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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 ($[H_2O_2]$) significantly increases after spinal cord injury (SCI). The present study explored (1) whether SCI-elevated $[H_2O_2]$ is sufficient to induce oxidation and cell death, (2) if apoptosis is a pathway of H_2O_2-induced cell death, and (3) whether H_2O_2-induced oxidation and cell death could be reversed by treatment with the catalytic antioxidant Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP). H_2O_2 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 H_2O_2-, or artificial cerebrospinal fluid (ACSF)-exposed with vehicle-, or MnTBAP-treated groups. Compared with ACSF-exposed animals, SCI-elevated $[H_2O_2]$ significantly increased intracellular protein and DNA oxidation by threefold and MLP by eightfold in neurons, respectively. H_2O_2-elevated extracellular malondialdehyde was measured by microdialysis sampling. We demonstrated that SCI-elevated $[H_2O_2]$ significantly increased extracellular malondialdehyde above pre-injury levels. H_2O_2 also significantly increased cell loss and the numbers of terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-(dUTP)-biotin nick end labeling (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 $[H_2O_2]$ contributes to post-SCI MLP, protein, and DNA oxidation to induce cell death. Therefore, we conclude that (1) the role of H_2O_2 in secondary SCI is pro-oxidation and pro-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.

Key words: 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 and Cuzzocrea, 2008; Hall, 2011). ROS include free radicals such as superoxide anions ($O_2^{\cdot-}$) and hydroxyl radicals ($^{\cdot}OH$) and non-radical oxidants such as hydrogen peroxide (H_2O_2). RNS include free radicals like nitric oxide ($^{\cdot}NO$) and non-radical oxidants such as peroxynitrite ($ONOO^-$). $O_2^{\cdot-}$ is produced through several aerobic pathways during normal metabolism, and superoxide dismutase converts $O_2^{\cdot-}$ into H_2O_2, which is reduced to H_2O by catalase, glutathione peroxidase, and thioredoxin/peroxiredoxin. There is a dynamic equilibrium between the

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Abbreviations: 2,3 and 2,5-DHBA, 2,3- and 2,5-dihydroxybenzoic acid; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; DNP, 2,4-dinitrophenyl; H_2O_2, hydrogen peroxide; $[H_2O_2]$, hydrogen peroxide concentration; HNE, 4-hydroxy-nonenal; HPLC, high-pressure liquid chromatography; i.p., intraperitoneally; MDA, malondialdehyde; MLP, membrane lipid peroxidation; MnTBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin; $^{\cdot}NO$, nitric oxide; NSE, neuron-specific enolase; $O_2^{\cdot-}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; $ONOO^-$, 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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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 $O_2^{\cdot-}$ and H_2O_2, which can produce ·OH via the metal-catalyzed Haber–Weiss/Fenton reaction through the sequence $O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow {}^{\cdot}OH$ (Halliwell, 2006). Elevated $O_2^{\cdot-}$ also rapidly reacts with ·NO to form $ONOO^-$ – a highly reactive oxidant. $ONOO^-$ can decompose to ·OH when protonated by the reaction $O_2^{\cdot-} + {}^{\cdot}NO \rightarrow ONOO^- + (H^+) \rightarrow ONOOH \rightarrow {}^{\cdot}OH$ (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 derivates, 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 $O_2^{\cdot-}$ (Liu et al., 1998), H_2O_2 (Liu et al., 1999a), ·OH (Liu et al., 2004), ·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 ·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 the rat spinal cord reduced $ONOO^-$-induced protein oxidation and nitration (Bao et al., 2003), and MLP (Liu et al., 2005), and ·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 ·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 ·OH.

The oxidant capacity of H_2O_2 is limited in comparison to highly oxidizing species such as $ONOO^-$ and ·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 have been described by others (Chiarugi, 2009; Groeger et al., 2009). Although a less potent oxidant than $ONOO^-$ or ·OH, H_2O_2 is more stable, contributing a longer period of elevated levels after SCI than $ONOO^-$ or ·OH (Liu et al., 1999a,, 2000, 2004). 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., 1999a), in the present study, we perfused H_2O_2 to replicate the concentrations and durations elevated 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 H_2O_2-scavenging ability of MnTBAP. Our results confirm that the levels/durations of H_2O_2 observed after SCI are sufficient to induce oxidative damage and cell death, suggesting that H_2O_2 contributes to secondary damage after SCI. The ability of MnTBAP to attenuate H_2O_2-induced oxidation and cell death further verify the H_2O_2-scavenging ability of MnTBAP *in vivo.*

EXPERIMENTAL PROCEDURES

Male Sprague–Dawley rats (250–300 g) 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.