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HYDROGEN PEROXIDE ADMINISTERED INTO THE RAT SPINAL CORD AT THE LEVEL ELEVATED BY CONTUSION SPINAL CORD INJURY OXIDIZES PROTEINS, DNA AND MEMBRANE PHOSPHOLIPIDS, AND INDUCES CELL DEATH: ATTENUATION BY A METALLOPORPHYRIN

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Abstract

We previously demonstrated that hydrogen peroxide concentration [H₂O₂] significantly increases after spinal cord injury (SCI). The present study explored 1) whether SCI-elevated [H₂O₂] is sufficient to induce oxidation and cell death, 2) if apoptosis is a pathway of H₂O₂-induced cell death, and 3) whether H₂O₂-induced oxidation and cell death could be reversed by treatment with the catalytic antioxidant Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP). H₂O₂ was perfused through a microcannula into the uninjured rat spinal cord to mimic the conditions induced by SCI. Protein and DNA oxidation, membrane phospholipids peroxidation (MLP), cell death and apoptosis were characterized by histochemical and immunohistochemical staining with antibodies against markers of oxidation and apoptosis. Stained cells were quantified in sections of H₂O₂-, or artificial cerebrospinal fluid (ACSF)-exposed with vehicle-, or MnTBAP-treated groups. Compared with ACSF-exposed animals, SCI-elevated [H₂O₂] significantly increased intracellular protein and DNA oxidation by three-fold and MLP by eight-fold in neurons, respectively. H₂O₂-elevated extracellular malondialdehyde was measured by microdialysis sampling. We demonstrated that SCI-elevated [H₂O₂] significantly increased extracellular malondialdehyde above pre-injury levels. H₂O₂ also significantly increased cell loss and the numbers of TUNEL-positive and active caspase-3-positive neurons by 2.3-, 2.8-, and 5.6-fold compared to ACSF controls, respectively. Our results directly and unequivocally demonstrate that SCI-elevated [H₂O₂] contributes to post-SCI MLP, protein, and DNA oxidation to induce cell death. Therefore, we conclude that 1) the role of H₂O₂ in secondary SCI is pro-oxidation and pro-

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cell death, 2) apoptosis is a pathway for SCI-elevated $[H_2O_2]$ to induce cell death, 3) caspase activation is a mechanism of H_2O_2 -induced apoptosis after SCI, and 4) MnTBAP treatment significantly decreased H_2O_2 -induced oxidation, cell loss, and apoptosis to the levels of ACSF controls, further supporting MnTBAP's ability to scavenge H_2O_2 by *in vivo* evidence.

Keywords

hydrogen peroxide; spinal cord injury; Mn (III) tetrakis (4-benzoic acid) porphyrin; proteins and DNA oxidation; membrane lipid peroxidation; apoptotic cell death

INTRODUCTION

Spinal cord injury (SCI) is a devastating neurological disorder that affects individuals of all ages and causes lifelong disability. Acute traumatic SCI is worsened by secondary damage processes involving the overproduction of endogenous deleterious substances (Young, 1993). Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to secondary destruction after traumatic central nervous system injury by oxidatively or nitratively damaging proteins, DNA, and phospholipids (Lewen et al, 2000; Genovese, 2008; Hall, 2011). ROS include free radicals such as superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($\bullet OH$) and non-radical oxidants such as hydrogen peroxide (H_2O_2). RNS include free radicals like nitric oxide ($\bullet NO$) and non-radical oxidants such as peroxynitrite ($ONOO^-$). $O_2^{\bullet-}$ is produced through several aerobic pathways during normal metabolism, and superoxide dismutase converts $O_2^{\bullet-}$ into H_2O_2 , which is reduced to H_2O by catalase, glutathione peroxidase, and thioredoxin/peroxiredoxin. There is a dynamic equilibrium between the potential for oxidative damage and cellular antioxidant defense capacity. ROS and RNS have physiological functions and play important roles in a range of biological processes such as mediating redox signaling (Halliwell, 2011; Murphy et al. 2011). Disruption of this balance produces excessive $O_2^{\bullet-}$ and H_2O_2 , which can produce $\bullet OH$ via the metal-catalyzed Haber-Weiss/Fenton reaction through the sequence $O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow \bullet OH$ (Halliwell, 2006). Elevated $O_2^{\bullet-}$ also rapidly reacts with $\bullet NO$ to form $ONOO^-$ – a highly reactive oxidant. $ONOO^-$ can decompose to $\bullet OH$ when protonated by the reaction $O_2^{\bullet-} + \bullet NO \rightarrow ONOO^- + (H^+) \rightarrow ONOOH \rightarrow \bullet OH$ (Beckman et al., 1990; Ischiropoulos et al., 1992). The overproduced ROS attack polyunsaturated fatty acids in cell membranes, triggering free radical chain reactions to cause membrane lipid peroxidation (MLP) to produce aldehydes, such as malondialdehyde (MDA, the end-products of MLP) and 4-hydroxy-nonenal (HNE, the byproducts of MLP; Baldwin et al., 1998). ROS attack on DNA triggers DNA strand breaks and modifies DNA bases to produce 8-hydroxy-2-deoxyguanosine (8-OHdG). ROS attack on proteins modifies amino acids converting them to carbonyl derivatives, fragments chains and generates cross links (Halliwell, 2006; Halliwell and Gutteridge, 2007). Therefore, the present study used MDA and HNE as specific indicators of MLP, protein carbonyl content as an indicator of protein oxidation, and 8-OHdG as an indicator of DNA oxidation.

As indicated by Halliwell (2009), a vast amount of knowledge about ROS/RNS has come from studies of cultured cells, an abnormal state that lacks the *in vivo* extracellular

environment and where cell culture media are contaminated with transition ions. Most importantly, the concentrations of ROS/RNS donors or oxidants applied to the cultured cells are not relevant to *in vivo* levels. Most *in vivo* studies indirectly evaluate the role of ROS/RNS in oxidative damage and cell death in central nervous system injury or disease by measuring reduction of oxidative damage markers in response to the administration of ROS/RNS inhibitors or scavengers. To avoid the limitations of *in vitro* approaches and directly assess the contribution of ROS/RNS overproduction to secondary damage after SCI, we employed a three-step strategy: 1) directly measuring extracellular concentrations of individual ROS/RNS over time following contusion injury to the rat spinal cord, 2) measuring oxidative damage and cell death markers after the administration of individual ROS/RNS into uninjured rat spinal cords at levels and over durations that replicated those observed following SCI *in vivo*, and 3) measuring oxidative damage and cell death markers after administering individual ROS/RNS along with their appropriate scavengers/inhibitors. This approach separates the damage caused by specific ROS/RNS from the effects of mechanical injury and the resulting pathological damage due to ischemia/reperfusion, edema, inflammation, etc. Assessments of scavenger/inhibitor efficacy confirm the role of ROS/RNS in secondary SCI and provide viable candidates for antioxidant therapy. We have previously used this 3-step strategy to 1) establish the time courses of $O_2^{\bullet-}$ (Liu et al., 1998), H_2O_2 (Liu et al., 1999), $\bullet OH$ (Liu et al., 2004), $\bullet NO$ and $ONOO^-$ (Liu et al., 2000) elevations in the extracellular space following contusion SCI, 2) demonstrate that administration of a donor of $ONOO^-$ or Fenton reagents to generate $\bullet OH$ in uninjured rat spinal cord at the concentrations and durations produced by SCI resulted in protein oxidation/nitration (Bao et al., 2003), MLP (Liu et al., 2005), apoptotic and necrotic cell death (Bao and Liu, 2002, 2003, 2004), and neurological dysfunction (Bao and Liu, 2002), and 3) demonstrate that administration of Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, a broad-spectrum ROS/RNS scavenger and a catalytic antioxidant) to rat spinal cord reduced $ONOO^-$ -induced protein oxidation and nitration (Bao et al., 2003), and MLP (Liu et al., 2005), and $\bullet OH$ -induced cell death (Bao and Liu, 2004). These *in vivo* results from the 3-step strategy directly and unequivocally demonstrate that SCI-elevated levels of $ONOO^-$ and $\bullet OH$ are sufficient to cause oxidative damage and consequent secondary cell death; moreover, the catalytic antioxidant MnTBAP ameliorated damage by scavenging the administered $ONOO^-$ and $\bullet OH$.

The oxidant capacity of H_2O_2 is limited in comparison to highly oxidizing species such as $ONOO^-$ and $\bullet OH$, and H_2O_2 has been reported as a redox signaling agent. The paradoxical roles of H_2O_2 in regulating cell survival and mediating oxidative damage has been described by others (Chiarugi, 2009; Groeger et al., 2009). Although a less potent oxidant than $ONOO^-$ or $\bullet OH$, H_2O_2 is more stable, contributing a longer period of elevated levels after SCI than $ONOO^-$ or $\bullet OH$ (Liu et al., 1999, 2000, 2003). However, whether SCI-elevated extracellular H_2O_2 contributes to oxidative damage and cell death or is involved in cell survival signaling after SCI has never been explored. Based on our previously established time course of extracellular H_2O_2 elevation following SCI (Liu et al., 1999), in the present study, we perfused H_2O_2 to replicate the concentrations and durations following SCI into the spinal cords of uninjured rats to 1) characterize oxidative damage to proteins, DNA, and membrane phospholipids; 2) examine cell death including apoptosis and possible apoptotic

pathways; 3) evaluate the H₂O₂-scavenging ability of MnTBAP. Our results confirm that the levels/durations of H₂O₂ observed after SCI are sufficient to induce oxidative damage and cell death, suggesting that H₂O₂ contributes to secondary damage after SCI. The ability of MnTBAP to attenuate H₂O₂-induced oxidation and cell death further verify the H₂O₂-scavenging ability of MnTBAP *in vivo*.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (250-300g) were used for all animal experiments. All procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee and were in accordance with the National Institutes of Health guide for the *Care and Use of Laboratory Animals*. All possible efforts were made to minimize the numbers of animals used and their suffering.

1. Animal experiment groups

One group animal was used to establish extracellular H₂O₂ concentration ([H₂O₂]) to be administered, which replicates the actual extracellular [H₂O₂] released after contusion SCI. This established extracellular [H₂O₂] was administered into a normal rat spinal cord to explore whether it induce oxidative damage and cell death. The detail is described in section 3 of the Experimental Procedures.

To explore whether the SCI-elevated [H₂O₂] is sufficient to induce oxidative damage, cell death, and apoptosis and whether MnTBAP treatment reverses such damage, three sets of animal experiments were performed: 1) H₂O₂-exposed with vehicle treatment (vehicle-treated), 2) H₂O₂-exposed with MnTBAP treatment (MnTBAP-treated), and 3) artificial cerebrospinal fluid (ACSF)-exposed with ACSF treatment (ACSF control). In the vehicle- and MnTBAP-treated groups, the established extracellular [H₂O₂] mimicking that released following SCI was perfused through a microcannula inserted laterally through the gray matter of the rat spinal cord, and MnTBAP or ACSF (the vehicle for MnTBAP) was perfused through the second cannula inserted together with the first cannula for treatments. In the ACSF control group, ACSF was perfused through both cannulas without H₂O₂ exposure. H₂O₂, ACSF or MnTBAP were perfused for 10 h, the same amount of time as [H₂O₂] elevation after SCI. At 14 h post-H₂O₂ (24 h from the beginning of H₂O₂ administration) or ACSF exposure, the spinal cords were harvested and processed for staining. Because caspase activation precedes apoptosis, the SCI-elevated level of H₂O₂ was perfused for 6 h, and the cords were harvested at the end of perfusion to evaluate cellular apoptosis by caspase activation. Therefore another three sets of animal experiments were performed. n = 5/group to characterize intracellular oxidation and cell death.

H₂O₂-induced apoptosis was confirmed by transmission electron microscopy (TEM) in three groups of animal (n = 2/group): 1) laminectomy only without cannulae implantation (sham control), 2) ACSF control (perfused only with ACSF), and 3) H₂O₂-exposed (perfused only with H₂O₂).

In addition to immunohistochemically assessing intracellular MLP, we also measured the time course of extracellular MDA production by performing microcannula sampling in

response to SCI-elevated H_2O_2 exposure in MnTBAP- and ACSF-treated groups. The SCI-elevated $[\text{H}_2\text{O}_2]$ was perfused through one cannula, and MnTBAP or ACSF was administered through the second cannula for the MnTBAP- and ACSF-treated groups, respectively ($n = 3/\text{group}$ to determine extracellular MLP).

2. Animal preparation, microcannula insertion, and H_2O_2 administration

The rats were anesthetized intraperitoneally (i.p.) first with sodium pentobarbital (35 mg/kg) followed by urethane (780 mg/kg i.p.) for maintenance. When the rat was fully anesthetized, its back was shaved and a laminectomy performed on vertebra T13 and the dura was kept intact. Care was taken not to injure the cord. After exposing the cord, two microcannulae were implanted. The microcannula was made from a microdialysis fiber (Spectra/Por RC; 200 μm inside diameter, Spectrum Laboratories, Inc, Houston, TX, USA) coated with a thin layer of silicone rubber (3140 MIL-A-46146 RTV, Dow Corning Corporation, Midland, MI, USA) with a final external diameter of 220 μm . Two pairs of holes were made within the 2-mm zone through the wall of a completely coated fiber using heated needles as described previously (Liu et al., 1998, 2000). The double microcannulae were implanted into the gray matter of the cord as described in our previous publications (Liu and McAdoo, 1993; Liu et al., 2000, 2005). The animal was then clamped in a small animal stereotaxic frame (David Kopf Instruments, Tujunuga, CA, USA) by attachments to its dorsal vertebral processes. A saline pool was created around the exposed cord, and the pool and body temperatures were maintained at 36-37°C and 37-38°C, respectively, throughout the experiment by utilizing feedback from a thermosensor placed in the pool to a heating lamp set above the pool (Physitemp Instruments G15, Inc., Clifton, NJ, USA) and from a rectal probe to a heating blanket (Harvard homeothermic blanket control unit; Les Ulis, France). The microcannulae were attached to a microdialysis syringe pump (CMA, Microdialysis AB, Solna, Sweden) with a length of polyethylene-50 tubing for administration. ACSF (composition in mM: Na^+ 151.1, K^+ 2.6, Mg^{2+} 0.9, Ca^{2+} 1.3, Cl^- 122.7, HCO_3^- 21.0, HPO_4^{2-} 2.5 and glucose 3.87) was pumped through the cannulae for 2 h at a flow rate of 5 $\mu\text{l}/\text{min}$ to allow the release of substances due to cannulae insertion to subside to a stable level. ACSF was bubbled with 95% $\text{O}_2/5\%$ CO_2 prior to each experiment to adjust the pH to 7.2. The perfusion solution was then switched to H_2O_2 in ACSF in one cannula at the flow rate of 5 $\mu\text{l}/\text{min}$ and MnTBAP (2.5 mM) or its vehicle (ACSF) in the other cannula at a flow rate of 2 $\mu\text{l}/\text{min}$ for MnTBAP- or vehicle-treated groups. The perfusion was continued for 10 h, corresponding to a period of H_2O_2 elevation after SCI that we measured previously (Liu et al., 1999). At the end of perfusion, the surgical site was closed in layers and the animals were awakened.

3. Establishment of extracellular H_2O_2 concentration to be administered to induce damage

In our previous studies, we measured the time course of $[\text{H}_2\text{O}_2]$ elevations in the extracellular space after SCI by microdialysis sampling (Liu et al., 1999). However, the $[\text{H}_2\text{O}_2]$ measured by microdialysis sampling is much lower than the actual extracellular $[\text{H}_2\text{O}_2]$ because of dilution by perfused ACSF for sampling and the low ratio of the penetrating microdialysis membrane as we reported previously (Liu and McAdoo, 1993; Liu et al., 1999, 2001). To determine $[\text{H}_2\text{O}_2]$ that yields a $[\text{H}_2\text{O}_2]$ in the extracellular space replicating the extracellular $[\text{H}_2\text{O}_2]$ measured by microdialysis sampling after SCI, a microdialysis fiber was glued together with a microcannula and inserted into the gray matter

of the cord as described in section 2. The microdialysis fiber was made from the same material as the microcannula, but the 2-mm dialysis zone was uncoated for microdialysis sampling. The 2-mm dialysis zones in the fiber and the 2-mm area with holes in the cannula were overlapped in the gray matter of the cord. Fig. 1A shows the location of microcannula and microdialysis fiber in a cross section of spinal cord stained with cresyl violet. H_2O_2 in ACSF administered through the microcannula freely diffused through the holes in the cannula into the extracellular space of the cord, passed through the membrane in the dialysis zone in the microdialysis fiber, and sampled through the dialysis fiber at a flow rate of 5 $\mu\text{l}/\text{min}$ — the same flow rate used to measure the time course of H_2O_2 elevation following contusion SCI. The microdialysates were collected from the outlet end of the fiber in plastic vials in ice as shown in Fig. 1A. ACSF was perfused through both the fiber and cannula for 2 h as described in section 2. After three samples were collected every 30 min to establish the basal level, the perfusion solution through the cannula was switched to H_2O_2 for administration for 5 h, and ACSF was continuously administered through the microdialysis fiber to sampling H_2O_2 . A series of $[\text{H}_2\text{O}_2]$ were administered into number of rats (one $[\text{H}_2\text{O}_2]$ per rat) and a series of time courses in response to each $[\text{H}_2\text{O}_2]$ administered were measured from the dialysates to identify which regimen replicated the extracellular $[\text{H}_2\text{O}_2]$ released after contusion SCI as we reported (Liu et al., 1999).

H_2O_2 in microdialysates was analyzed by high-pressure liquid chromatography (HPLC) to obtain the time courses of $[\text{H}_2\text{O}_2]$ changes. The sampled $[\text{H}_2\text{O}_2]$ was measured by converting H_2O_2 to $\cdot\text{OH}$ in the collecting vials by the Fenton reaction using a unique method developed in our laboratory (Liu et al., 1999). Briefly, the Fenton reagent FeCl_2 (0.2 mM) and salicylate (1 mM) in water (pH 3.0) were pre-added to the collecting vial (1:1, microdialysate:Fenton reagents). H_2O_2 sampled through the microdialysis fiber immediately reacts with FeCl_2 while reaching the Fenton reagent in the collecting vials to generate $\cdot\text{OH}$ in the vials by the Fenton reaction. The $\cdot\text{OH}$ produced in the collecting vials rapidly attacks salicylate to produce 2,3- and 2,5- dihydroxybenzoic acid (2,3- and 2,5-DHBA). The DHBAs in the collecting vials were measured by HPLC with electrochemical detection (Floyd et al., 1986) using our previously reported method (Liu et al., 1999; 2013)

4. Tissue processing

To determine whether H_2O_2 at the SCI-elevated level induces oxidative damage and cell death, the established extracellular $[\text{H}_2\text{O}_2]$ from Section 3 was administered for 10 h, then the incision was surgically repaired, and the animals were allowed to wake up. At 14 h post- H_2O_2 or ACSF exposure (or at the end of 6 h exposure to evaluate caspase activation as described in section 1), animals were re-anesthetized with pentobarbital (50 mg/kg i.p.) and sacrificed by transcardial perfusion fixation with 0.9% saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). A 1-cm segment of cord centered at the cannula track was removed and post-fixed in the same fixative for at least 24 h at 4°C , and then dehydrated in graded alcohol and xylene, embedded in paraffin, and cut transversely into 10- μm thick serial sections for staining. The paraffin-embedded sections containing the cannula track and sections 2-mm caudal were deparaffinized in two changes of xylene for 5 min each and then sequentially washed in a gradient of ethanol to water for staining.

5. Evaluating protein and DNA oxidation, and MLP

5.1. Immunohistochemical labeling of oxidative markers—To evaluate intracellular oxidation of protein and DNA and MLP, the deparaffinized sections were immunohistochemically labeled with antibodies against the oxidative markers DNP, 8-OHdG, and HNE as we used previously (Bao et al., 2003; Liu et al., 2005, 2013; Valluru et al., 2012). The markers of oxidized proteins and DNA and MLP in the extracellular space were washed out during tissue processing. Briefly, the deparaffinized sections were washed in PBS (0.14 M NaCl, 0.003 M KCl, and 0.01 M phosphate, pH 7.4) for 5–10 min, incubated in 0.6% H₂O₂ in methanol to quench endogenous peroxidase activity, rinsed in PBS (3 × 5 min), incubated for 30 min in 5% normal goat serum (Sigma, St. Louis, MO, USA), and then incubated overnight at 4° C with a monoclonal antibody (1:500) against 8-OHdG (QED Bioscience Inc., San Diego, CA, USA), or with anti-HNE (1:200) monoclonal antibody (Alpha Diagnostic, San Antonio, TX, USA). To label oxidized proteins, the sections were first incubated with 0.1% 2,4-dinitrophenylhydrazine in 2N HCl (Smith et al., 1998; Laski et al., 2001; Bao et al., 2003) over night at 4° C to label carbonyl groups in proteins, then in 0.6% H₂O₂ and methanol followed by incubation with a monoclonal antibody (1:100) to 2,4-dinitrophenyl (DNP, Zymed, San Francisco, CA, USA) overnight at 4° C. The antibody labeled sections were then incubated with biotinylated goat anti-mouse-IgG (1:200, Vector Laboratories, Inc., Burlingame, CA, USA) followed by incubation with avidin-biotin-horseradish peroxidase complex (1:200, Vector Laboratories, Inc.). After washing in PBS (3 × 5 min), the sections were rinsed in 0.1% acetate buffer (pH 6.0) for 10 min and developed in a glucose-diaminobenzidine-nickel solution (Yang et al., 1995; Leski et al., 2001) for 5–20 min. The visualized sections were rinsed in 0.1% acetate buffer (pH 6.0) for 10 min and in PBS (3 × 5 min) before they were dehydrated with a gradient of ethanol, cleared, and coverslipped with Permount to evaluate intracellular oxidation. The negative controls were treated similarly, but an equivalent volume of PBS was substituted for the primary antibody solution. Control sections 2-mm from the cannula track were stained with cresyl violet as described in section 6.1.

5.2. HPLC analysis of extracellular [MDA] in perfusates—The time course of extracellular MDA production in response to SCI-elevated H₂O₂ exposure was measured in the perfusates collected from the cannula in which H₂O₂ was administered. Two hours after double fiber insertion and ACSF perfusion, the perfusates were begun to collect from the outlet end of the cannula. After 60 min of fluid collection at 30 min per sample to obtain a basal level, the ACSF was changed to the established extracellular [H₂O₂], and perfusate collection continued for another 5.5 h. In the MnTBAP-treated or vehicle-treated group, 2.5 mM MnTBAP or ACSF, respectively, was pumped through another cannula into the cord. H₂O₂ in the perfusates was analyzed by HPLC with fluorescence detection using our previously reported method (Qian and Liu, 1997).

6. Evaluating cell death and apoptosis

6.1. Histochemical staining of surviving cells—To evaluate the cell loss, the deparaffinized sections were stained with 0.1% cresyl violet in a sodium acetate buffer for 30 min as described previously (Bao and Liu, 2004). The cresyl violet-stained sections were

dehydrated through an ethanol gradient, cleared, and coverslipped with Permount. A cresyl violet-stained sections was shown in Fig. 1A.

6.2. Immunohistochemical labeling of active caspase—To evaluate cellular apoptosis, active caspases were immuno-labeled in sections removed 6 h post- H_2O_2 exposure using the procedures described in section 5.1. They were immuno-labeled with an antibody to rabbit anti-human/mouse active caspase-3 (fragment p17 from R&D Systems, Minneapolis, MN, USA) and a monoclonal antibody to cleaved caspase-8 (Asp384, from Cell Signaling, Danvers, MA, USA), which were diluted in 2% goat serum in PBS (1:500) as described previously (Bao and Liu, 2003; Ling et al., 2013).

6.3. TUNEL and TUNEL plus immunohistochemical labeling—DNA fragmentation in the nucleus is an indicator of apoptosis. Fragmented DNA in cell nuclei was assessed with terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-(dUTP)-biotin nick end labeling (TUNEL) staining. The deparaffinized sections containing the cannula tracks were incubated with 20 μ g/ml proteinase K (Sigma) for 15 min, and then endogenous peroxidase was quenched in 3.0% hydrogen peroxide in PBS for 5 min at room temperature. TUNEL staining was accomplished using The ApopTag in Situ Apoptosis Detection Kit (Intergen Company, Purchase, NY, USA) following the manufacturer's instruction. The sections were incubated with TUNEL in a humidified chamber at 37°C for 1 h after immersion in equilibration buffer for 10 min and then incubated in the stop/wash buffer at room temperature for 10 min to stop the reaction. The sections were washed with 3 changes of PBS for 1 min each and then incubated in anti-digoxigenin peroxidase conjugate for 30 min. After washing in PBS, the sections were visualized with 0.05% diaminobenzidine in staining buffer (0.05% M PBS, pH 7.6) as previously described (Bao and Liu, 2003, 2004; Ling and Liu, 2007; Ling et al., 2013). Nonspecific labeling was assessed by using water or equilibration buffer to substitute for the volume of TdT in the TUNEL reaction mixture.

To identify TUNEL-positive neurons, the deparaffinized TUNEL-stained sections were immunohistochemically and immunofluorescently labeled for neuron-specific enolase (NSE) using the procedures that we used previously (Bao and Liu, 2003, 2004; Ling and Liu, 2007; Ling et al., 2013). For immunohistochemical staining, following TUNEL staining, the sections were washed with three changes of PBS for 5 min each, blocked with 5% goat serum, and incubated with anti-NSE (1:100) at 4°C for 24 h. After washing in PBS, the sections were incubated with biotinylated goat anti-mouse-IgG (1:200, Sigma) followed by a solution of an avidin-biotin-horseradish peroxidase complex (1:200, Vector Laboratories, Inc.) for 50 min. The sections were then visualized with a Vector SG Substrate Kit (Vector Laboratories, Inc.), washed in water, dehydrated, cleaned, and coverslipped with Permount. The control sections 2-mm from the cannula tracks were immunohistochemically stained with the anti-NSE antibody. Negative controls were similarly processed without a primary antibody.

The apoptotic neurons were further characterized by TUNEL + immuno-fluorescence double labeling. Following TUNEL staining with the ApopTag Fluorescein Direct in Situ Apoptosis Detection Kit (Intergen Company), the sections were washed in three changes of

PBS for 5 min each, blocked with 5% goat serum, incubated with anti-NSE (1:50, DAKO, Glostrup, Denmark), and washed in PBS. Then the sections were incubated in crystalline tetramethylrhodamine isothiocyanate-labeled goat anti-mouse IgG (1:100, Sigma) in PBS with 0.2% Triton X-100 and 0.1% BSA for 1 h in the dark at room temperature. The slides were coverslipped with PBS/glycerol and stored at 4°C. The fluorescent-labeled sections were observed under a Nikon epifluorescence microscope (Nikon, Tokyo, Japan) with standard Texas-Red optics (excitation: BP 540-580 nm) to observe NSE-labeled neurons or FITC optics (excitation: BP 450-490) to observe TUNEL-positive cells.

6.4. TEM confirmation of H₂O₂-induced apoptosis—neuronal apoptosis was confirmed by TEM on the basis of morphological characteristics as we described previously (Bao and Liu, 2003, 2004; Ling et al., 2013). At 14 h post-H₂O₂ or ACSF exposure or laminectomy for sham control, animals were re-anesthetized and sacrificed by transcardial perfusion-fixation with a solution containing 4% paraformaldehyde with 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). An approximately 0.5-cm segment of spinal cord containing the cannula track was removed and kept in the same fixative overnight at 4°C. After washing with cacodylate buffer, tissue blocks (0.1 mm³) taken from the ventral horn (Rexed layer VIII) were further fixed in 4% osmium tetroxide in 0.1 M cacodylate buffer for 1 h under a hood. The blocks then were stained with 2% uranyl acetate overnight at 4°C, dehydrated, and embedded in epoxy resin. The resin section was cut into semi-thin (1 µm) sections and stained with 1% toluidine blue to locate apoptotic neurons. Semi-thin sections containing apoptotic neurons were cut into ultra-thin sections on an ultramicrotome (Reichert Ultra-cut S; Reichert, Depew, NY, USA). The ultra-thin sections were observed under TEM (Philips CM-100; Philips, Amsterdam, The Netherlands) and photographed.

7. Quantitative evaluation of oxidation and cell death

To quantitatively evaluate H₂O₂-induced intracellular oxidation and cell death, the histochemically and immunohistochemically stained cells were counted at 100× magnification. Immuno-positive neurons were identified based on the presence of positive cells with neuronal morphology. TUNEL-positive cells were identified with dark nuclei in TUNEL-stained sections. Cresyl violet-stained neurons in control sections were identified by the presence of bright nuclei with enrichment of Nissl bodies. There were no significant differences in neuron counts between cresyl violet-stained sections counted by applying these criteria and anti-NSE immuno-labeled sections, validating the criteria for identifying neurons in cresyl violet-stained sections (Bao and Liu, 2004). The numbers of DNP-, 8-OHdG-, HNE-, and caspase-positive neurons in the immuno-labeled sections were counted along the cannula tracks. The corresponding areas in the same animal 2-mm caudal to the cannula track in the cresyl violet-stained sections were similarly counted to provide control values. Surviving cells were quantitated by counting the number of cells in the cresyl violet-stained sections along the cannula tracks and 2-mm caudal to the tracks. TUNEL-positive neurons were counted in TUNEL plus NSE double-stained sections along the cannula tracks and 2-mm caudal to the tracks in NSE-stained sections. Neurons were counted in areas 0.2-mm wide by 0.9-mm long (starting from the central canal and extending to the edge of the gray matter) parallel to and immediately adjacent to the cannula track in the gray matter of

the ventral and dorsal sides of the cannula track as the treated areas, as shown in Fig. 1A. Counts were converted to density (number of cells per unit area). The percentages of densities of the immuno-positive and TUNEL-positive neurons in each animal were calculated with the following formula: density of immuno- or TUNEL-positive neurons along the fiber track divided by densities of total neurons in the corresponding control area times 100%. The percentage of cells lost in each animal was calculated using the formula: % loss = ([control cell density – treated cell density]/control cell density) × 100%. Using the percentage of cell loss or percentage of immuno- or TUNEL-positive neurons obtained by comparing the densities along the cannula track and the corresponding control area offsets the variation caused by counting different populations of neurons caused by different position of perfusing cannula in different section. The cells on the ventral and dorsal sides of the cannula track in each section were counted 3 times to obtain an average count. Three sequential sections in each animal were counted, and the mean counts from three sections were also averaged.

8. Statistical analysis

The average percentages of densities were compared among groups using one-way analysis of variance (ANOVA) followed by post-hoc Tukey testing for intergroup differences. The average percentages of densities are presented as mean ± SD. Paired t-tests were used to assess [MDA] differences between the average post-H₂O₂ exposure and average pre-H₂O₂ exposure. The time course of extracellular [MDA] changes in response to exogenous H₂O₂ was compared between the ACSF- and MnTBAP-treated groups by repeated measures ANOVA.

RESULTS

Determination of extracellular [H₂O₂] released following SCI

As described in section 3 of the Experimental Procedures, the time course of [H₂O₂] elevations in the extracellular space after contusion SCI that we previously measured by microdialysis sampling (Liu et al., 1999) was not the actual extracellular [H₂O₂] in the cord. To measure the actual SCI-elevated extracellular [H₂O₂], H₂O₂ was perfused through a microcannula into a normal rat spinal cord, and the extracellular H₂O₂ was sampled through a microdialysis fiber inserted together with the perfusion cannula. As described in section 3 of the Experimental Procedures, a series of [H₂O₂] were administered and a series of time courses were measured from the dialysates collected through the dialysis fiber corresponding to each [H₂O₂] administered through the cannula. The time course of 2,3-DHBA production measured in response to expose to each [H₂O₂] was compared with the time courses of 2,3-DHBA production measured after SCI (Liu et al., 1999). We determined that 150 μM H₂O₂ applied into the extracellular space of the cord through the cannula yielded a time course of extracellular [H₂O₂] sampled by the microdialysis fiber replicated the time course of extracellular [H₂O₂] in the dialysates collected after contusion SCI. This time course is shown in Fig. 1B. Therefore, 150 μM H₂O₂ was the established extracellular [H₂O₂] elevated by contusion SCI. So, this [H₂O₂] was administered through the cannula into gray matter of the cord to evaluate its effects on oxidative damage and cell death.

The SCI-elevated level of H₂O₂ induces and MnTBAP attenuates protein oxidation

Fig. 2 presents the experimental evidence that 150 μ M H₂O₂ perfused through the cannula for 10 h induced intracellular protein oxidation. The photomicrographs of the immuno-labeled sections (upper panel) show that at 14 h post-ACSF exposure, few anti-DNP-labeled neurons were visible (Fig. 2, A and A'). In contrast, numbers of DNP-positive neurons were observed in the H₂O₂-exposed section (Fig. 2, B and B'). MnTBAP treatment clearly reduced the number of DNP-positive neurons (Fig. 2, C and C'). DNP-positive neurons along the cannula tracks and cresyl violet-stained neurons 2-mm caudal to the tracks were counted. The lower panel of Fig. 2 shows the quantitative results of H₂O₂-induced and MnTBAP-reduced protein oxidation. The average percentage of DNP-positive neurons in the immuno-labeled cross sections along the cannula tracks was 10.8 ± 3.1 in the H₂O₂-exposed sections compared with only 3.8 ± 1.3 and 6.4 ± 1.5 in ACSF- and MnTBAP-treated sections, respectively. H₂O₂ exposure significantly ($p < 0.001$) increased the average density percentage of DNP-positive neurons compared to ACSF-exposed controls. MnTBAP treatment significantly ($p = 0.02$) reduced the density percentage of DNP-positive neurons compared to the vehicle-treated sections. There were no significant differences in the % of DNP-positive neurons between MnTBAP-treated and ACSF-control groups ($p = 0.2$), indicating that MnTBAP effectively reduced intracellular protein oxidation to a level near that observed in the ACSF-control level.

The SCI-elevated level of H₂O₂ induces and MnTBAP attenuates DNA oxidation

Fig. 3 shows the experimental evidence that 150 μ M H₂O₂ perfused through the cannula for 10 h induced intracellular DNA oxidation. The photomicrographs of the immuno-labeled sections (upper panel) show at 14 h post-ACSF exposure, few anti-8-OHdG-labeled neurons were visible (Fig. 3, A and A'). In contrast, many 8-OHdG-positive neurons were observed in the H₂O₂-exposed sections (Fig. 3, B and B'). MnTBAP treatment dramatically reduced the numbers of 8-OHdG-positive neurons (Fig. 3, C and C'). By counting the immuno-positive neurons along the cannula tracks and cresyl violet-stained neurons 2-mm caudal from the tracks, the results shown in the lower panel demonstrate that H₂O₂-exposure significantly ($p < 0.001$) increased the average density percentage of 8-OHdG-positive neurons from 4.2 ± 2 in the ACSF-control sections to 13.6 ± 4.1 in the H₂O₂-exposed with vehicle-treated sections. MnTBAP treatment significantly ($p = 0.03$) reduced the percentage to 8.0 ± 2.5 compared with the vehicle-treated sections. There were no significant differences in the average percentages of 8-OHdG-positive neurons between MnTBAP-treated and ACSF control groups ($p = 0.2$), indicating that MnTBAP effectively reduced the DNA oxidation to near the ACSF control levels.

SCI-elevated level of H₂O₂ induces and MnTBAP attenuates MLP

Fig. 4 depicts the morphological and quantitative results of H₂O₂-induced HNE production and the effect of MnTBAP. The photomicrographs of the immuno-labeled sections (Fig. 4, upper panel) indicated that at 14 h post-ACSF exposure, a few HNE-positive cells were visible (Fig. 4, A and A'). In contrast, numbers of HNE-positive neurons appeared in the H₂O₂-exposed sections (Fig. 4, B and B'). MnTBAP treatment clearly reduced the number of HNE-positive neurons (Fig. 4, C and C'). As in Figs 2 and 3, quantitative results in the

lower panel show that the average density percentage of HNE-positive neurons in the immuno-labeled cross sections along the cannula tracks was 31.1 ± 6 in H_2O_2 -exposed sections versus 3.8 ± 3.2 in ACSF control sections. Compared to ACSF control, H_2O_2 exposure significantly increased the % of HNE-positive neurons ($p < 0.001$). MnTBAP treatment significantly ($p < 0.001$) reduced the % of HNE-positive neurons to 8.4 ± 3.4 compared to vehicle-treated sections. There were no significant differences in the average % of HNE-positive neurons between MnTBAP-treated and ACSF control groups ($p = 0.3$), indicating that MnTBAP effectively reduced neuronal MLP to near the ACSF control levels.

The time course of MDA elevation in extracellular space, in response to $150 \mu\text{M}$ H_2O_2 administered through the microcannula, was measured by microdialysis sampling. As shown in Fig. 5, SCI-elevated level of H_2O_2 significantly increased the concentration of MDA compared with the pre-injury levels ($p = 0.03$, paired t-test). MnTBAP administered through the second cannula significantly attenuated MDA production compared with vehicle treatment ($p = 0.02$, repeated measures ANOVA). Notably, MnTBAP treatment effectively reduced H_2O_2 -induced MDA elevation to the pre-injury level.

SCI-elevated level of H_2O_2 induces and MnTBAP attenuates cell death

To determine whether an SCI-relevant H_2O_2 concentration and duration is sufficient to induce cell death, spinal cord sections were stained with cresyl violet. Fig. 6 presents the morphological and quantitative results of H_2O_2 -induced cell loss and the effect of MnTBAP. The photomicrographs of the cresyl violet-stained sections (upper panel) show, in the control section obtained 2-mm caudal from the cannula tracks, many cresyl violet-stained cells and neurons with typical neuronal morphology were observed (Fig. 6, A and A'); in the section obtained at 14 h post-ACSF exposure, many cresyl violet-stained neurons and other cells appeared (Fig. 6, B and B'); H_2O_2 exposure apparently reduced the number of cresyl violet-stained cells (Fig. 6, C and C'), but MnTBAP treatment increased the number of surviving cells (Fig. 6, D and D'). The quantitative results (lower panel) show that H_2O_2 exposure significantly ($p < 0.001$) increased the percentage of cell loss from 10.9 ± 2.3 in the ACSF control sections to 25.3 ± 6.2 in H_2O_2 -exposed with vehicle-treated sections. MnTBAP treatment significantly ($p = 0.01$) decreased the % of cell loss to 16.1 ± 2.3 compared to the vehicle-treated sections. There were no significant differences in the average % of cell loss between MnTBAP-treated and ACSF control sections ($p = 0.1$), suggesting that MnTBAP protection against H_2O_2 -induced cell loss.

The SCI-elevated level of H_2O_2 mediates and MnTBAP attenuates apoptotic DNA fragmentation

To determine whether treatment with an SCI-elevated [H_2O_2] induces cell death via apoptosis, fragmented DNA (a marker of apoptosis) was stained with TUNEL in spinal cord sections. To observe neuronal apoptosis, TUNEL and NSE double staining were performed in the same sections. The upper panel of Fig. 7 shows photomicrographs of TUNEL-stained sections showing all TUNEL-positive cells, and the middle panel depicts TUNEL + NSE-double-stained sections, showing TUNEL-positive neurons. In the ACSF control section, there were few TUNEL-positive cells (Fig. 7, A –A'' in the upper panel) and TUNEL-positive neurons (Fig. 7, A and A' in the middle panel). The neurons along the fiber track in

the ACSF control sections (Fig. 7, A, A' in the middle panel) appear normal with bright nuclei and typical neuronal morphology and TUNEL-negative as indicated by arrows. Many TUNEL-positive cells appeared in the H₂O₂-exposed sections (Fig. 7, B-B'' in the upper panel). TUNEL-positive neurons with dark nuclei indicated by arrows, and some TUNEL-positive nuclei representing glial cells appeared in the H₂O₂-exposed sections (Fig. 7, B and B' in the middle panel). MnTBAP treatment reduced the number of TUNEL-positive cells (Fig. 7, C – C'' in the upper panel) and TUNEL-positive neurons (Fig. 7, C and C' in the middle panel). TUNEL and NSE immuno-fluorescence double staining co-localized H₂O₂-induced apoptotic DNA fragmentation in neurons (Fig. 7, D - F). The arrow heads indicate the same neurons in D, E, and F. By counting the TUNEL-positive neurons in the TUNEL + NSE double-stained sections, we demonstrated that H₂O₂ exposure significantly ($p = 0.002$) increased the percentage of TUNEL-positive neurons from 10.4 ± 3.7 in ACSF control sections to 29.7 ± 8.9 in H₂O₂-exposed sections. MnTBAP treatment significantly ($p = 0.02$) reduced the H₂O₂-induced increase to 16.7 ± 5.8 . There were no significant differences in the % of TUNEL-positive neurons between MnTBAP-treated and ACSF control sections ($p = 0.3$), suggesting that MnTBAP protected against H₂O₂-induced apoptosis.

The SCI-elevated level of H₂O₂ mediates and MnTBAP attenuates caspase activation

To determine whether caspase activation is a mechanism by which the SCI-elevated level of H₂O₂ induces apoptosis, caspase activation were observed by immuno-labeling with antibodies against specific active fragments of caspase-3 and caspase-8 on sections harvested at 6 h H₂O₂-exposure. The upper panel of Fig. 8 shows few caspase-3 positive neurons in the ACSF control section (Fig. 8, A and A') but apparent caspase-3-positive cells and neurons in the H₂O₂-exposed section (Fig. 8, B and B'). The arrows indicate the typical caspase-3-positive neurons. MnTBAP treatment reduced the number of active caspase-3-positive neurons (Fig. 8, C and C'). Caspase-8 active fragment immuno-labeling was negative even in the H₂O₂-exposed section (Fig. 8, D and D') at 6 h post-H₂O₂ exposure. The quantitative results revealed that H₂O₂ exposure significantly ($p < 0.001$) increased the active caspase-3-positive neurons from 3.3 ± 2.2 in ACSF control sections to 18.4 ± 4.6 in H₂O₂-exposed sections. MnTBAP treatment significantly ($p = 0.003$) reduced the H₂O₂-mediated increase in active caspase-3-positive neurons to 8.8 ± 3.4 . There were no significant differences in the average percentage of caspase-3-positive neurons between MnTBAP-treated and ACSF control sections ($p = 0.07$), suggesting that MnTBAP reduced H₂O₂-induced apoptosis through attenuation of H₂O₂-induced caspase activation.

TEM confirmation of H₂O₂-induced neuronal apoptosis

Neuronal apoptosis was confirmed by TEM observation in ultra-thin sections of the ventral gray matter of the spinal cord based on the specific morphological features as shown in Fig. 9. A normal neuron in a sham-operated control rat shows a large nucleus (pale-stained euchromatin) with abundant rough endoplasmic reticulum (RER), mitochondria, and Golgi complexes in the cytoplasm (Fig. 9A). At 14 h post-ACSF exposure, a neuron shows a slight loss of ribosomes from the RER, but the nucleus and other organelles are intact (Fig. 9B). At 14 h post-H₂O₂ exposure, neurons at different stages of apoptosis were observed (Fig. 9, C - F). A very early stage apoptotic neuron with condensed chromatin accumulated mainly at

the nuclear rim is shown in C. Chromatin condensation produced chromatin masses in the nucleus at the later stages of apoptosis (Fig. 9, D and E) in H₂O₂-exposed sections. The neuronal membrane became convoluted, and was drawn deep into the cytoplasm (Fig. 9E), an indentation that might finally pinch off to form an apoptotic body. Fig. 9F shows an apoptotic cell being engulfed by a phagocyte.

DISCUSSION

To explore the role of SCI-elevated level of H₂O₂ in secondary SCI, it is critical to administer H₂O₂ at the concentration and duration replicating that observed following SCI. The present study established that 150 μ M H₂O₂ perfused through the microcannula into the extracellular space of the gray matter of a rat spinal cord for 10 h produced a time course of extracellular [H₂O₂] similar to that observed after SCI as shown in Fig. 1B. Unlike O₂^{•-}, the extracellularly administered H₂O₂ can freely cross cell membranes and reacts with intracellular molecules. Therefore, this established extracellular [H₂O₂] was administered using this paradigm to determine whether it induces intraand extracellular oxidation of major cellular components, total cell death and apoptosis.

By counting DNP-, 8-OHdG-, and HNE-positive neurons along the cannula tracks in the spinal cord sections treated with 150 μ M H₂O₂ or ACSF for 10 h, we demonstrated that H₂O₂ exposure significantly increased oxidation of proteins and DNA in neurons to the levels of approximately three times the levels observed in ACSF control sections (Figs 2 and 3), and significantly increased MLP in neurons eight times above that in ACSF control sections (Fig. 4). MLP was also examined extracellularly by measuring the time course of MDA elevation in the extracellular space in response to 150 μ M H₂O₂ administration. We found a continuous elevation of [MDA] during the entire period of H₂O₂ administration (Fig. 5). The fact that H₂O₂ induced much more intracellular MLP than intracellular protein and DNA oxidation (eight-fold versus three-fold) suggests that membrane lipids are more vulnerable to H₂O₂-induced oxidative modification. Notably treatment with the broad spectrum ROS/RNS scavenger MnTBAP significantly attenuated the H₂O₂-induced increases in MLP and the oxidation of proteins and DNA to levels that were not significantly different from those in ACSF controls. Our results demonstrated that H₂O₂ at the concentration associated with SCI is sufficient to induce extra- and intracellular MLP and oxidation of proteins and DNA, and that MnTBAP effectively reduced H₂O₂-induced oxidative damage to the major cellular components by scavenging the administered H₂O₂.

By counting total surviving cells in the cresyl violet-stained sections along the cannula tracks in sections treated with 150 μ M H₂O₂ or ACSF for 10 h, we demonstrated that H₂O₂ exposure induced significantly more (2.3 times) cell loss than quantified in ACSF control sections (Fig. 6), indicating that [H₂O₂] elevated by SCI is sufficient to cause secondary cell death. This sound *in vivo* evidence supports the hypothesis that H₂O₂ is a secondary damaging agent in SCI. Our observation that H₂O₂-induced cell death was attenuated by MnTBAP is the first direct *in vivo* evidence that H₂O₂-induced oxidative damage is one of the causes of secondary cell death following SCI. It also makes MnTBAP a potential agent for treating SCI and other neurotraumas.

DNA fragmentation is an indicator of apoptosis. Apoptotic DNA fragmentation can be observed by TUNEL staining. By counting TUNEL + NSE double-stained neurons along the cannula tracks in response to 150 μ M H₂O₂ administration, we demonstrated that H₂O₂ exposure induced significantly more (2.8 times) neuronal DNA fragmentation than observed in ACSF controls (Fig. 7). The discovery that some apoptotic cells do not necessarily undergo DNA fragmentation and that cells with DNA fragmentation may become necrotic led to the use of caspase activation as another indicator of apoptosis in addition to TUNEL-stained fragmented DNA. Many endogenous agents formed upon initial insult can cause apoptosis through different pathways, many of which converge at the caspase activation cascade (Sastry and Rao, 2000). Caspases are cysteine proteases that cleave certain proteins, eventually leading to apoptotic cell death. Therefore caspase activation precedes apoptotic DNA fragmentation. In the present study, caspase activation was evaluated immediately after 6 h H₂O₂ exposure. By counting active caspase-3-positive neurons along the cannula tracks post-H₂O₂ or post-ACSF exposure, we demonstrated that H₂O₂ exposure induced significantly more (5.6 times) caspase-3 activation in neurons than observed in ACSF controls (Fig. 8). No caspase-8 activation was observed at this time point. Because caspase-3 is a downstream executioner and caspase-8 is upstream of caspase-3 in the caspase activation cascade (Eldadah and Faden, 2000), it is possible that caspase-8 was activated earlier than the 6 h time point. It was reported that caspase-3 activation follows SCI (Springer et al., 1999; Xu et al., 2005; Impellizzeri et al., 2012; Moon et al., 2012) and that ROS/RNS play roles in different apoptotic pathways (Wu and Bratton, 2013). The present study demonstrated that SCI-elevated H₂O₂ contribute to caspase activation after SCI, suggesting that H₂O₂ plays a role in at least one of apoptotic pathways by activating caspases after SCI.

TEM is a gold-standard approach to determine apoptosis based on specific ultra-structural morphologic changes such as cell surface blebbing, cell shrinkage, apoptotic body formation, nuclear chromatin condensation, cytoplasmic organelle compaction, and nuclear lobation (Wood and Youle, 1994). By using TEM, we previously confirmed that SCI induced neuronal and glial apoptosis (Ling et al., 2013). In the present study, we also confirmed that SCI-elevated H₂O₂ induced neuronal apoptosis by using TEM (Fig. 9). By assessing DNA fragmentation and caspase-3 activation as two indicators of cellular apoptosis and performing TEM confirmation of apoptosis, our data provide the first direct *in vivo* evidence that the SCI-elevated H₂O₂ contributes to the apoptotic cell death after SCI, that apoptosis is a pathway by which SCI-elevated [H₂O₂] can cause cell death, and that caspase activation is a mechanism of apoptosis induced by SCI-elevated H₂O₂. Notably, MnTBAP significantly reduced apoptotic DNA fragmentation and caspase activation, providing further evidence that links H₂O₂-induced intracellular oxidation with apoptosis.

H₂O₂ is an emerging signaling molecule with diverse regulatory functions (Groeger et al., 2009; Veal and Day, 2011) that are largely mediated through reversible protein oxidation on cysteine residues (Enyedi et al., 2013). H₂O₂ production promotes disulfide bond formation, thereby stabilizing protein structures (Margittai et al. 2012; Ruddock, 2012). We believe that whether H₂O₂ acts as a pro-survival or pro-death molecule in pathological conditions depends on its concentration and location of overproduction. The present study demonstrates

for the first time that the SCI-elevated $[H_2O_2]$ contributes to MLP and oxidation of DNA and proteins in neurons, as well as cell death including neuronal apoptosis in secondary SCI. These findings suggest that SCI-elevated H_2O_2 possesses pro-oxidation and pro-cell death functions and that the pro-oxidation function caused irreversible damage to the cellular component, thereby impairing their normal functions and causing cell death.

Numerous studies have shown that SCI significantly increases the oxidation of proteins (Leski et al., 2001; Aksenova et al., 2002; Huang et al., 2007) and DNA (Leski et al., 2001; King et al., 2006; Huang et al., 2007), MLP (Qian and Liu 1997; Springer et al., 1997; Lucas et al., 2002; Christie et al., 2008), cell death, and neuronal and glial apoptosis (Crowe et al., 1997; Liu et al., 1997; Yong et al., 1998; Ling and Liu, 2007; Moon et al., 2012; Ling et al., 2013). Using a contusion SCI model, we previously observed significant increases in MLP, protein oxidation and nitration, cell death and apoptosis in all cell types examined (neurons, motoneurons, astrocytes, and oligodendrocytes) (Ling and Liu, 2007; Valluru et al., 2012; Ling et al., 2013; Liu et al., 2013). Increased ROS production, oxidative damage, cell death, and apoptosis were also reported in different cell types in a compression SCI model (Xu et al., 2005; Nakajima et al., 2010; Chen et al., 2011). However, these *in vivo* studies using various SCI models to investigate different cell types do not provide direct evidence of a causal relationship from ROS elevation, to oxidative indicator overproduction, to cell death following SCI. Most results directly correlating H_2O_2 with oxidative damage and cell death come from cultured cells in which various $[H_2O_2]$ can be easily applied to various cell types in the settings of different insults (Hill et al., 2010; Siriphorn et al., 2010; Sakka et al., 2014; Zhao et al., 2014). However, because cultured cells lack normal extracellular environments, these results do not accurately represent the actual pathological conditions. To fill in the gap of lacking direct *in vivo* evidence, the present study utilized a unique *in vivo* approach to directly address the role of SCI-elevated H_2O_2 in secondary SCI by directly correlating H_2O_2 elevation, oxidative damage, and cell death and neuronal apoptosis. However, this study focused on the roles of H_2O_2 in neurons; further exploration of the action of SCI-elevated H_2O_2 in other cell types is needed.

In the present study, the spinal cord tissue were harvested and processed for histochemical and immunohistochemical stained at 14 h post- H_2O_2 exposure. The selection of this time point for evaluating the role of H_2O_2 in secondary SCI based on the previous reports. Protein carbonyls increase up to 1 month post-SCI (Aksenova et al., 2002). HNE-positive staining increased starting from 3 h to as long as 2 weeks post-SCI (Carrico et al., 2009). HNE-protein adducts increased in the damaged cord as early as 4 h after SCI, reached a peak level at 24 h, and remained significantly elevated up to 7 days after SCI (Luo et al., 2005). Immuno-labeled sections harvested 24 h post-SCI indicated that DNA oxidation was still significantly higher in injured sections than those in controls (King et al., 2006; Huang et al., 2007). Compared to sham controls, we found significantly higher levels of protein oxidation and nitration, and MLP 24 h post-SCI (Hachmeister et al., 2006; Valluru et al., 2012; Liu et al., 2013). Therefore, in the present study, H_2O_2 -induced oxidative damage was measured at 14 h post- H_2O_2 exposure. This was within the time frame of HNE and HNE-protein adducts formation, and elevation of protein and DNA oxidation. Our previously described temporal and spatial profiles of total cell loss and apoptosis indicated that significant neuron loss

(compared with sham control) starting at 1 h, most neurons near the epicenter have already been lost 12 to 24 h post-SCI, and neuronal apoptosis peaked at 12 – 48 h post-SCI (Ling and Liu, 2007; Ling et al., 2013). Therefore, 12-24 h post-SCI is a reasonable time point to examine cell death and apoptosis. The present study examined both oxidative damage and cell death at 14 h post-H₂O₂ exposure using one set of animals.

It was reported that MnTBAP possesses superoxide dismutase and catalase-like activities to convert O₂^{•-} to H₂O₂, and catalyze dissociation of H₂O₂ to water (Day 2004; Day et al., 1997). It also scavenges ONOO⁻ (Crow, 2000) and inhibits MLP (Day et al., 1999). In the central nervous system, cerebroventricular injection of MnTBAP inhibited kainate-induced mitochondrial O₂^{•-} production, DNA oxidation, and hippocampal neuronal loss in rats (Liang et al., 2000). Using a contusion SCI model, we demonstrated that intrathecal MnTBAP administration reduced MLP, decreased protein oxidation and nitration, and increased the number of surviving cells (Hachmeister et al., 2006; Valluru et al., 2012; Liu et al., 2013). Intraperitoneal injection of MnTBAP significantly reduced cell death and improved neurological recovery (Ling and Liu, 2007; Ling et al., 2013; Liu et al., 2013). Collectively, these results demonstrate the beneficial effects of the catalytic antioxidant MnTBAP in treating SCI. Although the mechanisms by which MnTBAP attenuates oxidative damage and cell death are not completely understood, our demonstration that MnTBAP effectively reduced SCI-elevated level of H₂O₂-induced oxidation and cell death indicates that directly scavenges administered H₂O₂, thereby reducing H₂O₂-induced oxidation and cell death, is one of its action to ameliorate secondary SCI.

CONCLUSION

In summary, our results show that 150 μM H₂O₂ is the extracellular [H₂O₂] released after SCI. This [H₂O₂] was then administered into normal rat spinal cord to observe the effects of this ROS without the confounding effects of mechanical injury. We demonstrated that: 1) the SCI-elevated level of H₂O₂ induced protein and DNA oxidation, MLP, and cell death, indicating that H₂O₂ is a likely mediator of posttraumatic oxidation to pro-oxidation, thereby destroying cells in the secondary SCI cascade; 2) the SCI-elevated level of H₂O₂ meets the criterion of a secondary damage agent in that it oxidatively damages major cellular components; 3) apoptosis is a pathway by which an SCI-elevated level of H₂O₂ induces cell death; 4) caspase activation is a mechanism for SCI-elevated H₂O₂ to induce apoptosis; and 5) H₂O₂ scavenging ability is a mechanism by which MnTBAP ameliorates H₂O₂-induced oxidative damage and cell death in our model, although this does not exclude other possible functions.

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Abbreviations

ANOVA	analysis of variance
ACSF	artificial cerebrospinal fluid
2,3 and 2,5-DHBA	2,3- and 2,5-dihydroxybenzoic acid
DNP	2,4-dinitrophenyl
H₂O₂, hydrogen peroxide	[H ₂ O ₂], H ₂ O ₂ concentration
HNE	4-hydroxy-nonenal
HPLC	high-pressure liquid chromatography
i.p.	intraperitoneally
MDA	malondialdehyde
MnTBAP	Mn (III) tetrakis (4-benzoic acid) porphyrin
MLP	membrane lipid peroxidation
•NO	nitric oxide
NSE	neuron-specific enolase
O₂^{•-}	superoxide anion
•OH	hydroxyl radical
8-OHdG	8-hydroxy-2-deoxyguanosine
ONOO⁻	peroxynitrite
PBS	phosphate-buffered saline
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCI	spinal cord injury
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-(dUTP)-biotin nick end labeling

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Highlights-revised

- Establish spinal cord injury (SCI) elevated extracellular H₂O₂ levels.
- SCI-elevated H₂O₂ induces oxidation and cell death in normal rat spinal cord.
- SCI-elevated H₂O₂ induces apoptosis in normal rat spinal cord.
- Caspase activation is a mechanism of SCI-elevated H₂O₂-induced apoptosis.
- MnTBAP attenuates oxidation and cell death by scavenging excess H₂O₂.

Fig. 1.

Establishment of the SCI-elevated extracellular [H₂O₂]. Following anesthesia and laminectomy on vertebra T13 of the rat spinal cord, a microdialysis fiber and a microcannula were inserted together laterally through the gray matter of the cord. A shows the location of microcannula and microdialysis fiber in a cresyl violet-stained cross section of spinal cord. H₂O₂ in ACSF was perfused through the microcannula, diffused into the extracellular space of the cord, sampled through the microdialysis fiber, and measured by HPLC, as described in sections 2 and 3 in Experimental Procedures. Different [H₂O₂] were administered, and a series of time courses were measured from collected dialysates, corresponding to each [H₂O₂] administered. B shows the time course of [H₂O₂] measured from the dialysates (presented as [2,3-DHBA]) in responding to 150 μM H₂O₂ perfused through the cannula. This time course replicates that of post-SCI [H₂O₂] and was subsequently administered through the cannula to test whether it induces oxidation and cell death.

Fig. 2.

H₂O₂-induces and MnTBAP attenuates protein oxidation: The animal experiment procedures were similar to those in Fig 1, except two cannulas were inserted. Then, 150 μ M H₂O₂ in ACSF was perfused through a microcannula into the gray matter of the cord for 10 h to mimic the concentration and duration produced in the extracellular space of the cord by SCI. ACSF (vehicle) or MnTBAP (2.5 mM in ACSF) was perfused in the second cannula in the vehicle- or MnTBAP-treated groups. ACSF was perfused through both cannulas in the ACSF control group. At 14 h post-H₂O₂ or ACSF exposure, the cord was collected and processed. The sections containing the cannula tracks were immuno-labeled with anti-DNP antibody and the sections 2-mm caudal from the tracks were cresyl violet-stained to serve as controls using the methods described in sections 5.1 and 6.1 in Experimental procedures. Upper panel, photomicrographs of anti-DNP antibody immuno-labeled cross sections. A-C: lower magnification; A'-C': higher magnification of A-C. B' shows the higher magnification of the dash box in B. The scale bars indicate 100 μ m. A and A', ACSF-exposed section; B and B', H₂O₂-exposed and vehicle-treated section; C and C', H₂O₂-exposed and MnTBAP-treated section. The arrow heads in A-C indicate the cannula tracks. The DNP-positive neurons and cresyl violet-stained neurons were counted and analyzed and are presented in the lower panel. Clearly, 150 μ M H₂O₂ exposure significantly increased the density % of DNP-positive neurons compared to ACSF control (indicated by *), whereas MnTBAP treatment significantly reduced the density % of H₂O₂-induced DNP-positive neurons (indicated by #) compared with vehicle treatment.

Fig. 3.

H₂O₂ induces and MnTBAP attenuates DNA oxidation. Sections obtained as in Fig. 2 were immuno-labeled with anti-8-OHdG antibody or stained with cresyl violet (2-mm from cannula tracks as control sections). Upper panel, photomicrographs of anti-8-OHdG antibody immuno-labeled cross sections. A-C, lower magnification; A'-C', higher magnification of A-C, Scale bars: 100 μ m. B' shows the higher magnification of the dash box in B. The frame in B' shows a typical 8-OHdG-positive neuron enlarged at high magnification. A and A', ACSF control section; B and B', H₂O₂-exposed with vehicle-treated section; C and C', H₂O₂-exposed with MnTBAP-treated section. The arrow heads in A-C indicate the cannula tracks. The 8-OHdG-positive neurons and cresyl violet-stained neurons were counted and analyzed and are presented in the lower panel. H₂O₂ exposure significantly increased the % of 8-OHdG-positive neurons as indicated by *, whereas MnTBAP treatment significantly reduced the 8-OHdG-positive neurons (#).

Fig. 4.

H₂O₂ induces and MnTBAP attenuates intracellular MLP. Sections obtained as in Fig. 2 were immuno-labeled with anti-HNE antibody or stained with cresyl violet. Upper panel: photomicrographs of anti-HNE antibody immuno-labeled cross sections. A-C, lower magnification; A'-C', higher magnification of A-C, Scale bars indicate 100 μ m. B' shows the higher magnification of the dash box in B. A and A', ACSF control section; B and B', H₂O₂-exposed with vehicle-treated section; C and C', H₂O₂-exposed with MnTBAP-treated section. The hollow areas in center of section A-C are the cannula tracks as indicated by arrow heads in Figs. 2 and 3. Counting the HNE-positive neurons along the cannula tracks and cresyl violet-stained neurons in the 2-mm control sections from the tracks and comparing the density % demonstrated that H₂O₂ significantly increased the densities % of HNE-positive neurons (indicated by *), but this increase was significantly attenuated by MnTBAP (indicated by #).

Fig. 5.

H₂O₂ induces and MnTBAP attenuates extracellular MLP. Two microcannulas were inserted laterally through the gray matter of the cord as described in section 2 in the Experimental Procedures. 150 μ M H₂O₂ was perfused through one cannula and ACSF or MnTBAP (2.5 mM) was perfused through the second cannula in the vehicle- or MnTBAP-treated group. H₂O₂-elevated MDA in the perfusates sampled by the first microcannula was analyzed by HPLC with fluorescence detection. Perfusates collection and analysis were described in section 5.2 in the Experimental Procedures. H₂O₂ administration significantly increased extracellular [MDA] compared to pre-administration level, and MnTBAP significantly attenuated this increase.

Fig. 6.

H₂O₂ induces and MnTBAP attenuates cell loss. Sections obtained as in Fig. 2 were cresyl violet-stained along the cannula tracks and 2-mm caudal from the tracks. Upper panel, the photomicrographs of cresyl violet-stained sections along the cannula tracks. A-D, lower magnification; A'-D', higher magnification of A-D, scale bars = 100 μm. B' shows the higher magnification of the dash box in B. A and A', control section 2-mm from the cannula tracks; B and B', ACSF-control section; C and C', H₂O₂-exposed with vehicle-treated section; D and D', H₂O₂-exposed with MnTBAP-treated section. Lower panel, the quantitative results indicated that H₂O₂ significantly (*) increased the % of cell loss compared with ACSF control, and MnTBAP significantly (#) reduced such loss compared with vehicle-treated sections.

Fig. 7.

H₂O₂ induces and MnTBAP attenuates DNA fragmentation. Sections obtained as in Fig. 2 were stained with TUNEL or double stained with TUNEL + NSE. Upper panel, photomicrographs of TUNEL-stained sections at three different magnifications to show the cannula tracks at lower magnification and TUNEL-positive cells at higher magnification. A-C, lower magnification; A'-C', higher magnification of A-C; A''-C'', higher magnification of A'-C'; A-A'', ACSF-exposed control section; B-B'' H₂O₂-exposed with vehicle-treated section; C-C'' H₂O₂-exposed with MnTBAP-treated section. Middle panel, photomicrographs of TUNEL + NSE double-stained sections at three different magnifications as described for the upper panel. A' shows the higher magnification of the dash box in A. D-F, TUNEL and NSE double-immunofluorescence-stained H₂O₂-exposed section. D, TUNEL fluorescence staining, the arrow heads indicate TUNEL-positive nuclei (green). E, the same section as in D with anti-NSE antibody immuno-fluorescence staining, the arrow heads indicate the same neurons (red) as in D. F, overlapping of D and E (yellow) indicates TUNEL-positive neurons. Scale bars indicate 100 μ m. The quantitative results shown in the lower panel demonstrate that H₂O₂ significantly (*) increased the density % of TUNEL-positive neurons compared with ACSF control and MnTBAP treatment significantly (#) reduced neuronal apoptosis compared with vehicle treatment.

Fig. 8.

H₂O₂ induces and MnTBAP attenuates caspase activation in neurons. Sections obtained as in Fig. 2 were immunohistochemically labeled with anti-active fragments of caspase-3 and caspase-8 antibodies. The upper panel shows photomicrographs of immuno-labeled sections with anti-active caspase-3 (A-C) and caspase-8 (D) fragment antibodies. A-D, lower magnification; A'-D', higher magnification of A-D, scale bars indicate 100 μ m. B' shows the higher magnification of the dash box in B. A-A' ACSF-exposed control section; B-B', H₂O₂-exposed with vehicle-treated section, C-C', H₂O₂-exposed with MnTBAP-treated section, D and D', active caspase-8-immuno-labeled H₂O₂-exposed section. The quantitative results (lower panel) reveal that H₂O₂ significantly (*) increased the density % of active caspase-3-positive neurons compared with ACSF control, and MnTBAP treatment significantly (#) reduced the H₂O₂-induced increase compared with vehicle-treated sections.

Fig. 9.

TEM confirmation of neuronal apoptosis. H_2O_2 was perfused as in Figs. 2-8, and the cord was processed for TEM as described in section 6.4 of the Experimental Procedures. A, a normal neuron from a sham control (magnification: $\times 14,300$); B, a neuron exposed to ACSF ($\times 14,300$); C-F, different stages of apoptotic neurons in H_2O_2 -exposed sections (C, $\times 57,750$, D, $\times 14,300$, E, $\times 42,625$ and F, $\times 31,625$). H_2O_2 exposure induced specific morphological features indicative of neuronal apoptosis. No apoptotic neurons were found in sham or ACSF control sections. Symbols: N, nucleus; M, mitochondria; R, rough endoplasmic reticulum; G, Golgi complex; Ch, chromatin.