondrial protein was loaded into a constantly stirred, thermostatically controlled (37°C) chamber (Oxytherm System, Hansatech Instruments Ltd) containing 250 μL 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

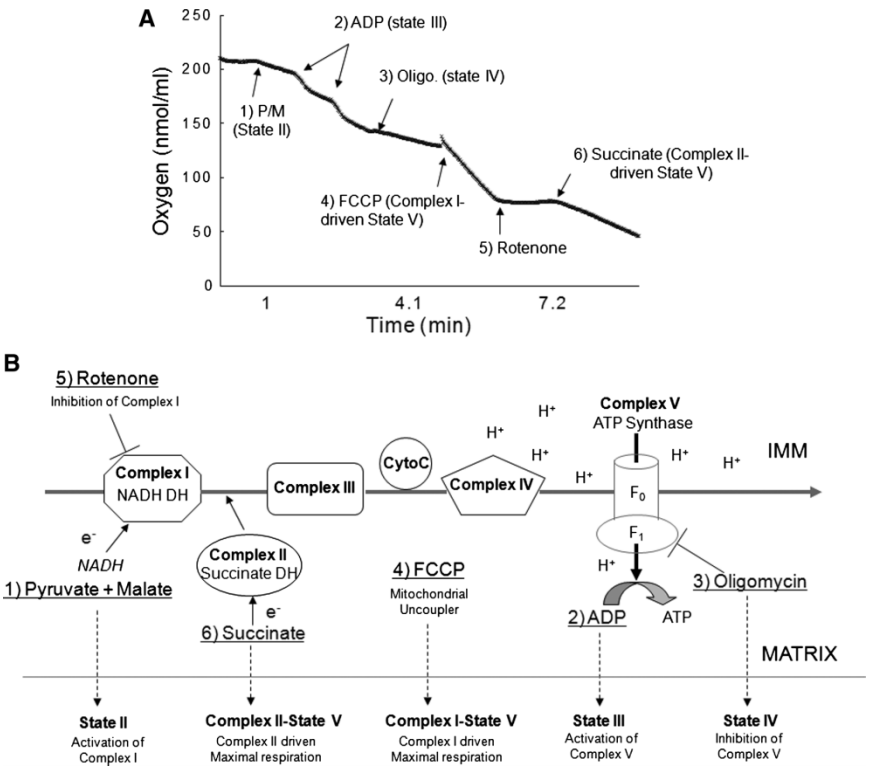

Figure 1. (A) A representative trace demonstrates the typical respiratory responses of healthy spinal cord mitochondria to a series of substrates. Addition of pyruvate and malate fuel Complex I and initiates respiratory state II; then 2 boli of ADP activate ATP synthase and initiate State III. Next, oligomycin, which is an inhibitor of ATP synthase, largely inhibits respiration so little ADP or O_2 is consumed; this is called state IV. FCCP, which is a mitochondrial uncoupler, dissipates the proton gradient and electron transport then works at its maximal extent to make up the loss of the gradient, therefore initiating complex I-driven State V. Finally, rotenone is used to shut down Complex I-driven respiration. Subsequent addition of succinate fuels Complex II to initiate Complex II-driven State V. (B) Simplified illustration of the flow of electron through electron transport chain (ETC) and oxidative phosphorylation. See text for the detailed explanations for the interaction of the substrates with mitochondrial oxidative phosphorylation and the concepts of respiratory states I–V.

the mitochondria allowed to equilibrate for 2 min. This was followed by the sequential additions of: (1) complex I substrates (5 mmol/L pyruvate and 2.5 mmol/L malate) to initiate the state II respiratory state; (2) two boli of 150 μmol/L ADP to initiate the state III respiratory rate for 2 min; (3) 2 μmol/L oligomycin to monitor the state IV respiration rate for an additional 2 min; (4) 2 μmol/L FCCP to measure the uncoupled respiratory rate (state V) for another 2 min; (5) rotenone (1 μM) to completely block complex I-driven respiration; and (6) 10 mmol/L succinate to activate complex II-driven respiration. The respiratory control ratio (RCR) was calculated by dividing state III oxygen consumption (the respiratory rate in the presence of ADP, second bolus addition) by state IV oxygen consumption (the respiratory rate in the presence of oligomycin). Mitochondria were prepared freshly and used immediately for each experiment.

Slot-blotting measurement of oxidative damage markers in isolated mitochondria

Mitochondrial protein from different treated groups (control, SIN-1 (5 μM) alone and SIN-1 (5 μM) + Tempol (5 μM)) was used for quantitative measurement of mitochondrial oxidative damage. 3-nitrotyrosine (3-NT) and 4-HNE were applied as markers for protein nitration and lipid peroxidation by using the slot-blot apparatus as previously described [3,4,16–18,25]. In all, ~2 μg of mitochondrial protein was transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) by slot-blot apparatus (Schleicher & Schuell, Dassel, Germany). The slot was loaded with 2 μg of mitochondrial protein and 300 μL TBS and the mitochondrial proteins were then filtered through the membrane by gravity. For detection of protein tyrosine nitration, a rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Milford, MA) was used (1:2000) for 2 h at room temperature. For detection of 4-HNE, a rabbit polyclonal anti-HNE antibody (Alpha Diagnostics International) was used (1:5000). IR-Dye 800CW goat anti-rabbit antibody was applied as the secondary antibody (1:5000; Vector Laboratories, Burlingame, CA) in the dark for 1 h. The intensity of the bands was then detected and quantified by using the Li-Cor Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska). On all blots, a blank slot without any mitochondrial protein was employed to correct for non-specific binding. The value of the blank was background-subtracted from the values for all the other samples.

Statistical analysis

Statistical analysis was performed using the STATVIEW package (JMP software, Cary, NC). All results are expressed as means ± SEMs. Data were analysed by one-way analysis of variance (ANOVA) first, if the ANOVA revealed a significant effect, post-hoc analysis was carried out by using the Student-Neuman-Keuls (SNK) test. A difference was considered significant at $p < 0.05$.

Results

Peroxynitrite donor SIN-1 impairs spinal cord mitochondrial complex I—driven respiration

We first investigated the ability and potency of exogenous PN (SIN-1) to cause mitochondrial respiratory dysfunction in isolated healthy spinal cord (SC) mitochondria. Ficoll-purified SC mitochondria (30–35 μg) were suspended in respiration buffer and pre-treated with different concentrations (1, 2.5, 5 and 10 μM) of PN donor SIN-1. The representative respiratory traces in Figure 2A demonstrate the dose-dependent alterations of SC mitochondrial respiratory rates after exposure to different concentrations of SIN-1. With the increase of SIN-1 dosage, SC mitochondrial respiration was compromised and the oxygen consumption was decreased progressively. At a dosage of 10 μM SIN-1, mitochondrial function was severely impaired with little response to substrates as shown in the representative respiratory traces. Figure 2B displays the dose–response analysis of SIN-1-induced mitochondrial dysfunction by the quantification of the RCR, which is the ratio of state III to state IV oxygen consumption rates and is considered as a sensitive measure of how well the electron transport is coupled with ADP phosphorylation. One-way ANOVA showed treatments produced

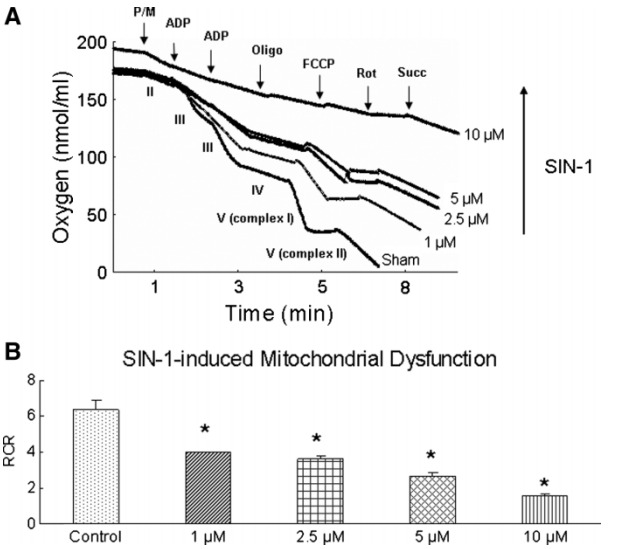

Figure 2. (A) The representative oxymetric traces show the progressive deterioration in SC mitochondrial bioenergetics following exposure to different dosages of SIN-1 (1–10 μM). (B) Compared to the control group, all SIN-1 treated groups result in significant decreases of RCR and SIN-1 induced mitochondrial dysfunction is dose-dependent (*$p < 0.0001$ vs control by SNK).

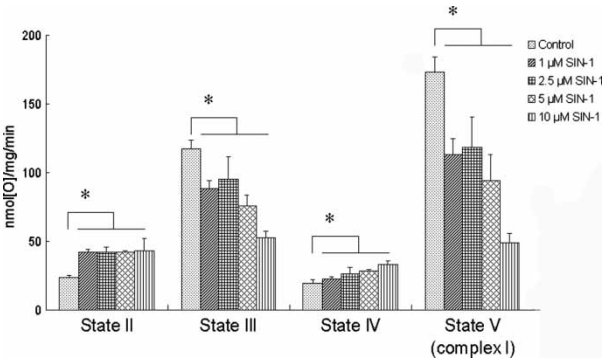

Figure 3. Quantification of all respiratory states of SIN-1 induced mitochondrial dysfunction. SIN-1 resulted in significant increases of State II and IV respiration and decreases of State III and Complex I-driven State V respiration, suggesting the occurrences of mitochondrial uncoupling and dysfunction after SIN-1 exposure ($\star p < 0.0001$ vs control by SNK).

a significant effect in terms of the RCR across all groups [$F(4,16) = 24.026$; $p < 0.0001$]. Mitochondria isolated from the SC tissue of healthy animals were found to be metabolically intact and well coupled (RCR > 6.0). Pre-treatment of mitochondria with PN donor SIN-1 for 5 min resulted in a progressive decrease in RCR compared with that in the untreated mitochondria (control group). Data are presented as means ± SEMs; $\star p < 0.0001$ compared with control as determined by one-way ANOVA and the Student-Neuman-Keuls post-hoc test.

Quantification of mitochondrial bioenergetics (Figure 3) showed significant, but not dose-related, increases in state II respiration after SIN-1 exposure at all tested concentrations ($\star p < 0.001$ vs control). State III (RCR numerator, the rate in the presence of ADP) and complex I driven-state V (uncoupled in the presence of FCCP) showed significant dose-related decreases with increasing SIN-1 concentration. In contrast, state IV respiration (RCR denominator, the rate in the presence of the ATP synthase inhibitor oligomycin) was increased by SIN-1 ($\star p < 0.001$ vs control), suggesting the compromised coupling of ETC and ATP production. Therefore, the dose-dependent decrease of RCR shown in Figure 2B was attributable to the simultaneous decrease of State III and increase of State IV following SIN-1 exposure.

Tempol partially protects spinal cord mitochondria from PN-induced respiratory dysfunction

We then carried out a dose–response analysis of the ability of tempol pre-treatment to antagonize SIN-1(PN)-induced mitochondrial dysfunction. The concentration of SIN-1 we applied was 5 μM since at this level the impaired RCR is closest to the mitochondrial RCR 24 h following SCI [4,18,27]. In attempting to titrate the optimal dosage of tempol, five concentrations (1.25, 2.5, 3.75, 5, 10 μM) were employed for an analysis of its protective effects against SIN-1. As demonstrated in Figure 4, one-way ANOVA shows that tempol treatment produced a significant effect across all groups [$F(6,18) = 30.043$, $p < 0.0001$]. Post-hoc analysis then revealed that pre-treatment with tempol at concentrations of 2.5, 3.75, 5 and 10 μM significantly increased the RCR compared to the SIN-1 (5 μM) alone group ($\#p < 0.001$, $n = 5$), showing a partial preservation of mitochondrial respiration. However, the mean RCR was lower at 10 μM than it was at 5 μM, indicative of a U-shaped dose–response curve. Additionally, in all tempol-treated groups, the RCR remained significantly suppressed when compared to the control untreated mitochondria ($\star p < 0.001$). We also tested whether the addition of tempol alone to the isolated SC mitochondria causes any alterations in mitochondrial respiratory states. Pre-incubation of isolated SC mitochondria with 0.5, 1.0, 5 or 10 μM concentrations of tempol did not cause any changes in mitochondrial respiratory rates (data not shown).

As indicated in Figure 5, quantification of all the respiratory states showed that 5 μM tempol, but not lower or higher concentrations, significantly improved state III (RCR numerator) ($\#p < 0.001$, $n = 5$). In all tempol-treated groups, there was a downward trend in the SIN-1 increased state IV respiration (RCR denominator) compared to SIN-1 alone, but no significant differences were detected. However, the state IV rate was not significantly higher than control after tempol concentrations at or above 2.5 μM. As for the SIN-1 attenuation of the State V respiratory rate, 3.75 μM tempol treatment produced a significant increase compared to the SIN-1 alone group ($\#p < 0.001$). However, in regards to the

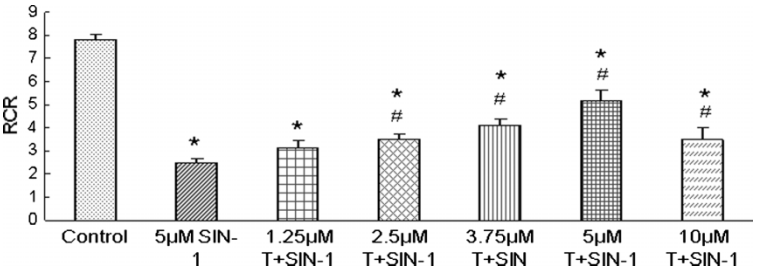

Figure 4. Tempol pre-treatments, starting from 2.5 μM to 10 μM, effectively antagonized SIN-1 induced (5 μM) mitochondrial dysfunction in terms of RCR. This protective effect is partial and shows a U-shaped dose–response ($\star p < 0.0001$ vs control; $\#p < 0.001$ vs SIN-1 alone treated group by SNK).

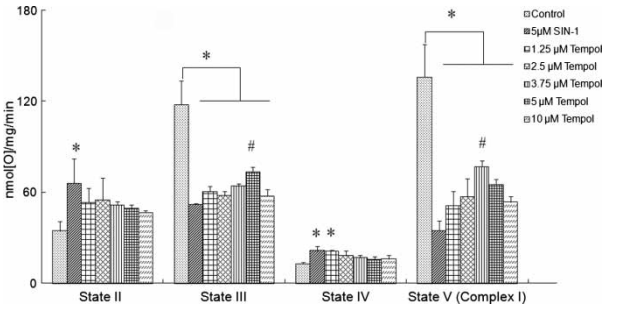

Figure 5. Quantification of the respiratory states showed that 5 μM tempol significantly improved state III respiration and 3.75 μM tempol significantly increased Complex I-driven State V respiration (*$p < 0.0001$ vs control; #$p < 0.001$ vs SIN-1 alone treated group by SNK).

tempol effects on States III and V, an inverted U-shaped dose–response curve was seen, although not observed for States II and IV.

Tempol protects spinal cord mitochondria against PN-induced 3-NT formation

In a separate set of experiments, we determined the oxidative damaging effects of the previously demonstrated mitotoxic concentration of SIN-1 (5 μM) in spinal cord mitochondria. As shown in Figure 6A, 5 min of exposure of the mitochondria to 5 μM SIN-1 produced a significant increase in 3-NT formation, as measured by immunoblotting. The representative slot blots (Figure 6A) show that SIN-1 pre-treatment dramatically increased 3-NT immunoreactivity in SC mitochondrial protein and tempol (5 μM)-pre-treatment almost completely blocked the SIN-1-induced formation of 3-NT. Densitometric quantification of the blots (Figure 6B) showed that the PN donor SIN-1 significantly increased 3-NT level in SC mitochondria, with a 6-fold increase compared to the control group (*$p < 0.0001$ vs control, $n = 6$). Tempol pre-treatment effectively prevented SIN-1-induced 3-NT formation in SC mitochondria, by significantly decreasing the level when compared to the SIN-1 alone group (#$p < 0.0001$ vs SIN-1 alone group, $n = 6$). This effect is considered complete since there is no significant difference between the control and tempol treated mitochondria.

Although 5 min incubation of SIN-1 induced 3-NT formation in mitochondrial protein, it had no effects on the LP marker 4-HNE. The representative blots demonstrated (Figure 6B) that the 4-HNE levels in mitochondrial proteins were similar among all groups. A basal level of 4-HNE formation could be detected in control untreated mitochondrial protein, indicating the existence of LP-modifications in healthy spinal cord mitochondria. No evidence of elevated 4-HNE was observed in mitochondrial treated with SIN-1 alone or after SIN-1 plus tempol treated groups.

Discussion

In the current study, application of SIN-1 to isolated healthy rat spinal cord mitochondria produced rapid impairment of mitochondrial respiratory function. Specifically, there was a dose-dependent decrease of the RCR that was attributable to the simultaneous decrease of State III and increase of State IV respiratory rates following SIN-1 exposure. In a similar fashion, others have reported that there is a significant elevation in state IV respiration after three sequential additions of 200 μM peroxynitrite to isolated mitochondria [28]. Therefore complex I-driven mitochondrial respiration was sensitively affected by SIN-1 exposure, supporting the notion that the complex I component of the ETC is a selective target for PN-mediated oxidative damage as previously documented [12,13,20,28,29]. The effects of tempol were associated with an increase in 3-NT levels in the

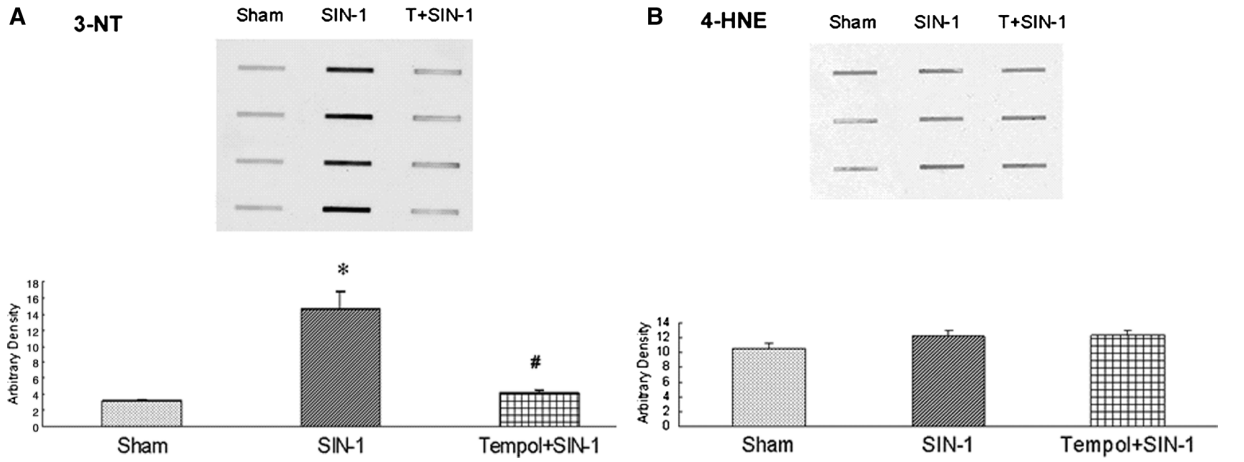

Figure 6. (A) Immuno-blotting results showed tempol effectively opposed SIN-1 induced 3-NT formation, suggesting its PN-scavenging properties (*$p < 0.0001$ vs control by SNK). (B) No statistical differences were detected among all treated group in terms of 4-HNE production.

mitochondrial proteins, but not in the levels of the LP byproduct 4-HNE.

The detrimental effects of SIN-1 on the RCR, the complex I-driven States II, III, IV and V were only partially inhibited by tempol pre-treatment, while at the same time tempol completely blunted the increase in 3-NT. More specifically, for State III, the protective effect of tempol was dose-related up to 5 μM and for State V it was dose-related up to 3.75 μM. Above these concentrations, the efficacy decreased. While the current results do not definitively explain the partial protection of respiratory function in contrast to the complete attenuation of the increased 3-NT or the inverted U-shaped dose–response, the explanation may be based upon tempol's demonstrated ability to divert nitration toward another potentially damaging oxidative damage mechanism, nitrosation [22]. Thus, while tempol can prevent PN-induced nitration, it may be countered somewhat by a simultaneous increase in nitrosation which becomes more pronounced at higher tempol concentrations resulting in an inverted U-shaped dose–response curve at the higher tempol concentrations in regards to States III and V.

It has been well established that mitochondrial dysfunction and free radical damage are important pathological events following acute CNS injury [1–4,17,30–35]. Recent studies from our laboratories strongly suggest that the principal ROS involved in the secondary damage after CNS injury is PN [16,17,24,25,35,36]. The mitochondrion is both a major source of post-traumatic PN formation and a vulnerable target of its free radical-mediated damaging effects [3,4,10–12,28,29,37]. PN-associated reactions modulate mitochondrial function in various pathways including, but not limited to, inhibition of mitochondrial electron transport [37], inactivation of the pyruvate dehydrogenase complex [38] and inhibition of mitochondrial NADH:ubiquinone reductase activity [13]. Inactivation of mitochondrial electron-transport enzymes also increases the leakage of $O_2^{\bullet -}$ and hydrogen peroxide generated by the mitochondria, which may further contribute to cellular injury, in an additive or synergistic fashion [39].

Peroxynitrite anion ($ONOO^-$) is formed by the diffusion-limited combination of $^{\bullet}NO$ and $O_2^{\bullet -}$ with a rate constant on the order of 10^9 or 10^{10} mol/s [6,39]. The PN anion is largely protonated at physiological pH to form peroxynitrous acid (ONOOH), which undergoes homolytic decomposition and generation of the highly reactive nitrogen dioxide radical ($^{\bullet}NO_2$) and hydroxyl radical ($^{\bullet}OH$) [40]. In a more physiologically relevant condition, PN will react with carbon dioxide (CO_2) to form nitrosoperoxocarbonate ($ONOOCO_2^-$) which homolytically decomposes to generate $^{\bullet}NO_2$ and carbonate radical ($^{\bullet}CO_3$) [41,42]. It is widely accepted that the potent oxidizing ability of PN is actually due to its decomposition free radicals. The PN-derived $^{\bullet}OH$, $^{\bullet}NO_2$, and $CO_3^{\bullet -}$ can initiate LP cellular damage by attacking polyunsaturated fatty acids or cause protein carbonylation by reacting with susceptible amino acids [42]. Additionally, $^{\bullet}NO_2$ can nitrate the 3-position of tyrosine residues in proteins and thereby inactivate important components of mitochondrial electron transport chain and disrupt tyrosine signalling pathways [13,28,43].

The understanding of the importance of PN in post-traumatic mitochondrial dysfunction provides a promising therapeutic target for acute treatment after CNS injury. Increasing evidence has shown that compounds which interfere with PN-mediated reactions such as uric acid [44], FeTSPP [45] and tempol [18,25,27] can protect spinal cord tissue from secondary injury after SCI. In the present study, we provided direct evidence that tempol, which has also been shown to have protective effects in traumatic [23,24,46] and ischemic [15] brain injury models, is probably exerting its beneficial effects through scavenging of PN-derived radicals [31]. Although tempol is a well-documented neuroprotective nitroxide, its neuroprotective effects have been previously attributed to its superoxide dismutase (SOD) mimicry [20,46]. However, other studies have shown that tempol can catalytically decompose the PN-derived free radicals $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ [22]. This latter property is probably reflected in the decrease in 3-NT, a specific marker for PN formation, in tempol-treated spinal cord mitochondria, suggesting a linkage between tempol's mitochondrial protection and scavenging of PN-derived free radicals.

Although this mechanistic relationship has been partially confirmed by investigating PN-mediated oxidative damage and the protective effects of tempol in brain mitochondria [20], the current study has more completely examined the responses of spinal cord mitochondria to exogenous PN. Comparing our current results with spinal cord mitochondria to previous studies with brain mitochondria [20], it appears that spinal cord mitochondria are as much as five times more sensitive to PN delivered via SIN-1. In the case of spinal cord mitochondria, a 1 μM concentration is sufficient to attenuate the RCR while, in brain mitochondria, a 5 μM concentration was required [20]. Likewise, twice as much tempol (5 μM) was required to maximally protect against SIN-1 damage to spinal mitochondria compared to brain mitochondria in which 2.5 μM was the optimal protective concentration. These differences are consistent with reports of physiological differences in the mitochondria isolated from different organs or subregions [47–49]. More to the point, there are intrinsic differences between brain and spinal cord mitochondria, with the latter producing significantly more oxygen radicals during respiration [21]. Thus, spinal cord mitochondria may be more sensitive to PN due to the fact that they are chronically exposed to a greater degree of background oxidative stress.

It was surprising to us that exposure of isolated normal, healthy spinal cord mitochondria to PN delivered via SIN-1 caused rapid protein nitration-related, but not LP-derived, 4-HNE-mediated protein modification. In *in vivo* neurotrauma models, LP has been conclusively demonstrated to be a significant oxidative damage mechanism [30–34]. However, in the present experiments, the brief 5 min exposure of SC mitochondria to PN was probably too short to allow LP to progress sufficiently to generate enough 4-HNE to measurably increase the protein-bound levels assessed by immunoblot. In isolated brain mitochondria, we [20] similarly observed only a non-significant increase in 4-HNE in contrast to a statistically significant increase in 3-NT formation after 5 min exposure to SIN-1. Thus, for both brain and SC mitochondria, it appears that the initial PN-mediated mitochondrial dysfunction is mainly attributable to PN-mediated nitration. Indeed, PN-induced nitration has been extensively studied as a crucial mechanism underlying the PN's toxicity [42,50,51]. For example, it is known that nitration is involved in the PN-induced inactivation of Mn superoxide dismutase (MnSOD), therefore amplifying mitochondrial injury by depleting this important anti-oxidant defense mechanism [50]. Furthermore, recent data has revealed that another important mitochondrial target for PN is cytochrome c. Nitration of cytochrome c results in a marked increase in its peroxidase activity, which may exacerbate the oxidative damage to mitochondrial proteins and membranes after PN exposure [43,51].

In summary, the protective effects of tempol on mitochondrial respiration were accompanied by a decrease in 3-NT, strongly suggesting that the beneficial effects of tempol we observed *in vivo*, including attenuation of oxidative damage, mitochondrial dysfunction and cytoskeletal degradation [27], resulted from direct scavenging of PN-derived free radicals.

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