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Age increases reactive oxygen species production in macrophages and potentiates oxidative damage after spinal cord injury

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Abstract

Age potentiates neurodegeneration and impairs recovery from spinal cord injury (SCI). Previously, we observed that age alters the balance of destructive (M1) and protective (M2) macrophages, however, the age-related pathophysiology in SCI is poorly understood. NADPH oxidase (NOX) contributes to reactive oxygen species (ROS)-mediated damage and macrophage activation in neurotrauma. Further, NOX/ROS increase with CNS age. Here, we found significantly higher ROS generation in 14 vs. 4-month-old (MO) mice after contusion SCI. Notably, NOX2 increased in 14 MO ROS-producing macrophages suggesting that macrophages and NOX contribute to SCI oxidative stress. Indicators of lipid peroxidation, a downstream cytotoxic effect of ROS accumulation, were significantly higher in 14 vs. 4 MO SCI mice. We also detected a higher percentage of ROS-producing M2 (Arginase-1-positive) macrophages in 14 vs. 4 MO mice, a previously unreported SCI phenotype, and increased M1 (CD16/32-positive) macrophages with age. Thus, NOX and ROS are age-related mediators of SCI pathophysiology and normally protective M2 macrophages may potentiate secondary injury through ROS generation in the aged injured spinal cord.

Keywords

Aging; Arginase-1; Microglia; Macrophage polarization; Dihydroethidium; gp91^{phox}

1. Introduction

The average age at the time of spinal cord injury (SCI) has steadily increased since the mid-1970s. According to National Spinal Cord Injury Statistical Center (NSCISC), the average age at the time of SCI has shifted from 29 years old, in the 1970's, to the current age of 42 years (NSCISC, 2013). Elderly people have a substantially higher mortality rate than

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younger patients during the first year after SCI (Furlan and Fehlings, 2009). In addition, older subjects with SCI have less ability to translate a neurological improvement into daily functional recovery than younger individuals (Jakob et al., 2009). We, and others, have observed similar results in rodent SCI models; middle-aged animals have increased tissue pathology and worse functional recovery after SCI compared to young controls (Fenn et al., 2014; Genovese et al., 2006; Hooshmand et al., 2014; Siegenthaler et al., 2008a; 2008b; Zhang et al., 2015a). Despite these observations, little is known about the mechanisms involved in age-related pathology following traumatic SCI.

SCI triggers reactive oxygen species (ROS) production including hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl ($\text{OH}^\bullet$) radicals. Significant decreases in antioxidant levels and increases in biomarkers of oxidative stress are detectable in plasma and urine samples from patients at 1, 3, and 12 months post-SCI (Bastani et al., 2012). ROS have important pathophysiological effects on both acute and chronic SCI (Bains and Hall, 2012; Bastani et al., 2012; Carrico et al., 2009; Ordonez et al., 2013; Xiong et al., 2007). Increased ROS formation overwhelms antioxidant defenses and causes oxidative damage (e.g. lipid peroxidation, protein nitration) thereby propagating tissue loss subsequent to the primary mechanical SCI (Hall, 2011).

SCI triggers ROS production in activated macrophages and microglia (Fleming et al., 2006). Macrophage ROS production is facilitated through upregulation of NOX2, one of seven members of the NOX (Nicotinamide adenine dinucleotide phosphate oxidase) enzyme family. NOX is a multi-subunit enzyme that transfers electrons across membranes and generates superoxide (Brandes et al., 2014). Activation of NOX2 requires translocation of cytosolic components to the cell membrane, including p47^{phox} , p67^{phox} and the small GTP binding protein, Rac; these are then assembled to the transmembrane components $\text{gp91}^{\text{phox}}$ and p22^{phox} (Sareila et al., 2011). In response to CNS trauma, the catalytic component of NOX2, also known as $\text{gp91}^{\text{phox}}$, increases in macrophage/microglia (Cooney et al., 2013; Kumar et al., 2012). In addition, increases in NOX2 expression, ROS generation, and microglia activation in the brain are age-related following systemic LPS challenge and contribute to chronic neurodegeneration (Qin et al., 2013). However, the effect of age on NOX2 activation, ROS formation, and macrophage activation in response to SCI is unclear.

Recently, ROS and NOX have been implicated in the modulation of macrophage/microglia activation. For example, increased superoxide production blocks anti-inflammatory IL-4 from decreasing LPS-induced pro-inflammatory cytokines (Ferber et al., 2010). In contrast, pharmacological inhibition NOX2 or genetic deletion of $\text{gp91}^{\text{phox}}$ or p47^{phox} decreases pro-inflammatory cytokine expression and increases anti-inflammatory mediators in response to LPS treatment (Choi et al., 2012; Pawate et al., 2004; Qin et al., 2005). Depending on their phenotype and activation status, macrophages may initiate secondary injury mechanisms and/or promote regeneration and repair in SCI. Pro-inflammatory, “M1 macrophages” are neurotoxic, release proteases and pro-inflammatory molecules, and cause axon retraction; whereas anti-inflammatory, “M2” macrophages, are non-neurotoxic, release anti-inflammatory cytokines, and promote axon regeneration (Horn et al., 2008; Kigerl et al., 2009; Kroner et al., 2014). Age plays a key role in how macrophage/microglia respond to stimuli (Damani et al., 2010; Mahub et al., 2012) and we recently reported that age skews

SCI macrophage activation toward a pro-inflammatory, M1-status (Fenn et al., 2014; Zhang et al., 2015a).

In the current study, we hypothesize that age-related activation of NOX2 in macrophage/microglia contributes to enhanced ROS production and oxidative damage in SCI. Additionally we investigate how ROS contributes to SCI macrophage activation states. Age is a key regulator of macrophage function. Understanding the differences in the inflammatory response and oxidative stress after SCI is important to determine how age at time of injury affects endogenous repair processes, pathology, and clinical therapies.

2. Materials and Methods

2.1. Animals

C57BL/6 mice (female, 4 and 14 months of age) were obtained from National Institute of Aging to model young (~18 years old) and middle-age (~45 years old) humans respectively (Quinn, 2005). These ages represent the demographic shift in the SCI population (DeVivo and Chen, 2011). Animals were housed in IVC cages with ad libitum access to food and water. A total of 62 mice received SCI in the current study. One mouse died after SCI due to anesthesia complication. All experiments were performed in accordance with the guidelines of the Office of Responsible Research Practices and with approval of the Institutional Animal Care and Use Committees at the University of Kentucky.

2.2. Spinal Cord Injury

Animals were anesthetized via intraperitoneal (i.p.) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). After a T9 laminectomy, mice received a mild to moderate mid-thoracic contusion SCI using the Infinite Horizons injury device (50 kdyn displacement; Precision Systems and Instrumentation) (Scheff et al., 2003). The skin incision was then closed using monofilament suture after injury. Animals were allowed to recover from the surgery in warmed housing unit (cage on ~37 °C warm pad) overnight before returning to home cages. Post surgically, mice were immediately given one subcutaneous injection of buprenorphine-SR (1 mg/kg) and antibiotic (5 mg/kg, Enroloxacin 2.27%: Norbook Inc, Lenexa, KS) dissolved in 2 ml of saline and continued to receive antibiotic subcutaneously in 1 ml saline for 5 days. Manual bladder expression was performed on injured mice twice daily or until autonomic bladder expression returned.

2.3. Tissue processing and immunohistochemistry

At 3, 7 or 14 days post-SCI, mice were injected (i.p.) with dihydroethidium (DHE, ThermoFisher Scientific; Cat# D-1168) at 0.01mg/g body weight. 4 hours after injection, animals were anesthetized by i.p. injection of ketamine (120 mg/kg) and xylazine (10 mg/kg) and then sacrificed by transcardial perfusion with PBS and fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS. Spinal cords were dissected and post-fixed for 2 h in 4% PFA and then rinsed and stored in phosphate buffer (0.2 M, pH 7.4) overnight at 4 °C. Tissues were then cryoprotected by immersion in 30% sucrose for 3-4 days at 4 °C. Spinal cord tissue (8 mm in length, 4 mm rostral and 4 mm caudal from the lesion) blocks were rapidly frozen in optimal cutting temperature compound (OCT, Sakura Finetek USA, Inc.)

on dry ice and stored at -20°C prior to sectioning. The spinal cords from the different experimental groups were randomly distributed (by experimenters blinded to group inclusion) in each tissue block to ensure that equal numbers of 4 and 14 MO samples were present on every slide. Transverse serial sections ($10\text{ }\mu\text{m}$) were cut through each block, mounted on coated slides, and then stored at -80°C before staining.

The details of primary and secondary antibodies used in this study are listed in Table 1. Spinal cord sections were warmed for 1 h at 37°C and rinsed with 0.1M PBS. Then, slides were incubated in blocking buffer (0.1 M PBS containing 1% bovine serum albumin (BSA, Fisher Scientific, Cat# BP1605), 0.1% Triton X-100 (Sigma-aldrich, Cat# X-100), 0.1% fish gelatin (Sigma-aldrich, Cat# G7765), and 5% normal goat or donkey serum (Sigma-aldrich, Cat# G9203; D9663) at room temperature for 1 h, followed by incubation in blocking buffer containing primary antibodies overnight at 4°C . On the second day, slides were rinsed in 0.1M PBS and then incubated with secondary antibodies at room temperature for 1 h. After the last rinse, all the slides were coverslipped with Immu-Mount (ThermoFisher Scientific). Antibody specificity was confirmed using non-primary controls (for example, see Supplementary Fig. 1). All the fluorescent images were taken using a C2+ laser scanning confocal microscope (Nikon Instruments Inc, Melville, NY).

2.4. Tissue analysis

Investigators blind to experimental groups performed all data acquisition and tissue analysis. The lesion epicenter for each animal was identified as the tissue section with the least amount of axon and myelin staining on cross-sections double-stained with Eriochrome cyanine/neurofilament (EC/NF) as described previously (Zhang et al., 2015a; 2015b).

The production of superoxide *in vivo* was detected by injecting mice with dihydroethidium (DHE) 4 h before sacrificing as described above. DHE is able to freely permeate cell membranes and sensitive to superoxide, which oxidizes DHE to ethidium bromide (Kim et al., 2010). Ethidium bromide then intercalates with the DNA in the nucleus and emits a bright red fluorescence that can be detected at 570 nm (Aoyama et al., 2008; Nazarewicz et al., 2013). Two mice (one 4 MO and one 14 MO) were excluded from 14 dpi ox-DHE and 4-HNE quantification because of lack of sufficient tissue on the slides after staining. The proportion of oxidized-DHE (ox-DHE) signals or positive 4-HNE staining was quantified using threshold-based measurements to identify positive fluorescent signals above background within the lesion area with the MetaMorph analysis program (Molecular Devices, Sunnyvale, CA). The MetaMorph colocalization plugin was applied to analyze the colocalization of DHE signals with cellular markers, including microglia/macrophages (TomL), neurons (NeuN), and astrocytes (GFAP); the colocalization of DHE and NOX2 (gp91^{phox}) immunoreactivity; and the colocalization of DHE and macrophage phenotype markers Arg-1 and CD16/32. Lesion areas were identified based upon adjacent EC/NF stained sections and TomL immunoreactivity and the proportion of the sampled area above threshold or double-positive (colocalization) were determined using three adjacent sections centered on the lesion epicenter for each animal. Individual measures for each animal are the result of averaging the values across these three adjacent sections.

2.5. Western blotting and ELISA

At 3 or 7 dpi, mice were sacrificed by an overdose i.p. injection of ketamine (120 mg/kg) and xylazine (10 mg/kg) and then transcardially perfused with 0.1M PBS. The spinal cords (8 mm in length centered at lesion epicenter) were then rapidly dissected. Spinal cord tissue was sonicated in 400 μ L Triton lysis buffer (1.0% Triton, 20.0 mM Tris HCL, 150.0 mM NaCl, 5.0 mM EGTA, 10.0 mM EDTA, and 10.0% glycerol) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics, Indianapolis, IN, USA) and then centrifuged for 15 minutes at 13,000 rpm at 4°C. The supernatant was collected and the protein concentration was measured using a BCA Protein Assay (Pierce; Rockford, IL, USA).

For Western blotting, protein samples (50 μ g per sample) were separated on SDS-PAGE precast gels (Bio-Rad Laboratories, Hercules, CA) using XT-MES running buffer (Bio-Rad), and then blotted on nitrocellulose membranes (Bio-Rad) using a semi-dry electro-transferring system at constant voltage (15 volts) for 1 hour at room temperature. After transferring, nitrocellulose membranes were blocked with 5% fat-free milk/TBS blocking buffer for 1 hour and then incubated with primary antibodies (Table 1) overnight at 4°C in blocking buffer containing 0.5% Tween-20 (TBS-T). On the following day, membranes were washed in TBS-T, incubated with secondary antibodies (Table 1), and imaged using Odyssey Infra Red Imaging System (Li-COR Biosciences, Lincoln, NE, USA). Band immunoreactivity was quantified using ImageJ software. 4-HNE signals in each lane were normalized to corresponding GAPDH signals.

Quantification of 4-HNE protein adducts was performed using the OxiSelect HNE Adduct Competitive ELISA Kit (Cell Biolabs, San Diego, CA). Dilution series of 4-HNE-BSA standards were prepared in the concentration range of 0 to 100 μ g/mL according to manufacture instructions. Standards and protein samples (200 μ g per sample) were loaded into individual 4-HNE conjugate coated wells. After incubation for 10 minutes at room temperature, the diluted anti-4-HNE antibody was added to each well and incubated at room temperature for 1 hour on an orbital shaker, followed by three times washing with wash buffer. Then, the diluted secondary antibody-HRP conjugate was added into wells and incubated at room temperature for 1 hour with shaking. Each well was washed three times following secondary antibody incubation, and incubated with substrate solution for 15 minutes. The reaction was stopped by the stop solution, and the absorbance was measured immediately at 450 nm using Epoch microplate spectrophotometer (BioTek, Winooski, VT).

2.6. Statistical analysis

Investigators blinded to group inclusion performed data analyses. Data were analyzed using unpaired t-test or Mann-Whitney test as appropriate to compare differences between 4 and 14 MO mice and two-way ANOVA followed by Bonferroni's test were used for multiple comparisons. Results were considered statistically significant at $p < 0.05$. Statistical analyses and quantification graphs were generated using GraphPad Prism 6.0 (GraphPad Software). All results are presented as mean $\pm$ SEM.

3. Results

3.1. Age increases ROS production and enhances oxidative damage in the injured spinal cord

Previously we reported increased tissue damage after SCI in 14 vs. 4 MO mice (Zhang et al., 2015a). Since oxidative stress plays a vital role in the evolution of secondary damage in SCI (Bains and Hall, 2012; Xiong et al., 2007), the purpose of the current study was to determine if oxidative stress is a contributing factor to age-related differences in SCI pathology. First we investigated the temporal profile of ROS production in injured spinal cords from 14 and 4 MO SCI mice. By tracking the signals of oxidized dihydroethidine (ox-DHE), a marker for intracellular superoxide, we identified that ROS production in the injured spinal cord is significantly higher in 14 MO vs. 4 MO mice at 3 ($p=0.04$) and 7 dpi ($p=0.03$) (Fig. 1). Levels were still higher in 14 MO at 14 dpi, but this difference was not statistically significant ($p=0.2$) (Fig. 1).

Superoxide can further give rise to the formation of hydrogen peroxide, a cell membrane permeable molecule that has cytotoxic effects and also leads to other free radical generation (e.g. hydroxyl radical) (Jia et al., 2012). Overproduction of ROS may result in lipid peroxidation which is a hallmark of oxidative damage (Braugher and Hall, 1992). Lipid peroxidation can be assessed by quantifying the end products, such as 4-hydroxynonenal (4-HNE), an aldehyde that can attack amino groups on proteins and compromise protein structure and/or function (Devasagayam et al., 2003). After SCI, 4-HNE is detectable acutely at the lesion epicenter and surrounding gray and white matter and is increased up to 2 weeks post-SCI (Carrico et al., 2009). We used spinal cord homogenates to examine overall 4-HNE production using immunoblotting and ELISA and observed significantly increased lipid peroxidation after 14 vs. 4 MO SCI at 7 dpi (Fig. 2A-B). This delayed expression is consistent with the downstream effects of acute and maintained ROS production. Next, we examined the distribution of lipid peroxidation in the lesion epicenter using immunohistochemistry. There were no differences in 4-HNE staining at 3 and 14 dpi between age groups ($p=1.0$ and 0.5 , respectively, Fig. 2C-C' & 2E-E'). However, 4-HNE staining was significantly increased in the injured spinal cord from 14 vs. 4 MO mice at 7dpi ($p<0.05$, Fig. 2D-D'), consistent with our immunoblotting and ELISA results. Thus, constitutive higher ROS generation in older injured spinal cords is associated with increased indices of lipid peroxidation and may contribute to the previously observed increases in tissue loss and decreases in motor recovery in 14 MO SCI animals (Zhang et al., 2015a).

3.2. Macrophage/microglia are the main source of ROS acutely after SCI

Increased carbonylation of protein, an oxidative stress marker, is detectable in rat spinal cord homogenates 24 hours post contusion injury (Cooney et al., 2014). In addition, superoxide generation occurs in neurons 1 hour after incomplete SCI (Aoyama et al., 2008). The time course of ROS production in other cell types in the injured spinal cord, however, has not been fully characterized. To identify ROS-producing cells we double-labeled with cell type specific markers: macrophage/microglia (tomato lectin, TomL), neurons (NeuN), and astrocytes (GFAP) and ox-DHE. As shown in Figure 3 and Supplementary Figure 2, oxidized DHE, indicative of ROS production, was mainly detectable in macrophage/

microglia at 3 and 7 days after injury. We observed no difference in the cellular distribution of DHE as a function of age (data not shown). At 3 dpi, in both age groups, macrophage/microglia were responsible for ~80% of ROS production (Fig. 3A-B, G). By 7 dpi, the percentage of ROS produced by astrocytes (GFAP+cells) increased; however, macrophage/microglia are still the major cells (~50%) that contribute to the ROS production in the injured spinal cord tissue (Fig. 3H).

3.3. Age upregulates NOX2 activation in macrophage/microglia following SCI

Various enzyme systems, such as inducible nitric oxide synthase and cyclooxygenase-2 (COX2) are involved in SCI-triggered ROS production and these systems may vary with age (David and Kroner, 2011; Genovese et al., 2006; Trivedi et al., 2006). Thus, we examined the expression of NOX2, iNOS and COX2 in injured spinal cords of 4 MO and 14 MO mice using whole cord homogenates. Among all the enzymes, only the expression of NOX2 was elevated from 3 to 7 dpi (Supplementary Fig. 3A-C). Although there was no significant difference in NOX2 gene expression or protein level due to age (Supplementary Fig. 3A&D-D'), NOX2 is the main superoxide-generating enzyme found in macrophages and has been detected in microglia/macrophages following rat contusion SCI (Cooney et al., 2014). To further investigate whether age alters macrophage-specific NOX2 expression and ROS production, we then performed triple-labeling with TomL, DHE, and the anti-gp91^{phox} antibody, which identifies the membrane component of NOX2 in cells. Gp91^{phox} is the membrane component of NOX2 and staining alone does not necessarily indicate enzyme activity; therefore we used gp91^{phox} co-labeling with ox-DHE to identify NOX2 activation. As shown in Figure 4I, ox-DHE and gp91^{phox} double labeling was significantly higher in 14 MO than 4 MO mice and interestingly, NOX2 immunoreactivity was almost exclusively observed in ROS-producing macrophages, especially in 14 MO SCI mice (see arrows in Fig. 4 of NOX2 and DHE in TomL-positive cells). This indicates that during the acute phase of SCI, NOX2 contributes to cellular ROS generation in macrophage/microglia at the lesion epicenter and its activity is increased with age at the time of SCI.

3.4. Age potentiates ROS production from M2 SCI macrophages

We previously observed that age decreases potentially reparative M2 SCI macrophage activation (Fenn et al., 2014; Zhang et al., 2015a). We next examined whether the disproportionate macrophage activation of NOX2 in 14MO animals may be contributing to the different macrophage polarization states. Specifically, we examined DHE-colocalization with markers of M2 (Arginase-1, ARG-1) and M1 (CD16/32) macrophages in 14 and 4 MO animals after SCI. As reported previously, both M1 and M2 macrophages are prevalent in the lesion during the first week after SCI (Supplementary Fig. 4B-C) (Kigerl et al., 2009). There was no significant difference in M2 activation between 4 and 14 MO mice at 3 or 7 dpi (indicated by ARG-1 positively stained cells, Fig. 5A; D vs. G; J vs. M). Interestingly, the proportion of ARG-1-positive macrophages that double-stained with ox-DHE was significantly higher in 14 vs. 4 MO SCI mice at 3 and 7 dpi (Fig. 5 B-C; F vs. I; L vs. O; Supplementary Fig. 4A). Although there was a three-fold increase in CD16/32+ M1 macrophages in 14 vs. 4 MO SCI mice at 3 dpi (proportional area=1.5±0.3% vs. 0.5±0.2%, respectively, p<0.05), there was no significant age difference in the percentage of CD16/32 + ox-DHE double-positive macrophages at either 3 (11.8±2.6 vs. 7.3±1.9, p=0.11) or 7 dpi

(2.4 ± 0.3 vs. 1.7 ± 0.4 , $p=0.41$) (14 and 4 MO SCI animals respectively, $n=4-5$). These data suggest that age plays an important role in macrophage phenotype alteration following SCI by enhancing M1 polarization and potentiating the contribution of M2 cells to ROS generation.

4. Discussion

The central nervous system (CNS) is specifically vulnerable to oxidative stress and reactive oxygen species (ROS) are postulated to be a major factor in age-related deterioration in neuronal function (Finkel and Holbrook, 2000). Although ROS-mediated oxidative damage following CNS injury is a widely studied secondary injury mechanism, here we report the effects of age on the cellular/subcellular sources of ROS generation and temporal induction of oxidative damage following SCI. Using dihydroethyidium (DHE), a sensitive dye that allows in situ superoxide detection in live cells, we found that superoxide production was significantly increased in the spinal cord from animals injured at 14 vs. 4 months of age. Our data also demonstrate that macrophage/microglia and NOX are cellular and subcellular sources of ROS production following SCI. Importantly, enhanced NOX2 activation contributes to age-related oxidative stress in the injured spinal cord.

In addition to various early events that contribute to superoxide production after traumatic SCI, such as the arachidonic acid cascade, xanthine oxidase activity and “mitochondrial leak” (Hall, 2011), activated microglia and infiltrating macrophages are major sources of superoxide following SCI (David and Kroner, 2011; Hall, 2011; Kim et al., 2010). In the current study, we observed that at 3 and 7 dpi, during the onset and peak of SCI macrophage infiltration (Kigerl et al., 2009), the majority of superoxide is produced by activated macrophage/microglia. Following SCI, macrophage/microglia exhibit marked increases in oxygen consumption and generation of superoxide from membranes-associated NADPH oxidase (Jia et al., 2012). Through co-labeling of ox-DHE and gp91^{phox} we observed a significant increase in NOX2 activation in macrophage/microglia at the lesion epicenter of 14 MO vs. 4 MO SCI mice. Low levels of gp91^{phox} expression have also been shown in neurons and astrocytes following traumatic brain injury (TBI) and SCI (Cooney et al., 2014; Dohi et al., 2010), and we confirmed expression of gp91^{phox} in these cells is unaffected by age. We also observed that other unidentified cells also account for ~20% ROS production at 7 dpi. These unidentified cells may be oligodendrocytes, as activation of NOX has been detected in oligodendrocytes after SCI and inhibition of NOX activity attenuates excitotoxicity of oligodendrocytes (Johnstone et al., 2013). However, the most prominent gp91^{phox} co-localization occurred in macrophage/microglia (TomL-positive cells) at 3 and 7 dpi.

Increased NOX2 (gp91^{phox} expression) in activated macrophage/microglia has been detected as early as 24 h following SCI. This elevation peaks at 7 dpi and maintained up to 28 dpi (Cooney et al., 2014). Moreover, chronically expressed NOX2 in highly activated microglia has been observed 1 year after TBI (Loane et al., 2014). In TBI, age is associated with upregulation of NOX subunits and enhanced microglia activation and tissue damage (Kumar et al., 2012). To our knowledge, the current report is the first of enhanced NOX2 activation in middle-aged vs. adult mice after mild-to-moderate SCI. NOX2 activation in macrophage/

microglia give rise to extracellular ROS buildup which is toxic to neighboring cells, especially neurons (Angeloni et al., 2015). Additionally, NOX2 contributes to intracellular ROS production in activated macrophage/microglia (Bylund et al., 2010). It has been suggested that NOX2-mediated ROS production is a key driver of self-propagating cycles of microglial-mediated neurodegeneration since NOX2 activation induces changes in microglia morphology and proinflammatory gene expression (Qin et al., 2013). Given the dual effect of NOX2 on neurotoxicity and macrophage/microglia activation, early-enhanced activation of NOX2 in macrophage/microglia with age may be a causative factor that contributes to oxidative damage, neuronal cell death, and impaired functional recovery in response to SCI.

Another novel observation in the current study is the increased proportion of ROS-producing Arginase-1 (Arg-1)-positive macrophages in 14 MO vs. 4 MO SCI animals. While indicators of oxidative stress have been reported on M2-like (Ly6C^{lo}) SCI macrophages (Donnelly et al., 2011), Arg-1 is typically used as a phenotypic marker of M2 macrophage activation. Following SCI, Arg-1 expression in the lesion epicenter peaks at 7 dpi and is then down-regulated (Kigerl et al., 2009). We previously reported that Arg-1 immunoreactivity decreases with age after SCI using the BALB/c mouse strain (Fenn et al., 2014). In the current study, the overall protein level of Arg-1 at the lesion epicenter was not significant between 4 and 14 MO C57BL/6 mice at 3 or 7 dpi. This discrepancy is likely due to strain differences, however, in the current study, we found that the percentage of Arg-1-positive macrophages co-labeling with ox-DHE was higher in 14 MO mice. ROS production is a key effector of cytotoxic microglia (Banati et al., 1993) and is involved in the activation of M1 macrophages (Brüne et al., 2013). Moreover, NOX-derived superoxide is closely associated with M1 macrophage activation, inhibition of NOX activity reduces M1 polarization and oxidative stress, and NOX inhibition enhances M2 macrophage activation (Khayrullina et al., 2015; Padgett et al., 2015). Shifting macrophages from an M1 to M2 response using cyclic AMP and IL-4 is accompanied by reduced oxidative stress (Ghosh et al., 2016) and ROS inhibition disproportionately affects M2 vs. M1 polarization *in vitro* (Zhang et al., 2013). These reports suggest that NOX activation and subsequent ROS production may be key effectors that regulate the dynamic equilibrium of M1 vs. M2 macrophage activation states. Thus, age-related ROS production in Arg-1-positive cells might shift macrophage polarization toward a pro-inflammatory, M1 status in SCI. We have observed these age-related alterations in M2 induction and polarization across different animal strains and across different M2 macrophage phenotypes (Fenn et al., 2014; Zhang et al., 2015a). Collectively, these results suggest that ROS may be a key effector that regulates the imbalanced macrophage responses in the aged microenvironment after SCI.

SCI-triggered ROS accumulation results in the oxidative degradation of DNA, proteins, and lipids (Cui et al., 2004). Accordingly, our data demonstrated that overproduction of ROS in 14 MO SCI mice is associated with exacerbated 4-HNE production, a hallmark of lipid peroxidation. It is possible that in addition to free radicals, ROS-induced products, such as oxidized lipids, also participate in the modulation of the macrophage response. For example, it has been shown that alternatively activated M2 macrophages rapidly accumulate oxidized LDL, which simultaneously reduces the expression of the anti-inflammatory transcription factor, kruppel-like factor 2 and shifts M2 macrophages toward a pro-inflammatory profile (van Tits et al., 2011). Although the role of oxidized lipids in SCI macrophage activation has

not been fully investigated, ROS buildup in Arg-1-positive cells may drive the phenotypic conversion of M2 to M1 macrophages through lipid oxidation. To facilitate age-related optimization of anti-inflammatory and/or antioxidant SCI treatments, further studies are needed to determine the consequences of having more ROS producing Arg-1 macrophages in the aged injured spinal cord. In addition, although a recent publication reports that Arg-1 is exclusively expressed in infiltrating macrophages, not microglia, after SCI in young mice (Greenhalgh et al., 2016); investigations into the sources of M1 vs. M2 macrophages in older mice may give further insights in age-related neuroinflammation.

In conclusion, we demonstrated that age enhances oxidative damage through upregulation of NOX2 levels after SCI. In addition, age-related ROS production in macrophage/microglia may shift the dynamic equilibrium of macrophage activation toward a proinflammatory state further contributing to age-related SCI pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(4-HNE)	4-hydroxynonenal
(ARG-1)	Arginase-1
(DPI)	Days post injury
(DHE)	Dihydroethidium
(NOX)	Nicotinamide adenine dinucleotide phosphate oxidase
(ROS)	Reactive oxygen species
(SCI)	Spinal cord injury
(TBI)	Traumatic Brain Injury

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Highlights

- Macrophages and NOX are major sources of ROS generation in the acute phase of SCI.
- Age increases oxidative stress through upregulation of NOX2 levels following SCI.
- Age plays an important role in macrophage phenotypic changes after SCI.

Fig. 1. Reactive oxygen species (ROS) production is higher after 14 vs. 4 MO SCI

Representative images of spinal cord sections at the lesion epicenter stained with the superoxide-sensitive dye DHE from 4 and 14 MO (month old) mice at 3 (A-B), 7 (C-D), and 14 (E-F) days post injury (dpi). Quantification of oxidized DHE fluorescence labeling (red) reveals significantly higher ROS production in 14 vs. 4 MO injured spinal cords at 3 (G) and 7 (H) dpi. Scale bar= 100 μ m. Results are mean $\pm$ SEM, n=4-5/group. * $p<0.05$.

Fig. 2. Lipid peroxidation is increased after 14 vs. 4 MO SCI

Representative images and densitometry quantification of immunoblotting of 4-HNE (A-A'). ELISA quantification of 4-HNE adducts in injured spinal cords of 4 and 14 MO mice (B). Cross sections at the lesion epicenter were labeled with anti-4-HNE antibody. Representative images and quantification of 4-HNE immunoreactivity of 4 MO and 14 MO at 3 (C-C''), 7 (D-D'') and 14 (E-E'') dpi. 4-HNE immunoreactivity significantly increased in lesion epicenter of 14 vs. 4 MO SCI mice at 7 dpi. Results are mean $\pm$ SEM, n=4-5/group. *p<0.05. scale bar= 100 μ m.

Fig. 3. ROS are primarily detected in the macrophages/microglia following SCI

Superoxide generation was detected by oxidized-DHE (ox-DHE; A, C & E). Representative confocal images show colocalization of ox-DHE with TomL-positive (A-B; arrowheads), NeuN-positive (C-D; arrowheads), and GFAP-positive (E-F; arrowheads) cells in the lesion epicenter from 4 MO mice at 3 dpi. Notice the high degree of red-blue overlap in B vs. D and F. The percentage of ROS production by different cell types at 3 dpi (G) and 7 dpi (H) was quantified at the three continuous sections at the lesion epicenter. (G) Macrophage/microglia (TomL-positive cells) accounts for ~80% of ROS production in the lesion epicenter at 3 dpi, while astrocytes (GFAP-positive cells) = 5%, and neurons (NeuN-positive cells) = 10%. Other ROS-producing cells (4%) were not phenotyped. See Supplementary Fig. 2 for the specificity of ox-DHE double labeling with cellular markers. (H) Macrophage/microglia account ~50% of ROS production at 7 dpi, while astrocytes (GFAP-positive cells) = 23%, neurons (NeuN-positive cells) = 6.3%, and other non-phenotyped = 23%. There was no

observable difference in the cellular distribution of DHE between 4 and 14 MO after SCI, n=4-5/group. Scale bar= 10 μ m.

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Fig. 4. NOX2 activity is increased in 14 vs. 4 MO spinal cords after SCI

Representative images of the lesion epicenter from 4 and 14 MO mice at 3 dpi stained with gp91^{phox} (A&E, green), DHE (B&F, red), and TomL (C&G, blue). NOX2 activation was confirmed through co-labeling of gp91^{phox} and DHE. The majority of activated NOX2 was detected in TomL-positive macrophages (D&H, arrowheads). (I) NOX2 activation is significantly upregulated in 14 MO as compared to 4 MO SCI. Results are mean $\pm$ SEM, n=4-5/group. *p<0.05. Scale bar= 20 μm .

Fig. 5. Age increases ROS-production in M2 SCI macrophages

Sections from the lesion epicenter were immunolabeled with anti-Arg-1 antibody (green; D, G, J&M) and DHE (red; E, H, K&N). (A) Arg-1-positive staining is not significant different between 4 and 14 MO mice at either 3 or 7 dpi. High-powered confocal images reveal significantly more ARG-1-positive macrophages (green) producing ROS after 14 vs. 4 MO SCI at both 3 (B, F&I, arrowheads) and 7 (C, L&O, arrowheads) dpi. The percentage of ARG-1-positive macrophages expressing ROS was quantified by co-labeling of DHE and ARG-1. Results are mean $\pm$ SEM, $n=4-5$ /group. * $p<0.05$. Scale bar= 10 μ m.

Fig. 6. Age enhances NOX2-mediated oxidative stress following SCI

(1) Spinal cord injury triggers macrophage NOX2 enzyme activity, a primary cellular and subcellular source of reactive oxygen species (ROS). We detected significantly increased NOX2 activation with age after SCI. (2) This further gives rise to lipid peroxidation and resulting aldehyde formation, such as 4-hydroxynonenal (4-HNE), and subsequent secondary injury. Our results of molecular and histochemical analyses of 4-HNE indicate that age potentiates this ROS-induced oxidative damage in injured spinal cord. (3) Age also potentiated M1 activation (CD16/32) and increased ROS production in normally protective Arginase-1 (Arg-1)-positive M2 macrophages. (4) Lipid peroxidation may also facilitate an M2 to M1 conversion thereby further increasing age-related, macrophage-mediated SCI tissue damage.

Table 1
Antibodies used in the current study

Antibodies	Host	Dilution	Vendor	Cat #
Immunohistochemistry-Primary Antibodies				
1. Biotinylated Tomato Lectin (TomL)		1:1000	Sigma	L0651
2. NeuN (2 ⁰ : #11 below)	Rabbit	1:1000	Novus	NBP1-77686
3. GFAP (2 ⁰ : #11)	Rabbit	1:500	Novus	NB300-141
4 gp91 ^{phox} (NOX2) (2 ⁰ : #10)	Goat	1:100	Santa Cruz	SC5826
5. CD16/32 (2 ⁰ : #12)	Rat	1:100	BD Pharmingen	553142
6. Arginase-1 (Arg-1) (2 ⁰ : #10)	Goat	1:200	Santa Cruz	SC18354
7. 4-hydroxynonenal (4-HNE)(2 ⁰ : #11)	Rabbit	1:500	Millipore	393207
8. Neurofilament (2 ⁰ : #13)	Chicken	1:500	Aves Lab	NFH
Immunohistochemistry-Secondary Antibodies				
9. Alexa Fluor 488 anti-rabbit (IgG)	Donkey	1:500	ThermoFisher	R37118
10. Alexa Fluor 488 anti-goat (IgG)	Donkey	1:1000	ThermoFisher	A11056
11. Biotinylated anti-rabbit (IgG)	Donkey	1:1000	Jackson ImmunoResearch Laboratories	711-065-152
12. Biotinylated anti-rat	Donkey	1:500	ThermoFisher	A18749
13. Biotinylated anti-chicken (IgY)	Goat	1:1000	Aves Lab	B-1005
14. Streptavidin, Alexa Fluor 633 conjugate (used for #11-13)		1:1000	ThermoFisher	S-21375
Western Blotting-Primary Antibodies				
4-HNE (western blotting)	Rabbit	1:2000	Alpha Diagnostic International	HNE11-S
gp91 ^{phox}	Goat	1:500	Santa Cruz	SC5826
GAPDH	Rabbit	1:5000	Abcam	Ab9485
Western Blotting-Secondary Antibodies				
IRDye 800CW anti-rabbit	Goat	1:20000	LI-COR	926-68071
IRDye 680CW anti-rabbit	Goat	1:20000	LI-COR	926-32211
IRDye 800CW anti-goat	Donkey	1:20000	LI-COR	925-32214
ELISA Antibodies				
4-HNE (ELISA)	Goat	1:1000	Cell Biolabs	STA-838