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ROLE OF REACTIVE OXYGEN SPECIES PEROXYNITRITE IN TRAUMATIC SPINAL CORD INJURY

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ABSTRACT OF DISSERTATION

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The Graduate School

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ROLE OF REACTIVE OXYGEN SPECIES PEROXYNITRITE IN TRAUMATIC
SPINAL CORD INJURY

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ABSTRACT OF DISSERTATION
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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Yiqin Xiong

Lexington, Kentucky

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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

ROLE OF REACTIVE OXYGEN SPECIES

PEROXYNITRITE IN TRAUMATIC SPINAL CORD INJURY

Peroxynitrite (PN, ONOO⁻), formed by nitric oxide radical (•NO) and superoxide radical ($O_2^{•-}$), plays an important role in post-traumatic oxidative damage. In the early work, we determined the temporal characteristics of PN-derived oxidative damage in a rat spinal cord injury (SCI) model. Our results showed 3-nitrotyrosine (3-NT), a specific marker for PN, rapidly accumulated at early time points (1 hr, 3 hrs), after when it plateaued and the high level was sustained to 1 week post injury. The co-localization of 3-NT and lipid peroxidation derived-4-HNE observed in immunohistochemistry indicates PN is involved in lipid peroxidative as well as protein nitrative damage. PN-oxidative damage exacerbates intracellular Ca^{2+} overload, which activates Ca^{2+}-dependent calpain-mediated cytoskeletal protein (α-spectrin) degradation. The 145 kD fragments of α-spectrin (SBDP 145), which are specifically generated by calpain, increased dramatically as early as 1 hr after injury although the peak increase did not occur until 72 hrs post injury. The high level waned back toward sham level at one week post injury.

We then carried out experiments to evaluate the beneficial effects of tempol, a scavenger of PN-derived radicals, following SCI. Three pathological events including PN-induced oxidative damage, mitochondrial dysfunction and cytoskeletal degradation were investigated. Immunoblotting and immunohistochemical studies indicated PN-mediated oxidative damage including protein nitration, protein oxidation and lipid peroxidation, were all reduced by a single dose of tempol (300mg/kg, i.p) after SCI. Spinal cord (SC) mitochondrial dysfunction in terms of the respiratory control ratio (RCR) significantly improved by both 150 mg/kg and 300 mg/kg tempol treatments. Moreover, calpain-mediated proteolysis was significantly decreased by tempol, with greater effects on calpain-specific SBDP 145 observed.

Direct PN-scavenging effect of tempol was confirmed in vitro. Exposure of healthy SC mitochondria to SIN-1, a PN donor in vitro, impaired mitochondrial respiration in a dose-dependent manner. Tempol was able to protect mitochondria against SIN-1-induced damage by improving mitochondrial function and decreasing mitochondrial 3-NT

formation. These findings strongly support the concept that PN is a crucial player in the secondary damage following SCI. And tempol, by scavenging PN-induced free radicals, provides a promising pharmocotherapeutic strategy for treating acute SCI.

KEYWORDS: Reactive Oxygen Species, Peroxynitrite, Tempol, Oxidative Damage, Spinal Cord Injury

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ROLE OF REACTIVE OXYGEN SPECIES PEROXYNITRITE IN TRAUMATIC
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To my parents and brother

For their never ending love and support

谨以此文献给我亲爱的父亲幼成,母亲淑惠和哥哥一曲

您们宽厚深沉的亲情和坚定永久的支持是我不断前行的动力

ACKNOWLEDGEMENTS

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CHAPTER ONE

INTRODUCTION OF SPINAL CORD INJURY

I. Statistics of Spinal Cord Injury

Spinal cord injury (SCI) is a devastating neurological disorder that not only creates enormous physical and emotional cost to individuals but also is a significant financial burden to society at large. Presently, there are approximately 253,000 Americans, with a range of 225,000 to 296,000 persons, living with SCI. Additionally, the annual incidence of spinal cord injury, not including those who die at the scene of the accident, is approximately 12,000 new cases each year (Nobunaga et al., 1999). In other words, every 44 minutes another person sustains an SCI. Although SCI can victimize active individuals at any age, it primarily affects young male adults. 2006 Annual report from National Spinal Cord Injury Statistical Center (NSCISC) shows the average age at injury is 33 years, and 81% of total patients are male.

The most common cause of SCI is motor vehicle crashes, which accounts for 43.4% of reported cases, followed by falls (19.9%), acts of violence (18%), sports (10.7%) and other reasons (8%). Although those who survive their initial injuries can expect to live long lives nowadays, the majority of the patients end up with differing extents of neurological deficits that diminish their quality of life. As shown in **Figure1.1**, paraplegia, which means losses of movement

Figure 1.1 Neurological Outcomes of Spinal Cord Injury

and sensation in the lower body, affects 42.6% of the SCI population and 56.4% are affected by quadriplegia, which involves losses of movement and sensation in both the arms and legs. (www.spinalcord.uab.edu).
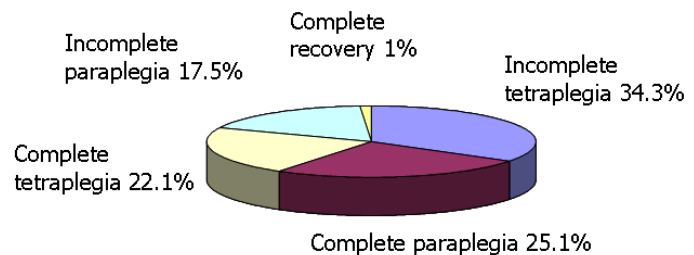
While the emotional and physical costs are overwhelming to a SCI individual, the financial costs to the patient, his/her family and society are enormous. The estimated lifetime costs for health care and living expenses for those individual with SCI varies from a half million dollars to well over two million dollars as shown in **Table 1.1** (adapted from www.spinalcord.uab.edu). More so, these cost estimates do not include the indirect costs from the loss of employment, wages, fringe benefits and productivity, which averages $52,900 annually per SCI-individual but can vary widely depending on a patient's education, injury severity and pre-injury employment.

Table 1.1. Estimated lifetime costs by Age at injury

Severity of Injury	25 years old	50 years old
High Tetraplegia (C1-C4)	$2,924,513	$1,721,677
Low Tetraplegia (C5-C8)	$1,653,607	$1,047,189
Paraplegia	$977,142	$666,473
Incomplete Motor Functional at any Level	$651,827	$472,392

Given the high financial costs as well as the severe physical and emotional consequences of SCI, research aimed at exploring effective ways of lessening injury severity, maintaining or recovering function, which would markedly improve the independence and productivity of the individuals with traumatic SCI is greatly desired by society.

II. Pathophysiology of Traumatic Spinal Cord Injury

Primary Damage

As outlined in **Figure 1.2**, upon initial insults, there is immediate mechanical contusive, compressive, shearing or stretch injury to the spinal cord elements including the neuronal cell body and axons, glial cells and endothelial cells of the spinal vascular system, which is known as the primary damage after SCI. Primary damage mainly affects spinal gray matter due to its softer consistency and greater vascularity, with relatively little damage to white matter outside the injury epicenter (Wolman, 1965). Those primary damage events occur immediately after injury and are usually irreversible and inevitable, therefore no clinically useful therapeutic targets are provided at this stage other than neurorestorative to regenerate or replace the damaged cells.

Figure 1.2 Principal mechanisms in the pathophysiology of SCI
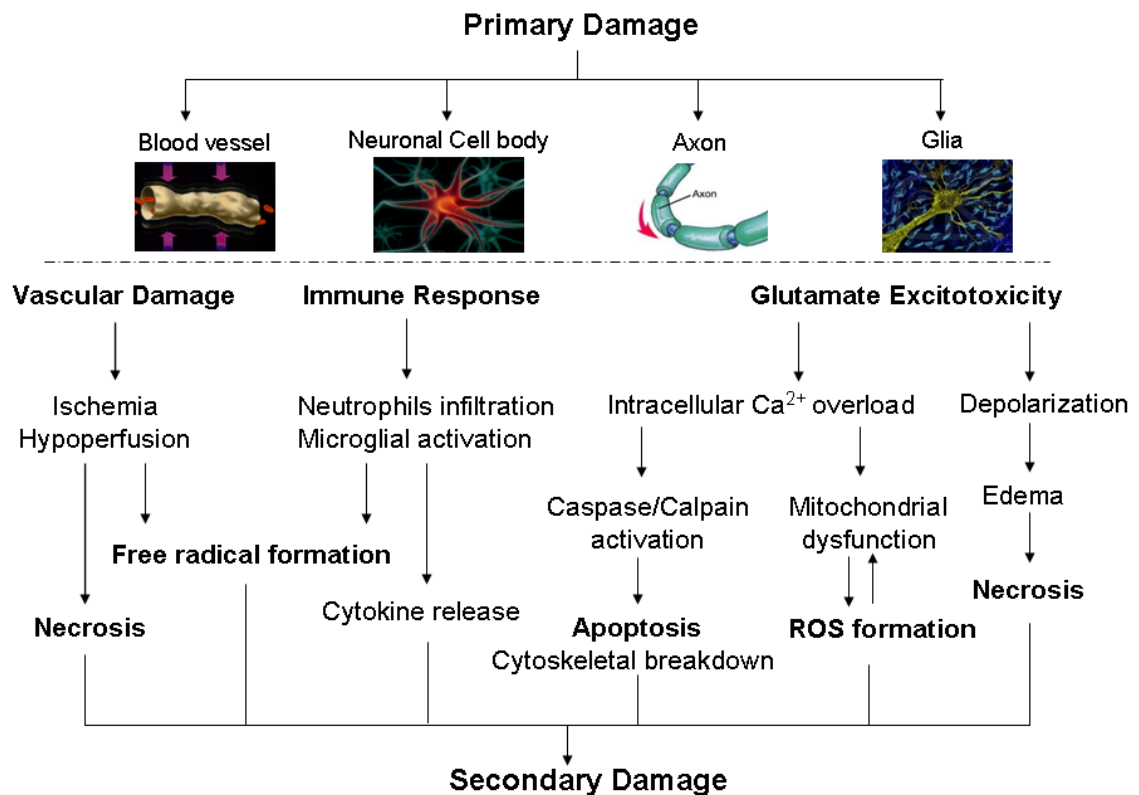
Secondary Damage

Typically after primary damage, residual white matter contains some portions of intact motor or sensory tracts, allowing for the possibility of neurological recovery. However, during the first minutes and hours following injury, a secondary degenerative process is initiated involving vascular abnormalities, ischemia-reperfusion, glutamate excitotoxicity and disturbances in ionic homeostasis, oxidative cell injury, and a robust inflammatory response (Anderson and Hall, 1993; Lewen et al., 2000; Tator and Fehlings, 1991; Dumont et al., 2001), which leads to a continued period of delayed and prolonged damage after the primary injuries. This leads to the notion that pharmacological treatments which interrupt the secondary cascade, if applied in a timely fashion, could improve spinal cord tissue survival. Therefore, a knowledge of these secondary events involved in SCI is essential for developing beneficial pharmacological interventions. Principal secondary damage mechanisms (**Figure 1.2** above) are addressed in this section one by one.

Vascular Damage

The vascular insults after SCI produce both hemorrhagic and ischemic damage. The large spinal vessels such as the anterior and posterior spinal arteries are typically spared while small intramedullary arteries and veins appear to be very vulnerable to damage following SCI, both at the site of injury and for some distances rostral and caudal to the injury epicenter (Tator, 1991; Tator and Fehlings, 1991; Tator and Koyanagi, 1997). Small areas of hemorrhage due to the disruption of microcirculation progress to regions of hemorrhagic necrosis with time (Tator and Koyanagi, 1997). Additionally, post-traumatic ischemia occurs progressively and may result from vasospasm, intravascular thrombosis (Nemecek, 1978), microcirculation decreases and systemic hypoperfusion (Tator, 1991; Tator and Fehlings, 1991). Following ischemia and hypoperfusion, any subsequent improvement in spinal cord blood flow will result in post-ischemic reperfusion that may exacerbate injury through the production of deleterious free radicals and other toxic byproducts (Lewen et al., 2000; Piantadosi and

Zhang, 1996). Furthermore, breakdown of the blood-spinal cord barrier (BSCB) at the injury epicenter not only leads to the protein extravasation (Nobel and Wrathall, 1989), but also results in the inflammatory invasion of neutrophils, macrophages and T-cells (Bareyre and Schwab, 2003), which potentially contribute to the secondary injury mechanisms (Kakulas, 1984; Blight, 1992; 1994; Park et al., 2004*).*

Inflammatory Response

A biphasic inflammatory response occurs following SCI. First, neutrophils infiltrate the injured cord, release lytic enzymes and reactive oxygen species (ROS) and potentially exacerbate injury to neurons, glia and blood vessels (Hausmann, 2003). It is reported that neutrophil accumulation in the injury area is significantly increased within 3 hrs and peaks within 24 hrs of SCI (Carlson et al., 1998; Dusart and Schwab, 1994; Popovich et al., 1997). Neutrophil infiltration is followed by the activation of resident microglia and the migration of bone-derived macrophages into the injured region. These cells then phagocytose debris and release cytokines mediating inflammation that may paradoxically promote tissue repair.

Microglial activation is maximal between 3 and 7 days post SCI and precedes the majority of monocyte and macrophage infiltration (Popovich et al., 1997). These inflammatory responses probably contribute to subsequent secondary injury as evidence have demonstrated improved behavioral recovery upon deletion of leukocytes prior to compressive SCI (Taoka et al., 1997). The production of cytokines by invading inflammatory cells has been implicated as mediators of damage through generation of free radicals (Blight, 1994). However, the numerous types of cytokines and their multi-faceted effects complicate the interpretation of their exact roles (Park et al., 2004). For example, tumor necrosis factor alpha (TNF-α), a key inflammatory mediator produced by microglia, macrophages and potentially by neurons as well, increased as early as 30 min and the elevated level maintained to several hours after SCI (Yakovlev and Faden, 1994; Wang et al., 1996). The increase of TNF-α level and subsequent activation of TNF-receptor-nuclear factor κB (NF-κB) can be suppressed by methylprednisolone (MP), the only proven drug for treating SCI, suggesting the

effectiveness of inhibiting post-traumatic inflammatory responses (Xu et al., 1998). Additionally, an in vitro study showed that the application of TNF-α resulted in lysis of oligodendrocytes, which is presumably mediated by the production of another important inflammatory mediator, nitric oxide ($^{\bullet}$NO) (Merrill et al., 1993). However, conflicting results exist concerning the pathophysiological significance of cytokines after SCI. For example, worse functional recovery, larger lesion size and an increase of apoptotic cell death were demonstrated after SCI in mice lacking TNF-receptor (TNFR) (Kim et al., 2001). Furthermore, TNF-α knock-out mice showed slightly decreased white matter preservation after SCI, without any beneficial effects on functional outcome (Farooque et al., 2001). These results suggest that inflammatory mechanisms mediated by cytokines such as TNF-α may have deleterious effects at the early phase post injury, but may also play a role in neural repair at later time points (Park et al., 2004).

Glutamate Excitotoxicity

Glutamate is the major excitatory neurotransmitter of the CNS and is involved in the synaptic plasticity underlying learning and memory as well as the formation of neuronal networks during CNS development. However, excessive or prolonged exposure to glutamate is toxic to CNS can lead to neuronal cell death (Doble, 1999). Following SCI, extracellular concentrations of excitatory amino acid including glutamate, aspartate, glutamine and asparagines, increase to neurotoxic levels rapidly (Liu et al., 1991; Farooque et al., 1996; McAdoo et al., 1999). The observed rise in glutamate is responsible for sustained activation of glutamate receptors in CNS resulting in neuronal cell death (Lipton and Rosenberg, 1994; Park et al., 2004). The subsequent over-activation of glutamate receptors allows excessive Ca^{2+} influx into the cell, either through the activation of ligand-gated channels or opening of voltage-gated Ca^{2+} channels due to cell membrane depolarization. Excessive Ca^{2+} entry results in cell death via secondary messenger cascades including activation of protein kinases, phospholipases, proteases, ROS and mitochondrial dysfunction (Lipton and Rosenberg, 1994). In particular, high intracellular Ca^{2+} levels activate the Ca^{2+}-dependent cysteine protease calpain, resulting in myelin and cytoskeletal degradation, thereby compromising axonal integrity and function (Banik et al., 1997ab; Schumacher et al., 1999; Park et al., 2004). It

has also been proposed that mitochondria play a key role in glutamate neurotoxicity. Glutamate-induced intracellular Ca^{2+} overload overwhelms the mitochondrial function by decreasing mitochondrial membrane potential and ATP synthesis, increasing ROS generation and ultimately leads to necrotic and/or apoptotic cell death (Nicholls and Budd, 1998).

Apoptosis

Apoptosis, or programmed cell death, has been seen after ischemic or traumatic injury to the central nervous system (CNS), suggesting that active cell death may also mediate secondary damage after CNS injury (Springer et al., 1999; Beattie et al., 2002). In SCI rat models, apoptotic cells were found from 6 hours to 3 weeks after injury, many of which are oligodendrocytes (Crowe et al., 1997; Shuman et al., 1997). It has been suggested that SCI-induced apoptosis has two stages, an early glial and neuronal apoptosis within 24 hrs of injury at the lesion site, and a later phase involving white matter microglia and oligodendrocytes outside the injury epicenter (Liu et al., 1997; Warden et al., 2001). The later phase of apoptotic cell death, which is maximal at 8 days post injury, may result from the loss of trophic support after axonal degeneration or it may be the consequence of microglial activation (Shuman et al., 1997). Undoubtedly, apoptotic cell death of oligodendrocytes will induce myelin degeneration and cause additional disturbances of axonal function.

On the other hand, spinal cord trauma leads to increased expression of death receptors and their ligands as well as activation of caspases and calpain (Banik et al., 1997b; Springer et al., 1999; Casha et al., 2001). Evidence indicates that caspases play important roles in mediation of apoptosis following SCI (Springer et al., 1999). Activation of caspases has been proposed to induce apoptosis simultaneously through extrinsic or intrinsic pathways (Ray and Banik, 2003; Ray et al., 2003). Following injury, increased production of TNF-α may induce the extrinsic caspase cascades leading to apoptosis. Also, SCI increases production of intracellular signaling molecules, such as Ca^{2+} and ROS, which activate Ca^{2+}-sensitive calpain and intrinsic caspase pathways. These receptor mediated-extrinsic and mitochondria-mediated intrinsic pathways

converge to caspase-3, the key executioner of apoptosis, and culminate in programmed cell death (Ray et al., 2003).

Free radical damage

Free radicals are highly reactive molecules with unpaired electons in their outer orbit and are thought to play a large and important role in trauma-induced CNS damage and stroke or ischemia (Hall, 1989; Hall and Braughler, 1993; Lewen et al., 2000). Oxygen-derived free radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$) are generated at least through the following cellular pathways: 1) SCI-induced ischemia-reperfusion of the vascular system (Oliver et al., 1990); 2) mitochondrial dysfunction due to intracellular Ca^{2+} overload (Nicholls and Budd, 1998) and 3) the inflammatory responses following SCI (Blight, 1994). Other resources for free radicals include, but not limited to, nitric oxide synthase (NOS), Ca^{2+}-mediated activation of phospholipases, xanthine oxidase, inflammatory cells and the Fenton and Harber-Weiss reactions (Lewen et al., 2000). Highly reactive oxygen and nitrogen species can be utilized by immune system to kill pathogen, but excessive production will contribute to cellular damage by oxidizing and nitrating proteins, lipids and nucleic acids. Free radicals can also initiate cell membrane damage by oxidizing the polyunsaturated fatty acids (PUFAs) in the lipid bilayer and induce lipid peroxidation (LP). Moreover, other oxidative mechanisms involving protein/DNA nitration and oxidation are important contributors to secondary damage after CNS injury.

Free radical attenuation has long been investigated as a major therapeutic target. Methylprednisolone (MP), the only proven pharmacological treatment used clinically for acute SCI, is believed to exert its beneficial effects in large part by suppressing free radical-induced damage (Hall, 1993). In the next section, I will focus on the secondary injury cascades that are involved in oxidative damage and the crucial role of a potent oxidizing ROS: peroxynitrite (PN) and its derived free radicals following SCI.

III. Free Radical Production and Oxidative Damage in Traumatic Spinal Cord Injury

Key Concepts

Oxidative Stress and Oxidative Damage

Oxygen is necessary for life. However, it can also be deleterious. Among all the O_2 taken up by mammalian cells, about 85-90% is effectively utilized by their mitochondria for energy production. The remainder may convert to reactive oxygen species (ROS) or free radicals. In normal conditions, the production of reactive species (RS) is approximately balanced by antioxidant defense systems including, but not limited to, mitochondrial manganese-containing superoxide dismutase and reduced glutathione. However, this balance is not perfect, especially under pathological conditions, so that some RS-mediated damaging occurs. The term 'oxidative stress' refers to a serious imbalance between RS production and antioxidant defenses and Sies defined it as "a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage" (Sies, 1991). Such damage is often called 'oxidative damage'. Halliwell and Whiteman have defined oxidative damage as "the biomolecular damage caused by attack of RS upon the constituents of living organisms" (Halliwell and Whiteman, 2004). Increased levels of oxidative damage can result not only from oxidative stress, but also from failure of repair or replacement systems.

Free Radical and Reactive Species

A free radical can be defined as "any molecular species capable of independent existence (hence the term "free") that contains one or more unpaired electrons" (Halliwell and Gutteridge, 2006).

Reactive oxygen species (ROS) is a term used to describe a number of oxygen-derived radicals, such as $O_2^{\bullet-}$ and $^{\bullet}OH$, and some non-radical derivatives of O_2.

Non-radicals ROS have no unpaired electron, but they are oxidizing agents and/or are readily converted into radicals (Halliwell, 2006). Note that all oxygen radicals are ROS, but not all ROS are oxygen radicals.

Reactive nitrogen species (RNS) is a similar descriptor that includes radicals $^{\bullet}NO$ and $^{\bullet}NO_2$, and nonradicals derivatives of $^{\bullet}NO$ such as HNO_2 and N_2O_4 (Halliwell and Gutteridge, 2006). Due to some overlapping between ROS and RNS, reactive species (RS) is used as a collective term to include both. It is important to know that the reactivity differs from each other among ROS. For instance, H_2O_2, $^{\bullet}NO$ and $O_2^{\bullet-}$ react quickly with only a few molecules, whereas $^{\bullet}OH$ is a strong oxidant that reacts rapidly with almost everything it comes into contact with (Halliwell, 2006). The classification of RS is shown in **Table 1.2** (Modified from Halliwell, 2006).

Table 1.2. Oxygen and Nitrogen-derived Reactive Species (RS) and Radicals

ROS that are radicals	ROS that are non-radicals
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydoxyl, $^{\bullet}OH$	Ozone, O_3
Hydroperoxyl, $HO_2^{\bullet}$	Singlet oxygen, $^1O_2$
Carbonate, $CO_3^{\bullet-}$	* Peroxynitrite, $ONOO^-$
	* Peroxynitrous acid, $ONOOH$
	Nitrosoperoxycarbonate, $ONOOCO_2^-$
RNS that are radicals	RNS that are non-radicals
Nitric oxide, $^{\bullet}NO$	Nitrous acid, HNO_2
Nitrogen dioxide, $^{\bullet}NO_2$	Dinitrogen trioxide, N_2O_3

* also classified as RNS

Important Reactive Species

Superoxide Radical ($O_2^{\bullet-}$) and Hydrogen Peroxide (H_2O_2)

Superoxide radical ($O_2^{\bullet-}$) is one of the most important primary ROS and can be produced from a variety of sources (Kontos, 1989). One major source of $O_2^{\bullet-}$ in vivo is through the mitochondrial electron transport chain (ETC). It is estimated that up to 1% of total mitochondrial O_2 consumption goes toward the production of $O_2^{\bullet-}$ or hydrogen peroxide (H_2O_2) at intermediate steps of the mitochondrial electron transport (Ischiropoulos and Beckman, 2003). ETC complex I (NADH dehydrogense) and coenzyme Q (ubiquinone-cytochrome b) appear to be the two primary sites of $O_2^{\bullet-}$ generation by proton "leakage" from the ETC to O_2 (Turrens and Boveris, 1980). The rate of leakage at physiological O_2 concentration is probably less than 5% of total electron flow of ETC, but the leaking rate rises as the O_2 concentration is increased (Halliwell, 1992; Turrens, 2003).

In addition to mitochondrial leakage, there are also other sources of $O_2^{\bullet-}$. Some $O_2^{\bullet-}$ is produced by activated phagocytic cells to kill pathogens (Halliwell, 2006). Activated phagocytic cells are capable of reducing O_2 into $O_2^{\bullet-}$ through the activity of NAD(P)H oxidase (Bianca et al., 1999). Another source of $O_2^{\bullet-}$ is the largely endothelial cell-contained xanthine oxidase (Kontos, 1989; Fridovich, 1970). Most of xanthine/hypoxanthine oxidation in vivo is catalysed by xanthine dehydrogense, which transfers electrons from the substrates onto NAD^+ and will not generated $O_2^{\bullet-}$. However, under ischemic and post-ischemic reoxygenation conditions, xanthine dehydrogenase can be converted to the oxidase form, and therefore produce $O_2^{\bullet-}$ and H_2O_2 while oxidizing xanthine or hypoxanthine (**Figure 1.3**)
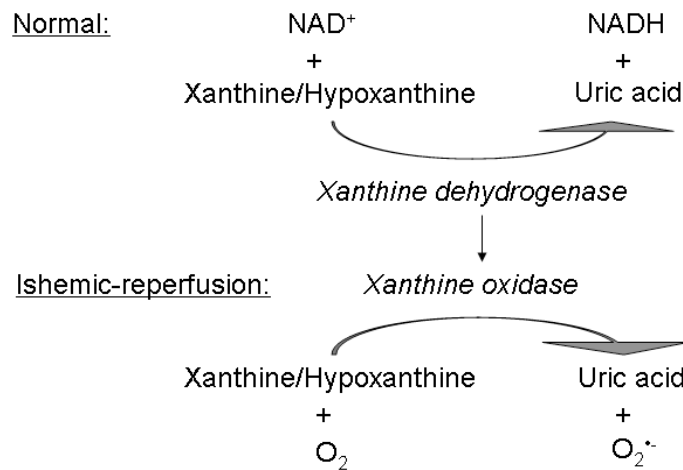
Figure 1.3. Superoxide radical production ($O_2^{\bullet -}$) via Xanthine oxidase

The transport of O_2 by erythrocytes is another possible source of superoxide. It is believed that hemoglobin, the preponderant protein in erythrocytes, is susceptible to a slow autoxidation (Misra and Fridovich, 1972). Normally, the iron in the heme rings of hemoglobin remains in the ferrous state (Fe^{2+}) for O_2 binding. However, some delocalization of the electron takes place and results in an intermediate structure (methemoglobin), producing ferric iron (Fe^{3+}) in the heme ring. Ferric ion present in the heme ring is unable to bind O_2, thereby releases a molecule of superoxide radical.
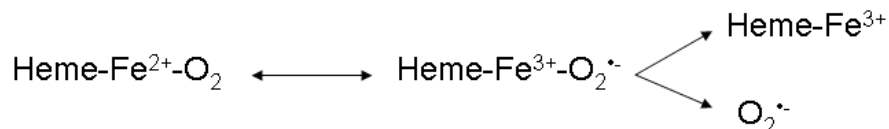
Moreover, $O_2^{\bullet -}$ can be produced from the arachidonic acid cascade through prostaglandin hydroperoxidase (PGH) and 5-lipoxygenase activity as a side-chain reaction, depending on the presence of NADH or NADPH (Kukreja et al., 1986). More recently, Ca^{2+} influx was suggested to be a source of mitochondrial $O_2^{\bullet -}$ as well. Grijalba et al proposed that Ca^{2+} alters the lipid organization of mitochondrial intermembrane and affects ETC function such as coenzyme Q mobility, leading to mono-electronic oxygen reduction which promotes the production of $O_2^{\bullet -}$ (Grijalba et al.,1999).

Although $O_2^{\bullet-}$ is generated from a variety of sources, several scavenging mechanisms exist to antagonize its potential damaging effects. Superoxide dismustase (SOD), a potent antioxidant enzyme, is specific for $O_2^{\bullet-}$ and effectively neutralize $O_2^{\bullet-}$ by the dismutation. Indeed, owing to this spontaneous or enzyme-catalyzed dismutation, most of $O_2^{\bullet-}$ ends up as H_2O_2 (Kontos, 1989). H_2O_2 is also one major form of ROS and can be produced directly by divalent reduction of oxygen (Chance et al., 1979). However, it is poorly reactive at physiological level and is usually removed by catalase or glutathione peroxidase (GPx) (**Figure 1.4**).

Figure 1.4. Detoxification of superoxide
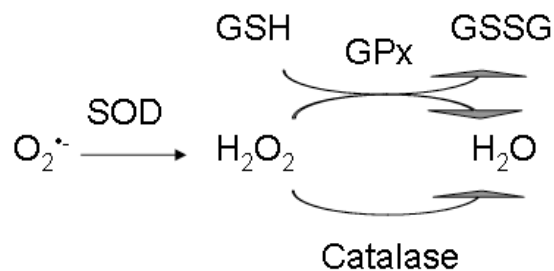
As illustrated, $O_2^{\bullet-}$ can deplete the endogenous antioxidant enzyme reservoir by direct reactions, such as catalase and GPx, as well as some redox enzymes involved in energy metabolism (Zhang et al., 1990; Winterbourn, 1993; Imlay, 2003). However, $O_2^{\bullet-}$ is not a strong oxidant, only reacts rapidly with a few molecules (Halliwell, 2006). Neither $O_2^{\bullet-}$ nor H_2O_2 is sufficiently reactive to account for much of post-traumatic oxidative damage found in vivo. Indeed, it is known that $O_2^{\bullet-}$-derived secondary ROS are much more cytotoxic than $O_2^{\bullet-}$ itself.

Hydroxyl radical ($^{\bullet}OH$)

In contrast to $O_2^{\bullet-}$, hydroxyl radical ($^{\bullet}OH$) is an extremely reactive form of ROS and can be generated directly in tissues by radiation or secondarily from the reaction between $O_2^{\bullet-}$ and H_2O_2:

$$H_2O_2 + O_2^{\bullet-} \longrightarrow {}^{\bullet}OH + OH^- + O_2$$
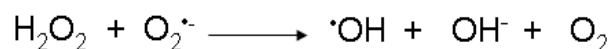
However, this reaction is not considered as a significant source of $^\bullet$OH unless in the presence of a catalytic transitional metal, usually iron. In that case, H_2O_2 generate $^\bullet$OH via the Fenton reaction (Halliwell, 1978; McCord and Day, 1978):

$$H_2O_2 + Fe^{2+} \longrightarrow {}^\bullet OH + OH^- + Fe^{3+}$$
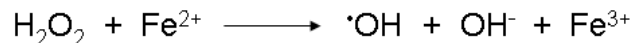
The resulting Fe^{3+} may be reduced by $O_2^{\bullet-}$, making possible the repetition of the cycle:

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2$$

Hydroxyl radical can also be produced from decomposition of peroxynitrite ($ONOO^-$), which is not dependent on the presence of transition metal ions (Beckman et al., 1990; Radi et al., 1991ab; Crow et al., 1994). Detailed explanation will be provided in the 'Peroxynitrite' section below.

Although $^\bullet$OH is a highly reactive ROS and capable of destroying various biomolecules, the reaction with organic molecules is diffusion rate-limited, with a rate ranging from 10^9 to $10^{10} \bullet M^{-1} S^{-1}$ (Halliwell and Gutteridge, 1989). Therefore, it will react as fast as it is formed and disappear quicky, which cannot explain any form of oxidative damage that occurs remotely from its site of formation. Moreover, far greater concentrations of free metals and H_2O_2 than those present in vivo would be required to cause oxidative damage through Fenton reaction pathway (Beckman, 1994; Crow and Beckman, 1996). For this and many other reasons, $^\bullet$OH may not be as important a cause of oxidative stress in vivo as thought in the past (Beckman, 1994; Crow and Beckman, 1996; Murphy et al, 1998).

Nitric oxide (NO, $^\bullet$NO)

Nitric oxide ($^\bullet$NO) is a ubiquitous diffusible messenger that can regulate vascular tone, modulate neuronal signaling and kill pathogens (Moncada et al., 1991). It is formed from arginine, O_2 and NADPH by the enzyme nitric oxide synthase (NOS):
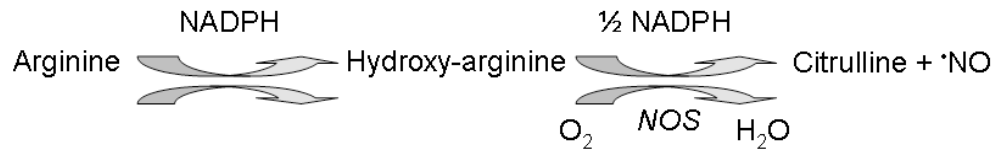
There are three identified isoforms of NOS, which include endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), all of which appear to be involved in the pathology of CNS injury. Studies have shown an early increase of nNOS activity and expression in resident spinal cord cells, followed by a late increase of iNOS activity and expression in inflammtory infiltrating cells following SCI (Conti et al., 2007). Recently a novel isoform of NOS was described located within mitochondria, thus named mitochondrial NOS (mtNOS) (Bates et al., 1995; Lopez-Figueroa et al., 2000), which was also responsible for $^{\bullet}$NO formation upon intra-mitochondrial Ca^{2+} increase.

Nitric oxide is a free radical because it is composed of seven electrons from nitrogen and eight electrons from oxygen:

$$\bullet\, N \equiv O$$
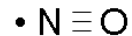
This odd number of electrons means that one is unpaired causing $^{\bullet}$NO to be a free radical. However, $^{\bullet}$NO is not highly reactive simply because it is a free radical. It only reacts rapidly with a select range of molecules that have unpaired electrons, which are typically other free radicals and with transition metals like heme iron. The biological chemistry of $^{\bullet}$NO can be simplified in a reasonable approximation to three major reactions (**Figure 1.5**): its activation of guanylate cyclase, which is responsible for signal transduction; its elimination by reaction with oxyhemoproteins such as oxyhemoglobin or oxymyoglobin (P-Fe^{2+}-O$_2$) (Goretski and Hollocher, 1988) and its transformation to peroxynitrite by reaction with O$_2^{\bullet-}$ (Beckman et al., 1990; Beckman and Koppenol, 1996).

Figure 1.5. Principal reactions of nitric oxide ($^{\bullet}$NO)

$$NOS \longrightarrow {}^{\bullet}NO$$

Guanylate cyclase (GTP → cGMP)

$$^{\bullet}NO \longrightarrow P\text{-}Fe^{2+}O_2 + {}^{\bullet}NO + O_2 \longrightarrow P\text{-}Fe^{3+} + NO_3^-$$

$$^{\bullet}NO + O_2^{\bullet-} \longrightarrow ONOO^-$$
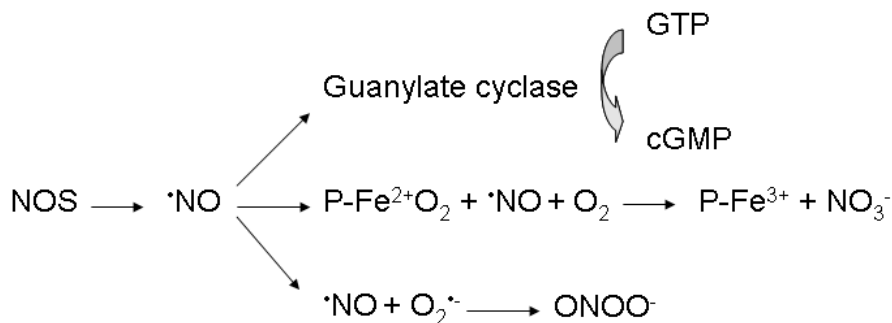
The reaction rate between $O_2^{\bullet-}$ and $^{\bullet}NO$ occurs at the near-diffusion-controlled rate of 6.7×10^9 $M^{-1} \bullet S^{-1}$ (Huie and Padmaja, 1993), so that nearly every collision between $O_2^{\bullet-}$ and $^{\bullet}NO$ results in the irreversible formation of peroxynitrite (ONOO$^-$). As shown in **Figure 1.6**, this reaction rate is approximately three times faster than the scavenging of $O_2^{\bullet-}$ by superoxide dismutase (SOD) (Cudd and Fridovich, 1982; Beckman and Koppenol, 1996).

$O_2^{\bullet-}$

$^{\bullet}NO$ — 6.7×10^9 $M^{-1} \bullet s^{-1}$ → ONOO$^-$ → Oxidative damage

SOD — 2×10^9 $M^{-1} \bullet s^{-1}$ → H_2O_2 + O_2 — Catalase → H_2O
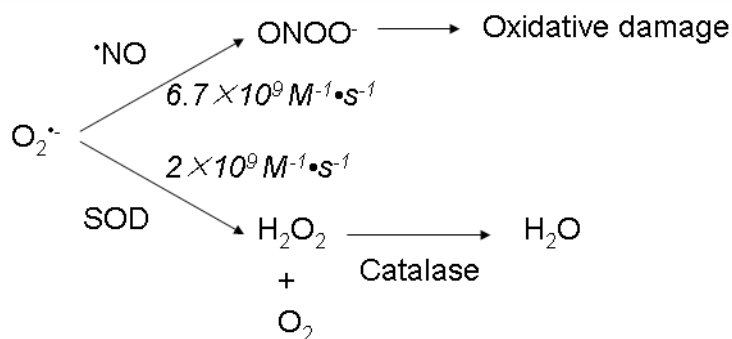
Figure 1.6. Excessive $^{\bullet}$NO production out-competes SOD for $O_2^{\bullet-}$

Thus $^{\bullet}$NO is the only biological molecule that can outcompete endogenous SOD for $O_2^{\bullet-}$, if being produced in high enough concentrations under pathological conditions (Beckman and Koppenol, 1996). It has been demonstrated that $^{\bullet}$NO can exert both protective and detrimental effects in several disease states of the CNS, including SCI. Low nitric oxide ($^{\bullet}$NO) concentrations are required in physiologic processes, whereas large amounts of $^{\bullet}$NO may be detrimental by increasing oxidative stress (Conti et al., 2007). For example, concentrations of $^{\bullet}$NO that cause vasodilatation are on the order of 5-10 nM and will not effectively compete with SOD for $O_2^{\bullet-}$; when the concentration of $^{\bullet}$NO rises to

micromolar concentrations under pathological conditions such as ischemia brain injury or spinal cord injury (SCI) (Malinski et al., 1993; Nakahara et al., 2002), it can effectively compete with SOD because of its higher reaction rate with $O_2^{\bullet-}$ and form the potent oxidant $ONOO^-$. This is probably an important mechanism that underlies the toxic effects of excessive $^{\bullet}NO$ production in pathological conditions. Direct evidence has been shown that NO inhibits mitochondrial ETC through peroxynitrite formation (Riobo et al., 2001).

Peroxynitrite (ONOO⁻, PN)

*i. **Biochemistry***

Peroxynitrite (PN) is a term used to refer to the sum of the peroxynitrite anion ($ONOO^-$) and its conjugate acid peroxynitrous acid ($ONOOH$) (Szabo et al., 2007). After Beckman and colleagues proposed PN to be the most important player in biological oxidative damage (Beckman et al., 1990), mounting evidence has been shown to support this notion.

As described previously, the peroxynitrite anion ($ONOO^-$) is produced by the reaction of $^{\bullet}NO$ and $O_2^{\bullet-}$ radicals at a diffusion-controlled rate (Huie and Padmaja, 1993). The peroxynitrite anion ($ONOO^-$) is relatively stable in alkaline solutions, slowly breaking down to nitrite and oxygen (Murphy et el., 1998).

$$ONOO^- \longrightarrow NO_2^- + \tfrac{1}{2} O_2$$
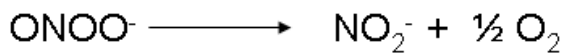
Around neutral pH, peroxynitrite anion ($ONOO^-$) is protonated to its conjugate acid $ONOOH$, which is unstable and rapidly decomposes to nitrate (Murphy et al., 1998; Koppenol et al., 1992).

$$ONOO^- \underset{pKa=6.75}{\rightleftharpoons} ONOOH \xrightarrow{k_1 = 4.5 \text{ s}^{-1}} H^+ + NO_3^-$$
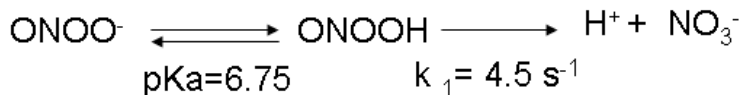
Both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid ($ONOOH$) are reactive and can participate directly in one- and two-electron oxidation reactions with biomolecules.

Inside a cell, the intracellular free thiol concentration is relatively high (5-10 mM), PN will prefererably react with reduced glutathione (GSH) and forms thiol radicals and sulphenic acid derivatives (Reed, 1990). These products are unstable and react rapidly with reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG will be recycled by glutathione reductase, and $O_2^{\bullet-}$ will be neutralized by SOD (Murphy et al., 1998; Winterbourn, 1993). See **Figure 1.7** (modified from Murphy et al., 1998) for the pathways involved in this reaction:
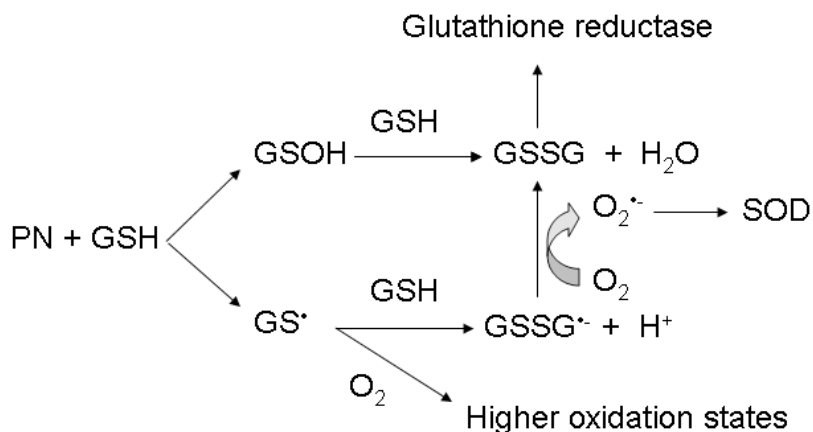
Figure 1.7. Detoxification of peroxynitrite by thiol and glutathione system

Therefore, glutathione may be an effective antioxidant defense system to scavenge peroxynitrite generated inside a cell. However, when excessive PN is formed or the intracellular thiols are depleted, the thiol radical will attack other compounds and form protein cross-linking and the mixed disulphides. Moreover, thiol radicals react very rapidly with oxygen to form peroxyl radicals and other sulphur-derived oxidants, leading to subsequent oxidative stress (Murphy et al., 1998). It has been shown that the depletion of the glutathione reservoir makes mitochondria more susceptible to PN damage (Scarlett et al., 1996; Barker et al., 1996).

Detoxification of PN by thiols will be much less effective in plasma because plasma thiol concentration ($\sim$0.5 mM) is about 10-fold less than inside cells (Murphy et al., 1998). Alternatively, due to the high concentration of carbon dioxide (CO_2) in plasma ($\sim$ 1.3 mM), the reaction of PN with CO_2 or bicarbonate anion (25 mM) becomes more

biologically significant (Denicola et al., 1996). Indeed, in plasma the reaction rate of PN with CO_2 is about 60-fold faster than that with thiols (Murphy et al., 1998), which leads to the formation of one-electron oxidants carbonate ($CO_3^{•-}$) and nitrogen dioxide ($^•NO_2$) radicals (yield～35%) (Szabo et al., 2007; Radi et al., 2001):

$$ONOO^- \xrightarrow{\;CO_2\;} ONOOCO_2^- \begin{cases} NO_3^- + CO_2 \text{ (isomerization, 65\% yield)} \\ ^•NO_2 + CO_3^{•-} \text{ (homolysis, 35\% yield)} \end{cases}$$
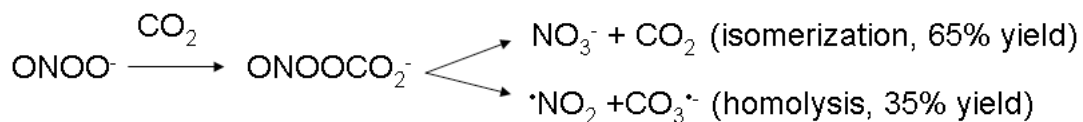
Nitrogen dioxide ($^•NO_2$) can undergo diffusion-controlled radical-radical termination reactions with biomolecules, resulting in nitrated compounds. Radical-radical termination refers to a reaction between two radicals leading to a non-radical adduct as end product and thus stops radical propagation reactions (Szabo et al., 2007).

Additionally, ONOOH can undergo homolytic fission, which means rupture of a covalent bond in a molecule, in which the two resulting products keep one of the bond electrons, to generate one-electron oxidants, $^•OH$ and $^•NO_2$ radicals with a 30% yield (Szabo et al., 2007):

$$ONOOH \begin{cases} NO_3^- \text{ (isomerization, 70\% yield)} \\ ^•NO_2 + {}^•OH \text{ (homolysis, 30\% yield)} \end{cases}$$
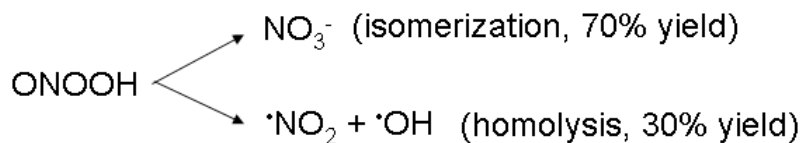
However, this reaction is relatively slow compared with the other PN-derived reactions and therefore is a modest component of the in vivo reactivity of PN in aqueous compartments. However, ONOOH can readily cross cell membranes (Denicola et al., 1998) and its decomposed $^•NO_2$ and $^•OH$ radicals therefore initiate the lipid peroxidation, protein nitration and other oxidative reactions (Szabo et al., 2007; Radi et al., 1991).

Figure 1.8 Biochemistry of peroxynitrite: reaction targets and fate
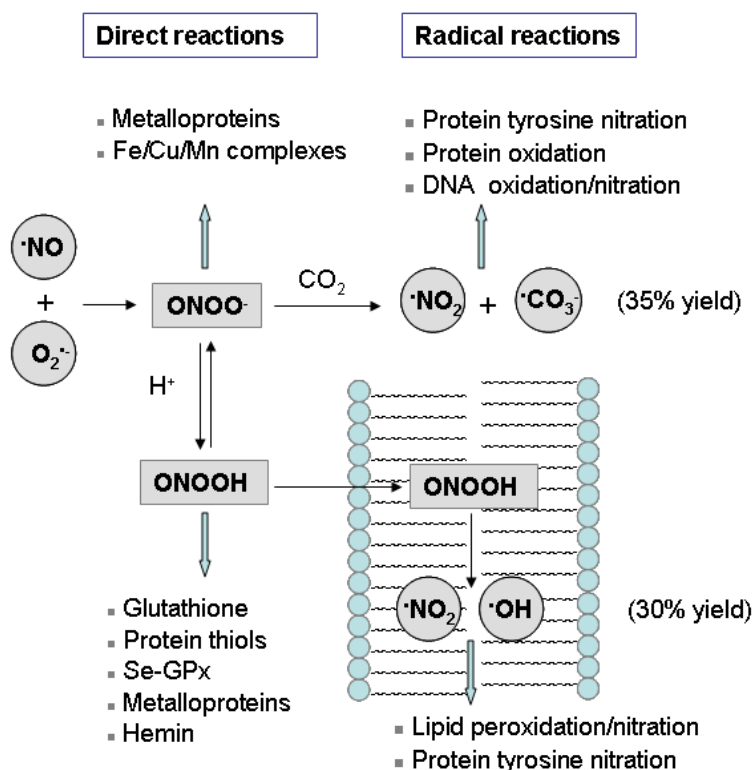
The biochemistry of PN is summarized in **Figure 1.8** (modified from Szabo et al., 2007). As illustrated, nitric oxide ($^\bullet$NO) and superoxide ($O_2^{\bullet-}$) react rapidly to form peroxynitrite anion (ONOO$^-$), which is in equilibrium with its conjugated acid-peroxynitrous acid (ONOOH). Both ONOO$^-$ and ONOOH can undergo direct reactons with biomolecules and deplete the endogenous antioxidant defense system. But more importantly, in the presence of CO_2, ONOO$^-$ and CO_2 will react to form nitrosoperoxocarbonate (ONOOCO$_2^-$) which can decompose to potent oxidants carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide radical ($^\bullet$NO$_2$) (with a 35% yield) that lead to nitration and oxidation of protein and DNA. Alternatively, ONOOH can generate hydroxyl ($^\bullet$OH) and $^\bullet$NO$_2$ radicals by homolytic fission with a 30% yield. PN-derived $^\bullet$OH plays a key role in initiating lipid peroxidation and PN-derived $^\bullet$NO$_2$ can undergo termination reactions with other biomolecule-derived radicals, resulting in nitrated proteins, which is currently considered as a biomarker or footprint for PN formation (Szabo et al., 2007; Beckman, 1996).

ii. Oxidative Mechanisms

Peroxynitrite is a biologically significant oxidant that is capable to inflict cellular damage through a variety of pathways, the principal biological effects of PN are outlined in **Figure 1.9**. Three principal oxdiative mechanisms including lipid peroxidation, protein modification by nitration and oxidation, oxidative damage to DNA, are discussed respectively in this section.

Figure 1.9 Selected biological effects of Peroxynitrite

Lipid Peroxidation
- 4-Hydroxynonenal (HNE)
- Malondialdehyde (MDA)

Mitochondria-induce Ca^{2+} release and PTP formation

Protein Oxidation
- Carbonyl formation

ONOO⁻

DNA single-stranded DNA breaks

Protein Modification
- Tyrosine nitration-disrupt tyrosine kinase signaling pathway

Potent inducer of apoptotic cell death
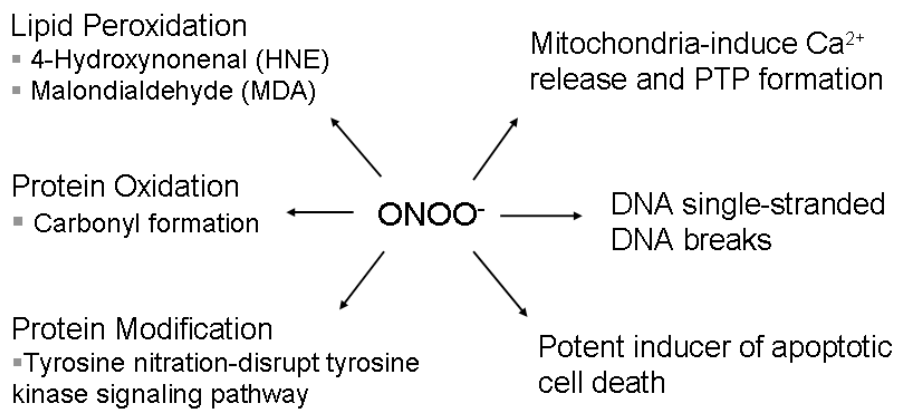
Lipid Peroxidation: Lipid peroxidation (LP) is generally thought to be a major mechanism for inducing cellular oxidative damage. Free radicals, either from PN decomposition or other sources, can react with lipids resulting in the peroxidation of fatty acids. Central nervous system (CNS) tissue is particularly susceptible to LP due to its high content in poly-unsaturated fatty acids (PUFAs), which serve as excellent substrates for LP because of the presence of reactive bis-allylic methylene groups. In these groups, the hydrogen atoms are easily abstracted due to the low bond dissociation energies on the carbon-hydrogen bonds (Kelly et al., 1998).

LP in biological systems proceeds through complex reactions consisting of three phases: initiation, propagation, and termination. Initiation starts when a free radical comes along, it can easily remove the hydrogen atom and the associated electron. Peroxidation therefore occurs by abstraction of a hydrogen atom from a methylene

carbon in the lipid (LH) to generate a highly reactive lipid radical (L˙), termed alkyl radical. In the propagation phase, oxygen adds rapidly to L˙ at a diffusion-controlled rate to produce the lipid peroxyl radical (LOO˙). The resulting LOO˙ is able to attack other biomolecules such as DNA and proteins, and form the primary oxidation product, a lipid hydroperoxide (LOOH). In the absence of antioxidants (i.e. α-tocopherol), LOO˙ can also abstract a hydrogen from neighboring lipid molecule (LH), producing another alkyl radical (L˙), therefore the LP propagates throughout the cell membrane (Kelly et al., 1998). Termination of lipid peroxidation occurs via the coupling of any two radicals to form nonradical products (radical-radical elimination). Nonradical products are stable and unable to propagate lipid peroxidation chains. An overview of lipid peroxidation processes is provided in **Figure 1.10** (modified from Kelly et al., 1998).
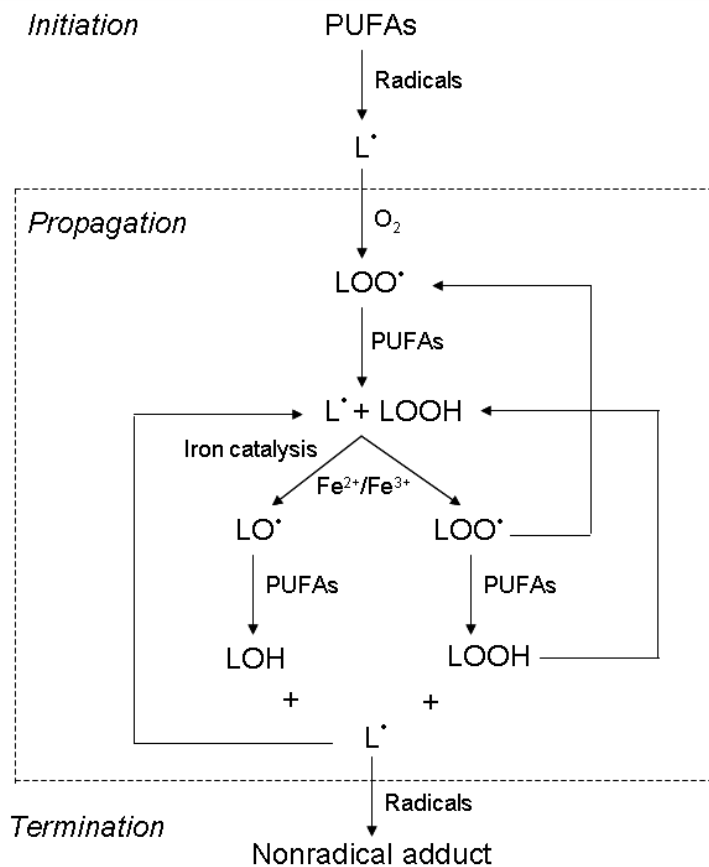
Figure 1.10 Chemistry of cell membrane lipid peroxidation (L˙ Alkyl radical, LOO˙ Peroxyl radical, LO˙ Alkoxyl radical, LOH Lipid hydroxide, LOOH Lipid hydroperoxide)

PN-derived radicals $^{\bullet}NO_2$, $CO_3^{\bullet-}$ and $^{\bullet}OH$ are potent oxidants that are capable of inducing intensive tissue damage by initiating LP. As a result, LP decreases membrane fluidity, increases permeability of the membrane bilayer to various substances that are normally impermeable and inactivates certain membrane-bound enzymes (Kelly et al., 1998). The continuation of oxidation of fatty acid side chains and their fragmentations to produce aldehydic breakdown products eventually leads to the loss of membrane integrity. Moreover, the products of LP, such as $LOO^{\bullet}$, $LO^{\bullet}$ and downstream aldehydes can induce severe damage to proteins. For example, 4-hydroxynonenal (HNE), the main end-product of LP, can conjugate onto proteins causing toxic protein aggregates within membranes (Keller et al., 1997; Kruman et al., 1997). Furthermore, it has been shown that another LP product 4-hydroxyhexenal (HHE) is a potent mediator of mitochondrial permeability transition (Kristal et al., 1996).

The primary lipid oxidation products, LOOH, are unstable and decompose to form secondary products such as aldehydes and ketones through various reaction pathways. The resulting diverse breakdown products, coupled with a small concentration of these products in vivo, complicate the accurate quantification of lipid peroxidation. The widely applied bioassay for LP is to measure the aldehyde "end-products". Reactive aldehydes, among which 4-HNE is one of the most cytotoxic aldehydes (Keller et al., 1997), are able to bind rapidly to proteins, therefore providing for immunoblotting measurement of such adducts within cells and tissues. Moreover, immunohistochemical methods can be used to locate the distribution of such adducts to demonstrate the spatial characteristics of LP-induced oxidative damage (Niki et al., 2005).

Protein Nitration/Oxidation: Many biomolecules are susceptible to PN-derived radicals by nitration or/and oxidation, including tyrosine residues, thiols, DNA and PUFAs. Indeed, 3-nitrotyrosine (NT), 3,3'-dityrosine and 3,4'-dihydrophenylalanine formed by PN through tyrosine nitration, dimerization and hydroxylation respectively, are entirely dependent on free-radical pathways (Radi, 2004). The nitration of protein tyrosine residues to 3-NT by PN also contributes relatively specifically to the toxicity of PN and is widely used as a bioassay to detect PN formation in vivo (Beckman, 1996). Tyrosine

nitration can inactivate enzymes, for example MnSOD (MacMillan-Crow et al., 1996), thus increasing the amount of $O_2^{\bullet-}$ available to react with $^{\bullet}$NO and establishing an autocatalytic spiral of increasing mitochondrial PN formation. Furthermore, tyrosine nitration blocks tyrosine phosphorylation and thus disrupts downstream tyrosine kinase signaling pathways (Kong et al., 1996), resulting in permanent impairment of cyclic cascades that control signal transduction processes and regulate cell cycles. Additionally, the nitrated lipids may lead to the secondary inhibition of protein function and assist in protein tyrosine oxidation and nitration in biomembranes and lipoproteins (Bartesaghi et al., 2006).

The general assay of oxidative protein damage is to measure the protein carbonyls. The rationale is based on the fact that several reactive species attach amino acid residues to generate products with carbonyl groups, which can be measured after reaction with 2,4-dinitrophenylhydrazine (DNPH). The immuno-detection is designed to recognize the protein with carbonyl group that is conjugated to DNPH.

Oxidative damage to DNA: DNA is another key cellular component that is particularly susceptible to oxidative damage. Before the exploration the importance of PN, the primary ROS responsible for DNA damage was believed to be $^{\bullet}$OH, as neither $O_2^{\bullet-}$ nor H_2O_2 radicals react directly with DNA (Halliwell and Aruoma, 1991). Generally speaking, $^{\bullet}$OH can attack on DNA bases leading to three classes of damage: hydroxylation, ring opening, and fragmentation. The resulting lesions are usually products of the secondary reactions that occur after the initial radical attack. In addition to producing direct damage to bases, a transient radical species generated within the DNA base may covalently bind with other macromolecules within the cell, forming protein-DNA cross-link. Not only this, $^{\bullet}$OH may also attack the sugar-phosphate backbone of DNA, causing a different variety of lesions. Another indicator for radical attack on the DNA backbone is the fragmentation of deoxyribose. Single strand breaks occur via hydrogen abstraction at the C-4 position, leading to oxidation of the sugar moiety. This may be coupled with a second sugar oxidation on the complimentary strand, causing a double strand break. Strand breaks in the DNA molecule may prove mutagenic or even lethal for the cell (Halliwell and Aruoma, 1991). PN is another biologically

important oxidant to induce DNA damage. Evidence showed peroxynitrite causes single-strand DNA strand breaks in both plasmid supercoiled DNA (Salgo et al., 1995a) and viable DNA in rat thymocytes in a dose-dependent fashion (Salgo et al., 1995b). It is believed PN reacts preferentially with guanine in DNA (Douki and Cadet, 1996), and the majority of mutations caused by PN occur at G:C base pairs (Juedes and Wogan, 1996). PN-mediated DNA strand breakage resulted in a significant decrease activation of DNA repair enzyme poly-(ADP-ribose) polymerase, which depletes celluar NADH and disrupts energy metabolism (Szabo et al., 1996).

IV. Role of Mitochondria in Traumatic Spinal Cord Injury

The mitochondrion is the 'powerhouse' of the cell. Maintaining mitochondrial bioenergetics in neurons is critical, because their energy supply is almost solely dependent on mitochondrial-derived ATP (Budd and Nicholls, 1998; Nicholls and Budd, 2000; Sullivan et al., 1998; Sullivan et al., 2005). After CNS injury, compromised mitochondrial function is believed to play a pivotal role in neuronal death due to, but not limited to the following mechanisms: a) mediating glutamate excitotoxicity through the regulation of Ca^{2+} signaling (Schinder et al., 1996); b) giving rise to more generation of ROS and thus inducing further oxidative damage; c) initiating the necrotic or apoptotic pathways by opening mitochondrial permeability transition pore (mPTP) (Fiskum, 2000; Friberg and Wieloch, 2002; Wieloch, 2001). Each topic is addressed briefly in this section.

Mitochondria as a Ca^{2+} Sink

Disruption of ionic balance, especially Ca^{2+} homeostasis has been observed in the lesion site after experimental SCI (Park et al., 2004). Apart from physical shearing of cell membranes resulting in the loss of ionic normal gradients, more subtle effects, such as Na^+-K^+ ATPase failure after experimental SCI (Clendenon et al., 1978) will greatly increase the cytosolic Ca^{2+} concentration (Young and Koreh, 1986). Failure of Na^+-K^+ ATPase depolarizes the cell membrane, which in turn activates the voltage-gated Ca^{2+} channel and causes subsequent Ca^{2+} influx. Additionally, impaired ATPase causes intracellular Na^+ accumulation and the reversal of Na^+-Ca^{2+} exchangers (Stys et al., 1992), further exacerbating the intracellular Ca^{2+} overload. More importantly, previously described glutamate excitotoxicity, an important pathological event after CNS injury, is triggered by massive Ca^{2+} influx arising from over-stimulation of the NMDA subtype of glutamate receptors (Faden et al., 1989). Major sources of intracellular Ca^{2+} after CNS trauma are illustrated in **Figure 1.11.**

Figure 1.11. Major sources of intracellular Ca^{2+} after CNS injury
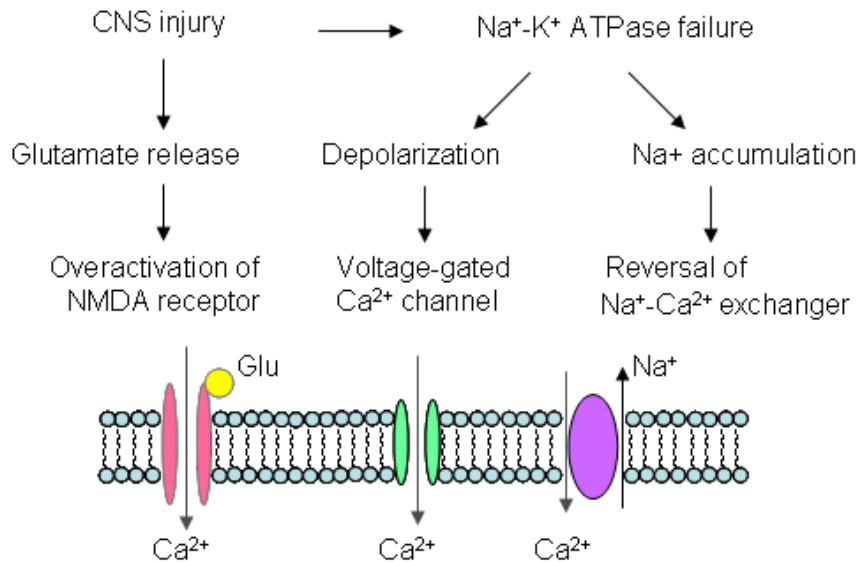
Mitochondria serve as high capacity Ca^{2+} sink and help in maintaining cellular Ca^{2+} homeostasis which is essential for normal neuronal function (Ichas and Mazat, 1998; Rizzuto et al., 1999; Rizzuto et al., 2000). Specifically, when the intracellular Ca^{2+} reaches a "set-point" of 500 nm, the unique features of mitochondria allow them to take up Ca^{2+} in order to modulate Ca^{2+} homeostasis (Schinder et al., 1996). However, if the increase of intracellular Ca^{2+} is beyond the mitochondrial buffering capability or mitochondrial function is impaired under abnormal conditions such as CNS injury, intracellular Ca^{2+} overload occurs and initiate a chain of pathological events. For instance, excessive mitochondrial Ca^{2+} accumulation uncouples electron transport from ATP synthesis and leads to mitochondrial dysfunction (Beatrice et al., 1980; Gunter and Pfeiffer, 1990; Bernardi et al., 1994). Indeed, mitochondrial dysfunction is believed as the primary event and an essential mediator of glutamate excitotoxicity (Schinder et al., 1996).

Mitochondria and ROS formation

Mitochondrial production of energy is accompanied by the generation of ROS as by-products of the oxidative phosphorylation process. Oxidants such as $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$

are constantly generated even during normal metabolism as the intrinsic rate of proton leakage (Shigenaga et al., 1994). There are some cellular anti-oxidant defenses including glutathione, SOD and catalase that neutralize the production of oxidants. However, under pathological conditions such as SCI, the rate of ROS production within mitochondria is exacerbated and if the amount of ROS unbalances the endogeous antioxidants, oxidative stress occurs, followed by cellular oxidative damage. The CNS is particularly susceptible to ROS-induced damage because a) it has a high consumption of oxygen; b) it contains high levels of membrane polyunsaturated fatty acids (PUFAs) susceptible to free radical attack; c) it is relatively deficient in oxidative defenses (e.g. poor catalase activity and moderate superoxide dismutase and glutathione peroxidase activities) and d) a high content in iron and ascorbate can be found in some regions of the CNS (Halliwell, 1992). Tissue injury, for example by ischemia or trauma, can cause increased metal ion availability and cerebrospinal fluid (CSF) cannot bind released iron, which will in turn enable more generation of ROS through the Fenton and Haber-Weiss reactions (Halliwell, 1992).

Moreover, the disturbance of energy metabolism during Ca^{2+} overload results in an increase in the mitochondrial leak of oxygen radicals, which can overwhelm the antioxidant defense mechanisms and contribute to cell death (Nicholls and Budd, 2000). It has been shown that Ca^{2+} influx induces inner mitochondrial membrane (IMM) lipid re-organization by interacting with the anionic head of cardiolipin, which is abundant in IMM. As a result, single electrons delivered to oxygen at intermediate steps in the ETC results in to the production of $O_2^{\bullet-}$ (Grijalba et al., 1999). In addition, $^{\bullet}NO$ is also produced intra-mitochondrially through the activation of an isoform of NOS located within the mitochondria. It was suggested that this isoform of NOS is activated upon mitochondrial Ca^{2+} uptake (Giulivi, 1998). The simultaneous increases of $O_2^{\bullet-}$ and $^{\bullet}NO$ then promote the diffusion-controlled formation of PN, which is now proposed as an essential player mediating oxidative damage following CNS injury. Although $^{\bullet}NO$ is believed to have a potentially toxic effect by itself, the toxicity that some have attributed to $^{\bullet}NO$ is more likely mediated by PN and its decomposition products rather than $^{\bullet}NO$ itself (Szabo et al., 2007; Bringold et al., 2000). For example, early work indicated that in

cells exposed to ˙NO, mitochondrial respiration was inhibited by the inactivation of electron-transport complex I and III (NADH-dependent and succinate-dependent respiration, respectively) without affecting complex IV (cytochrome c oxidase, the site of oxygen consumption). Those effects were later demonstrated both in isolated mitochondria and in cells to occur mainly through generation of PN (Radi et al., 1994; Riobo et al., 2001). Moreover, the inactivation of MnSOD by PN, due to nitration of the critical tyrosine-34 residue, could also amplify mitochondrial injury (MacMillan-Crow et al., 1996). Therefore, the mitochondrion serves as a primary source of ROS formation in pathological conditions and it is also a vulnerable target of self-derived ROS.

Mitochondrial permeability transition pore (mPTP)

Post-traumatic excessive mitochondrial sequestration of Ca^{2+} can lead to a sudden increase in inner membrane permeability to compounds with a molecular mass less than 1,500 Daltons, defined as the formation of mitochondrial permeability transition pore (mPTP) (Bernardi et al., 1994; Crompton, 1999). The mPTP formation is an underlying mechanism for causing mitochondrial swelling, metabolic failure and ultimately cell death (Zamzami et al., 1997). Although it was considered to be a non-specific membrane opening or megachannel (estimated to be 2-3 nm in diameter), evidence showing that the onset of the mPTP can be prevented by the immunosuppressant cyclosporine A (CsA) suggested it is more than a non-specific membrane rupture (Bernardi et al., 1994; Bernardi 1996; Sullivan et al., 1999). Multiple studies have indicated that the oxidation of mPTP components may play a role in promoting mPTP formation. Direct evidence showed mPTP formation is induced by the depletion of glutathione and the oxidation of pyridine nucleotides (Nieminen et al., 1997). Some evidence has indicated that mPTP occurs when thiol (-SH-) groups of inner membrane proteins are oxidized (McStay et al., 2002). The mPTP appears to have two open conformations in corresponding physiological pathways (Ichas and Mazat, 1998), that is, a low-conductance state which is involved in the regulation of the Ca^{2+} homeostasis during the life of the cell, and a high-conductance state which initiates the apoptotic cascade during Ca^{2+}-dependent cell death. A low-conductance state, that allows the diffusion of small ions like Ca^{2+}, is

pH-operated, promoting spontaneous closure of the channel. A high-conductance state, that allows the unselective diffusion of big molecules, stabilizes the channel in the open conformation, disrupting in turn the mitochondrial structure and causing the release of proapoptotic factors.

An interesting hypothesis about mPTP formation is that it might serve as a defense mechanism to ameliorate or eliminate high ROS-producing cells and prevent further oxidative damage (Starkov, 1997). In this theory, mPTP is formed upon Ca^{2+} influx, which assists O_2 consumption through dissipating membrane potential ($\Delta\Psi_m$) and proton force (ΔpH) across the membrane. By increasing the proton leak through the inner mitochondrial membrane (IMM) or uncoupling, mitochondrial respiration is promoted and less oxygen is available for ROS production. Therefore mPTP formation appears to be a modulator responding to increased oxidative stress. However, complete uncoupling of mitochondrial respiration and ADP phosphorylation or prolonged uncoupling through mPTP will exhaust ADP in the matrix and lead to the inhibition of respiration. Highly oxidized mPTP will not be able to recover and result in disruption of outer mitochondrial membrane. Another theory is, excessive Ca^{2+} will saturate the internal Ca^{2+}-binding sites of the mPTP and induce the irreversible switching from low- to high-conductance state (Ichas and Mazat, 1998). Once reaching this point of no return, small proteins that trigger the initiation of apoptosis, such as cytochrome c and apoptosis-inducing factor (AIF), are released from the mitochondrial intermembrane space into the cytoplasm (Brustovetsky et al., 2002; Brustovetsky et al., 2003; Green and Kroemer, 2004; Sullivan et al., 2005). Released cytochrome c binds apoptotic protease activating factor 1 (Apaf-1) and activates the caspase cascade (Hengartner, 2000).

It is important to note that compared to brain, spinal cord (SC) mitochondria are more susceptible to oxidative damage and intracellular Ca^{2+} overload based on the fact that after traumatic brain injury (TBI), cyclosporine A reduces damage (Sullivan et al, 1999) but is ineffective following SCI (Rabchevsky et al., 2001). This disparity is found to be attributable to: 1) significantly higher levels of O_2^- production, lipid peroxidation and mitochondrial DNA oxidation in normal SC neurons; 2) decreased complex I enzyme activity and respiration in normal SC mitochondria; 3) substantially reduced threshold for

Ca^{2+}-induced mitochondrial permeability transition in SC (Sullivan et al., 2004). Because of above reasons, mitochondrial dysfunction and oxidative damage in SCI, compared to TBI, are more important issues to address and to prevent.

V. Calpain-Mediated Cytoskeletal Proteolysis in Traumatic Spinal Cord Injury

SCI evokes an increase in intracellular free Ca^{2+} level resulting in activation of calpain, a Ca^{2+}-dependent cysteine protease which cleaves a broad spectrum of substrates. Mounting evidence indicates that uncontrolled calpain activity mediates the breakdown of many cytoskeletal and membrane proteins leading to neuronal death and is considered as another crucial mediator in the pathophysiology of SCI (Ray and Banik, 2003; Banik et al., 1992; 1997ab; Springer et al., 1997b).

Calpain family

Calpain, discovered by Guroff in the CNS (Guroff, 1964), is a family of Ca^{2+}-activated cysteinyl/thiol (neutral) proteases, in which cysteine residues are present in its active site. The calpain family consists of several tissue-specific isoforms (n-calpain) and two ubiquitous isoforms (μ-calpain and m-calpain). Ubiquitous calpain isoforms are abundantly expressed in the CNS and are regulated by calpastatin, an endogenous calpain-specific inhibitor. Calpains are expressed in all vertebrates in which they are highly conserved across species. They are also found in various cell types and tissues (Sorimachi et al., 1997). The two ubiquitous calpain isoforms are categorized mainly on the basis of their sensitivity to $[Ca^{2+}]$ upon activation in their purified state in vitro. In other word, μ-calpain (or calpain 1) has micro-molar sensitivity to Ca^{2+} and is located primarily in the neuronal soma and dendrites. m-Calpain (or calpain 2) has a milli-molar sensitivity to Ca^{2+} activation and is primarily located in axons and glia. Moreover, because of the distinct subcellular localization of the calpain subtypes, they may serve specific physiological roles (Hamakubo et al., 1986). More recently, μ-calpain, which has higher affinity for Ca^{2+} binding, has been shown to locate in mitochondria (Garcia et al., 2005).

Calpain structure and activation

Both μ-calpain and m-calpain consist of two parts: a non-identical 80 kDa catalytic subunit and an identical 30 kDa regulatory subunit (Ray et al., 2003). The 80 kDa large subunit can be divided in four domains (I, II, III, and IV), and the 30 kDa small subunit in two domains (V and VI) (Croall and Demartino, 1991). The domain structure of calpain subunits is illustrated in **Figure 1.12** (modified from Ray et al., 2003).

Figure 1.12 Domain structure of calpain subunits

Domain I: Autolytic cleavage site
Domain II: Cysteine protease activity, bind with calpastain
Domain III: Mediator of Ca^{2+} binding with domain V
Domain IV: Five Ca^{2+} binding sites
Domain V: Membrane anchor
Domain VI: Five Ca^{2+} binding sites
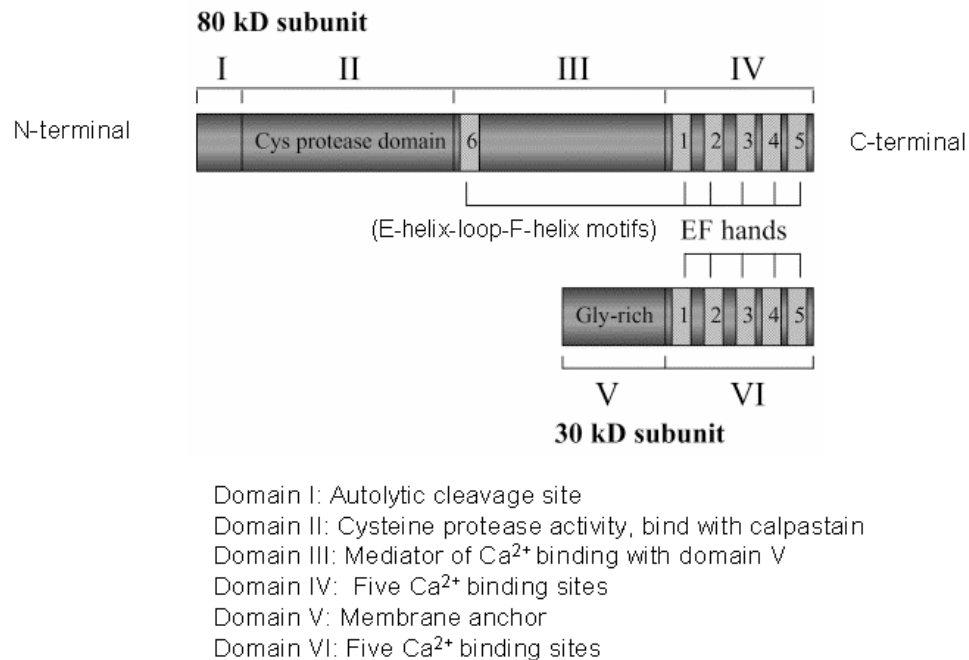
In resting cells, calpain is an inactive proenzyme in the cytosol where the intracellular free Ca^{2+} concentration stays 50–100 nM (Pietrobon et al., 1990). Calpain activation is triggered by an increase in intracellular free Ca^{2+} concentration in the cytosol. Some researchers believed calpain activation is accomplished by autolysis of N-terminal propeptide portions of both subunits, which results in a conformational change in the molecule and separation of truncated subunits leading to activation (Ray et al., 2003; Imajoh et al., 1986; Inomata et al., 1988). However, another mechanism proposes that elevated intracellular free Ca^{2+} concentration triggers the translocation of inactive calpain from the cytosol to the cell membrane (Molinari et al., 1994; Suzuki et al., 1987), where calpain is activated with the aid of membrane effectors such as phospholipids (Saido et al., 1991) and calpain activator protein (Melloni et al., 1998; Ray et al., 2003). Once activated

on the membrane, calpain diffuses back into the cytosol and becomes resistant to the inhibition of calpastatin. Depending on the location of activation, calpain isoforms generated from activation processes may be involved in the physiological functions (Ray et al., 2003). Simplifed illustration of calpain activation hypotheses and major calpain substrates is displayed in **Figure 1.13** (modified from Dr. Geddes).

Figure 1.13. Simplified illustration of Ca^{2+}-activated Calpain-mediated Proteolysis
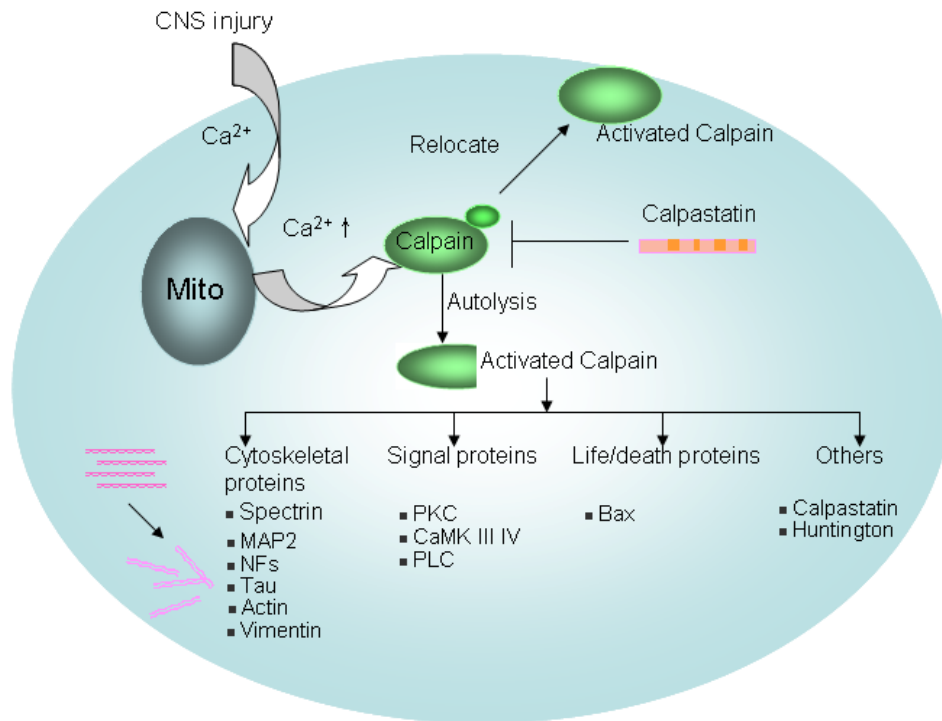
Role of Calpain in SCI

Early studies on SCI found that progressive breakdown of myelin proteins such as myelin basic protein (MBP) and proteolipid protein (PLP), and axonal proteins such as neurofilament protein (NFP) and microtubule-associate protein 2 (MAP2) are associated with the structural degeneration of myelin and axons in the spinal cord after trauma (Banik et al., 1980). Increased calpain immunoreactivity has also been detected in macrophages, reactive astrocytes, microglia, and neurons in the SCI lesion as well as the adjacent areas (Ray et al., 2003; Li et al., 1995). The early increase in calpain expression and activity

following SCI is probably provoked by elevation of intracellular Ca^{2+} levels. Later increase of calpain activity is likely to stem from the inflammatory cells through production of cytokines and activation of the arachidonic acid cascade (Ray et al., 2003). Since calpain plays a key role in the pathophysiology of neurodegenerative disorders and diseases, many studies have been performed to identify calpain inhibitors and their potential beneficial effects. In traumatic SCI models, protease inhibitors that block calpains have been shown to attenuate the cytoskeletal protein loss, enhance the axonal survival and thus improve the neurological function after injury (Schumacher et al., 2000; Ray et al., 2000; Zhang et al., 2003).

Since subunit autolysis seems to be an early event in the intramolecular activation of μ-calpain, the ratio of the activated, autolyzed 76 kD isoform to the 80 kD precursor form has been used as an index for μ-calpain activation. However due to the concept that calpain activation does not require autolysis, a more reliable and commonly used method of measuring calpain activity is to detect the presence of breakdown products (BDPs). Preferred substrates for calpain include cytoskeletal proteins spectrin, MAP2 and neurofilament proteins (NF), which are major components of neuronal cytoskeleton and membrane. The breakdown fragments of the 280 kD α-spectrin include calpain-specific 145kD proteins, so called signature breakdown protein (SBDP) and 150 kD fragments which are generated by calpain and/or caspase 3. Therefore calpain activity can be indirectly detected by using antibody-based immuno-detection (Wang, 2000). The neuronal cytoskeleton is an important component maintaining cell architecture, as well as axonal transport, and possibly neuronal plasticity (Ludin and Matus, 1993). Studies have been shown that after experimental SCI, the cytoskeletal disrupton has 2 phases: a rapid loss of MAP2, Tau or nonphosphorylated NF, and a delayed loss of phosphorylated NF at 1 week post injury (Zhang et al., 2000). Our time course study of calpain-mediated spectrin breakdown also revealed rapid accumulations of SBDPs but the peak did not occur until 72 hours post injury (Xiong et al., 2007). These various studies indicate the important role of calpain in cytoskeletal de-arrangement and degradation after SCI.

VI. Hypotheses and Specific Aims

Based on described rationales and mechanisms, we hypothesized that:

Hypothesis 1: Peroxynitrite-mediated oxidative stress is an important factor in secondary SCI, which causes cellular damage by lipid peroxidation and protein oxidation and nitration. (oxidative damage).

Hypothesis 2: Cellular and mitochondrial oxidative damage contributes to an increase in intracellular Ca^{2+}, which triggers calpain-mediated cytoskeletal degradation.

Hypothesis 3: Tempol, a scavenger of peroxynitrite-derived free radicals, should be an effective neuroprotective agent for treating SCI that will reduce oxidative damage, preserve mitochondrial function, calpain-mediated cytoskeletal degradation.

To test our hypotheses, we came up with and finished working on the following specific aims:

Specific Aim 1: Investigate the time course of oxidative damage in the contused rat spinal cord using immunoblotting and immunohistochemical methods by applying three oxidative markers including peroxynitrite-derived 3-NT, lipid-peroxidized 4-HNE and protein-oxidized protein carbonyls.

Specific Aim 2: Investigate the time course of calpain-mediated cytoskeletal damage in the contused rat spinal cord by measuring α-spectrin breakdown.

The results of these two aims were published in the paper "Role of peroxynitrite in secondary oxidative damage after spinal cord injury" Xiong Y, Rabchevsky A and Hall ED. *J. Neurochem.* (2007) Feb;100(3):639-49. (Chapter Two)

Specific Aim 3: Examine the neuroprotective effects of tempol in the rat spinal cord contusion model by evaluating tempol's ability to inhibit oxidative damage, preserve the mitochondrial function and reduce cytoskeletal degradation after spinal cord injury.

The results of this specific aim were presented at the 36th Annual Meeting of the Society for Neuroscience in October, 2006 and 25th Annual National Neurotrauma Society Symposium in July, 2007 and are in the process of submitting to *Journal of Neuroscience* in a manuscript titled "Beneficial effects of tempol, a catalytic scavenger of peroxynitrite, in a rat spinal cord injury model". (Chapter Three)

Supplementary Specific Aim 4: Provide direct evidence that tempol exerts its neuroprotective effects at least partly through scavenging peroxynitrite-derived radicals by exposure isolated healthy spinal cord mitochondria to peroxynitrite donor SIN-1.

Some results of this specific aim were also presented at the 36th Annual Meeting of the Society for Neuroscience in October, 2006 and are in the process of submitting to *Free Radical Research* in a manuscript titled "Tempol protects spinal cord mitochonodria from oxidative damage induced by peroxynitrite donor SIN-1" (Chapter Four)

CHAPTER TWO

ROLE OF PEROXYNITRITE IN SECONDARY OXIDATIVE DAMAGE AFTER
SPINAL CORD INJURY

I. Introduction

Much of the damage that occurs in the spinal cord following traumatic injury is due to the secondary effects of glutamate excitotoxicity, Ca^{2+} overload, and oxidative stress, three mechanisms that take part in a spiraling interactive cascade ending in neuronal dysfunction and death (Tator and Fehlings, 1991; Anderson and Hall, 1993; Young, 1993; Lynch and Dawson, 1994). The role of reactive oxygen-induced oxidative damage to spinal cord lipids (i.e. lipid peroxidation, LP) and proteins has been strongly supported in previous work (Hall and Braughler, 1989; 1993; Azbill et al., 1997; Springer et al., 1997a; Baldwin et al., 1998; Juurlink and Paterson, 1998; Aksenova et al., 2002). Among the ROS species, hydrogen peroxide (H_2O_2) and its derived hydroxyl radical ($^{\bullet}OH$) are known to play an important role in post-traumatic oxidative damage (Braughler and Hall, 1984; 1989). However, in 1990, Beckman and coworkers (Beckman et al., 1990) introduced the theory that the principal ROS involved in producing secondary tissue injury in neurological disorders is peroxynitrite (PN, ONOO-), formed by the reaction of nitric oxide synthase (NOS) generated $^{\bullet}NO$ and $O_2^{\bullet-}$ at almost diffusion-limited rates ($6.7\times10^9\,M^{-1}\cdot S^{-1}$, Huie and Padmaja, 1993). Since that time, the biochemistry of PN (also referred to as a reactive nitrogen species) has been further identified. It is believed that the potent oxidizing ability of PN is actually due to its decomposition products that possess potent free radical characteristics. These products are formed in two pathways. As shown at left, the first involves the protonation of PN to form peroxynitrous acid (ONOOH), which can undergo hemolytic decomposition to form the highly reactive nitrogen dioxide radical ($^{\bullet}NO_2$) and hydroxyl radical ($^{\bullet}OH$). Probably more physiologically important, PN will react with carbon dioxide (CO_2) to form nitrosoperoxocarbonate ($ONOOCO_2^-$), which can decompose into nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radical ($CO_3^{\bullet-}$).
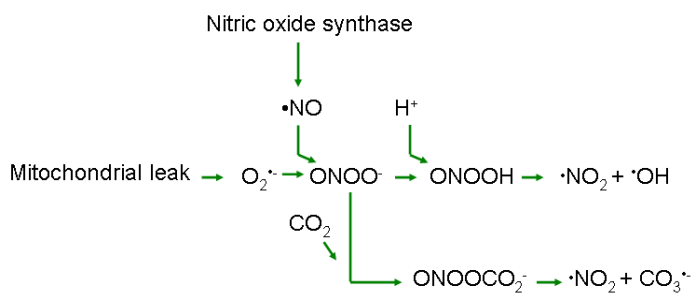
Peroxynitrite-derived radicals ($^\bullet$OH, $^\bullet$NO$_2$, CO$_3^{\bullet-}$) can initiate lipid peroxidation (LP) cellular damage by attacking unsaturated fatty acid or cause protein carbonylation by reacting with susceptible amino acids. LP products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) can therefore bind to cellular proteins compromising their structural and functional integrity. 4-Hydroxynonenal is the more important player in that it is actually itself neurotoxic (Kruman et al., 1997) and may be a more specific marker for LP than MDA (Uchida et al., 1992; Schmidt et al., 1996). Additionally, $^\bullet$NO$_2$ can nitrate the 3-position of tyrosine residues in proteins, the measurement of 3-nitrotyrosine (3-NT) accumulation thus provides a biomarker of peroxynitrite action (Beckman et al., 1996). These oxidative mechanisms undoubtedly underlie the demonstrated neurodegenerative effects of PN shown in neuronal cell culture models (Kruman et al., 1997; Neely et al., 1999).

Another critical mechanism in the evolution of secondary injury involves the activation of Ca^{2+}-dependent proteases (calpains) (Zimmerman and Schlaepfer, 1984; Suzuki et al., 1987; Saido et al., 1994) that can cleavage a broad spectrum of substrates, including the cytoskeletal proteins (Braughler and Hall, 1984; Banik et al., 1997ab; Bartus et al., 1995; Kampf et al., 1997), which are essential for normal cellular function and survival (Schlaepfer and Zimmerman, 1985; Siman et al., 1989; Johnson et al., 1991). One of the cytoskeletal substrates is α-spectrin, which has recently been proposed as a potential diagnostic biomarker for calpain activation in CNS injury (Wang, 2000; Hall et al., 2005; Ringger et al., 2004). The breakdown products of α-spectrin include the Ca^{2+}-specific 145 kD fragment (SBDP 145) and calpain/caspase 3-generated 150 kD fragment (SBDP 150). Over the past few years, the role of oxidative damage in post-traumatic Ca^{2+} overload and the resulting calpain activation has been strongly supported. Oxidative damage is known to exacerbate excitotoxic glutamate and to compromise the neuronal homeostatic mechanisms that either directly or indirectly regulate intracellular Ca^{2+} including, the Na$^+$-K$^+$ ATPase, Ca^{2+}-ATPase, Na$^+$-Ca^{2+} exchanger and mitochondrial Ca^{2+} sequestration (Siesjo and Bengtosson, 1989; Pellegrini-Giampietro et al., 1990; Hall, 1995; Sullivan et al., 1998). Thus, oxidative damage most likely contributes to calpain activation by disruption of Ca^{2+} homeostasis.

To investigate the hypothesis that PN is a crucial player in secondary oxidative damage in SCI, the present study was undertaken in a rat model of moderately severe contusion SCI to examine temporal changes in three oxidative markers, 3-NT, 4-HNE and protein carbonyl, over the first week post-injury. In parallel, the time course of calpain-mediated α-spectrin breakdown was also determined to provide a better understanding of the temporal relationship between oxidative damage and intracellular Ca^{2+} overload-mediated calpain activation.

II. Materials and methods

Subjects

This study employed young adult female Sprague-Dawley rats (Charles River, Portage, MI) weighing between 200 and 225 grams. 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) and xylazine (10 mg/kg) before a

IH Impactor *(Sheff et al., 2003)*

laminectomy of the T10 vertebrae was performed. Spinal cord injury was performed using the Infinite Horizon (IH) device shown at the left (Scheff et al., 2003), which creates a reliable contusion injury to the exposed spinal cord by rapidly applying a force-defined impact with a stainless steel-tipped impounder. Care was taken to perform laminectomies that were slightly larger than the 2.5 mm impactor tip. The vertebral column was stabilized by clamping the rostral T9 and caudal T11 vertebral bodies with forceps. The vertebral column and exposed spinal cord was carefully aligned in a level horizontal plane. During impact, the stepping motor drove the coupled rack toward the exposed spinal cord inflicting the contusion injury. The force applied to spinal cord was 200 kdyn, which produces a moderately severe injury. The impactor device was connected to a computer that records the impounder velocity, actual force and displacement of the spinal cord.
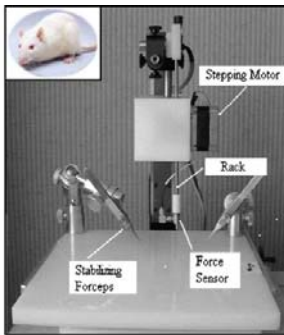
Immunoblotting Analysis for 4-HNE, 3-NT and Protein Carbonyl

At different time points following surgery (1hr, 3hr, 6hr, 24hr, 48hr, 72hr, 1week), a first set of animals (6 rats per time point) was killed by sodium pentobarbital overdose (150 mg/kg). A 20 mm segment of spinal cord containing the impact epicenter was removed rapidly by laminectomy. The harvested tissue was dissected on a chilled stage and

immediately transferred to a centrifuge tube containing 800 μl Triton lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 10 mM EDTA, 20 mM HEPES, 10% solution of glycerol and protease inhibitor cocktail (Roche Inc. Nutley, NJ)] and then briefly sonicated. Following dismembranation, the spinal cord tissue samples were centrifuged at 15,000×g for 30 min at 4°C, the supernatant was collected, protein levels were determined using the Protein Assay Kit (Pierce Biotechnology, Inc. Rockford, IL), the samples then diluted to a concentration of 1 μg/μl and stored at -80°C until assay.

Measurement of Oxidative damage by Slot immunoblotting

A 2 μl protein sample (2 μg) was loaded on slot blot apparatus for optimal antibody binding sensitivity. After addition of 200 ml Tris-buffered saline (TBS), protein was slowly brought down to the underneath nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) by gravity. For lipid peroxidation, rabbit polyclonal anti-HNE antibody was applied (1:5,000 Alpha Diagnostics International, Inc. San Antonio, TX). For PN-generated 3-NT, rabbit polyclonal anti-nitrotyrosine antibody was employed (1:2,000 Upstate USA, Inc. Charlottesville, VA). To detect protein oxidation, the oxy-blot technique was used (Oxy-blot Protein Oxidation Detection Kit; Chemicon International, Temecula, CA). The slot blots were analyzed using the Li-Cor Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska), which employs IRDye800 conjugated goat anti-rabbit IgG (1:5,000, Rockland; Gilbertsville, PA) as the secondary antibody. Preliminary studies in our laboratory were carried out to demonstrate that the 2 μg sample was within the linear range of densitometry curve for each of the oxidative markers. Thus, we were able to verify that the densitometric readings were not beyond the range of accurate quantitation. On all blots, a blank slot without any protein loading was employed to correct for nonspecific binding. The value of the blank was background-subtracted from the values for all the other samples.

Immunohistochemistry for 3-NT and 4-HNE

At different time points following surgery (1hr, 3hr, 6hr and 24hr), a second set of animals was overdosed with sodium pentobarbital (150 mg/kg) and perfused with 150 ml of 0.1M phosphate-buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde in PBS (PH=7.4). For cross sections, a 5 mm spinal cord segment, centered on the injury epicenter, was dissected at different time points. For longitudinal sections, a 15 mm spinal cord segment including the impact site was dissected 24 hrs after injury. After harvesting, the spinal cords were immersed in 4% paraformaldehyde in PBS for 4 hrs and transfer the tissues to PBS overnight and cryopreserved in phosphate buffered 20% sucrose for two days. Spinal cords were sectioned at 20 μm in a transverse or longitudinal plane, and the section was transferred directly onto a series of Superfrost plus slides (Fisher Scientific International Inc., Hampton, NH). After collecting all the spinal cord sections, the slides were placed on a tray and stored at 4°C to dehydrate overnight after which they were stored at -20°C until staining.

On the day of staining, the frozen slides were removed from -20°C and let thaw at room temperature for 30 min. After rinsing in 0.2 M phosphate-buffered saline (PBS), the sections were incubated in 3% H_2O_2 in 0.2 M PBS for 30 min, followed by incubation in blocking buffer (5% goat serum, 0.25% Triton-X, 1% dry milk in 0.2 M PBS) for 1 hr, followed by exposure to either the rabbit polyclonal anti-4-HNE (1:5000) or anti-3-NT antibody (1:2000) overnight. The following day, sections were incubated for 2 hrs at room temperature with biotinylated goat anti-rabbit secondary antibody (1:200, Vector ABC-AP Kit, Vector Labs, Burlingame, CA). After rinsing, the sections were incubated in VECTASTAIN ABC reagent (Vector Labs, Burlingame, CA) for 1 hr followed by development of the staining using the Vector blue method (Vector Blue Alkaline Phosphatase Substrate Kit, Vector Labs, Burlingame, CA) in the dark for 10-30 min. After reaction, spinal cord sections were counter-stained with nuclear fast red (Vector labs, Burlingame, CA), dehydrated and then photographed on an Olympus Provis A70 microscope with an Olympus Magnafire digital camera (Olympus America, Inc., Melville, NY).

Western Blot Analysis for α-Spectrin Breakdown

Five μg 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 electro transferring unit (Bio-Rad Laboratories, Hercules, CA) at 20 mA for 15 min. The blots were probed with mouse monoclonal anti-α-spectrin antibody (1:5,000, Affiniti, Inc., Ft. Lauderdale, FL; now part of Biomol, International, LP), which recognizes an epitope that is common to the 280 kilo-dalton (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 (1:5,000, Rockland, Gilbertsville, PA) for 1 hr 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. This quantitative method has been employed in this laboratory for multiple studies (Hall et al., 2005).

Statistical Analysis

Quantitative densitometry analysis was used for reading the slot blots and western immunoblots. Statistical analysis was done using the STATVIEW software package (JMP software, Cary, NC). All values are expressed as mean ± standard error (S.E.M.). A one-way analysis of variance (ANOVA) was first run. If the ANOVA revealed a significant ($p < 0.05$) effect, post-hoc testing was carried out to compare individual post-traumatic time points to the sham, non-injured group by Fisher's PLSD test. In all cases, a p value of < 0.05 was considered significant.

III. Results

Time Course of Peroxynitrite-induced Oxidative Damage after SCI

Rapid accumulation of 3-NT after spinal cord injury is evidenced in the representative slot blot **(Figure 2.1)**. Sham rats are non-injured animals that underwent laminectomy surgery followed by tissue harvesting 24 hrs later. Compared to the non-injured sham group, injury produced a significant increase in 3-NT measured in the injured spinal cord tissue across the post-traumatic time course [One-way ANOVA $F_{(7,40)}=22.784$; $p<0.0001$]. Figure 2.1 displays the time course of 3-NT production between 1 hr and 1 week after SCI. Post-hoc testing revealed a significantly higher level of 3-NT as early as 1 hr post injury, suggesting the rapid increase of PN formation after SCI. The peak in 3-NT occurred at 24 hrs post injury ($p<0.05$ vs. 6 hrs, n=6) and decreased at later time points ($p<0.05$ vs. 48 hrs, 72 hrs, 1 week, n=6). However, compared to sham rats, the significantly high level of 3-NT maintained up to 1 week after SCI ($p<0.0001$, n=6). Y axis indicates the arbitrary density ratio to control bands in each blot, which normalized the bands across different blots. Control tissues are combined SC tissues from rats that underwent laminectomies and were sacrificed at 3 hrs, 24 hrs and 48 hrs.

Figure 2.1 Time Course of 3-nitrotyrosine formation after SCI
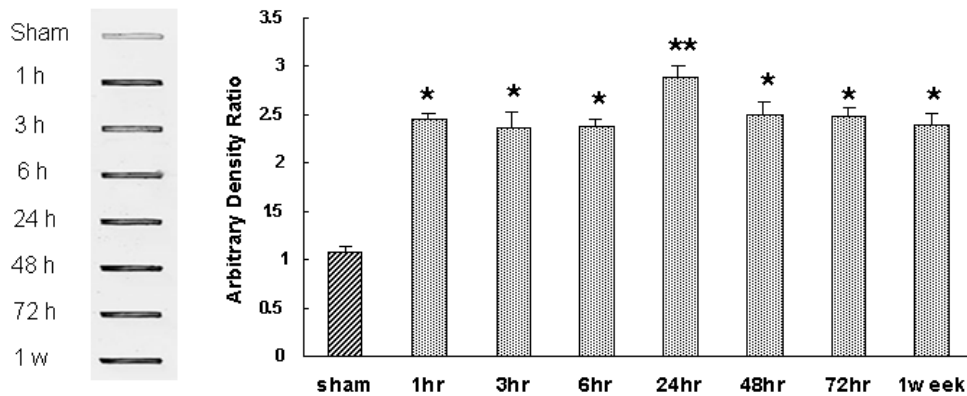
The production of protein oxidation-derived protein carbonyls after spinal cord injury showed a similar pattern **(Figure 2.2)**. Left panel demonstrated a representative blot of protein carbonyls staining. One-way ANOVA showed an overall increase in protein carbonyl contents was observed after injury [$F_{(7,40)}=16.978$, $p<0.0001$]. Post-hoc testing revealed that the level of protein carbonyls was significantly elevated at 1 hr post injury compared to the sham groups. The high levels were sustained to 1 week post injury ($p<0.0001$, n=6). Although the protein carbonyl level produced at 24 hrs post injury was slightly higher than other time points, no significant differences were detected among different time points.

Figure 2.2 Time course of Protein Carbonyls formation after SCI
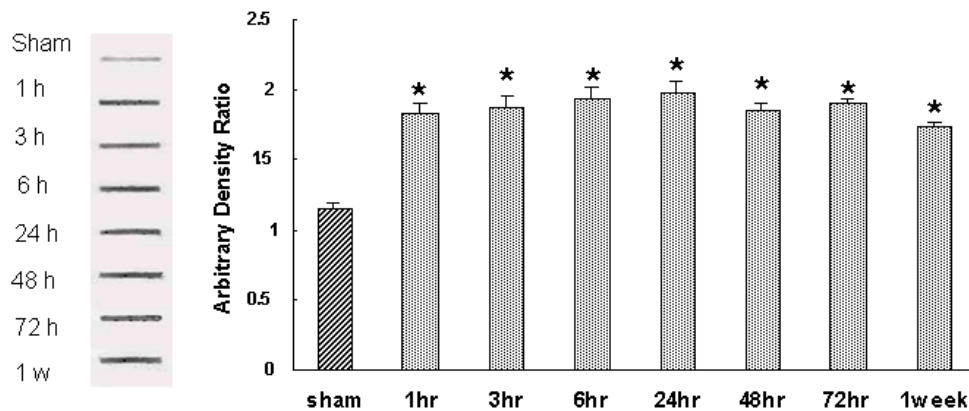
Figure 2.3 displays the time course of production of lipid peroxidation-generated 4-HNE between 1 hr and 1 week after moderate SCI. Injury resulted in a sustained increase in 4-HNE after injury [One-way ANOVA $F_{(7,40)}=6.822$, $p<0.0001$]. The significant elevation of 4-HNE was observed at all time points observed after SCI ($p<0.0001$, n=6). The peak increase was observed at 24 hrs post injury ($p<0.01$ vs. 6 hrs, n=6) and the high levels waned thereafter ($p<0.05$ vs. 48 hrs, 72 hrs, 1 week, n=6). Based on these results, the temporal characteristics of the formation of PN, protein carbonyls and 4-HNE after SCI are in excellent agreement with each other.

Figure 2.3 Time course of 4-hydroxynonenal formation after SCI
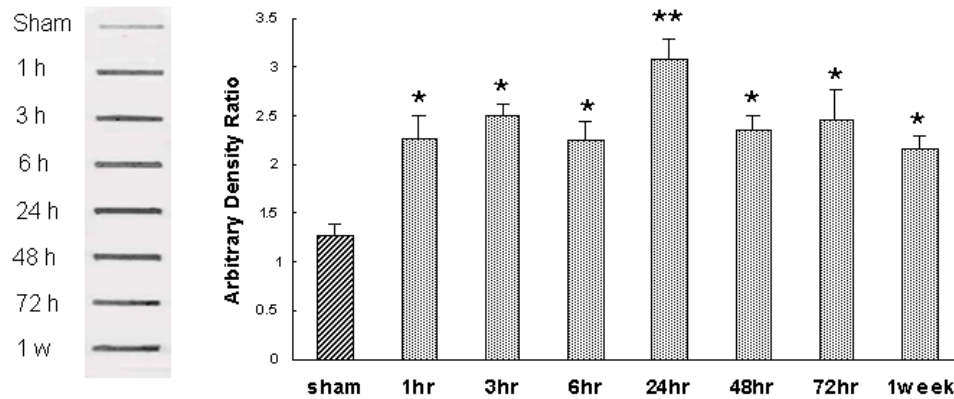
Immunohistochemical Analysis of Peroxynitrite-induced Oxidative Damage

As shown in **Figure 2.4**, 3-NT immunoreactivity (3-NT-IR) was absent in cross sections obtained from laminectomy control (sham) animals harvested at 24 hrs following surgery. In contrast, at 1 hr following injury, a massive increase in 3-NT-IR was detected in cross sections examined at the epicenter of the contusion injury. Staining was most intense in the gray matter, but was also seen to extend into the surrounding white matter. By 3 hrs post-injury, a necrotic (hemorrhagic) lesion appeared in the gray matter dorsal to the central canal. Between 3 and 6 hrs this lesion increased in size and was surrounded by 3-NT immunostaining. No 3-NT immunoreactivity was detected in the section in the absence of primary antibody exposure (Data not shown). Scale bar: 0.2 mm.

Figure 2.4 Immunohistochemistry of 3-NT staining at different time points after SCI

Sham   1 hr   3 hrs   6 hrs
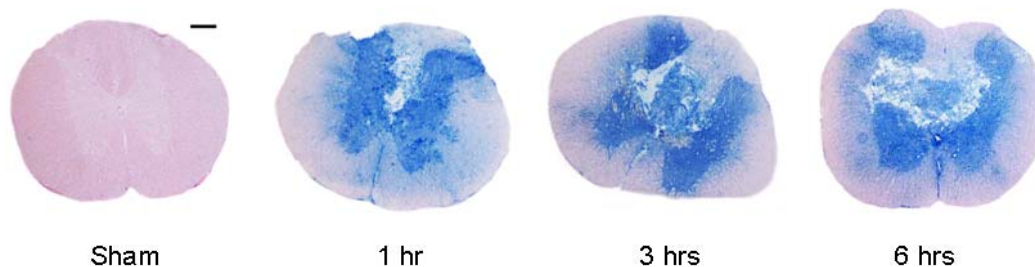
The cross sectional immunohistochemical analysis of 4-HNE accumulations after SCI is shown in the lower panel of **Figure 2.5**. The spatial and temporal staining patterns of 4-HNE are quite similar to those for 3-NT staining. Positive 4-HNE immunoreactivity (HNE-IR) was weak, but present, in the gray matter of sham non-injured rats. At 1 hr following injury, intense HNE-IR was detected around the impact site and was primarily associated with the gray matter, and a major necrotic cavitation formed by 6 hrs following SCI. The co-localization of lipid peroxidized 4-HNE and PN marker 3-NT suggested that lipid peroxidation, which is at least partly mediated by or associated with PN formation, is another important oxidative mechanism following acute SCI.

Figure 2.5 Immunohistochemistry of 4-HNE staining at different time points after SCI

Sham   1 hr   3 hrs   6 hrs
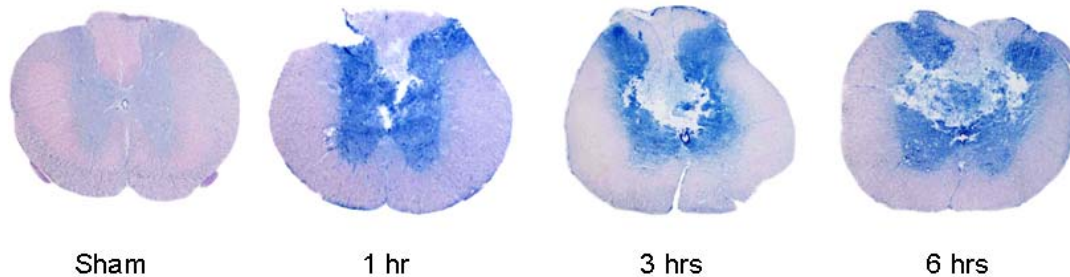
It should be noted that the apparent discrepancy between the slot blot studies which showed a substantial 3-NT and 4-HNE signal in the sham, non-injured animals and the immunohistochemical studies which showed little or no 3-NT or 4-HNE staining in the shams we believe is probably due to the difference in preparation of the animals. The slot blot measurements were made on samples obtained from animals that were not perfused. Thus, it is probable that the measured 3-NT and 4-HNE is largely due to presence of blood components in the samples. In contrast, the animals used for the immunohistochemistry were of necessity perfused with PBS and with paraformaldehyde which would eliminate blood contribution. In any case, even though the apparent magnitude of the oxidative damage increase is more dramatically seen in the

immunohistochemical studies compared to the immunoblot analyses, the extended time course of the post-traumatic increase in oxidative damage is observed with both methods.

To have a better understanding of the co-localized phenomenon of 3-NT and 4-HNE staining, higher power views (20X) of the 3-NT and 4-HNE immunohistochemistry at 6 hrs post injury are provided in **Figure 2.6**. Intense 3-NT and 4-HNE immunostaining are seen in motor neuron somata and axons that course through the ventral spinal white matter. Increased staining is also apparent throughout the neuropil. The staining patterns of 3-NT and 4-HNE observed in high power views are also resembled to each other. Scale bar: 0.2 mm.

Figure 2.6 Co-localization of 3-NT and 4-HNE Immunostaining

6 hr 3-NT

6 hr 4-HNE

4X   20X
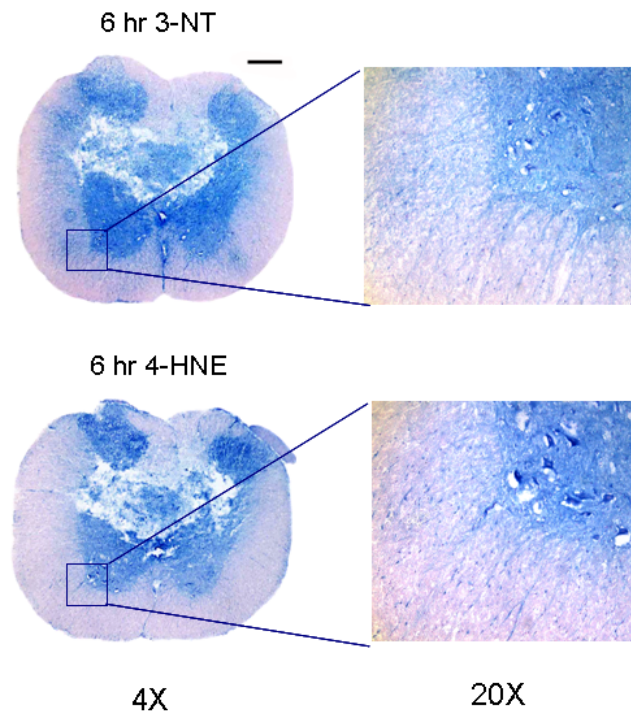
Careful observation of 3-NT staining showed PN formation is not just limited to the injury site, the ensuing oxidative stress spread within the SC tissue. **Figure 2.7** displays the distribution of 3-NT immunostaining in five sections over an 8 mm distance which includes the epicenter and the surrounding rostral (+) and caudal (-) tissues. The sections were obtained from animals that sacrificed at 6 hrs after SCI. Intense 3-NT

immunoreactivity was detected even at sections 4 mm rostral to the injury epicenter. Scale bar: 0.2 mm.

6 hr 3-NT

| +4 mm | +2 mm | Epicenter | -2 mm | -4 mm |
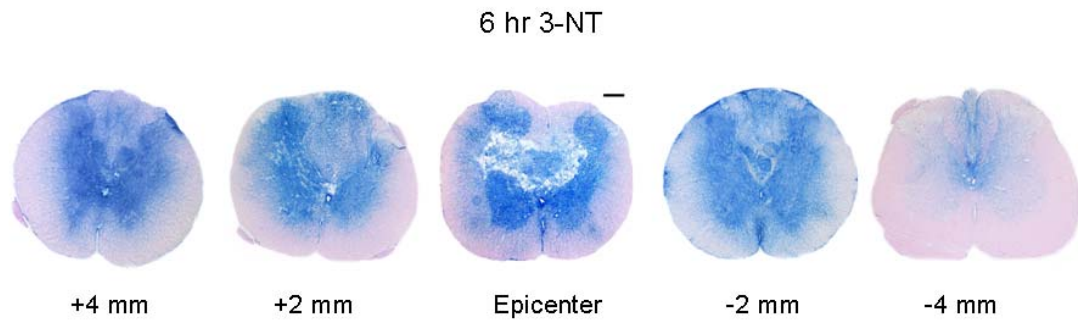
Figure 2.7 Distribution of 3-NT formation at different levels after SCI

In **Figure 2.8**, examples of 15 mm longitudinal SC sections are shown which demonstrate the extent and coincidence of 3-NT and 4-HNE accumulations at 24 hrs following injury. Obvious tissue loss and cavitation could be observed at the impact sites. The 3-NT-IR was most intense around the injury site, but was clearly detected up to 5 mm rostral and caudal to the injury epicenter. Although the staining was most intense in the gray matter, increased staining is also seen in the white matter. The 4-HNE-IR showed the similar staining pattern again underscoring the overlap and coincidence of nitrative and lipid peroxidative damage after SCI. Scale bar: 1 mm.

Figure 2.8 Co-localization of 3-NT and 4-HNE immunostaining in longitudinal sections

24 hr 3-NT

24 hr 4-HNE
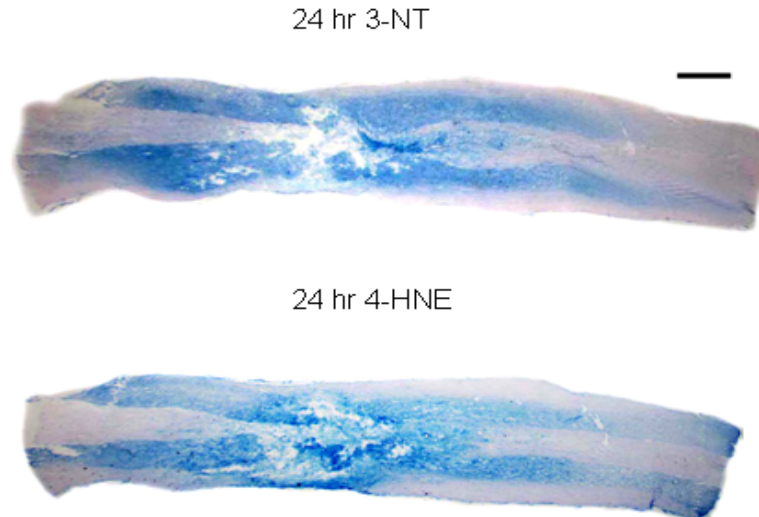
Time Course of Calpain-Mediated Cytoskeletal Degradation

We next carried out experiments to investigate the calpain activation and subsequent cytoskeletal disruption after SCI by measuring the breakdown of α-spectrin. **Figure 2.9** shows the time course of changes in the levels of the breakdown products of the cytoskeletal protein α-spectrin which has been demonstrated to be produced by the proteolytic action of calpain (Wang, 2000). A representative western blot is shown in the upper panel. The bands from the top to the bottom are stained for intact α-spectrin (280 kD), calpain/caspase 3-generated 150 kD and calpain-generated 145 kD fragments, respectively. In the present study, we focused on the 145 kD fragments. The accumulation of calpain-specific SBDP 145 was readily detected across the post-traumatic time course [One-way ANOVA $F(7,40)=22.620$, $p<0.0001$]. Post-hoc analysis showed that there was a significant increase in the 145 kD as early as 1 hr post-injury, indicating the rapid onset of calpain activation after SCI. Quantitative analysis of SBDP 145 showed the significant increases of all the injury groups compared to the sham group ($p<0.0001$, n=6). However the peak increases didn't occur until 72 hrs

post-injury (p<0.05 vs. 48 hrs, n=6), suggesting that maximal activation of calpain was somewhat delayed in comparison to the earlier peak in oxidative damage which occurred at 24 hours post injury (Figure 2.1 and 2.3). At 1 week post injury, the increase of SBDP 145 decreased back toward the control level (p<0.0001 vs. 72 hrs, n=6), but remained significantly higher than the non-injured sham group (p<0.0001, n=6).
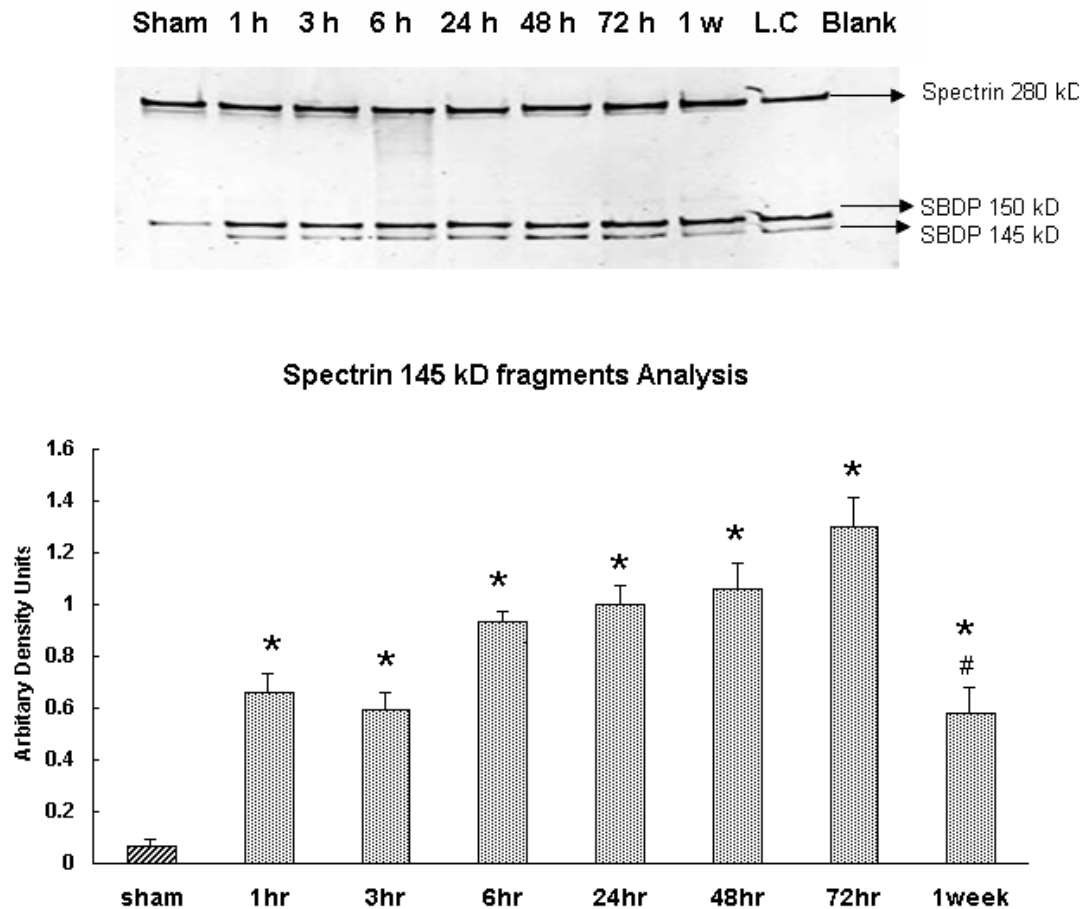
Figure 2.9 Time course of Spectrin breakdown after SCI

IV. Summary and discussion

Spatial and Temporal Pattern of PN-Mediated Oxidative Damage: Although the role of reactive oxygen species (ROS) and oxidative damage has been widely studied in spinal cord injury models (Hall and Braughler, 1989; 1993; Azbill et al., 1997; Springer et al., 1997; Juurlink and Paterson, 1998; Aksenova et al., 2002), only a few investigations have addressed the role of the ROS PN post spinal cord injury pathophysiology. In previous studies, neuronal NOS activity was shown to be upregulated at 5 hrs following SCI in rats (Sharma et al. 1996). Subsequently, it was shown that inducible NOS expression levels and activity are also increased after contusion SCI except this does not occur until 72 hrs after contusion SCI (Diaz-Ruiz et al., 2004). Evidences have shown that the administration of NOS inhibitors can exert a neuroprotective effect in the injured rat spinal cord and improve neurological recovery (Cohen, 1996). From these studies, it was originally proposed that $^\bullet$NO itself plays an important role in secondary damage following SCI (Sharma et al. 1996). However, interpretation of these data has changed since it is now clear that PN is the mediator of $^\bullet$NO cytotoxicity (Bartosz, 1996). Consistent with the hypothesis that PN is a critical player in post-traumatic oxidative damage, three different antioxidant compounds that either scavenger PN or its derived free radicals $^\bullet$OH, $^\bullet$NO$_2$ and CO$_3^{\bullet-}$, have been shown to be neuroprotective and/or to improve neurological recovery in rat SCI models. For instance, uric acid, a selective inhibitor of certain PN-mediated reactions, protected the spinal cord tissue from secondary damage in vitro and in a mouse SCI model (Scott et al., 2005). Tempol, a scavenger of PN-generated free radicals (Carroll et al., 2000), improves locomotor and histological outcomes after spinal cord contusion in rats (Hillard et al., 2004). More recent study has shown that exposure of rat spinal cord tissue in vivo to PN can produce oxidative damage like that found in the mechanically injured spinal cord (Liu et al., 2005). All these findings strongly support the concept that PN is an important mediator of secondary oxidative damage after acute SCI.

Despite these previous studies which imply that PN is involved in post-SCI secondary injury, an understanding of the spatial and temporal characteristics of

PN-induced oxidative damage has been lacking. The present results provide this much needed information. First of all, the time courses of protein nitration, LP and protein oxidation show an almost identical temporal pattern. Moreover, all three markers, as measured by either immunoblotting or immunohistochemistry, increased rapidly (as early as 1 hr) after spinal cord injury. In the case of lipid peroxidative (i.e. 4-HNE) and protein oxidative (i.e. protein carbonyl) damage, this is consistent with the findings of earlier researchers who have examined time courses of lipid and protein oxidative secondary injury in rat SCI models (Azbill et al., 1997; Springer et al., 1997; Baldwin et al., 1998; Aksenova et al., 2002). However, our current time course is more complete in that it includes both LP and protein oxidation in parallel as well as including protein nitrative damage after SCI.

In our immunohistochemical analysis, intense 3-NT and 4-HNE immunoreactivity was seen in the injury epicenter and adjacent area, and more importantly, in those areas, the 3-NT and 4-HNE staining are spatially and temporally coincident. It is well known that LP 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; Hall and Braughler, 1993; Anderson and Hall, 1993; Luo, 2005). It is a multi-step process that causes damage to membrane lipids and results in the changes in membrane fluidity and permeability, altered function of membrane-associated proteins and increased likelihood of organelle and cell lysis (Gutteridge and Halliwell, 1989; Halliwell and Chirico, 1993). Our finding that the spatial and temporal patterns 3-NT and 4-HNE go hand in hand suggests that PN may be a common mediator of LP as well as protein nitration. However, it is also likely that iron catalyzed, $^\cdot$OH-initiated LP mechanisms also play some role in post-traumatic LP and protein oxidation in injured spinal cord tissue (Braughler and Hall, 1989; Hall and Braughler, 1989; 1993) although they cannot directly contribute to protein nitration. Both protein nitration and LP are most intense in the central gray matter and progressively extend into the surrounding white matter. This extension of oxidative damage from the center of the cord toward its periphery is consistent with the long known centrifugal progression of post-traumatic neurodegeneration that occurs after blunt SCI. Furthermore, our longitudinal immunohistochemical observation at 24 hrs

after SCI demonstrated that both 3-NT and 4-HNE staining spreads a considerable distance from the injury site, suggesting oxidative damage is a crucial pathological event after SCI that leads to further damage by affecting adjacent area.

Secondly, our results show that the time course of PN-mediated oxidative damage extends out to at least a week after injury. This finding of sustained oxidative damage suggests that antioxidant strategies which target PN may need to be prolonged for multiple days in order to adequately protect the injured spinal cord. Thus, the single dose studies that have so far been carried out in rodent SCI models with either the PN scavenger uric acid (Scott et al., 2005) or the PN radical scavenger Tempol (Hillard et al., 2004) although sufficient to show some positive effects, are probably inadequate to produce optimal antioxidant protection.

Relationship of Oxidative Damage to the Temporal Pattern of Calpain-Mediated Cytoskeletal Degradation: Previous studies have shown calpain-mediated proteolysis is also a key contributor to the post-traumatic damage in the injured spinal cord (Braughler and Hall, 1984; Springer et al., 1997; Aksenova et al., 2002; Ray and Banik, 2003). Calpains, a family of Ca^{2+}-dependent cytosolic proteases, are abundantly expressed in the central nervous system. Pathological calpain activation is known to be triggered by excessive intracellular Ca^{2+} accumulation (Bartus, 1995; Kampfl et al., 1997), which is believed to be associated with the glutamate release and sustained NMDA activation as well as depolarization-induced opening of voltage-dependent Ca^{2+} channels after CNS trauma. After activation, calpains trigger multi-pattern neuronal death by cleavage of a broad spectrum of substrates including cytoskeletal proteins, membrane-associated proteins, enzymes involved in signal transduction, transcription factors and others (Carafoli and Molinari, 1998; Wang, 2000). Calpain-mediated proteolysis of the major membrane-skeletal protein, α-spectrin, results in the appearance of two highly stable breakdown products, calpain-specific SBDP 145 and the non-specific calpain/caspase 3 generated SBDP 150.

Several lines of evidence indicate that ROS and oxidative damage mechanisms participate in the exacerbation of post-traumatic Ca^{2+} overload and calpain-mediated cellular proteolysis. It has been generally reported that ROS cause a rapid increase in Ca^{2+} concentration in the cytoplasm of diverse cell types (Roveri et al., 1992; Chakraborti et al., 1999; Okabe et al., 2000). Growing evidence has shown that ROS regulate mitochondrial Ca^{2+} homeostasis and respiration (Richter, 1995). Specifically, PON formed by mitochondrial nitric oxide synthase (NOS) has been found to promote Ca^{2+} release from intact mitochondria (Bringold, 2000). All these studies support the notion that ROS probably promote calpain activation by disrupting intracellular Ca^{2+} regulation and thereby increasing Ca^{2+} overload.

On the other hand, our data suggests that the effect of ROS on calpain activation is more complicated than simply exacerbating its activity by compromising intracellular Ca^{2+} homeostasis and it has been hypothesized that the proteolysis of substrates by calpain is affected by the redox state of the local environment. Indeed, several investigators have reported that calpain activity can be strongly inhibited by ROS (Benuck et al., 1992; Rodney et al., 1997; Guttmann et al., 1997; Guttmann and Johnson, 1998; Jamie et al., 2001). To obtain a better understanding of the linkage between PN-mediated oxidative damage and calpain activation following SCI, a complete time course study of calpain-mediated cytoskeletal degradation, indicated by measurement of calpain-specific SBDP 145, was carried out. The results show that there is a biphasic time course of post-traumatic calpain-mediated α-spectrin degradation. Initially, there is a rapid onset of calpain activation and followed by a gradual secondary increase in α-spectrin degradation. The peak in cytoskeletal degradation is not achieved until 72 hrs following SCI even though it is well known that intracellular Ca^{2+} overload is triggered very early after acute SCI (Young et al., 1982; Stokes et al., 1983). A similar pattern of slowly progressive calpain-mediated α-spectrin degradation has also been observed in the hippocampus, cortex and striatum in transient ischemic injury model (Subert et al., 1989; Saido et al., 1993; Neumar et al., 2001). It is possible that even though calpain activation and cytoskeletal damage is initiated very early, it is partially mitigated by some initial

ROS-induced inhibition of calpain activity. Later, as oxidative damage progressively impairs the various Ca^{2+} homeostatic mechanisms, intracellular Ca^{2+} overload will increase and calpain activation will intensify.

Figure 2. 10 Hypothetical interrelationship between oxidative damage and calpain proteolytic degradative mechanisms following SCI
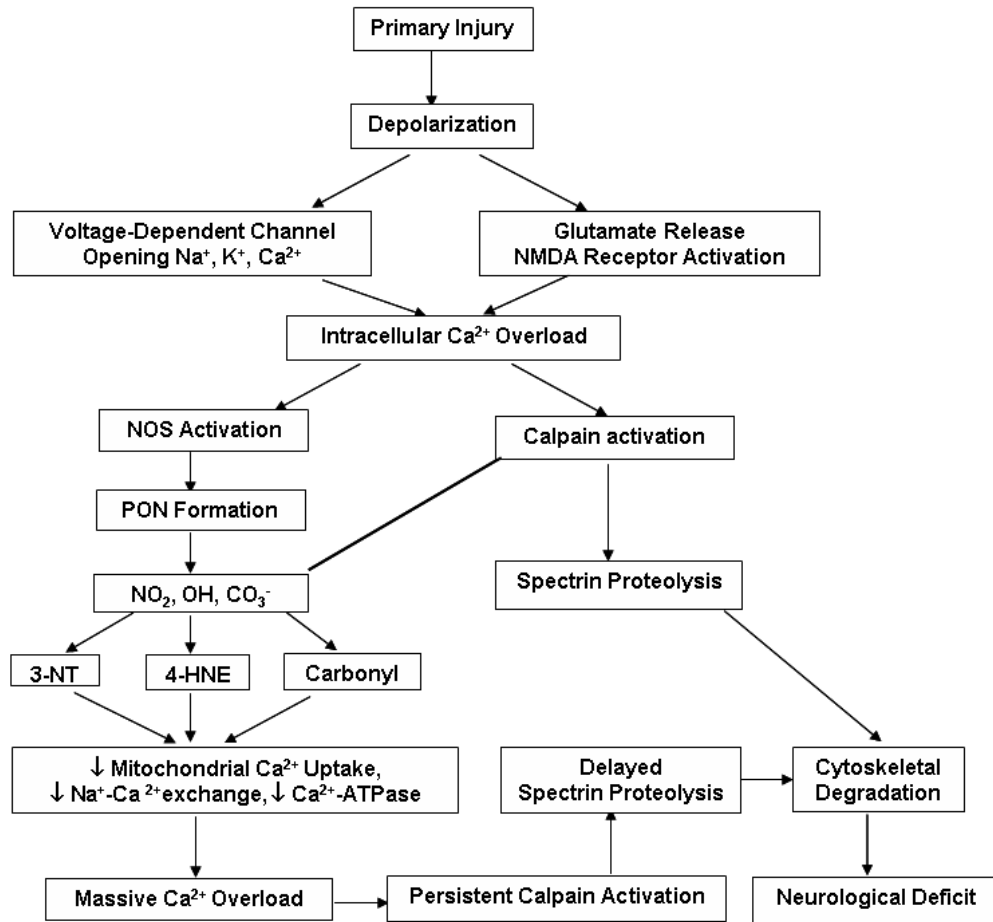
Hypothesis and Summary: Our overall hypothesis based upon the findings in this study is illustrated in **Figure 2.10**. Initial insults to the spinal cord cause membrane depolarization, resulting in the opening the voltage-dependent Na^+, K^+ and Ca^{2+} channels, and the release of glutamate into the extracellular spaces, which will lead to the sustained activation of NMDA receptor. Both mechanisms directly or indirectly elevate intracellular Ca^{2+} and therefore activate cytosolic calpain. This initial Ca^{2+} influx results

in the rapid onset of calpain activation and the early accumulation of α-spectrin fragments (e.g. SBDP 145). At the same time, mitochondria take up the excessive Ca^{2+} in the cell, which subsequently activates mitochondrial NOS and results in overproduction of $^\bullet$NO radical. Elevated $^\bullet$NO out-competes superoxide dismutase (SOD) for superoxide radical ($O_2^{\bullet-}$), leading to the formation of PN. Then, PN-derived free radicals initially produce a partial inhibition of calpain activity. However, prolonged oxidative stress induces cellular oxidative damage by lipid peroxidation (LP), protein oxidation and nitration, indicated as increased 4-HNE, protein carbonyls and 3-NT respectively. These cytotoxic products exacerbate Ca^{2+} overload by compromising the mitochondrial Ca^{2+} transport, reversing the Na^+-Ca^{2+} exchanger (Li et al., 2000) and impairment of the Ca^{2+}-ATPase (Kurnellas, 2005). This secondary disruption of Ca^{2+} homeostasis results in persistent calpain activation, which dramatically out-competes the initial inhibition of ROS, induces further cytoskeletal degradation, which is indicated as the delayed phase of our α-spectrin breakdown analysis.

In summary, the findings of this study strongly support the important role of PN-mediated oxidative damage following spinal cord injury. Prolonged oxidative damage and calpain activation after SCI, these two important events interact and are responsible for much of the secondary injury tissue loss after SCI. Accordingly, a combination of PN-targeted antioxidants and a calpain inhibitor might have a better neuroprotective action than either approach alone. Future studies in our laboratory will explore this possibility.

CHAPTER THREE

BENEFICIAL EFFECTS OF TEMPOL, A CATALYTIC SCAVENGER OF

PEROXYNITRITE-DERIVED FREE RADICALS, IN A RAT MODEL OF SPINAL

CORD CONTUSION INJURY

Abstract of some portions of this work has been published as:

Beneficial effects of tempol on oxidative damage, mitochondrial dysfunction and cytoskeletal degradation after spinal cord injury in rats. (2007) *J. Neurotrauma. 24(7): p229*

This manuscript is in the process of submitting to *J. Neuroscience.*

Note: I, Yiqin Xiong, contributed 100% to the research shown in the result section of Chapter Three. Thanks to Drs. Alexander G. Rabchevsky and Indrapal N. Singh for mitochondrial expertise.

I. Introduction

Oxidative damage is one of the well-documented secondary injury mechanisms after initial insults to CNS. Among all the reactive oxygen species (ROS), the role of peroxynitrite (PN), formed by nitric oxide ($^{\bullet}$NO) and superoxide ($O_2^{\bullet-}$) (Beckman et al., 1990), has been proposed as a key contributor of post-traumatic oxidative damage (Beckman, 1994; Crow and Beckman, 1996), mainly because its decomposition products ($^{\bullet}$NO$_2$, $^{\bullet}$OH, $CO_3^{\bullet-}$) possess potent free radical characteristics. PN-derived radicals exert toxic effects by oxidizing protein, modifying protein by tyrosine nitration and inducing lipid peroxidation (Hall and Braughler, 1989; 1993; Kruman et al., 1997; Alvarez and Radi, 2003; Xiong et al., 2007). Moreover, PN can react with DNA forming single-strand DNA breaks (Salgo et al., 1995a; Szabo et al., 1996) and directly inhibit mitochondrial respiration (Bolanos et al., 1995), resulting in further cellular and tissue damages. In agreement, a number of PN-targeting compounds protected against secondary damage, reduced inflammation and improved the functional recovery after spinal cord injury (SCI), either by selectively inhibiting PN-mediated reactions or scavenging PN (Scott et al., 2005; Genovese et al., 2007), which strongly supported the role of PN in mediating secondary damage and open up a novel direction to neuroprotective therapy after CNS trauma.

In the present study we focused on tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine -N-oxyl), a representative of a newly emerged antioxidant family: stable nitroxide radicals (Krishna and Samuni, 1994). Nitroxides are endowed with versatile antioxidant properties including but not limited to: (superoxide dismutase) SOD-mimic activity with better membrane permeability and potency than SOD (Krishna et al., 1996); catalase and peroxidase-like activities (Mehlhorn and Swanson, 1992; Krishna et al., 1996), inhibition of the production of hydroxyl radicals ($^{\bullet}$OH) during Fenton-type reactions (Zeltcer et al., 1997) and inhibition of lipid peroxidation by disrupting lipid radical chain reactions (Miura et al., 1993). Studies showed that 6-membered cyclic nitroxides, such as tempol, are more effective antioxidants since they react with superoxide ($O_2^{\bullet-}$) about 2 orders of magnitude faster than 5-membered ring nitroxides (Samuni et al., 1990). Moreover, several other unique characteristics of tempol make it superior including the fact that it has a small molecule weight (MW:172) and can cross the blood brain barrier readily (Mitchell et al.,

1991), and most importantly, tempol can catalytically decompose the PN-generated free radicals nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radical ($CO_3^{\bullet-}$) (Carroll et al., 2000). The oxidized forms of tempol can exchange among themselves without depletion in the reaction with PN-derived radicals. Consistent with this mechanism of action, it has been shown that the level of 3-nitrotyrosine (NT), a specific marker for PN formation, decreased in the tempol-treated tissue, supporting the notion that tempol may largely exert its protective effects through scavenging PN-derived radicals (Carroll et al., 2000).

We have previously reported that PN-mediated oxidative damage involved spinal cord tissue and downstream calpain-activated cytoskeletal breakdown (Chapter Two). Past studies demonstrated mitochondrial oxidative damage paralleled subsequent functional compromise in traumatic brain injury, strongly suggesting that mitochondria are the primary source and target of PN (Singh et al., 2006). Similar mitochondrial dysfunction was also observed following SCI (Sullivan et al., 2007). 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. By taking this pharmacological approach, we are able to establish a mechanistic linkage between these successive pathophysiological events; confirm the beneficial effects of tempol after SCI recently reported by others (Hillard et al., 2004) and perhaps advance a promising therapeutic approach for acute neuroprotection after SCI.

II. Materials and methods

Subjects

This study employed young adult female Sprague-Dawley rats (Charles River, Portage, MI) weighing between 200 and 225 grams. 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) and xylazine (10 mg/kg) before a laminectomy at T10 vertebrae level was performed. Spinal cord injury was carried out using the Infinite Horizon device (Scheff et al., 2003) as previously described (Chapter Two). The force applied to spinal cord was 200 kdyn, which produces a moderately severe injury.

Tempol Preparation and Dosing

Tempol was purchased from Sigma-Aldrich (Milwaukee, WI, USA) and freshly prepared in 0.9% saline before abdominal intraperitoneal (i.p.) injection. Rats were randomly allocated into 3 or 4 groups (n=6 per group): (I) Sham, subjected to T10 laminectomy and no treatment. (II) Vehicle, subjected to spinal cord injury and given 0.9% saline or DMSO as vehicle i.p. at the onset of injury. (III) Tempol-treated, subjected to SCI and given tempol (300 mg/kg) i.p. at the onset of injury. (IV) For initial experiments, lower dose of tempol (150 mg/kg, i.p.) group was also included to determine the optimal dosing.

Slot-Immunoblotting and Western-Blotting Analysis

Spinal cord was rapidly squirted out 24 hrs after laminectomy or SCI. 10 mm spinal cord (SC) tissue including impact epicenter was dissected and homogenated 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×g for 30 min and the supernatant was collected and normalized to 1 µg/µl by protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). For slot blots, 2 µg proteins was loaded in each well of the slot blot apparatus and collected on the nitrocellulose membrane by sedimentation. For western blot, 5 µg of protein was loaded on 3-8% SDS/PAGE gels and transferred to nitrocellulose membranes after electrophoresis. The membranes were blocked in odyssey blocking buffer for 1 hr followed by incubating in primary antibody at 4°C overnight. Rabbit polyclonal anti-hydroxynonenal (HNE) antibody (1:5000; Alpha Diagnostics International, Inc., San Antonio, TX, USA), rabbit anti-nitrotyrosine (NT) antibody (1:2000; Upstate USA, Inc., Charlottesville, VA, USA) were applied to detect lipid peroxidation and protein nitration, respectively. Oxy-blot detection kit (Chemicon International, Temecula, CA, USA) was employed to detect protein oxidation. For α-spectrin breakdown products, mouse monoclonal anti-α-spectrin antibody (1:5000; Biomol International, LP Plymouth, PA, USA) was applied. The blots were analyzed using the Li-cor odyssey infrared imaging system (Li-cor Biosciences, Lincoln, NE, USA), which employs IRDye800 conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000, Rockland) as the secondary antibodies.

Immunohistochemical Analysis for 3-NT

At 24 hrs post-injury another two sets of rats (2 per set), one was injured and the other was tempol treated (300 mg/kg i.p.), were overdosed with sodium pentobarbital (150 mg/kg) and perfused with 0.1M PBS followed by 4% Paraformaldehyde in 0.1M PBS. 10 mm SC segment containing impact center was then harvested and properly stored. Spinal cords from injured and tempol-treated group were paralleled and sectioned at 20 µm in a transverse plane. After rinsing at the day of staining, the sections were incubated, blocked and exposed to anti-3-NT antibody overnight. The following day, sections were incubated for 2 hrs 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 hr followed by development of the staining using the Vector blue method (Vector Blue Alkaline Phosphatase Substrate Kit; Vector Labs) in the dark for 10-30 min. After reaction, spinal

cord sections were counter-stained with nuclear fast red (Vector Labs, Burlingame, CA), dehydrated and then photographed on an Olympus Provis A70 microscope with an Olympus Magnafire digital camera (Olympus America, Inc., Melville, NY, USA).

Mitochondrial Ficoll Gradient Purification

Briefly, the whole spinal cord were rapidly squirted out and 20 mm segments with injury site in the center were 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℃. First, it was centrifuged twice at 1,300×g for 3 min in an eppendorf microcentrifuge at 4℃ to remove cellular debris and nuclei. Then the pellet was discarded, and the supernatant was further centrifuged at 13,000×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, Illinois) to release synaptic mitochondria and cooled to 4℃ under a pressure of 1,200 psi for 10 min (Brown et al., 2004; Singh et al., 2006). After nitrogen disruption, the mitochondria were placed atop a discontinuous Ficoll gradient (7.5%, 10%), and centrifuged at 100,000×g for 30 min (Sullivan et al., 2003, Singh et al., 2006). The mitochondrial pellets at the bottom were then transferred to microcentrifuge tubes and topped off with isolation buffer without EGTA and centrifuged at 10,000×g for 5 min at 4℃ to yield a tighter pellet (Singh et al., 2006). The final mitochondrial pellet was re-suspended in 35 μg 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) maintained at 37℃ as described previously (Sullivan et al., 2003). In all, about 30 μl of isolated mitochondrial protein was

placed in a chamber containing 250 μL of KCl-based respiration buffer (125 mmol/L KCl, 2 mmol/L MgCl$_2$, 2.5 mmol/L KH$_2$PO$_4$, 0.1% BSA, and 20 mmol/L HEPES at pH 7.2) and allowed to equilibrate for 1 min. State II respiration is fueled by complex I substrates, 5 mmol/L pyruvate and 2.5 mmol/L malate. Two boluses of 150 μmol/L ADP were added to the mitochondria to initiate the state III respiratory rate for 2 min, followed by the addition of 2 μmol/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 μmol/L 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 μM) to shut down state I-driven respiration completely. Complex II-driven respiration is then initiated by the addition of 10 mmol/L complex II activator succinate (Singh et al., 2006). The respiratory control ratio (RCR) was caculated by dividing state III oxygen consumption (rate obtained in the presence of ADP, second bolus addition) by state IV oxygen consumption (rate obtained after the addition of oligomycin) and serves as a sensitive index for mitochondrial functional analysis. Fresh mitochondria were prepared and run for respiration immediately for each experiment to assess the mitochondrial bioenergetics.

Electron Microscopy of Mitochondrial Ultra-structure

As described previously (Nukala et al., 2006; Singh et al., 2006), the purified mitochondrial pellets were fixed in 4% glutaraldehyde overnight at 4°C before being embedded for electron microscopy. The fixed mitochondrial pellets were then washed overnight at 4°C in 0.1 mol/L sodium cacodylate buffer, followed by 1 hr secondary fixation at room temperature in 1% osmium tetroxide (in sodium cacodylate buffer). Then, the mitochondrial pellets were rinsed with distilled water and dehydrated in a gradient ethanol series up to 100% and twice in propylene oxide. The pellets were then placed in a 1:1 mixture of propylene oxide and Epon/Araldite resin and infiltrated overnight on a rotator. Next, 100% Epon/Araldite resin was added and rotated for 1 hr at room temperature. Finally, fresh resin was prepared and degassed using a vacuum chamber. The mitochondrial pellets were added to the flat molds, filled with fresh resin, and baked overnight at 60°C. The 90nm ultra-thin sections were cut using an RMC MT-7000 ultra-microtome mounted on 150 mesh copper grids and stained with uranyl

acetate and lead citrate. A Zeiss 902 electron microscope was used to examine and photograph the samples.

Statistical Analysis

Quantitative densitometry analysis was used for reading the slot blots and western blots. Statistical analysis was performed using the STATVIEW package (JMP Software, Cary, NC). All values are expressed as mean ± standard error (S.E.M.). A one-way analysis of variance (ANOVA) was first run. If the ANOVA revealed a significant effect, post-hoc testing was then carried out to compare individual tempol or saline-treated groups to the sham, non-injured group by Student-Newman-Keuls (SNK) analysis. In all cases, a $p<0.05$ was considered significant.

III. Results

Tempol Effects on Peroxynitrite-mediated Oxidative Damage after SCI

A. Slot-Immunoblotting and Western-Blotting Analysis: There are at least three oxidative mechanisms including protein nitration, lipid peroxidation and protein oxidation involved in PN-mediated oxidative damage following SCI (Chapter Two). The specific marker for PN formation 3-nitrotyrosine (NT), the lipid peroxidation product 4-hydroxynenonal (HNE) and protein oxidation–derived protein carbonyls (PC) were employed as markers for these oxidative events respectively. Animals were sacrificed 24 hrs post-injury when the maximal oxidative damage occurred (Chapter Two). **Figure 3.1** shows the results obtained from quantitative antibody-based immuno-slotblotting. For each oxidative marker, One-way ANOVA revealed that there is 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: [$F_{(2,15)}=54.616$; $P<0.0001$]). Student-Newman-Keuls (SNK) analysis showed injury resulted in significantly increased levels of 3-NT, 4-HNE and PC compared to the sham group, and 300 mg/kg tempol administration reduced the injury-induced elevations of 3-NT and 4-HNE significantly compared to 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 better protective effect of tempol is observed in terms of protein oxidation since tempol was able to bring PC level back to normal level (# $p<0.0001$ vs. injured, n=6).

Figure 3.1 Beneficial effects of tempol on oxidative damage after SCI (Slot blotting)
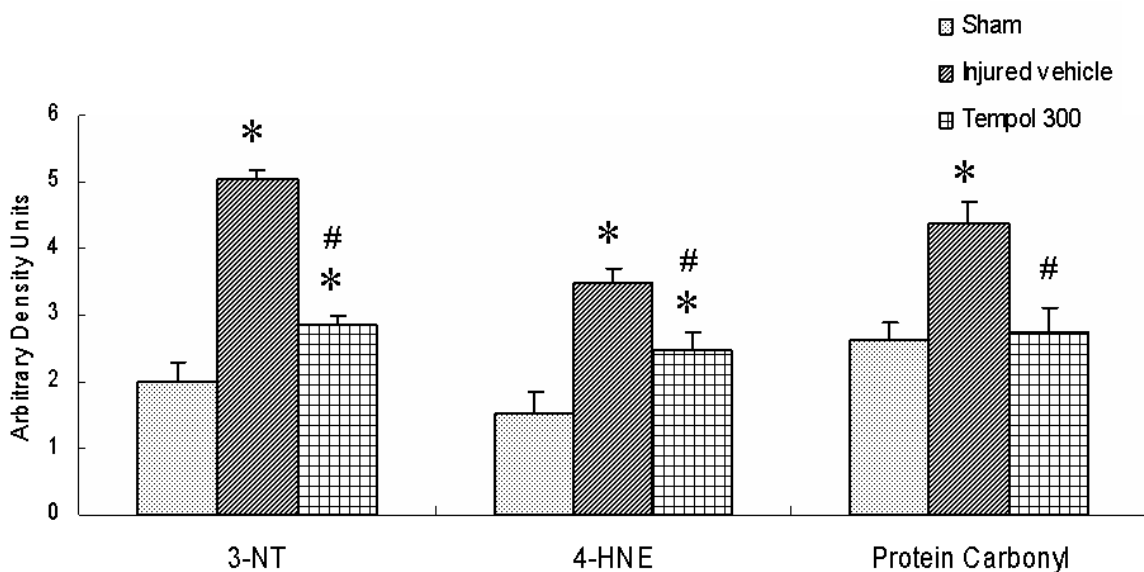
Western blot analysis of PN-mediated oxidative damage was also carried out to further confirm the effects of tempol. Representative blots of 3-NT, 4-HNE and PC shown in **Figure 3.2** provide a clear view of different staining patterns and side-by-side qualitative comparisons of protein nitration, lipid peroxidation and protein oxidation among different groups. As demonstrated, injury (I) produced a rise of staining intensity in all markers comparing to the sham groups (S) and tempol treatment (300 mg/kg i.p.) (T) reduced the degree of injury-induced positive staining. Quantifications were also performed by densitometrically measuring the full-length blot as indicated by the red box. The results shown in Figure 2B are similar to what we obtained in slot blotting. One-way ANOVA was first run to indicate a significant effect associated with treatment (for 3-NT: $F_{(2,15)}=14.697$; $P<0.001$]; for 4-HNE: [$F_{(2,15)}=12.550$; $P<0.001$]; for PC: [$F_{(2,15)}=32.515$; $P<0.0001$]). Post-hoc analysis showed injury produced a significant increases of 3-NT, 4-HNE and PC levels comparing 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 slot immunoblot measurements, the protective effects of tempol detected by western blot were incomplete since the tempol-treated groups remained

significant higher levels in 3-NT and PC when compared with sham groups (*$p<0.05$, n=6).

Figure 3.2 Beneficial effects of tempol on oxidative damage after SCI (western blotting)
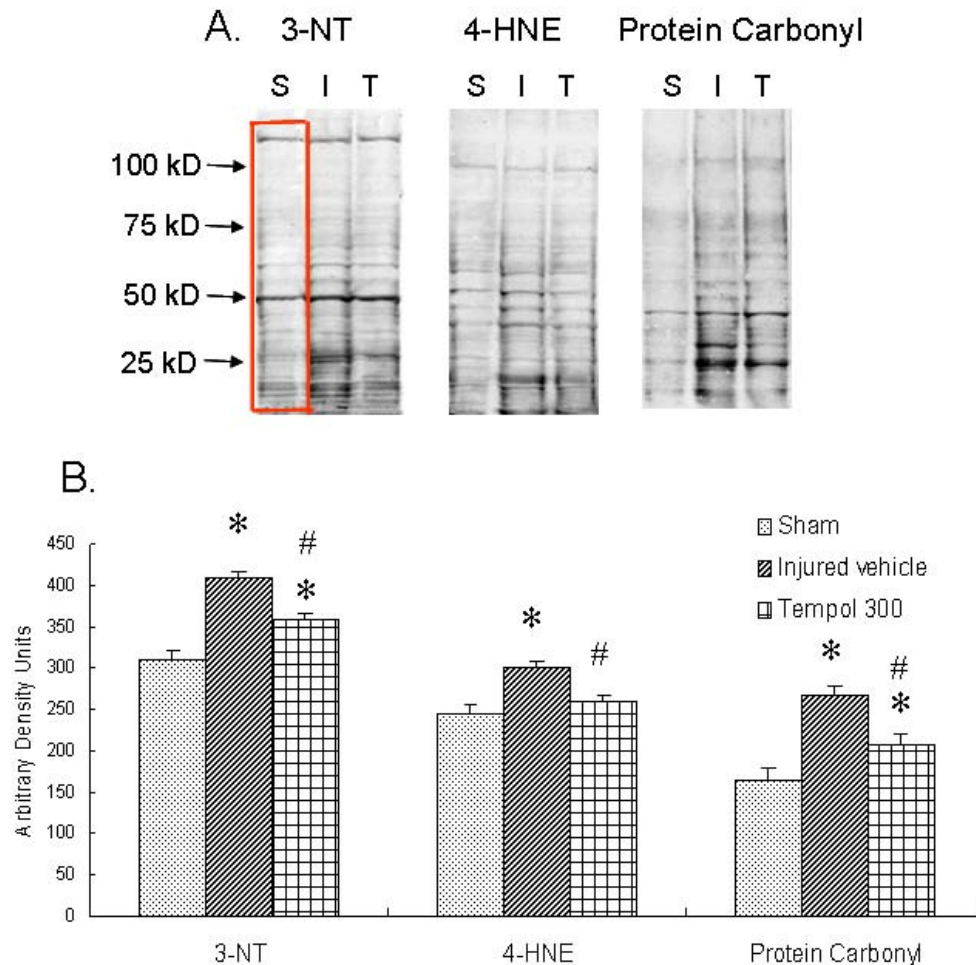
B. Immunohistochemical Analysis: **Figure 3.3** displays the cross sectional illustration of the distribution of 3-NT immunostaining at 24 hrs post-injury. 3-NT immuno-reactivity was compared between a saline and a tempol-treated animal side by side. In the upper panel, which showed sections obtained from the saline-treated vehicle group, contusion SCI resulted in the appearance of a central necrotic lesion at the epicenter, with sparing of a peripheral rim of tissue. Intense 3-NT immuno-staining 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 spreading for some distances along the spinal cord. As displayed, extensive 3-NT immuno-reactivity is still remarkable at the injured sections 2 mm rostral or caudal to the epicenter. In comparison, no necrotic cavity and less 3-NT immuno-reactivity in the gray and white matter were observed at the epicenter of tempol-treated spinal cord (lower panel). Tempol administration clearly limited the 3-NT immuno-reactivity observed in the sections 2 mm rostral and caudal to the epicenter. Scale bar: 0.2 mm.

Figure 3.3 Side-by-side comparison of 3-NT immunohistochemistry in vehicle- and tempol-treated SC
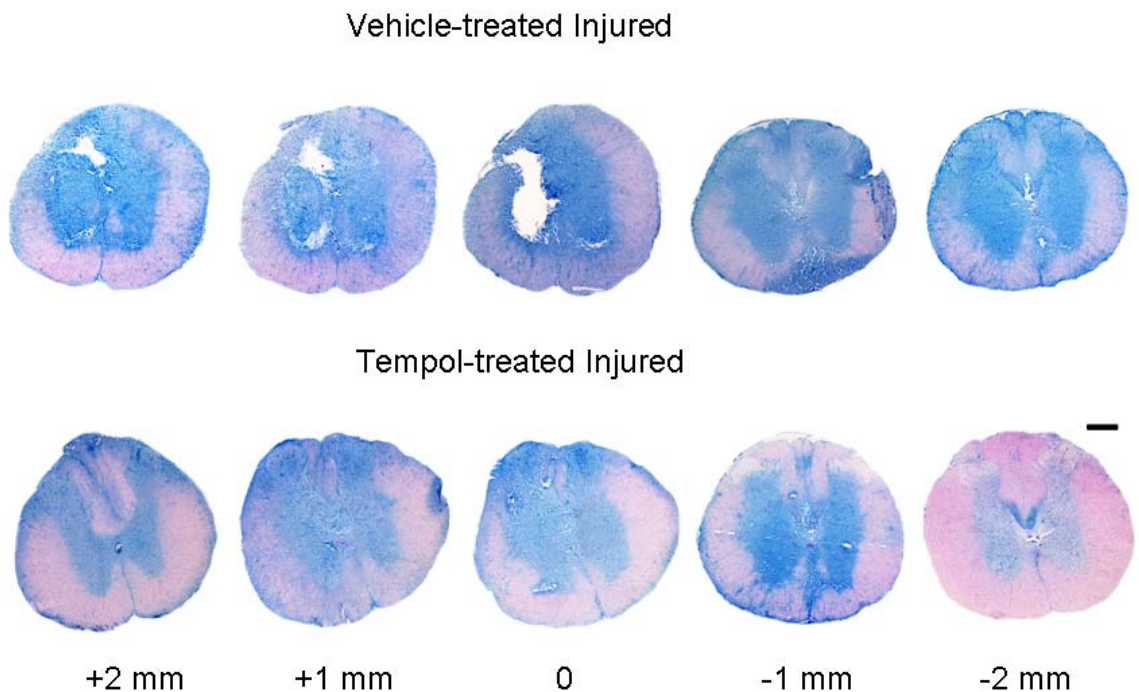
Tempol Effects on Mitochondrial Dysfunction after SCI

Previous studies showed evidence of mitochondrial oxidative damage and subsequent functional impairment following SCI and the injury-induced mitochondrial dysfunction is

at least partly attributable to PN-mediated oxidative damage (Chapter Two; Sullivan et al., 2007). 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 trempol-treated animals. **Figure 3.4** shows a simplified illustration of mitochondrial electron transport chain (ETC) and oxidative phosphorylation. Upper panel (A) displays a typical respiratory trace of spinal cord mitochondrial respiration. Lower panel (B) shows simplified pathways of electron transport through ETC. ETC consists of Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome reductase), Cytochrome b and c, Complex IV (cytochrome oxidase) and Complex V (F_0F_1 ATPase or ATP synthase). (For simplification, some other components such as ubiquinone, are not shown in this figure). Briefly, electrons are fed into Complex I by pyruvate and malate, or into Complex II by succinate, transported through Complex III, cytochrome c and finally reach Complex IV. Each electron is accepted by O_2 and reduced to H_2O. As the electron flows, protons (H^+) are pumped from the matrix into the mitochondrial intermembrane space (IMM) thus forming a proton gradient across the IMM. When H^+ flows back into the matrix through ATP synthase (Complex V), ADP is phorsphorylated to ATP. Knowledges of this process, known as oxidative phosphorylation, are crucial to understand the subsequent mitochondrial respiration studies we employed. Figure 3.4A shows typical responses or respiraton of healthy mitochodria obtained from SC. Addition of pyruvate and malate fuel Complex I and initiate respiratory state II; 2 bolus of ADP activate ATP synthase and initiate State III; Oligomycin is an inhibitor of ATP synthase, so little ADP or O_2 is consumed after addition of oligomycin, which is named State IV. FCCP is an uncoupler that 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. Rotenone shuts down Complex I-driven respiration and addition of succinate fuels Complex II to initiate Complex II-driven State V.

Figure 3.4 Illustration of ETC and respiratory states of Mitochondrial respiration

A. Typical spinal cord mitochondrial respiratory trace

B. Simplified schematic of electron transport chain (ETC) and Mitochondrial respiration
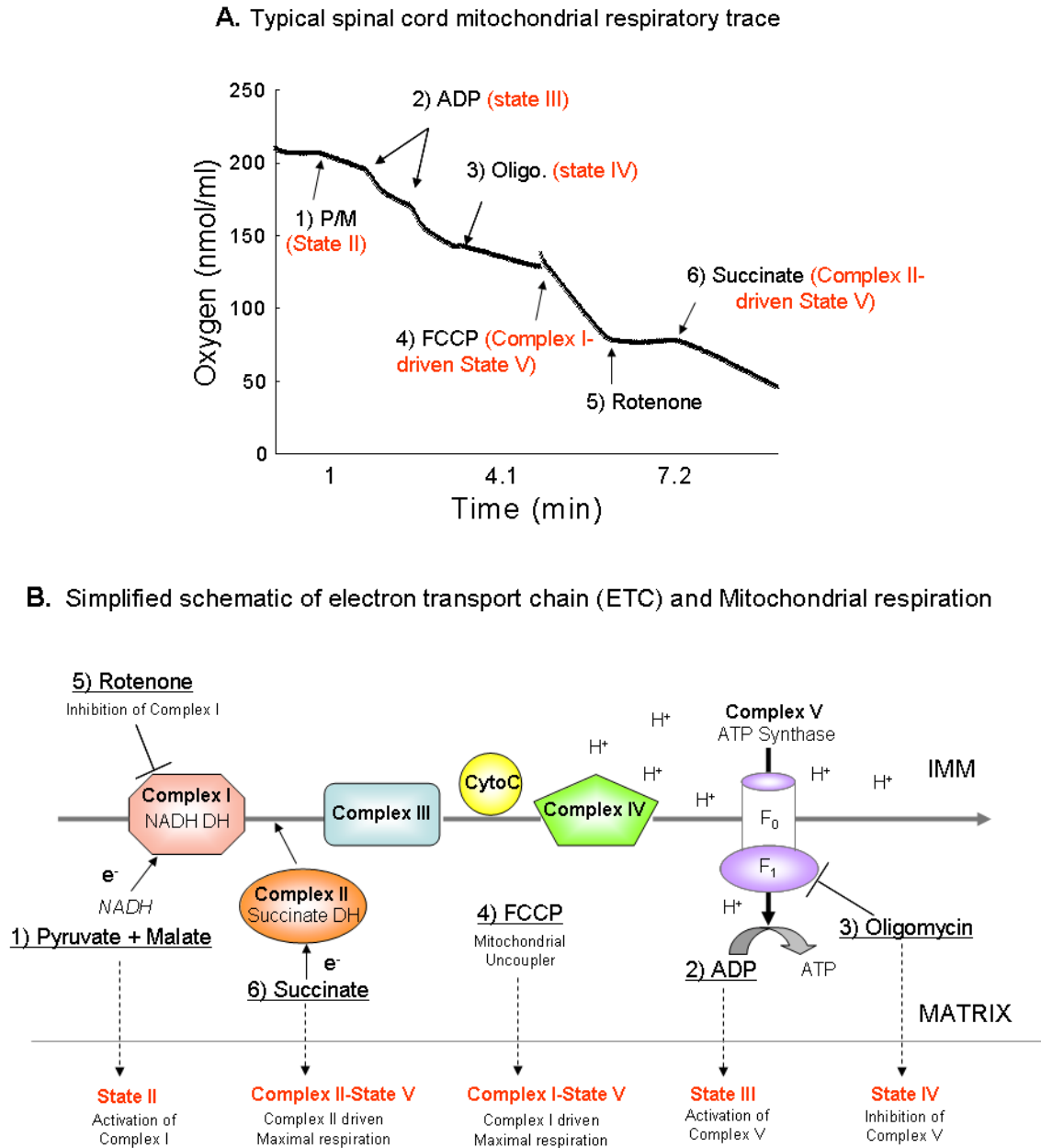
Figure 3.5 demonstrated the effects of tempol on mitochondrial respiration after SCI in terms of respiratory control ratio (RCR). RCR is a widely used index in mitochondrial functional analysis since it is a sensitive measure of mitochondrial respiration and how well the ETC is coupled to oxidative phosphorylation (i.e., efficiency of ATP production). It is calculated as the ratio of oxygen consumption in the presence of ADP (respiration state

III) to the addition of ATPase inhibitor oligomycin (respiration state IV). Mitochondria are considered healthy if presented with a RCR≥5.0. **Figure 3.5A** displays a representative respiratory trace obtained from sham non-injured SC for comparison. Quantification of RCR in **Figure 3.5C** indicated 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 [$F_{(3,20)}=31.501$, $p<0.0001$]. A significant drop of RCR was found 24 hrs after contusion injury with the mean value decreased from 7.4 down to 2.9 (*$p< 0.0001$ vs. sham, n=6). This mitochondrial functional failure was consistent with the ultrastructural changes observed in the following electron microscopic photomicrographs.

Sham non-injured   24 hrs after SCI
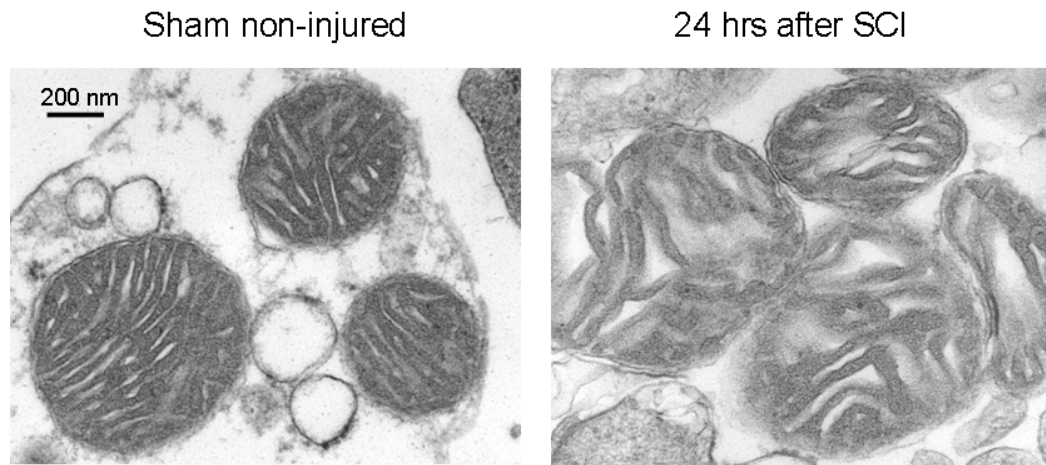
Injury inflicted severe ultra-structural damage to the spinal cord mitochondria, resulting in swelling, dilated cristae and disrupted inner and outer membranes. Quantifications of the RCR showed that a single bolus of tempol (150 mg/kg, i.p.) significantly improved mitochondrial bioenergetics by bringing back the mean RCR to 4.5 (#$p<0.001$ vs. injured, n=6), as indicated in **Figure 3.5C.** A higher dose of tempol (300 mg/kg) showed slightly better effects by increasing the mean RCR to 4.7 (#$p<0.001$ vs. injured, n=6). However, both tempol treatments can only improve injury-induced mitochondrial dysfunction partially, with RCRs remaining significantly lower compared to the sham group (*$p<0.001$, n=6). A representative overlay traces in **Figure 3.5B** demonstrated the impaired oxygen consumption of SC mitochondria isolated from vehicle-treated rats and improved respiration of mitochondria isolated from tempol-treated (300 mg/kg) rats. In agreement, the electron microscopic mitochondrial picture in **Figure**

3.6 appears to have more normal-looking mitochondria in tempol-treated (300 mg/kg) group comparing to vehicle-treated group.

Figure 3.5 Tempol partially preserved mitochondrial function after SCI
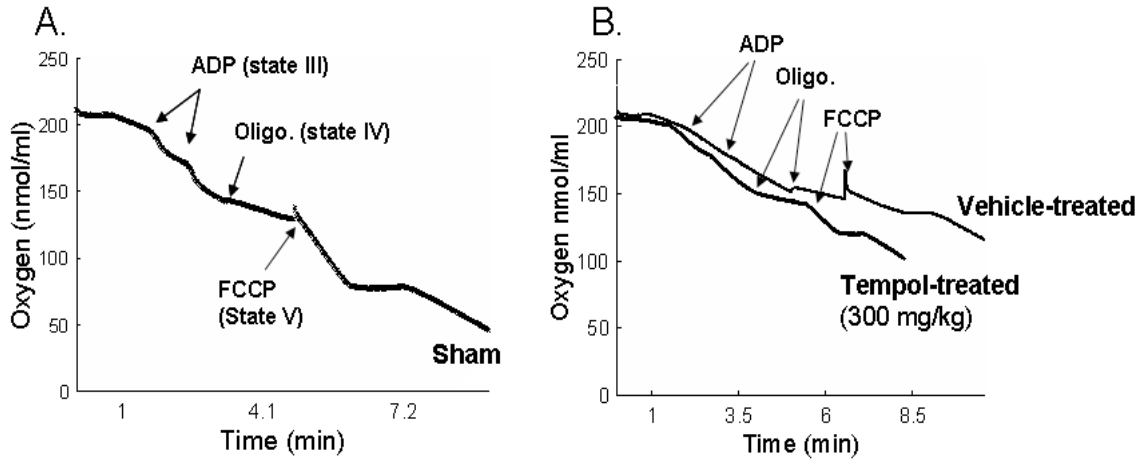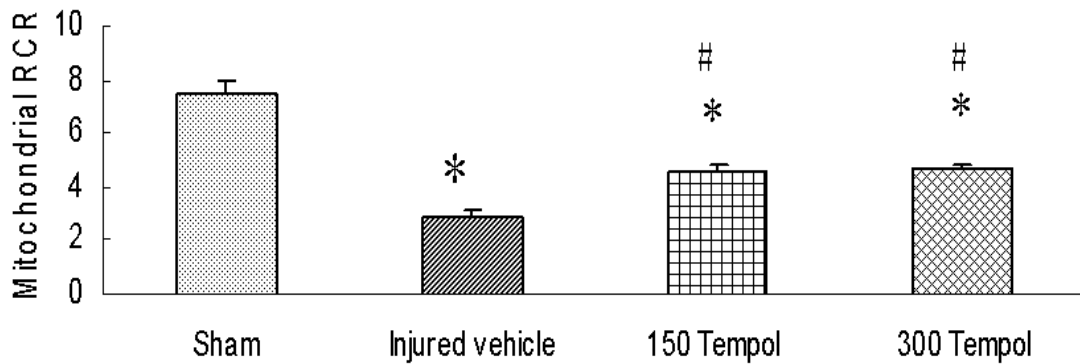
Figure 3.6 Ultra-structure of Mitochondria from different treatment groups
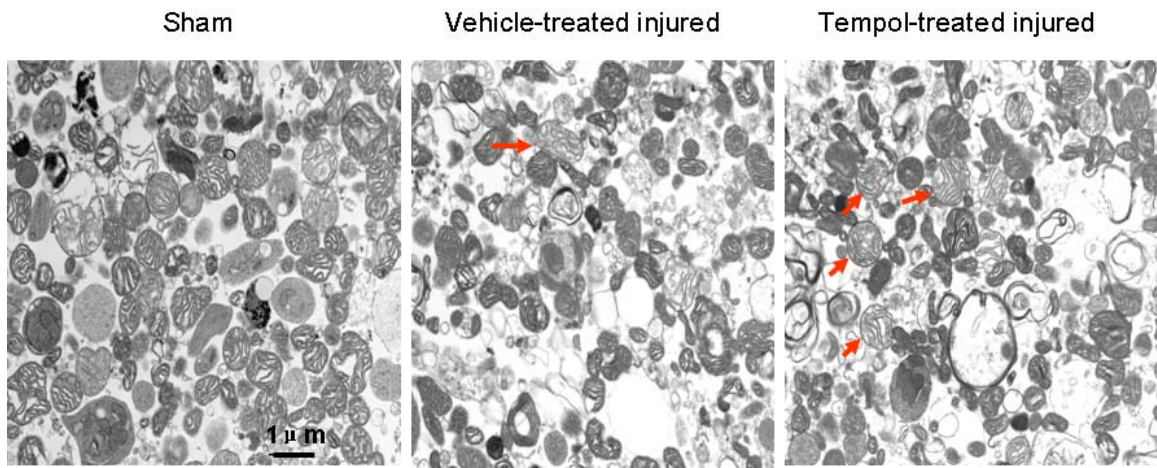
Quantification of mitochondrial bioenergetics **(Figure 3.7)** shows significant effects of tempol treatment in regards to the different mitochondrial respiratory states except complex II-driven state V, indicating that components of the ETC downstream of complex I were still functioning. (state II: $[F_{(3,20)}=3.515, p<0.05]$; 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 hrs post injury. Both tempol does simultaneously increased state III and decreased state IV with statistical significances compared to vehicle treated group, indicating better capacity of ATP production and coupling ($#p<0.001$,n=6). However, state IV and complex I-driven state V in the tempol-treated groups were still significantly lower compared to the sham group, suggesting that the salvage of mitochondrial function was significant, but incomplete. No significant differences in complex II-driven state V respiration could be detected across the experimental groups.

Figure 3.7 Quantification of respiration states showing beneficial effect
of tempol on mitochondrial dysfunction after SCI
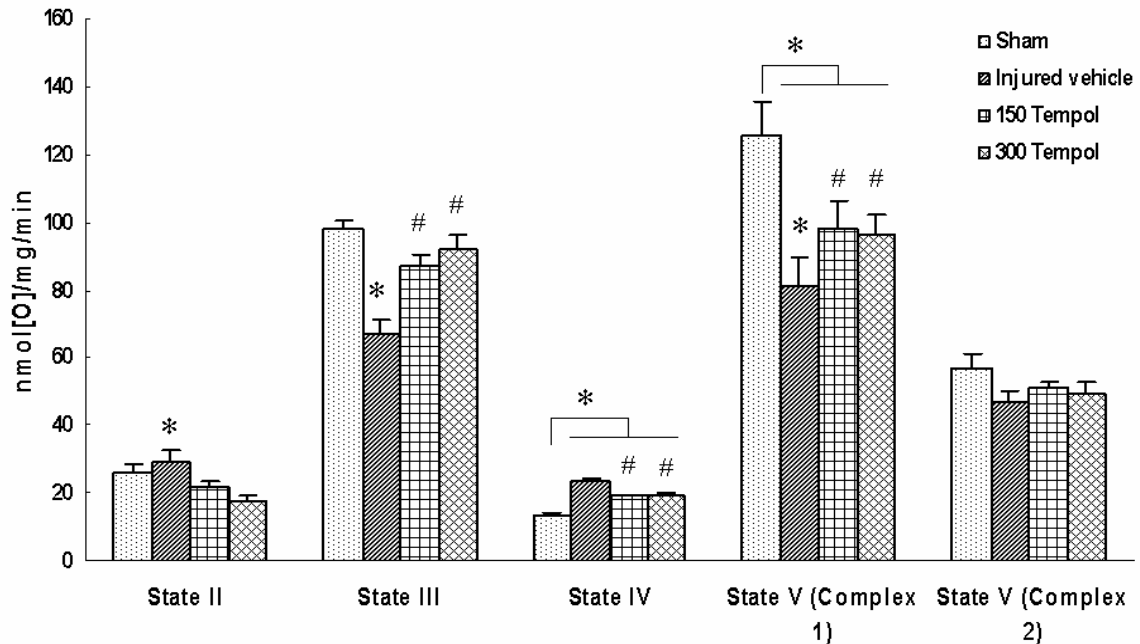
Tempol Effects on Calpain-mediated α-Spectrin Breakdown after SCI

PN-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. Fragment 145 kD, also known as signature breakdown protein 145 (SBDP 145), is generated specifically by calpain SBDP 150 and therefore considered as an indicator of calpain activation. In contrast, SBDP 150 is generated by two enzymes: calpain and caspase 3. Two groups of western blots are shown in **Figure 3.8A**. The three bands from the top to the bottom are intact spectrin (280 kD), SBDP 150 and SBDP 145 respectively. As shown, the elevated level of calpain-specific SBDP 145 was readily observed following injury and tempol administration (300 mg/kg, i.p.) reduced SBDP 145 accumulation. Quantification of SBDP 145 **(Figure 3.8B)** shows injury induced a ten-fold increase of this fragment (*$p<0.0001$ vs Sham, n=6), this Ca^{2+}-activated calpain-mediated spectrin proteolysis was significantly decreased in the tempol group

(#*p*<0.0001 vs Injured, n=6). Quantification of calpain/caspase 3-generated SBDP 150 **(Figure 3.8C)** displays that tempol also reduced this breakdown fragment significantly (#p<0.001 vs Injured, n=6), with a relatively high level detected in the sham group compared to SBDP 145.

Figure 3.8 Tempol effectively antagonized calpain-mediated spectrin breakdown after SCI
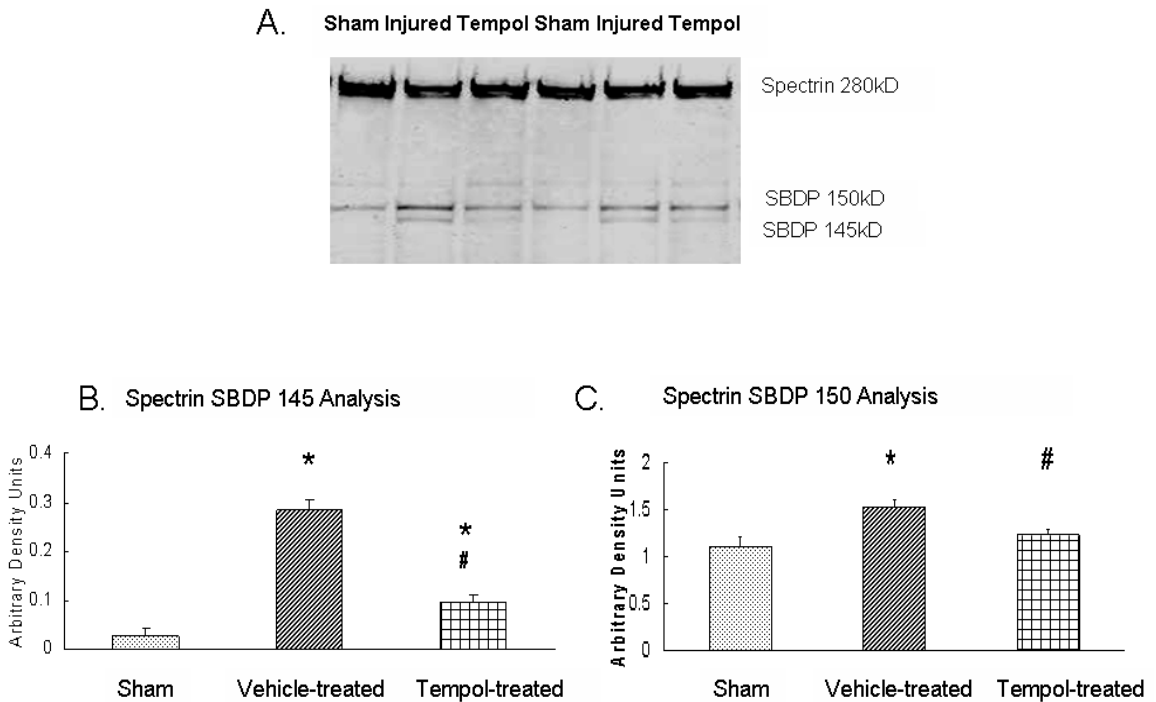
Therapeutic Time Window for Tempol Effect

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. We then carried out experiments to determine the time window for the efficacy of tempol, which will be an important step towards its clinical application. Treatment was begun at the time of injury or after 1, 2 or 4 hours delay and the attenuation of calpain-activated cytoskeletal breakdown by single dose of tempol (300 mg/kg, i.p.) was assessed at 24 hrs post injury. A representative western blot is displayed in the upper panel of **Figure 3.9**, in which six

experimental groups of α-spectrin proteolysis are compared. As demonstrated, injury induced intense accumulations of calpain-generated SBDP 145 and these accumulations were attenuated in acutely-treated tempol (<5min post injury) group and decreased to some extent in 1 hr delay group, whereas remarkable staining of SBDP 145 was easily detected in 2 hrs and 4 hrs delay groups. 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 the sham (*p<0.001, n=6). Tempol treatment immediately post-injury significantly attenuated calpain-mediated proteolysis (#$p<0.0001$ vs injured, n=6), which is consistent with our previous experiments (Figure 3.8). Although there is a downward trend in the 1 hr delay group observed in the blot, post-hoc SNK testing indicates no significant difference compared to the vehicle-treated injured group, and tempol treatment starting at 2 hrs or 4 hrs post injury showed no reductions of α-spectrin breakdown when compared to vehicle group. This suggests that tempol has a very short therapeutic window of < 1 hr.

Figure 3.9 Tempol has a short therapeutic window (<1 h) in preventing spectrin breakdown
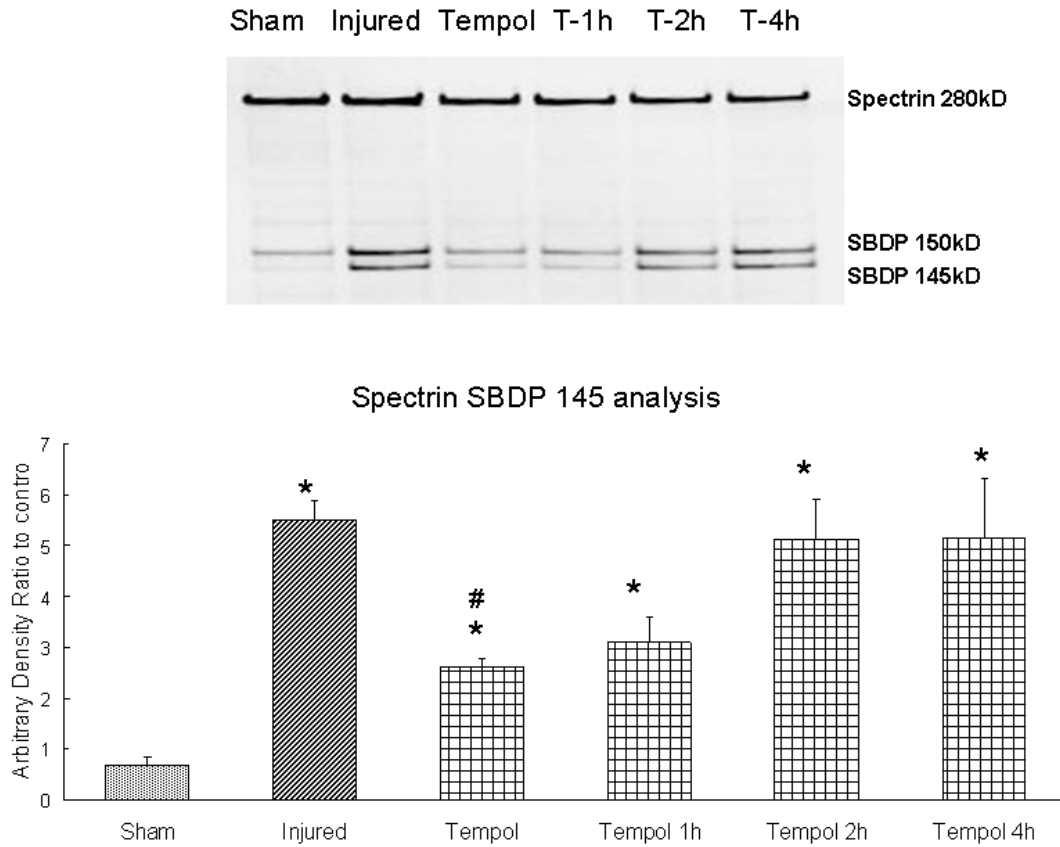
IV. Summary and Discussion

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; 3) calpain-mediated proteolysis following spinal cord contusion injury in a rat model. This pharmacological approach we employed not only strongly supports a pivotal role of PN-mediated oxidative damage in post-traumatic injury, as we previously reported (Chapter Two), but also helps to establish a mechanistic relationship between PN-induced oxidative damage and the downsream secondary injury events mitochondrial dysfunction and Ca^{2+}-activated, calpain-mediated cytoskeletal degradation after SCI. More importantly, the effectiveness of tempol in SCI rat model, by scavenging PN-derived free radicals, strongly suggests that PN is a promising therapeutic target after SCI.

In the past decade, although mounting evidence has verified the neuroprotective effects of tempol in various injury models (Beit-Yannai et al., 1996; Cuzzocrea et al., 2000; Kwon et al., 2003; Hillard et al., 2004), few studies were carried out to explore the mechanisms underlying the beneficial effects of tempol. In those studies, it was suggested that the protective effects of tempol were due to its catalytic scavenging of superoxide ($O_2^{\bullet-}$) as a SOD mimetic. However, with the unveiling of the important role of PN-mediated oxidative damage in secondary CNS injury, tempol's effects need to be re-evaluated considering its catalytic scavenging ability of PN-derived radicals $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (Carroll et al., 2000). To meet that aim, a stable, widely-used biomarker for PN oxidative damage, 3-nitrotyrosine (NT) was applied to investigate tempol's ability to reduce PN-mediated oxidative damage. Although specificity of 3-NT for PN was questioned since nitric oxide ($^{\bullet}NO$) could also give rise to tyrosine nitration, subsequent analyses indicated $^{\bullet}NO$-mediated nitration is probably from PN-derived $^{\bullet}NO_2$ and PN is the major source for 3-NT production (Denicola et al. 1996; Radi., 1998; Alvarez and Radi, 2003). Since PN-derived radicals can also lead to lipid peroxidative cellular damage (Radi et al., 1991b) 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 blotting showed that acutely treatment of animals with a single dose of tempol

(300 mg/kg, i.p.) effectively reduced PN-mediated oxidative damage 24 hrs after SCI in terms of protein nitration, lipid peroxidation and protein oxidation, as measured by 3-NT, 4-HNE and protein carbonyls levels, respectively. Western blots provided side-by-side comparisons of different groups and distinct staining patterns of three markers confirmed the non-overlapping, co-existence of these oxidative mechanisms following SCI. Detection of 3-NT formation by immunohistochemistry demonstrated single dose of tempol protected spinal cord against PN-induced oxidative damage following injury, which is similar to previous study (Hillard et al., 2004), further supporting our hypothesis that tempol exerts its effects at least partly by targeting PN-derived free radicals.

Peroxynitrite-derived free radicals not only lead to cellular oxidative damage, they also attack mitochondria, which are proposed as a key source of PN formation and a target of it's free radical-mediated damaging effects (Carroll et al., 2000). A high content of polyunsaturated fatty acids (PUFAs) in membrane makes mitochondria an especially vulnerable target for oxidative damage. PN has been reported to increase mitochondrial proton leak and electron transport uncoupling (Echtay et al., 2003). In vitro study showed exogenous PN increased levels of PN footprint 3-NT, LP-induced 4-HNE and increased protein carbonyls in isolated brain mitochondrial proteins after exposure to the PN generating compound SIN-1 (Singh et al., 2006). In the currently employed experimental SCI model, a progressive increase in mitochondrial oxidative damage, including increased 3-NT, is observed prior to the loss of mitochondrial bioenergetics (Sullivan et al., 2007). These studies strongly suggest that PN-derived free radical damage may be a major cause for mitochondrial dysfunction. To verify this theory, we then evaluated the efficacy of tempol, as a PN-scavenging compound, to antagonize mitochondrial dysfunction after SCI. Our results showed both tempol doses (150 mg/kg, 300 mg/kg, i.p.) administered immediately after injury preserved mitochondrial function following SCI, with slightly better effect observed at higher dosage of tempol treatment. Mitochondrial electron microscopic pictures showed tempol (300 mg/kg) increased the number of normal-looking mitochondria after SCI, which is consistent with and provides an ultrastructural correlated for our functional analysis. Quantification of mitochondrial respiration states showed tempol improved mitochondrial bioenergetics by increasing the ATP production capacity

and better coupling of ADP phosphorylation to electron transport, which is shown by the increase of state III and the decrease of state IV, respectively. While complex I-driven respiration is susceptible to PN damage and sensitive to tempol treatments, no differences regarding complex-II driven respiration are detected across the groups. This reiterated the fact that complex-I flavin mononucleotide (FMN) group and pyruvate dehydrogenase are very susceptible to oxidative damage and confirmed that components downstream of complex-II in the ETS are still functional, as previously reported (Starkov et al., 2002; Martin et al., 2005; Sullivan et al., 2007).

Severe injury to the spinal cord causes excitotoxic insult due to activation of the glutamatergic mechanisms (Stout et al., 1998). The consequent increases in physiological Ca^{2+} levels is buffered by mitochondria, which acts as a Ca^{2+} sink thus maintaining cellular homeostasis. However, this important aspect of Ca^{2+} homeostasis is lost with time when the Ca^{2+} load far exceeds the buffering capacity of the mitochondria, especially when mitochondrial function is compromised, eventually leading to cell death (Kristal and Dubinsky, 1997; Crompton, 1999). In the present study, we hypothesized that PN-oxidative damage and subsequent mitochondrial dysfunction exacerbate the disruption of Ca^{2+} homeostasis following SCI, resulting in excessive activation of calpain and proteolytic degradation of a number of neuronal proteins, which has been suggested to be final common pathway of neuronal cell death in CNS injuries and neurodegenerative diseases (Bartus, 1997). We previously reported the temporal characteristics of α-spectrin breakdown after spinal cord injury (Chapter Two), indicating cytoskeletal degradation is one of important post-traumatic pathological events following SCI. In this study, the experiments were then performed to investigate the effect of tempol on Ca^{2+}-activated, calpain-mediated α-spectrin breakdown. Results showed tempol significantly decreased the levels of calpain-specific SBDP 145 and calpain/caspase SBDP 150 after injury, with greater effects observed in SBDP 145 (Figure 3.8C), suggesting tempol's cytoskeletal-protective effect is mostly due to the attenuation of calpain activation. 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 inhibition of caspase-3 activation as being part of the protective effect of tempol. Indeed, the significant

reduction of calapin/caspase 3-generated SBDP 150 by tempol suggests tempol also inhibits the apoptosis after SCI, as caspase 3 is the main executioner of the apoptotic pathway. This data is not surprising to us based on the facts that PN can activate caspases 3, 2, 8, 9 and lead to delayed, programmed apoptotic cell death (Virag et al., 1998; Zhuang et al., 2000; Vicente et al., 2006).

Another important finding of this study is the investigation of therapeutic window of tempol's efficacy. Although tempol showed potent neuroprotective effects in various injury models (Cuzzocrea et al., 2000; Hillard et al., 2002), tempol treatments has been initiated acutely after the injury in these studies, which will be impractical in nearly all injured patients. Therefore, defining the therapeutic time window for tempol is an important step towards its clinical application. Unfortunately, our results indicated tempol has a very short time window and has to be given within one hour post injury to show its neurochemical benefits.

In conclusion, despite the possibly limited therapeutic potential of tempol due to its short time window, 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 as illustrated in **Figure 3.10**. It seems that in the future, antioxidant treatment may need to be given repeated dosages to prolong its benefits or combined with other agents that more directly target mitochondria and/or calpain activation in order to achieve a more pronounced and practical neuroprotective approach.

Figure 3.10 Hypothetic pathways of peroxynitrite-induced oxidative damage after SCI
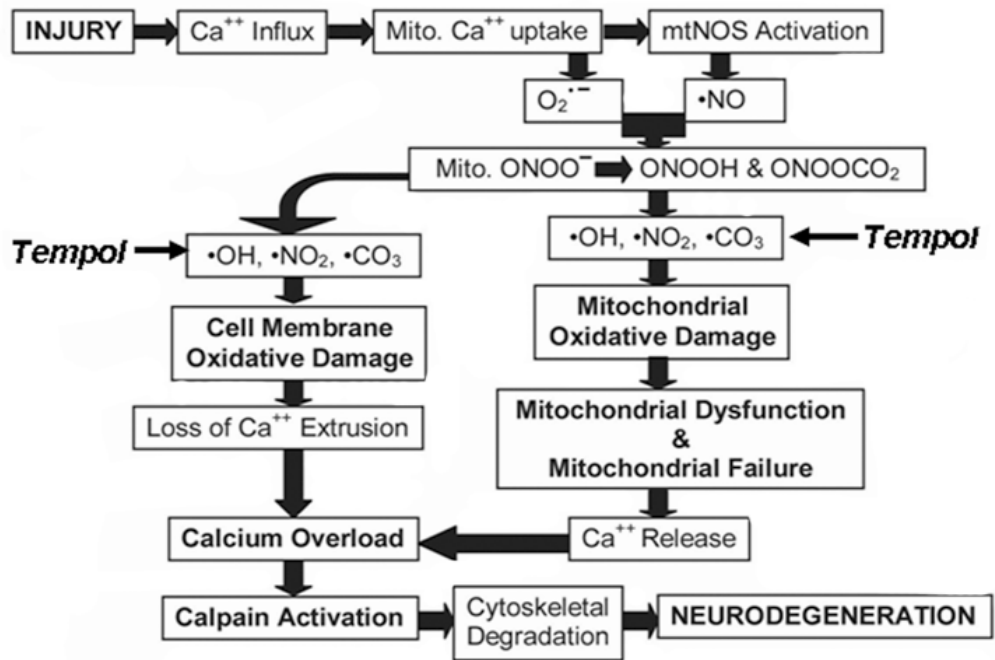
CHAPTER FOUR

TEMPOL PROTECTS SPINAL CORD MITOCHONDRIA FROM OXIDATIVE
DAMAGE INDUCED BY PEROXYNTIRTE DONOR SIN-1

Abstract of some portions of this work has been published as:

Effects of Tempol on mitochondrial dysfunction and cytoskeleton degradation after spinal cord contusion injury in rats. *Soc. Neurosci. Abstract, Atlanta, Georgia. (2006).*

This manuscript is in the process of submitting to *Free Radical Res.*

Note: I, Yiqin Xiong, contributed 100% to the research shown in the result section of Chapter Four although guidance in the performance of this work was provided by Dr. Indrapal N. Singh.

I. Introduction

Mitochondria are the specialized organelles for energy metabolism. The primary physiological function of the mitochondrion is to generate ATP through oxidative phosphorylation via the electron transport chain (ETC). However, they are also involved in both the necrotic and apoptotic cell death pathways by a number of oxidative or nitrosative reactions (Fujimura et al., 2000; Green and Kroemer, 2004). Maintaining mitochondrial bioenergetics in neurons is even more critical, since their energy supply almost completely depends on the mitochondria-derived ATP (Budd and Nicholls, 1998; Nicholls and Budd, 2000). Mitochondrial defects or failure have been well-documented as a crucial mechanism underlying a variety of neurodegenerative diseases (Baron et al., 2007; Bonilla et al., 1999; Schapira, 1999). Due to the fact that reactive oxygen species (ROS) are the by-products of the oxidative phosphorylation process, the direct association of mitochondrial impairments and oxidative damage has been studied extensively over the past decades (Patel, 2004; Albers and Beal, 2000; Beal, 2004). Indeed, the mitochondrion is believed to be the major source of increased oxidative stress in pathological conditions, as well as a vulnerable target of itself derived-free radicals (Trushina and McMurray, 2007).

Recently, there has been increasing appreciation of the role of ROS-induced mitochondrial dysfunction in the secondary injury cascades following CNS trauma (Singh et al., 2006; Sullivan et al., 2003; 2007; Lifshitz et al., 2004). Among these reactive species, the most important player has been proposed to be peroxynitrite (PN), a potent oxidizing agent formed by the diffusion-controlled combination of nitric oxide ($^\bullet$NO) and superoxide ($O_2^{\bullet-}$) (Beckman et al., 1990; Huie and Padmaja, 1993). PN exerts its cytotoxic effects mainly through mitochondrially-derived free radicals ($^\bullet$OH, $^\bullet$NO$_2$, $CO_3^{\bullet-}$), which lead to secondary oxidative damage by nitrating and oxidizing protein, lipids (Chapter Two) and DNA (Salgo et al., 1995ab; Szabo et al., 1996; 2007). Specifically, PN is considered as a central contributor to protein nitration and its major product 3-nitrotyrosine (NT) is widely used as the footprint for PN formation (Radi et al., 2001). Previous in vitro studies have demonstrated that PN impairs mitochondrial

respiration through at least three mechanisms: a) nitrating cytochrome c, an important componet of electron transport chain (ETC) (Cassina et al., 1996); b) selectively inhibiting NADH:ubiquinone reductase (ETC Complex I) (Riobo et al., 2001); 3) depleting mitochondrial anti-oxidant defense system (i.e. manganese superoxide dismutase, glutathione) and leading to mitochondrial oxidative damage (MacMillan-Crow et al., 1997; Radi et al., 2002). In agreement, in vivo studies using a variety of animal injury models have also supported a pivotal role of PN in mediating the progress of secondary injury cascades (Cuzzocrea et al., 2000; Szabo et al., 2002; Deng-bryant et al., 2007; Xiong et al., 2007). Additionally, evidence showed that SCI-induced mitochondrial dysfunction is correlated with the increase of oxidative damage, especially the elevation of the PN footprint 3-NT in mitochondrial proteins (Xiong et al., 2007; Sullivan et al., 2007), strongly suggesting the direct linkage of PN and post-traumatic mitochondrial oxidative damage.

In Chapters Two and Three, we have carried out systematic and in-depth analysis to examine the co-existence of three pathological events including PN-induced oxidative damage, mitochondrial dysfunction and Ca^{2+}-induced calpain-mediated cytoskeletal breakdown in rat spinal cord contusion injury model. Tempol, by scavenging PN-derived free radicals, was able to antagonize these pathological events, suggesting PN-induced oxidative damage might be an early event leading to subsequent mitochondrial impairment and cytoskeletal degradation following SCI. The effectiveness of targeting PN-derived radicals indeed provides a promising therapeutic strategy for the acute neuroprotection of SCI. To further understand the contribution of PN in SCI-induced mitochondrial dysfunction and the mechanism(s) underlying tempol's neuroprotective effects in SCI animal model, we applied PN donor SIN-1 (3-morpholinosydnonimine) directly to healthy spinal cord mitochondria to evaluate PN's toxic effects on mitochondrial respiration. SIN-1 is capable of generating PN precursors, $^{\bullet}NO$ and $O_2^{\bullet -}$ simultaneously and is therefore widely used as a PN donor in vitro (Hogg et al., 1992; Singh et al., 2007). We pretreated mitochondria with tempol prior to their exposure to SIN-1, by measuring mitochondrial bioenergetic parameters and oxidative damage in

mitochondrial protein and provided direct evidence that tempol is able to antagonize SIN-1-induced spinal cord mitochondrial damage.

II. Materials and Methods

Animals

Experiments were performed with isolated mitochondria from young adult female Sprague-Dawley rats (Charles River, Portage, MI) (200 to 225 grams) that were fed and watered ad libitum. The animals were randomly cycling and were not tested for stage of the estrus cycle. They were fed and watered ad libitum. 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 includeing 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-ethanesulfonic 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 (Sullivan et al., 2004; Singh et al., 2006). Briefly, the rats were decapitated,
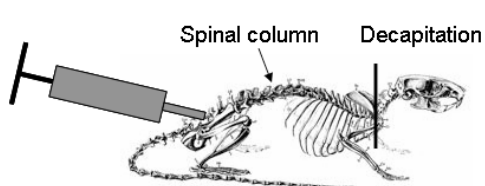
and the spinal cords were quickly squirted out by a 60 ml syringe filled with saline from the bottom of spinal column. By using the lumbar enlargement as a landmark, a 2 cm segment of spinal cord containg mid-thoracic region was dissected on a ice-cold glass plate and then homogenized in a Potter-Elvehjem 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, adjusted to a pH of 7.2 with KOH). The homogenate was first centrifuged twice at $1,300 \times g$ for 3 min in an Eppendorf microcentrifuge at $4°C$ to remove cellular debris and nuclei. Then the supernatant was collected and further centrifuged at $13,000 \times g$ for 10 min. The crude mitochondrial pellet was then obtained and subjected to nitrogen decompression to release synaptic mitochondria, using a nitrogen cell disruption bomb, and cooled to $4°C$ under a pressure of 1,200 psi for 10 min (Brown et al., 2004; Singh et al., 2006). 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. The mitochondrial pellets 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. The final mitochondrial pellet was resuspended in isolation buffer without EGTA and the mitochondrial protein concentration was determined using a BCA protein assay kit with a BioTek Synergy HT plate reader (Winooski, VT).

Mitochondrial Respiration Studies

Mitochondrial respiratory rates were measured using a Clark oxygen electrode in a continuously stirred sealed and thermostatically controlled (37 $°C$) chamber (Oxytherm System, Hansatech Instruments Ltd.) as described previously (Sullivan et al., 2003; Singh et al., 2006). Briefly, 30-35 μg of mitochondrial protein was loaded into the chamber 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 allowed the mitochondria to equilibrate for 1 min. This was followed by the 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 boluses 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; 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

A portion of the 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-hydroxynonenal (4-HNE) were applied as markers for protein nitration and lipid peroxidation by using the slot-blot apparatus. In all, approximately 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 antityrosine antibody (Upstate Biotechnology, Milford, MA) was used (1:2,000) with incubation overnight at 4℃. For detection of 4-HNE, a rabbit polyclonal anti-HNE antibody (Alpha Diagnostics International) was used (1:5,000) with incubation overnight at 4℃. The following morning a secondary IR-Dye 800CW goat anti-rabbit antibody was applied (1:5000; Vector Laboratories, Burlingame, CA) in the dark for 1 hr. 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 nonspecific 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 analyzed 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-Keul (SNK) test. A difference was considered significant at $p < 0.05$.

III. Results

Exposure of Spinal Cord Mitochondria to PN Donor SIN-1 Impairs Mitochondrial Complex I 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) was suspended in respiration buffer and pretreated with different concentrations (1 μM, 2.5 μM, 5 μM and 10 μM) of PN donor SIN-1. Mitochondrial states II, III, IV, and V respiratory rates were then measured and expressed in nanomoles of oxygen per milligram (nmol/ml) of isolated mitochondrial protein. The representative respiratory traces in **Figure 4.1A** demonstrate the dose-dependent alterations of SC mitochondrial bioenergetics after exposure to different concentrations of SIN-1. With the increase of SIN-1, 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 respiratory trace. **Figure 4.1B** displays the dose-response analysis of SIN-1-induced mitochondrial dysfunction by the quantification of RCR. RCR is the ratio of state III oxygen consumption (rate in the presence of ATP, second bolus) to state IV oxygen consumption (rate in the presence of ATPase inhibitor oligomycin). The RCR is considered as a sensitive measure of mitochondrial function and how well the electron transport is coupled with ADP phosphorylation. One-way ANOVA showed treatments produced 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). Pretreatment 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; *$p<0.0001$ compared with control as determined by one-way ANOVA and the Student-Neuman-Keuls post hoc test.

Figure 4.1 Peroxynitrite donor SIN-1 impaired spinal cord mitochondrial function in a dose-dependent manner
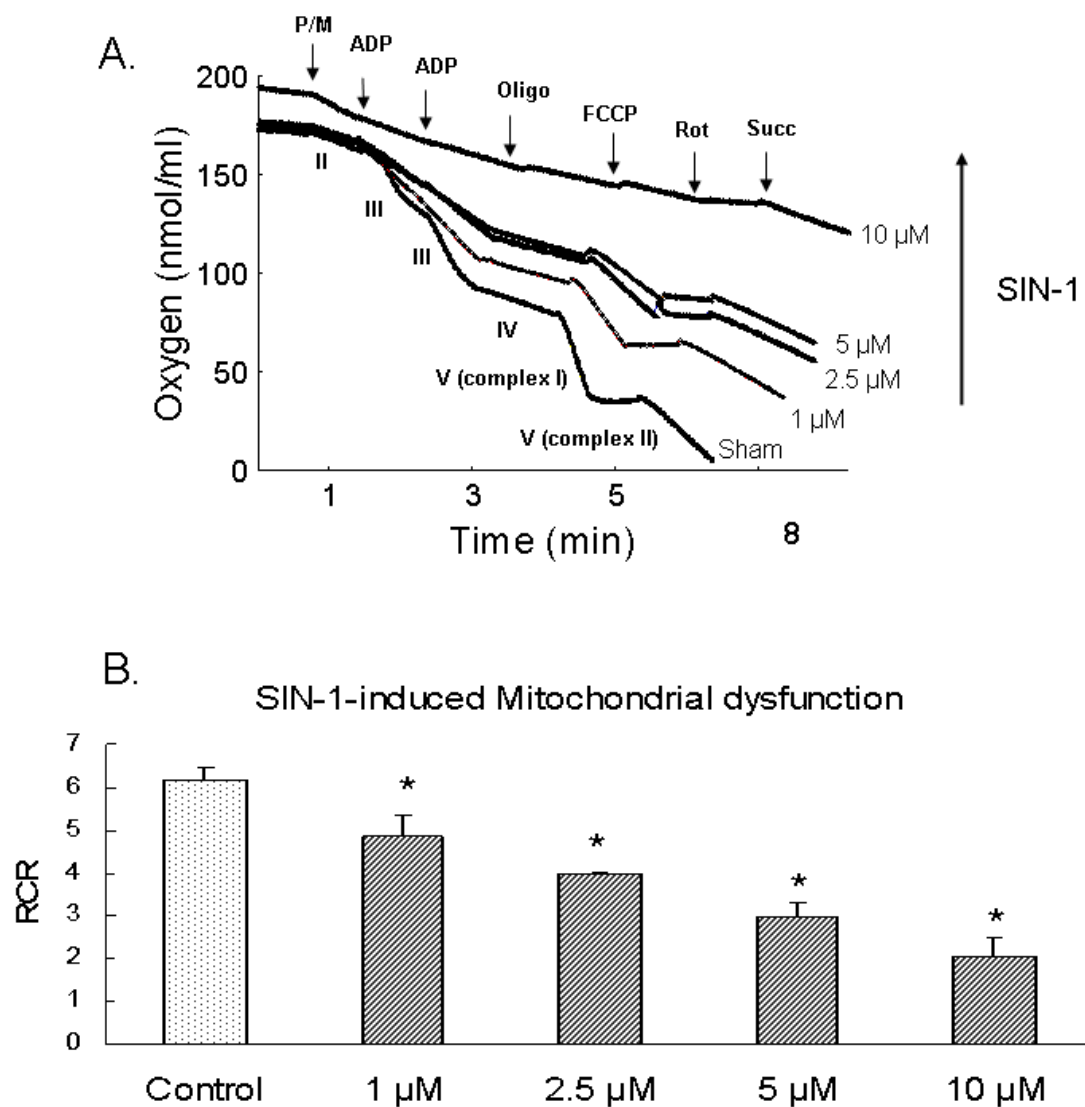
Figure 4.1 Dose-related effect of PN donor SIN-1 on respiratory dysfunction in Ficoll-purified isolated mitochondria. A: The representative oxymetric traces show the dose-dependent alterations in SC mitochondrial bioenergetics following exposure to different dosages of SIN-1. B: SIN-1 induced mitochondrial dysfunction in a dose-dependent fashion in terms of RCR (*p<0.0001 vs control by SNK).

Quantification of mitochondrial bioenergetics **(Figure 4.2)** shows significant increases in state II respiration after SIN-1 exposure of all concentrations (*p<0.001 vs control). State III (RCR numerator, rate of the presence of ADP) and complex 1 driven-state V (uncoupled in the presence of FCCP) showed significant dose-related decreases as the increase of SIN-1 concentration. In contrast, state IV respiration (RCR denominator, the rate in the presence of the ATP synthase inhibitor oligomyclin) was increased by SIN-1 (*p<0.001 vs control), suggesting the compromised coupling of ETC and ATP production due to PN formation. Therefore, the dose-dependent decrease of RCR showed in Figure 4.1B was attributable to the simultaneous decrease of State III and increase of State IV following SIN-1 exposure. Moreover, it deserves to be pointed out that although complex I-driven mitochondrial respiration was sensitively affected by SIN-1 exposure, maximum complex II-driven respiration (the rate in the presence of succinate) was not modified, supporting the notion that the complex I component in the ETC is a selective target for PN-mediated oxidative damage (Riobo et al., 2001; Murray et al., 2003; Singh et al., 2007).

Figure 4.2 Quantification of respiratory rates showing SIN-1-induced spinal cord mitochondrial dysfunction
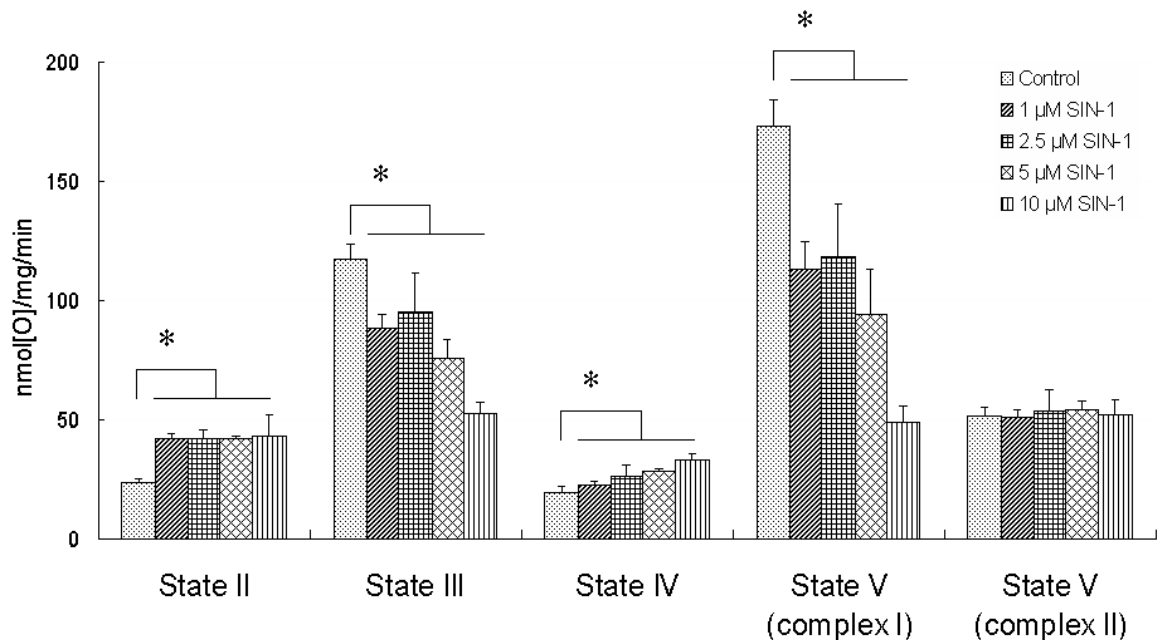
Tempol Partially Protects Spinal Cord Mitochondria from SIN-1-induced Respiratory Dysfunction

We then carried out a dose-response analysis of the ability of tempol pretreatment to antagonize SIN-1-induced mitochondrial dysfunction. The concentration of SIN-1 we applied was determined as 5 μM since at this dosage the impaired RCR is closest to the mitochondrial RCR 24 hrs following SCI (Sullivan et al., 2007). In attempting to titrate the optimal dosage of tempol, five concentrations (1.25 μM, 2.5 μM, 3.75 μM, 5 μM, 10 μM) were employed for a detailed analysis of its protective effects against SIN-1. As demonstated in **Figure 4.3A**, one-way ANOVA displays treatments produced a significant effect across all groups [$F_{(6,18)}=30.043$, $p<0.0001$]. Pretreatment with tempol at concentrations of 3.75 μM and 5 μM significantly increased the RCR compared to the SIN-1 (5 μM) alone group (#$p<0.001$, n=5), suggesting a preservation of mitochondrial respiration. However, tempol lost its efficacy at a higher concentration of 10 μM indicative of a U-shaped dose-response. Additionally, in all tempol-treated groups the RCR remained significantly suppressed when compared to the control untreated mitochondria (*$p<0.001$). Data are presented as means±SEMs; *$p<0.0001$ compared with control as determined by one-way ANOVA and SNK post hoc test.

Figure 4.3. Tempol partially protected SIN-1 induced mitochondrial dysfunction

A. Tempol effects on SIN-1 induced Mito. Dysfunction

B.
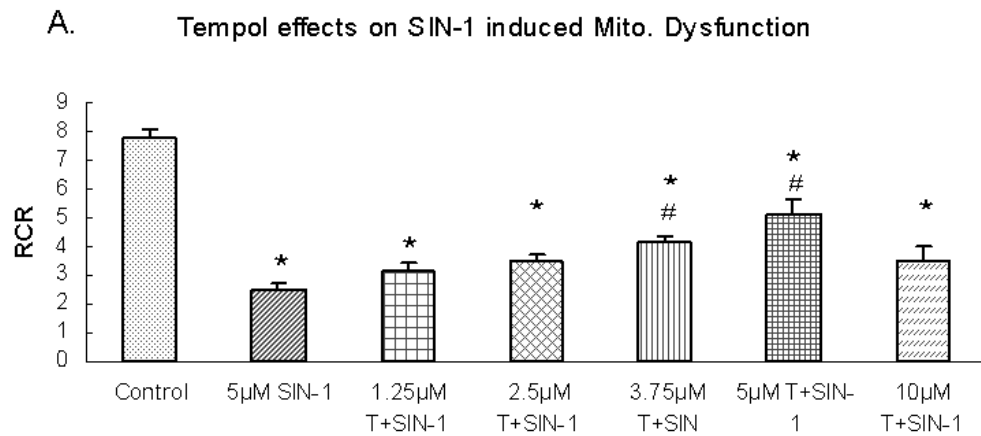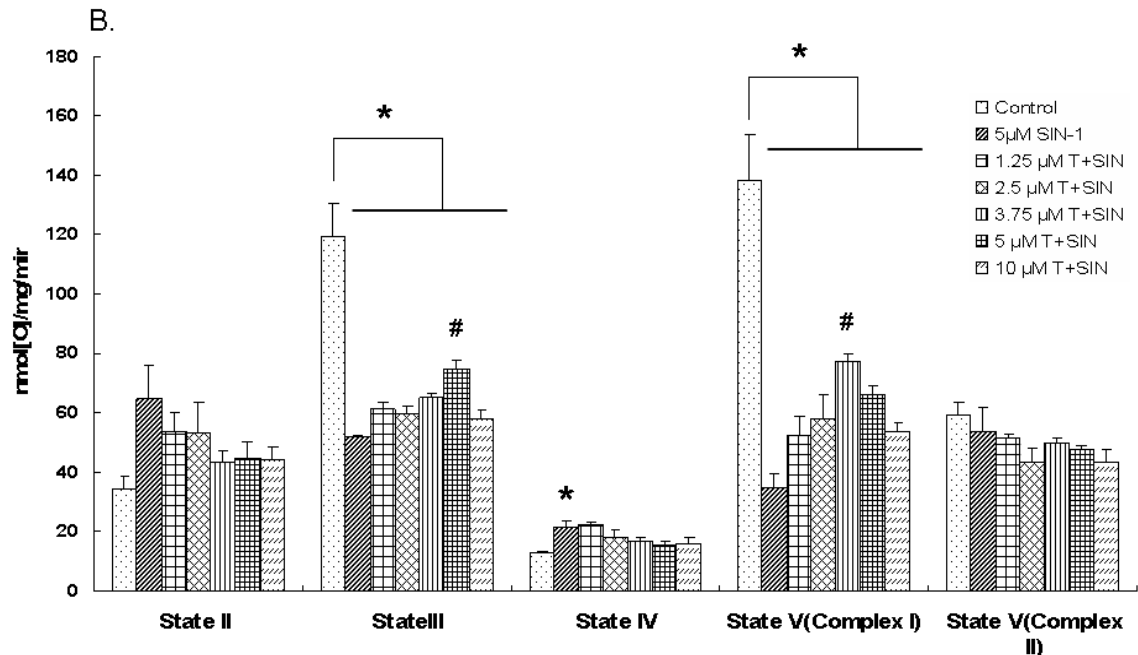
As indicated in **Figure 4.3B**, quantification of all respiratory states showed only tempol at 5 μM, but not at lower or higher concentrations, improved state III (RCR numerator) with a statistical significance (#$p<0.001$, n=5). In all tempol-treated groups, there was a downward trend in terms of state IV respiration (RCR denominator) compared to SIN-1 alone group, but no significantly differences detected. As for complex I-driven State V respiratory rates, 3.75 μM tempol treatment has a significant effect compared to the SIN-1 alone group (#$p<0.001$), but not other concentrations. No significant differences were detected across all groups in terms of complex II-driven respiration.

Tempol Protects Spinal Cord Mitochondria against SIN-1 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 4.4**, 5 min of exposure of the mitochondria to SIN-1 produced a significant increase in 3-NT formation, as measured by immunoblotting. The representative slot blots (Figure 4.4A) demonstrated SIN-1 pretreatment dramatically increased 3-NT immunoreactivity in SC mitochondrial protein and tempol (5

μM)-pretreated mitochondria almost completely blocked the SIN-1-induced formation of 3-NT in mitochondria by bringing the 3-NT immunoreactivity back to sham level. Densitometric quantification of the blots (Figure 4.4B) showed 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 pretreatment effectively prevented SIN-1-induced 3-NT formation in SC mitochondria, by significantly decreasing the level when compared to 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.

Figure 4.4 Tempol antagonized SIN-1-induced 3-NT formation in Mitochondria

A.   Control   SIN-1   T+SIN-1

B.   Tempol prevents 3-NT formation in Mito. after SIN-1 exposure
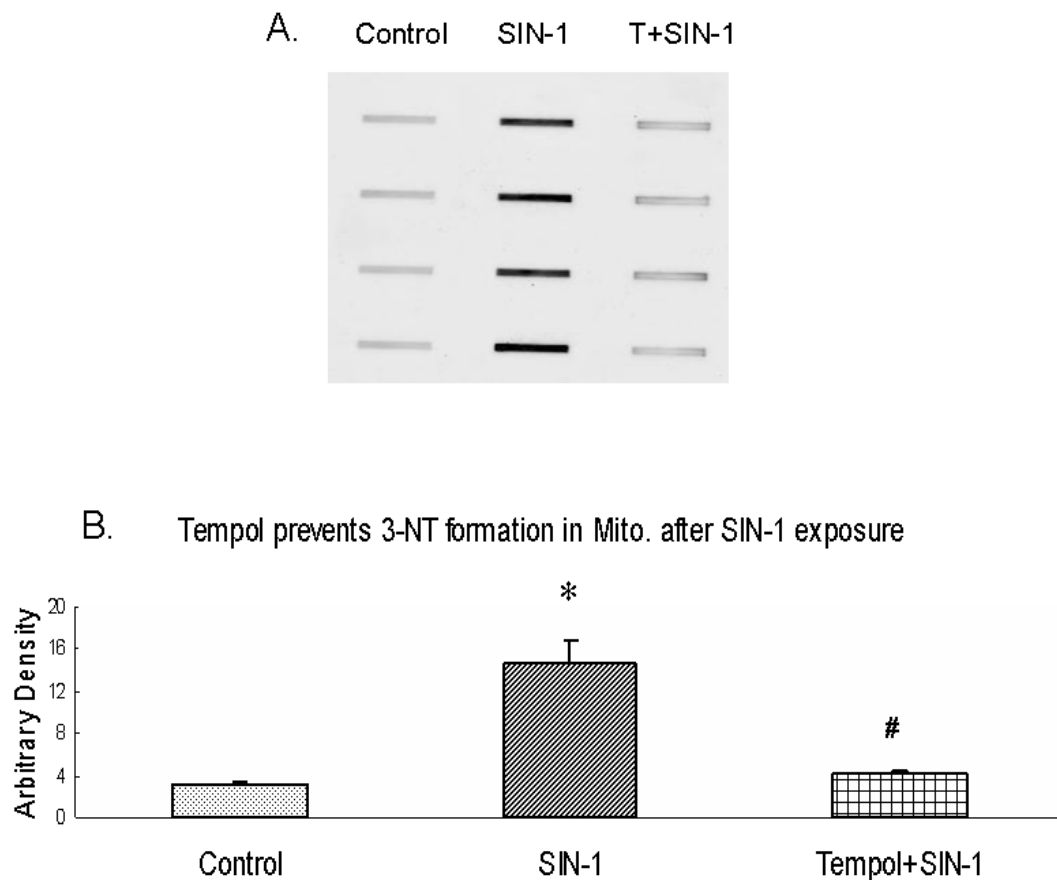
Exposure of SIN-1 to Spinal Cord Mitochondria has no effects on 4-HNE formation

Although 5 min incubation of SIN-1 induced 3-NT formation in mitochondrial protein, it had no effects on the lipid peroxidation marker 4-HNE. The representative blots demonstrated (**Figure 4.5A)** 4-HNE levels in mitochondria proteins among all groups. Note that a basal level of 4-HNE formation could be detected in control untreated mitochondrial protein, indicating the existence of lipid peroxidation in healthy spinal cord mitochondria. No evidence of elevated 4-HNE was observed in SIN-1 alone treated and SIN-1 and tempol treated groups. Quantification in **Figure 4.5B** showed there was no increase in 4-HNE after exposure to SIN-1 and there was no significant differences detected among three groups.

Figure 4.5. 5 min SIN-1 exposure does not affect 4-HNE formation in Mitochondria
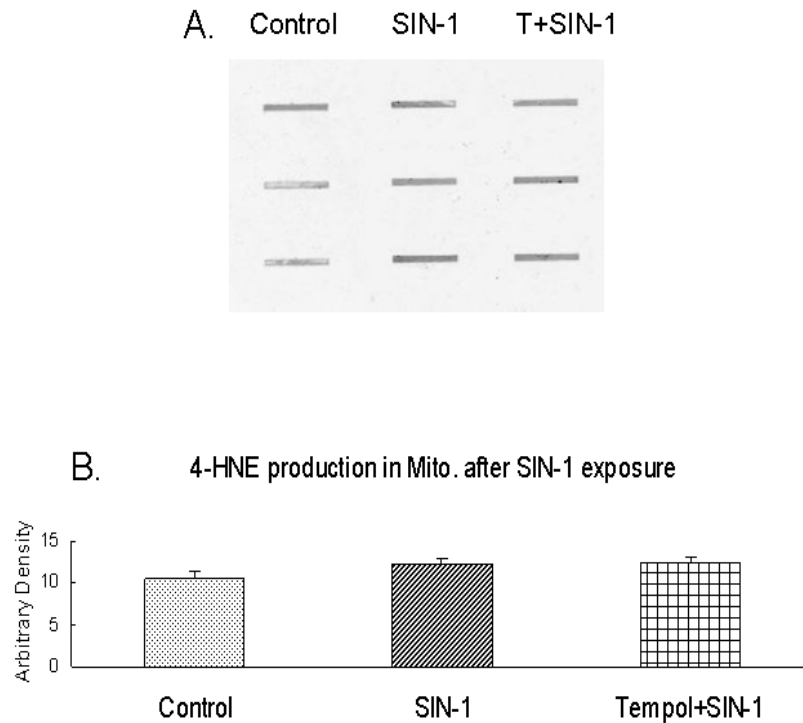
IV. Summary and Discussion

It has been well established that mitochondrial dysfunction and free radical damage are two important pathological events following acute central nervous system injury (Hall et al., 2004; Sullivan et al., 2000; 2004; 2007; Lifshitz et al., 2004). Recent studies from our laboratories strongly suggest that the principal ROS involved in the secondary damage after CNS injury is PN, often referred to as a reactive nitrogen species (Hall et al., 1999; 2004; Xiong et al., 2007; Deng-Bryant et al., 2007). The mitochondrion is both a major source of posttraumatic PN formation and a vulnerable target of its free radical-mediated damaging effects (Carroll et al., 2000; Szabo et al., 2007). PN-associated reactions modulate mitochondrial function in various pathways including, but not limited to, inhibition of mitochondrial electron transport (Radi et al., 1994), inactivation of the pyruvate dehydrogenase complex (Martin et al., 2005), inhibition of mitochondrial NADH:ubiquinone reductase activity (Riobo et al., 2001). Inactivation of mitochondrial electron-transport enzymes increases the amounts of superoxide and hydrogen peroxide generated by the mitochondria, which may further contribute to cellular injury, in an additive or synergistic fashion.

PN 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/sec (Huie and Padmaja, 1993; Halliwell and Gutteridge., 1998). As described previously, PN is protonated to form peroxynitrous acid (ONOOH), which can undergo homolytic decomposition and generation of the highly reactive nitrogen dioxide radical ($^\bullet$NO$_2$) and hydroxyl radical ($^\bullet$OH) (Radi et al., 1991). In a more physiologically relevant reaction, PN will react with carbon dioxide (CO_2) to form nitrosoperoxocarbonate ($ONOOCO_2^-$) which homolytically decompose to generate $^\bullet$NO$_2$ and carbonate radical ($^\bullet$CO$_3$) (DeNicola et al., 1996; Radi, 2004). It is widely accepted that the potent oxidizing ability of PN is actually due to its decomposition free radicals. PN-derived $^\bullet$OH, $^\bullet$NO$_2$, and $CO_3^{\bullet-}$ can initiate lipid peroxidative cellular damage by attacking polyunsaturated fatty acid or cause protein carbonylation by reacting with susceptible amion acids (Radi et al., 1991; Radi, 2004). Additionally, $^\bullet$NO$_2$ can nitrate the 3-position of tyrosine residues in proteins and inactivate important enzymes (i.e.

MnSOD), components of mitochondrial electron transport chain and disrupt tyrosine signaling pathways (Cassina et al., 2000; Riobo et al., 2001; Murray et al., 2003).

The understanding of the importance of PN in posttraumatic mitochondrial function impairment provides a promising therapeutic target for acute treatment after CNS injury. Increasing evidence has been shown compounds that interfere with PN-mediated reactions (Scott et al., 2005) or neutralize PN, effectively protect tissues and animals from oxidative stress conditions. In the present study, we provided direct evidence that tempol, a stable nitroxide antioxidant which has been shown to have protective effects in various experimental models (Cuzzocrea et al., 2000; Szabo et al., 2002; Hillard et al., 2004; Deng-Bryant et al., 2007 and Chapter Four), is actually exerting its beneficial effects through scavenging of PN-derived radicals. Although tempol is a well-documented neuroprotective nitroxide, its neuroprotective effects have been attributed simply to its superoxide dismutase (SOD) mimicry. However, recent studies showed tempol can catalytically decompose the PN-derived free radicals $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (Carroll et al., 2000). This latter property is probably reflected in the decrease in 3-NT, a specific marker for PN formation, in tempol-treated mitochondrial and spinal cord tissue, suggesting a linkage between tempol's mitochondrial protection and scavenging of PN-derived free radicals. Although this relationship has been partially confirmed by investigating PN-mediated oxidative damage and the protective effects of tempol in brain mitochondria (Singh et al., 2007), the current study has, for the first time, examined the responses of spinal cord mitochondria to exogenous PN. Previous studies have reported the physiological differences exist in the mitochondria isolated from different organs or subregions (Andreyev et al., 1998; Andreyev and Fiskum, 1999; Friberg et al., 1999). Indeed, there are intrinsical differences between the mitochondria localized in the brain and those in spinal cord (SC) (Sullivan et al., 2004). Our preliminary studies confirmed this concept (**Figure 4.6**). From the representative mitochondrial traces, the same amount of brain mitochondria showed better coupling and more O_2 consumption (i.e. more efficient ATP synthesis) to added substrates when compared to the mitochondria obtained from SC, and the maximal capacity of the electron transport chain in brain mitochondria is much higher than that in SC

mitochondria which can be seen after the addition of the uncoupler FCCP. Although both of them are considered healthy, the mean respiratory control ratio (RCR) showed significantly higher value in brain compared to SC mitochondria (*p<0.01 vs SC, n=8).

Figure 4.6 Comparison of mitochondrial bioenergetics from brain and spinal cord
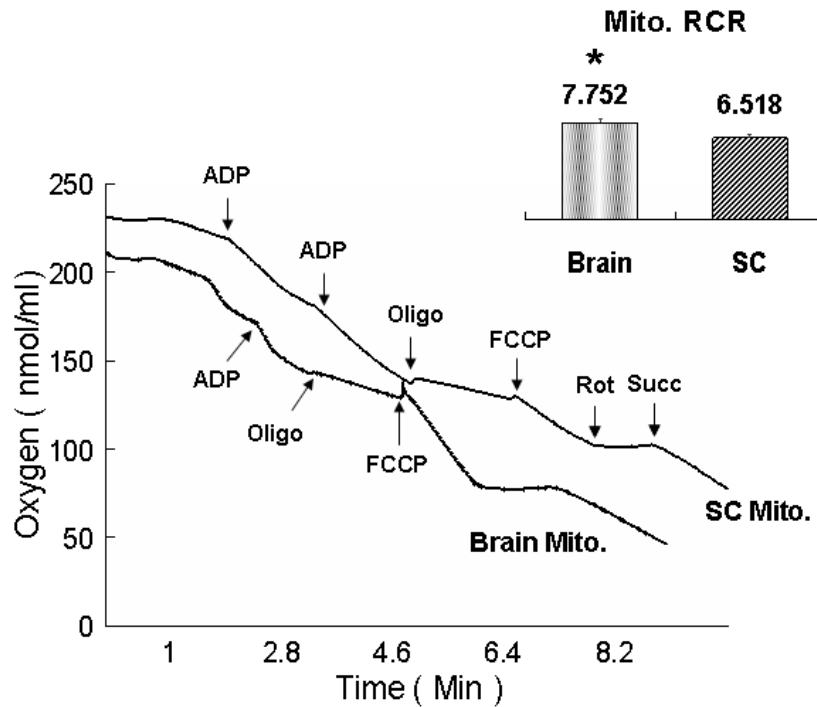
It was surprising to us that exposure of isolated normal, healthy spinal cord mitochondria to PN delivered via SIN-1 caused rapid protein nitration but not lipid peroxidation. The increase in 3-NT was coincident with severe impairment of mitochondrial respiration. However, the PN marker 3-NT, but not the lipid peroxidation product 4-HNE, dramatically increased in isolated SC mitochondria within minutes of SIN-1 exposure, suggesting the impaired mitochondrial respiratory function has a strong association with mitochondrial protein nitration but not lipid peroxidation. Lipid peroxidation may be a siginificant oxidative mechanism involved in PN-mediated oxidative damage in vivo, however, 5 min exposure of SC mitochondria to PN was probably too short to allow lipid peroxidation to take place. In isolated brain mitochondria, Singh et al. similarly observed only a non-significant increase in 4-HNE

signals but statistically significant increases in 3-NT formation after 5 min exposure to SIN-1. Thus, for both brain and SC mitochondria, it appears that the 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. Past studies have shown that the nitration was involved in the inactivation of Mn superoxide dismutase (MnSOD), therefore amplifying mitochondrial injury by depleting this important anti-oxidant defense mechanism (MacMillan-Crow et al., 1996). Furthermore, recent data have revealed that a crucial 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 (Cassina et al., 2000; Batthyany et al., 2005).

Although tempol is effective to antagonize PN-mediated 3-NT formation, the preservation of mitochondrial respiration is incomplete, suggesting that there are other mechanisms involved in PN-induced mitochondrial dysfunction which cannot be reversed by tempol; secondly, the neuroprotective effects shown by tempol in various vivo settings is largely due to its prevention of protein nitration. The first notion is easy to understand due to the multi-factorial effects of PN toxicity. The second notion is indeed consistent with previous biochemical studies based on tempol reaction with PN, which is, 3-NT is mainly from PN-dervived $^{\bullet}NO_2$ and tempol has the ability to divert nitration to nitrosation (Caroll et al., 2000) as demonstrated in following reaction:
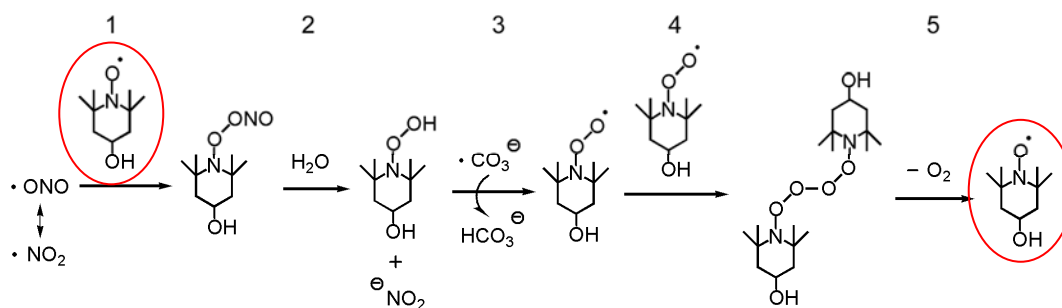
In summary, the protective effects of tempol on mitochondrial respiration were accompanied by a decrease in the 3-NT, strongly suggesting that the beneficial effects of tempol we observed in vivo, including attenuation of oxidative damage, mitochondrial dysfunction and cytoskeletal degradation, resulted from direct scavenging of PN-derived free radicals.

CHAPTER FIVE

GENERAL SUMMARY AND DISCUSSION

Traumatic spinal cord injury (SCI) is comprised of the primary injury and secondary injury. The initial mechanical insult to the spinal cord induces some instantaneous tissue damage, which is termed the primary injury. However, the primary injury induces a cascade of neurochemical changes, which lead to further neuronal cell loss, microvascular damage and dysfunction and impairment in behavioral function. Secondary injury begins immediately after injury, and this post-injury phase may last for days and weeks depending on the severity of the initial injury. Although little can be done to reduce the primary injury apart from decreasing the risk of SCI, it is feasible to ameliorate post-SCI secondary injury by timely pharmacological interventions. Thus, understanding pathological mechanisms responsible for post-traumatic neuronal damage will shed light on neuroprotective pharmaceutical approaches.

The current project has identified peroxynitrite (PN) as an important therapeutic target for pharmacological intervention. We evaluated the effect of PN-derived radical scavenger, tempol, on PN-induced oxidative damage, mitochondrial dysfunction and calpain-mediated proteolysis. Furthermore, the therapeutic window of tempol has also been investigated to determine the possibility to move tempol forward to its clinical transition. Finally, we explored the mechanism underlying tempol's mitochondrial protection and provided direct evidences showing tempol's ability to scavenge PN-derived free radicals.

The first study is a thorough comparative time course assessment of PN-induced oxidative damage and calpain-mediated α-spectrin breakdown in a rat spinal cord contusion model. Temporal and spatial co-existence of 3-NT and 4-HNE observed in both quantitative slot-blotting and immunohistochemical studies indicated that PN is the

main source of protein nitration and lipid peroxidation, strongly suggesting that PN is an important mediator of post-traumatic oxidative damage after SCI. The onset of PN-mediated oxidative damage measured in spinal cord tissue begins immediately after injury and peaks around 24 hr post injury. This is coincident with an increase of oxidative damage and functional deficit observed in spinal cord mitochondria (Sullivan et al., 2007), suggesting that mitochondria are perhaps the major source and target of post-traumatic production of PN. Furthermore, quantitative western-blots measured calpain-mediated cytoskeletal degradation which is also dramatically increased within the first hours after injury. The increased appearance of calpain-mediated spectrin breakdown (SBDPs) progresses slowly over time probably due to the inhibition of early production of oxidative stress and the peak occurred at 72 hrs post-injury. The second wave of calpain activataion is most likely due to the later compromised mitochondrial dysfunction and subsequent disruption of intracellular Ca^{2+} homeostasis. The increments of calpain/caspase 3-generated SBDP150 after SCI suggested a role of apoptosis in the progress of SCI pathophysiology.

Our time course study on PN-oxidative damage reveals a prolonged existence of oxidative stress following SCI. Considering the role of inflammation after acute injury, some aspect of the ROS production is most likely due to the invading neutrophils and macrophages. However, with the progress of secondary injury cascade, mitochondria, especially when their function get compromised by overwhelming intracellular Ca^{2+}, may become the major source for ROS formation and contribute, along with inflammatory mechanisms to the oxidative damage observed at later time points (72 hrs and 1 week post injury). Indeed, this two-phase oxidative damage notion is in excellent agreement with the time course study of Ca^{2+}-activated calpain-mediated spectrin breakdown, which reveals two phases of calpain activation. The first, rapid accumulation of SBDPs 145 and 150 at 1 hr post injury indicated an immediate influx of Ca^{2+} into the cell after SCI, which is attributable to the initial insults involving cellular and mitochondrial membrane ruptures. However, the later phase of calpain activation should due to distinct mechanisms that involve several interacting secondary injury events including PN-induced cellular and mitochondrial damage.

Aside from the concept that PN plays an important role after SCI by protein nitration and oxidation and lipid peroxidation, other points can be derived from our time course study. Firstly, the prolonged time course of PN-oxidative damage provides a practical time window for pharmacological intervention or at least a prolonged anti-PN therapeutic treatment should give us better protection over oxidative damage. Secondly, the quantitative measurement of the end products of oxidative damage and calpain-mediated proteolysis also provides measurable biomarkers for future therapeutic studies. Targeting PN-induced oxidative damage may have multiple beneficial effects through inhibition of several key mediators in the secondary injury process. However, the early onset of oxidative stress also makes PN-induced damage a difficult target. Timely treatment is critical in order optimally inhibited PN-mediated damage mechanisms.

To test the hypothesis that blockade of upstream PN-induced oxidative damage will produce a neuroprotective effect, and also to establish a mechanistic linkage of PN-induced damage, mitochondrial dysfunction and calpain over-activation, we pharmacologically targeted PN using a potent antioxidant, tempol, in a rat SCI model. Tempol is a membrane permeable, catalytic scavenger of PN-derived free radicals. In an initial study, we examined two dosages of tempol (150 mg/kg and 300 mg/kg) and evaluated the ability of tempol to preserve post injury mitochondrial dysfunction and found 300 mg/kg is a better working dosage for neuroprotection (see more detailed description in next paragraph). We then employed this effective dosage throughout the subsequent experiments and chose the PN-specific marker 3NT, the lipid peroxidation marker 4-HNE and protein oxidation-derived protein carbonyls as indices to examine the effects of tempol on PN-induced oxidative damage. In order to achieve maximal effect, tempol was administered i.p. immediately after injury (approximately within 5 min post injury) and tissue was harvested at 24 hr post-injury when oxidative damage is at its peak. Both immunoblotting (slot-blotting and western-blotting) and immunohistochemical evidences show that acute tempol treatment effectively inhibited PN-mediated oxidative damage.

To confirm our hypothesis that oxidative damage is an upstream element in the secondary injury cascade, we hypothesized that mitochondria are the main source of PN

production based upon previous studies (Singh et al., 2006; 2007; Sullivan et al., 2007) showing that PN-mediated damage occurs in mitochondria from the injured brain or spinal cord which parallels the loss of respiratory function and Ca^{2+} buffering capacity. Indeed, in response to glutamate-induced excitotoxic Ca^{2+} influx, mitochondrial Ca^{2+} uptake induces superoxide and nitric oxide radical production, which can combine with a diffusion rate-limited rate constant to produce PN. To test the association between mitochondrial PN oxidative damage and respiratory dysfunction, we administered two dosages of tempol at the onset of injury and isolated the mitochondria from the injured spinal cord at 24 hrs post-injury. Using Clarke-type electrode measurement of mitochondrial respiration, both tempol-treated (150 mg/kg and 300 mg/kg) mitochondria showed improved maintenance of respiratory function, with a slightly better effect seen in 300 mg/kg tempol-treated group. The protective effect of tempol on mitochondrial function reveals that: 1) tempol is membrane permeable and effective in penetrating into mitochondrial matrix; 2) mitochondria are a main source for PN production as well as the primary target of PN-mediated oxidative damage. Moreover, the success of using tempol in vivo to preserve mitochondrial function, strongly suggests that PN plays a significant role in mediating mitochondria Ca^{2+} influx-induced functional impairment.

Moving downstream in the hypothesized secondary injury cascade, we examined the effect of tempol on Ca^{2+}-dependent calpain activity. Calpain is activated upon Ca^{2+} influx and is shown to play an important role in cytoskeletal degradation, which is considered as a final common pathway to cell death. Because of the close relationship of calpain and cytosolic calcium level, we hypothesize that tempol, by preserving mitochondrial function and maintaining calcium homeostasis, will indirectly attenuate calpain activity. To test this hypothesis, we administered single i.p. dose of tempol immediately after injury and evaluated calpain-mediated SBDPs at 24 hrs post-injury measured by quantitative western-blotting. The results demonstrate that tempol significantly reduces calpain-specific SBPDs 145 and 150 after injury. The inhibitory effect of antioxidant tempol on calpain-mediated cytoskeletal proteolysis strongly confirms that PN-induced oxidative damage is upstream to calpain over-activation.

We then carried out a tempol therapeutic window study by measuring

calpain-mediated SBDPs after delaying tempol administration until 1 hr, 2 hrs and 4 hrs post injury. The results showed tempol lost its efficacy to antagonize calpain-mediated spectrin breakdown as early as 1 hr delay, suggesting tempol has a very short therapeutic window. Although it is apparent that the first one hour is crucial for PN-induced oxidative damage, this result does not rule out the possibility that by using a multiple dosing regimen or combination therapy with another compound that targets a different injury mechanism (i.e. calpain inhibitor, lipid peroxidation inhibitor) tempol will be able to demonstrate more clinically practical benefits. Based on the potent inhibition we observed of tempol on PN-induced oxidative damage after SCI, tempol, although having an impractically short therapeutic window, should be considered as an excellent tool drug to explore the role of PN in pathophsiology after SCI in animal models and also a possible candidate for combination therapy of SCI.

We have successfully proven that tempol, by preventing upstream PN-induced oxidative damage, ameliorated mitochondrial dysfunction and attenuated calpain-mediated cytoskeletal degradation in vivo. In the last study, we extend our investigation in vitro to better understand the underlying mechanisms involved in PN-mediated mitochondrial dysfunction and tempol's protective effects on mitochondria. This data is complementary to our vivo studies and provided direct evidences showing the ability of tempol to antagonize PN-induced damage to mitochondria. From the fact that SIN-1 induced impaired mitochondrial respiration in a dose-dependent manner in vitro, together with the evidences of PN-induced oxidative damage in vivo, we can conclude that the mitochondrial dysfunction we observed in vivo after SCI is largely caused by PN. Secondly, tempol effectively antagonize the 3-NT formation at the SIN-1 exposed mitochondria, suggesting the in vivo 3-NT is primarily from endogenous PN and tempol's benefits we observed in vivo strongly associated with its PN scavenging property.

In conclusion, in this dissertation research has successfully provided strong evidence for PN-induced oxidative damage being an important upstream mediator in the secondary cell death cascade in the injured spinal cord. By scavenging PN-derived free radicals with

the nitroxide antioxidant tempol, it is possible to protect mitochondrial function and probably Ca^{2+} buffering capacity which in turn reduces downstream calpain-mediated proteolysis. However, the short therapeutic window of tempol and the complexity of the secondary injury process after SCI challenge the idea that single therapy can achieve optimal neuroprotection and improvement in functional outcome. In the future, the combination therapies that simultaneously target PN-induced oxidative damage, along with either inhibition of lipid peroxidation, or mitochondrial permeability transition pore, or calpain activation, may be needed to provide optimal neuroprotections after acute SCI.

APPENDIX: ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
ΔpH	proton force
3-NT	3-nitrotyrosin
4-HNE	4-hydroxynonenal
8-OH-G	8-hydroxyguanine
8-OHdG	8-hydroxydeoxyguanosine
ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
AIF	apoptosis inducing factor
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
BSA	bovine serum albumin
BSCB	blood-spinal cord barrier
BDPs	breakdown products
CNS	central nervous system
CsA	cyclosporine A
CSF	cerebrospinal fluid
Cyt c	cytochrome c
DNPH	2,4-dinitrophenylhydrazine
eNOS	epithelial nitric oxide synthase
ETC	electron transport chain
EGTA	ethylene glycol tetraacetic acid
$FADH_2$	reduced flavin adenine dinucleotide
FCCP	p-trifluoromethoxy carbonyl cyanide phenyl hydrazone
Fisher's PLSD	Fisher's Protected Least Significant Difference
$FMNH_2$	reduced flavin mononucleotide
GPX	glutathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IMM	inner mitochondrial membrane
iNOS	inducible nitric oxide synthase
LP	lipid peroxidation
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
nNOS	neuronal nitric oxide synthase
MAP2	microtubule-associated protein 2
MBP	myelin basic protein
MDA	malondialdehyde
mPTP	mitochondrial permeability transition pore
MPTP	1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine
mtNOS	mitochondrial nitric oxide synthase
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

NF	neurofilament
NF-κB	TNF-receptor-nuclear factor κB
NFP	neurofilament protein
NMDA	N-Methyl-$_D$-aspartate
NO	nitric oxide
NOS	nitric oxide synthas
ONOO$^-$	peroxynitrite anion
ONOO	peroxynitrite anion
ONOOH	peroxynitrite acid
ONOOCO$_2$	nitrosoperoxo carbonate
PARS	ADP-ribose synthetase
PBS	phosphate-buffered saline
PGH	prostaglandin hydroperoxidase
PLP	proteolipid protein
PN	peroxynitrite
PUFAs	polyunsaturated fatty acids
RCR	respiratory control ratio
ROS	reactive oxygen species
RNS	reactive nitrogen species
SBDPs	α-spectrin breakdown products
SC	spinal cord
SCI	spinal cord injury
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIN-1	3-morpholinosydnonimine hydrochloride
SOD	superoxide dismutase
TBI	traumatic brain injury
TBS	tris-buffered saline
TNF-α	necrosis factor alpha
UCPs	uncoupling proteins
UQ	ubiquinone
XDH	xanthine dehydrogenase
XO	xanthine oxidase

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Research Experience

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(2001-2003) Electrophysiological methods were applied to study the habituation and desensitization of Hering-Breuer (HB) reflex in rabbit.

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Publications

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Xiong YQ, Rabchevsky AG, Sullivan PG, Hall ED. Beneficial effects of tempol, a catalytic scavenger of peroxynitrite-derived free radicals, in a rat contusion spinal cord injury model. *J. Neurosci. (In preparation)*

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Abstracts

Xiong YQ, Singh IN, Hall ED. Beneficial effects of tempol on oxidative damage, mitochondrial dysfunction and cytoskeletal degradation after spinal cord injury in rats. *J. Neurotrauma. 24(7): p229 (2007).*

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