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Pre-treatment with meloxicam prevents the spinal inflammation and oxidative stress in DRG neurons that accompany painful cervical radiculopathy

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Abstract

Painful neuropathic injuries are accompanied by robust inflammatory and oxidative stress responses that contribute to the development and maintenance of pain. After neural trauma the inflammatory enzyme cyclooxygenase-2 (COX-2) increases concurrent with pain onset. Although pre-treatment with the COX-2 inhibitor, meloxicam, before a painful nerve root compression prevents the development of pain, the pathophysiological mechanisms are unknown. This study evaluated if pre-treatment with meloxicam prior to painful root injury prevents pain by reducing spinal inflammation and peripheral oxidative stress. Glial activation and expression of the inflammatory mediator secreted phospholipase A2 (sPLA2) in the spinal cord were assessed at day 7 using immunohistochemistry. The extent of oxidative damage was measured using the oxidative stress marker, 8-hydroxyguanosine (8-OHG) and localization of 8-OHG with neurons, microglia and astrocytes in the spinal cord and peripherally in the dorsal root ganglion (DRG) at day 7. In addition to reducing pain, meloxicam reduced both spinal microglial and astrocytic activation at day 7 after nerve root compression. Spinal sPLA2 was also reduced with meloxicam treatment, with decreased production in neurons, microglia and astrocytes. Oxidative damage following nerve root compression was found predominantly in neurons rather than glial cells. The expression of 8-OHG in DRG neurons at day 7 was reduced with meloxicam. These findings suggest that meloxicam may prevent the onset of pain following nerve root compression by suppressing inflammation and oxidative stress both centrally in the spinal cord and peripherally in the DRG.

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Keywords

neuropathic pain; NSAID; phospholipase A2; inflammation; reactive oxygen species; radiculopathy

Introduction

Neuropathic pain has been estimated in 7–10% of the population, with chronic pain carrying societal costs of \$560–635 billion annually (Institute of Medicine (US) Committee on Advancing Pain Research, 2011; van Hecke et al., 2014; Holmes, 2016). In the cervical spine, the dorsal nerve roots are a common source of painful neuropathic injury since they are susceptible to loading from compression by disc herniation, spondylosis or other forms of trauma (Côté et al., 2004; Carette and Fehlings, 2005; Abbed and Coumans, 2007). Even a transient compression of the nerve root can produce chronic radiculopathy, which often manifests as pain or numbness that can radiate down the arm or leg (Abbed and Coumans, 2007; Kuijper et al., 2009; Caridi et al., 2011). Despite the high prevalence of painful neuropathy, current treatments are not effective in providing pain relief, partially due to an incomplete understanding of the mechanisms involved in pain cascades (Institute of Medicine (US) Committee on Advancing Pain Research, 2011). Both inflammatory and oxidative stress pathways at the injury site and in the spinal cord where nociceptive processing occurs, contribute to pain through the release of inflammatory mediators and reactive oxygen species (Abbed and Coumans, 2007; Goupille et al., 1998; Klein, 2016; Nagashima et al., 2009, Salvemini 2011). By inhibiting cyclooxygenase2 (COX-2) mechanisms, non-steroidal anti-inflammatory drugs (NSAIDs) can reduce both inflammation and oxidative stress (Takahashi et al., 2005; Ma et al., 2002); because of this, they are a common analgesic treatment but have side effects (Han et al., 2015; Wong et al., 2016). The NSAID meloxicam is, a known selective COX-2 inhibitor (Furst, 1997; Hawkey, 1999; Kimura and Kontani, 2009) that has been shown to cross the blood brain barrier (Dehouck et al., 1992; Novakova et al., 2014). It provides effective pain relief in orthodontic pain and animal models of temporomandibular joint pain without the same risk of adverse side effects (Zarif Najafi et al., 2015; Montesinos et al., 2016; Zhang and Gan, 2017). Although meloxicam administration before a painful nerve root compression has been shown to prevent pain onset (Philips et al., 2017), the pathophysiologic mechanisms responsible its effectiveness after nerve root injury are unknown.

After painful neural trauma, even a transient injury, the central nervous system mounts a robust inflammatory response contributing to pain onset and maintenance (Hubbard and Winkelstein, 2005; Nicholson et al., 2012; Rothman et al., 2009, 2010; Smith et al., 2013; Winkelstein et al., 2002). As part of that immune response, resident spinal glia become activated over different time courses (Hubbard and Winkelstein, 2005; Nicholson et al., 2014; Rothman et al., 2010; Sun et al., 2012; Takahata et al., 2011). Upon activation, both microglia and astrocytes release a host of pro-inflammatory cytokines as early as 1 hour after painful nerve root compression (Rothman et al., 2009b; Smith et al., 2016) that further exacerbate neuroinflammation in the spinal cord (Ren and Dubner, 2008; Crown, 2012; Thomas et al., 2015; Johnson et al., 2017). Activated glia and neurons also increase

production of phospholipase enzymes, including the subfamily phospholipase A2 (PLA2), (Lee et al., 1998; Svensson et al., 2005). PLA2 enzymes catalyze the hydrolysis of glycerophospholipids in the cellular membrane to produce free fatty acids, such as arachidonic acid (AA), which are used through the COX pathway to produce prostaglandins and other inflammatory mediators (Chacur et al., 2004; Svensson et al., 2005). sPLA2 increases in many painful conditions including discogenic pain (Ren et al., 2015), spinal cord injury (Titsworth et al., 2009), and inflammatory pain (Svensson et al., 2005) and its direct application to normal nerve tissue induces behavioral sensitivity (i.e. pain) and immune cell activation in the spinal cord (Chacur et al., 2004). The upregulation of sPLA2 during inflammation also increases activity of the COX enzymes, exacerbating inflammation (Chacur et al., 2004). Although COX-2 inhibition alleviates pain and suppresses neuroinflammation (Ripamonti et al., 1996.; Ma et al., 2002; Tzeng et al., 2005), it is not known if systemic COX-2 inhibition has effects on spinal sPLA2 production and associated glial activation.

In conjunction with inflammation, oxidative stress pathways also contribute to neuropathic pain through the production of reactive oxygen species (ROS) like hydroxyl radicals, superoxide and nitric oxide (Machelska and Celik, 2016; Geis et al., 2017; Kiasalari et al., 2017). ROS production early after neural trauma results from the dysregulation of many cellular metabolic processes including production by NADPH oxidase, mitochondrial respiratory chains and even from the COX-mediated metabolism of AA to produce prostaglandins (Simmons et al., 2004; Adibhatla and Hatcher, 2008). The accumulation of ROS after neural injury has been hypothesized to contribute to neuropathic pain by activating glia, through inflammatory mediator production (Mosley et al., 2006; Naziro lu et al., 2012; Areti et al., 2014) and by mediating spinal dorsal horn sensitization (Chung, 2004). In the spinal cord, activated microglia are the main sources of free radicals, which in parallel to their production of inflammatory cytokines, further exacerbates inflammation, and leads to tissue damage (Mosley et al., 2006). ROS-dependent oxidative damage is observed in neuropathic pain and neurodegenerative disorders (Mosley et al., 2006; Kim et al., 2010; Hoffman et al., 2011). In particular, increases in the oxidative stress protein and a marker of DNA damage and oxidation, 8-hydroxyguanosine (8-OHG), has been reported in the spinal cord between 3–14 days after a lumbar spinal nerve transection (Kim et al., 2010). Although 8-OHG has been studied in the context of diabetes, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), its involvement in pain is not well-characterized (Nunomura et al., 2004; Aguirre et al., 2005; Mosley et al., 2006; Hoffman et al., 2011). Further, whether COX-2 inhibition prevents pain by reducing oxidative damage during neural injury is unknown.

Since systemic administration of meloxicam prior to a painful nerve root injury has been shown to prevent the onset of both evoked and spontaneous pain for up to 7 days (Philips et al., 2017), this study assessed whether selective COX-2 inhibition with meloxicam before a painful nerve root compression modulates aspects of the spinal inflammatory cascades and/or oxidative stress in the dorsal root ganglion (DRG) at a time when pain is evident (Rothman et al., 2010; Nicholson et al., 2012; Smith et al., 2013). In order to assess if meloxicam reduces spinal inflammation, both microglial and astrocytic activation and sPLA2 production were evaluated at day 7 using immunohistochemistry in spinal tissue

from rats receiving meloxicam treatment prior to a painful nerve root compression (NRC). Spinal sPLA2 and its expression in spinal neurons, microglia and astrocytes were separately assessed at day 7. In addition, the extent of oxidative stress was also measured using immunohistochemistry by the DNA damage marker 8-OHG. The distribution of 8-OHG was first evaluated in spinal microglia, astrocytes and neurons. Based on those findings, 8-OHG expression was quantitatively assessed in the DRG neurons.

Experimental Procedures

Surgical Procedures

All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983). All surgical procedures were performed using male Holtzman rats (275–299g; Envigo; Indianapolis, IN) under inhalation isoflurane anesthesia (4% induction, 2–3% for maintenance). At the time of anesthetic induction, meloxicam (2mg/kg; Bimeda; Oakbrook Terrace, IL) was diluted in sterile saline to a volume of 1mL and administered subcutaneously immediately before applying the nerve root compression (NRC) (n=7, MxNRC). Additional rats underwent NRC only (n=5, NRC) or a sham surgical procedure (n=4, sham) to serve as controls as previously described (Weisshaar et al., 2011; Nicholson et al., 2014; Smith et al., 2016; Philips et al., 2017). Briefly, a midline incision was made to expose the cervical spine and a right dorsal hemilaminectomy at C6/C7 exposed the right C7 dorsal nerve root. A microvascular clip (10gf; World Precision Instruments; Sarasota, FL) was inserted through a small opening in the dura to compress the nerve root for 15 minutes. After 15 minutes, the clip was removed and the incision was closed using 3–0 polyester suture and surgical staples. Sham surgeries included all of the same procedures with no root compression in order to control for the effect of surgery. Rats were monitored during recovery in room air with a heating pad.

Behavioral Assessment

Sensitivity of the ipsilateral forepaw to mechanical stimuli was measured before surgery (baseline, day 0) and on postoperative days 1, 3, 5, and 7 as previously described (Crosby et al., 2015a; Kras et al., 2015; Smith and Winkelstein, 2017). The forepaw was stimulated using a series of von Frey filaments of increasing strengths ranging from 1.4g to 26g (Stoelting, Wood Dale, IL). The lowest strength filament to evoke a response was recorded as the response threshold if the next filament also elicited a positive response. If a rat was unresponsive to all filaments, the maximum filament strength (26g) was taken as the threshold. Each testing session consisted of 3 rounds with at least 10 minutes of rest between each round. The threshold for each rat on each day was determined by averaging the rounds and was normalized to each rat's own baseline threshold. A repeated measures ANOVA with post-hoc Tukey test compared response thresholds over time and between groups.

Tissue Harvest & Immunohistochemical Labeling of Spinal Cord & DRG

After behavioral assessment on day 7, the C7 spinal cord and DRGs were harvested to evaluate effects of meloxicam treatment on inflammation and oxidative stress. Rats were

deeply anesthetized with sodium pentobarbital (65mg/kg) and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. Tissues were post-fixed overnight and stored in 30% sucrose for 6 days at 4°C. Samples were axially sectioned (14¼m thick) onto slides for immunohistochemical labeling. Microglial and astrocytic activation in the C7 spinal cord at day 7 was assessed using markers of ionized calcium-binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP), respectively. Tissue sections were blocked for 2 hours in goat serum (Vector Labs; Burlingame, CA) and incubated in primary antibody solutions containing rabbit anti-Iba1 (1:1000; Wako; Richmond, VA) and mouse anti-GFAP (1:500; Millipore; Billerica, MA) overnight at 4°C. The next day, sections were incubated in a secondary antibody solution containing goat anti-rabbit 568 (1:1000; Life Technologies; Carlsbad, CA) and goat anti-mouse 488 (1:1000; Life Technologies; Carlsbad, CA).

To evaluate spinal sPLA2 and its cell-specific expression in each of neurons, microglia and astrocytes at day 7, sections were incubated overnight in antibodies to goat anti-sPLA2-IIA (1:500; Santa Cruz; Dallas, TX), mouse anti-MAP2 (1:250; Covance; Cumberland, VA), rabbit anti-Iba1 (1:500; Wako; Richmond, VA), and mouse anti-GFAP (1:500; Millipore, Billerica, MA). After incubation with primary antibodies, sections were washed in PBS and incubated in secondary antibodies of donkey (all from Invitrogen; Carlsbad, CA) anti-goat Alexa Fluor 488 (1:1000), donkey anti-rabbit Alexa Fluor 546 (1:1000), and donkey anti-mouse Alexa Fluor 350 and 546 (1:1000).

At the same time point (day 7), spinal and DRG sections also were evaluated for the cellular source of 8-OHG expression and the extent of neuronal 8-OHG in DRG neurons. Tissue sections were labeled to determine those cell types associated with expression of 8-OHG. In separate runs, tissue sections were blocked in goat serum (Vector Labs; Burlingame, CA) and then incubated overnight at 4°C in a primary antibody solution of mouse anti-8-OHG (1:200; Abcam, Cambridge, MA) and either rabbit anti-Iba1 (1:1000; Wako; Richmond, VA), rabbit anti-GFAP (1:500; Millipore; Billerica, MA), or mouse anti-NeuN 555 conjugate (1:500; Millipore; Billerica, MA). The next day, samples were incubated in secondary antibody solutions containing goat anti-mouse 488 (1:1000; Life Technologies; Carlsbad, CA) and goat anti-rabbit 568 (1:1000; Life Technologies, Carlsbad, CA). Samples labeled for 8-OHG and NeuN were only exposed to goat anti-mouse 488 (1:1000; Life Technologies; Carlsbad, CA). The distribution of 8-OHG in DNA and RNA was separately assessed on a subset of DRG tissue sections at day 7 (n=3 each group) as previously described (Nunomura et al., 1999, 2004; Lovell et al., 2011). Sections were first digested with Proteinase K (10¼g/mL; Sigma; St. Louis, MO) for 40 minutes at 37°C and then separately pre-treated with either RNase free DNase-I (10U¼L; Sigma) or DNase-I free RNase (0.5¼g¼L; Sigma) for 2 hours at 37°C prior to incubation with 8-OHG antibodies.

Immunohistochemical Analyses

For all analyses, spinal cord and DRG samples were collected also from normal un-operated rats (n=2) in order to provide reference for expression levels in naïve control tissues; samples with no primary antibody were included in all runs and analyses as controls and to verify specificity of each antibody. Tissue sections were imaged at 20x using a digital

camera and stereomicroscope with DP2-BSW software (Olympus; Center Valley, PA). Spinal cord images were cropped to include only the superficial dorsal horn (750×150 pixels) (Zhang et al., 2013; Nicholson et al., 2014b); densitometry was used to quantify the percentage of positive pixels as a measure of positive labeling (Zhang et al., 2013). For each label, the percentage of pixels above the threshold expression in normal samples was separately quantified in the dorsal horn on the side ipsilateral to the injury for each sample. The total percent positive pixels in the ipsilateral dorsal horn was normalized to that in the normal tissue sections; Spinal Iba1, GFAP and sPLA2 expression was separately averaged across each group and compared using separate ANOVAs with post-hoc Tukey tests to detect differences between groups.

To quantify colocalization of spinal sPLA2 in neurons, microglia and astrocytes, the total number of pixels positive for each of sPLA2 and MAP2, sPLA2 and Iba1, and sPLA2 and GFAP was separately quantified using a custom MATLAB script as previously described (Nicholson et al., 2012; Crosby et al., 2015b; Zeeman et al., 2016). Neuronal, microglial and astrocytic sPLA2 were each determined by dividing the total number pixels positive for sPLA2 and either MAP2, Iba1 and GFAP by the total number of positive pixels for sPLA2 (Zeeman et al., 2016) for each image and is presented as fold-change over normal. Each of neuronal, microglial and astrocytic sPLA2 expression was compared across groups using a two-way ANOVA with Tukey's post hoc test (groupXday).

Merged images of 8-OHG and Iba1, GFAP, and NeuN were evaluated qualitatively to identify which cell type in the spinal cord exhibited the greatest extent of DNA and RNA damage from oxidative stress. Since that evaluation revealed expression almost exclusively in neurons, neuronal 8-OHG expression was evaluated in the ipsilateral DRG. Images of the DRG were cropped (450×450 pixels) to include 10–20 random neurons per image for intensity analysis. The mean signal intensity and size of each neuron were determined by manually outlining each neuron in ImageJ (Kras et al., 2013). Neurons with an average diameter in the range of 4–21¼m were classified as small diameter neurons, and those with an average diameter ranging from 21–40¼m were taken as medium diameter neurons (Weisshaar et al., 2010; Dong et al., 2012). Neurons greater than 40¼m were considered large diameter neurons. Accumulation of 8-OHG was analyzed in all neuron sizes with separate follow-up analyses of small- and medium-diameter neurons since those neurons contribute to nociception (Weisshaar et al., 2010; Dong et al., 2012). Intensity of 8-OHG in the DRG was compared between groups using a one-way ANOVA with post hoc Tukey test, with separate tests run for small- and medium-diameter neuron sizes.

Results

A single injection of meloxicam before a nerve root compression prevents the development of mechanical hyperalgesia in the ipsilateral forepaw that is typically produced by a nerve root compression (Fig. 1). Behavioral outcomes in the MxNRC and sham groups are not different and the withdrawal thresholds for those groups are unchanged from baseline. Meloxicam treatment (MxNRC) prevents the reduction in paw withdrawal thresholds that is seen after a NRC ($p < 0.0251$), with thresholds for MxNRC significantly higher than those for NRC on days 1 ($p < 0.0056$), 5 ($p < 0.0083$), and 7 ($p < 0.0004$) (Fig. 1). In contrast, responses

in the NRC group significantly decrease from its baseline values on days 1 ($p<0.0059$), 5 ($p<0.0366$), and 7 ($p<0.0384$). In addition, although responses for the NRC group are significantly lower than sham overall ($p<0.0012$) and on each postoperative testing days ($p<0.0030$), there is no difference between MxNRC and sham on any day (Fig. 1).

As with the behavioral responses at day 7, meloxicam also prevents increases in Iba1 and GFAP in the ipsilateral C7 superficial dorsal horn that occur after nerve root compression (Fig. 2). Treatment with meloxicam reduces spinal Iba1 to sham and normal levels, which is a significant reduction from levels after a painful NRC ($p<0.0456$) (Fig. 2). Likewise, Iba1 expression for sham ($p<0.0016$) and normal ($p<0.0036$) is significantly lower than for a NRC (Fig. 2). GFAP expression in the spinal cord is also significantly lower than levels after a NRC for the MxNRC ($p<0.0010$), sham ($p<0.0002$), and normal ($p<0.0009$) groups (Fig. 2).

With meloxicam treatment, spinal sPLA2 is significantly decreased in the ipsilateral dorsal horn ($p<0.0001$) compared to that for NRC and has similar expression levels as those in spinal cords from rats receiving a sham procedure and from normal rats (Fig. 3). Spinal sPLA2 expression is significantly lower than that in the NRC group for both the sham ($p<0.0001$) and normal ($p=0.0012$) groups. Paralleling total sPLA2 expression, sPLA2 expression in each of neurons and astrocytes is also reduced to sham and normal levels and is significantly lower than expression after NRC for both neuronal sPLA2 ($p=0.0007$) and astrocytic sPLA2 ($p=0.002$) respectively (Fig. 3). Spinal microglial sPLA2 expression is also significantly lower ($p=0.0006$) in the MxNRC group than in the NRC group, but meloxicam treatment does not reduce microglial sPLA2 levels to normal expression ($p=0.0074$) (Fig. 3).

The marker of oxidative stress, 8-OHG, colocalizes predominantly with neurons in the spinal cord but not with microglia or astrocytes (Fig. 4). 8-OHG labeling in the spinal cord is not different from normal in any of the groups, regardless of injury or treatment. However, 8-OHG accumulation in the C7 DRG is modulated at day 7 following meloxicam treatment (Fig. 5). An average of 15 ± 6 neurons was measured in each image. For neurons of all sizes, meloxicam treatment significantly reduces 8-OHG immunoreactivity compared to NRC alone ($p<0.0004$) (Fig. 5). This trend is also observed in small- and medium- diameter ($p<0.0083$) neurons (Fig. 5). Similarly, 8-OHG labeling is lower ($p<0.0292$) in sham tissue compared to NRC for all sizes of neurons, as well as explicitly in small-diameter and medium-diameter diameter neurons (Fig. 5). In neurons of all sizes, 8-OHG expression is elevated in both DNA and RNA following painful compression, with greater 8-OHG expression observed in RNA than DNA (Fig. 6). Meloxicam treatment reduces 8-OHG expression in both DNA and RNA to sham levels in neurons of all sizes, including small-diameter and medium-diameter neurons (Fig. 6)

Discussion

This is the first study to show that in association with preventing pain onset (Fig. 1), systemic administration of meloxicam before an otherwise painful nerve root compression reduces spinal inflammation as well as peripheral oxidative damage in the DRG (Figs. 2–5).

Meloxicam reduces the microglial and astrocytic activation in the spinal cord that is typical after painful nerve root injury (Hubbard and Winkelstein, 2005; Rothman et al., 2010; Nicholson et al., 2014) (Fig. 2). Paralleling the reduction in glial activation at day 7, spinal sPLA2 production is also suppressed, with reductions in both glia and neurons (Fig. 3). Interestingly, these reductions in *spinal* inflammation occur even though meloxicam is given systemically, suggesting that centrally-mediated responses may be involved in the pain attenuation. Unlike the inflammatory mediator sPLA2, the oxidative stress marker 8-OHG is almost exclusively in spinal neurons (Fig. 4) after painful nerve root injury, suggesting that accumulation of oxidative damage in neurons, rather than immune cells, may drive pain cascades in the periphery. In addition to pain and spinal inflammation, systemic meloxicam treatment immediately prior to neural trauma prevented the increase in 8-OHG in both DNA and RNA in peripheral DRG neurons (Figs. 5 & 6), further supporting the notion that meloxicam may attenuate pain by reducing peripheral oxidative stress. Taken together, these results suggest meloxicam may prevent the development of pain by reducing neuroinflammation and oxidative stress both centrally and peripherally.

Meloxicam's reduction of spinal microglial and astrocytic activation a week after injury (Fig. 2) and which have been attributed to neuropathic pain maintenance (Winkelstein and DeLeo, 2002; Zhang and De Koninck, 2006; Rothman et al., 2010), suggests that its COX-2 inhibition may reduce the prolonged spinal neuroinflammation that is known to drive persistent neuropathic pain (Ripamonti et al., 1996; Ma et al., 2002; Tzeng et al., 2005). However, since meloxicam was given immediately before nerve root compression it is unclear if the associated analgesic effects were due to the prevention of glial activation at the later (day 7) time point or whether it mediated effects much earlier after the injury. Indeed, astrocytic activation in the spinal cord at day 7 or later has been associated with the maintenance of pain (Zhang and De Koninck, 2006; Rothman and Winkelstein, 2007; Nicholson et al., 2012; Smith et al., 2017). Of note, spinal microglia are activated robustly early (and before astrocytes) after neuropathic injury and so are associated with pain *onset* (Winkelstein et al., 2001; Scholz and Woolf, 2007; Rothman et al., 2010). Following spinal nerve ligation, which is a similar neuropathic injury to that used here, microglia and astrocyte activation occur sequentially with distinct roles in the temporal establishment of neuropathic pain (Zhuang et al., 2005). The absence of behavioral sensitivity with meloxicam treatment inhibiting COX-2 at day 7 when pain is typically evident after NRC may be due to the reduced spinal astrocytic activation at that time (Fig. 2). Similarly, microglial activation was also suppressed at that same time (Fig. 2); however, it is possible that the meloxicam pre-treatment inhibited earlier microglial activation that prevented both the pain and associated spinal inflammatory responses that were detected at the later time point (Figs. 1–3). Although early spinal microglial activation was not evaluated in this study, results do provide support for either a direct action by which meloxicam acts centrally to prevent pain or an indirect effect of reducing oxidative stress in peripheral afferents that drive spinal responses.

In conjunction with attenuating microglial activation, meloxicam also prevented the increase of overall and cell specific spinal sPLA2 expression after painful nerve root compression (Fig. 3), further supporting its effectiveness in suppressing spinal neuroinflammation. In addition, this is first study to show that spinal sPLA2 increases with a painful nerve root

compression, implicating spinal sPLA2 in the maintenance of neuropathic pain after root injury. Although sPLA2 expression was probed using quantitative immunohistochemistry, previous work has shown that sPLA2 protein quantification from immunohistochemistry in the spinal cord follows similar trends to those found by alternate methods such as Western Blot (Titsworth et al., 2009). Application of sPLA2 alone onto a healthy sciatic nerve alone induces pain in the rat, as well as promotes both microglial and astrocytic activation in the spinal dorsal horn (Chacur et al., 2004). Further, expression of the neurotransmitter, substance P, is abolished for up to 24 hours after sPLA2 inhibition in cultured DRG neurons that are stimulated with inflammatory cytokines (Morioka et al., 2002). The robust increase in spinal sPLA2 after a painful nerve root compression injury is attributed to increases in neurons, microglia and astrocytes (Fig. 3), which could be from either increased trafficking of activated glia into the spinal cord or increased activation of resident spinal glia and neurons (Rothman and Winkelstein, 2007; Sheng et al., 2011). Since early after neural trauma, increased production of pro-inflammatory cytokines and ROS from activated glia and neurons promote the production of sPLA2 (Chacur et al., 2004; Svensson et al., 2005) and early glial activation and cytokine production occur after painful nerve root compression (Rothman et al., 2010; Smith et al., 2013), it may be possible that meloxicam pre-treatment may prevent the early inflammatory response associated with painful root injury, suppressing spinal glial activation (Fig. 2) and decreasing neuronal and glial sPLA2 production in the spinal cord at day 7 (Fig. 3). However, given the distinct differences in temporal activation between microglia and astrocytes (Zhuang et al., 2005), it is unknown if the analgesic effects of meloxicam are due to the prevention of overall or cell-specific sPLA2 production earlier than day 7. Nevertheless, these glial and sPLA2 findings (Figs. 2 & 3) suggest that meloxicam suppresses spinal neuroinflammation by inhibiting *both* the activation of spinal glia *and* the production of sPLA2, which likely also mediate other centrally-mediated mechanisms of pain.

Although both spinal neurons and glia produce ROS in neuropathic pain states (Mosley et al., 2006), DNA and RNA oxidative damage was evident predominantly in neurons in this study (Fig. 3), suggesting neuronal toxicity and dysfunction may play a role in radicular neuropathic pain. Neuronal oxidative damage has been reported to increase due to excess ROS production of several neuropathic pain states, including traumatic brain injury (Zhang et al., 2012) and peripheral nerve injury (Kim et al., 2010). Although glial cells are the major source of ROS (Mosley et al., 2006), extracellular ROS produced from microglia has been shown to be directly toxic to neurons following neuropathic injury (Zhang et al., 2012; Manzanero et al., 2013). Further, intracellular ROS in microglia and macrophages can promote the production of neurotoxic cytokines (Block, 2008). After the same painful nerve root compression in this study, spinal 8-OHG is robustly elevated as early as 1 day lasting for up to 7 days (Karthi et al., 2018) which occurs concurrent with the timecourse of spinal glial activation (Rothman and Winkelstein, 2007; Rothman et al., 2010; Nicholson et al., 2012; Smith et al., 2016). As such, it may be possible that increased glial activation in the spinal cord contributes to the accumulation of spinal ROS and the subsequent increased spinal neuronal oxidative damage (Fig. 4). Spinal 8-OHG expression was not evaluated after meloxicam treatment in this study; so, that hypothesis remains only speculative.

In addition to spinal microglial activation, peripheral macrophages infiltrate the injured root after painful compression in this model (Chang and Winkelstein, 2011). Since those cells are a well-known source of extracellular ROS in neural and ischemic injuries (Hackel et al., 2013; Anwar et al., 2016), 8-OHG was also examined in peripheral DRG neurons. Systemic meloxicam that provided pain relief also reduced both the total 8-OHG and the 8-OHG that is specifically associated with DNA or RNA in the DRG at day 7 (Figs. 5 & 6), providing further evidence that peripheral oxidative stress, specifically oxidative DNA and RNA damage in neurons, is associated with nerve root compression as with neuropathic pain (Dirig et al., 1998; Takeda et al., 2005; Gmitterová et al., 2018). Interestingly, in addition to macrophage infiltration, painful compression of the nerve root also induces production of sPLA2 by DRG neurons (Zhang et al., 2017). Because the use of metabolites (i.e. arachidonic acid) from sPLA2 cell membrane hydrolysis are well-established sources of reactive oxygen species in the production of COX-2 in neurons and immune cells (Adibhatla et al., 2006; Nanda et al., 2007), it is possible that ROS production and the resulting peripheral oxidative stress are also due to the elevated sPLA2 activity in the DRG after painful root injury. Given the role of COX-2 production in ROS generation and oxidative stress (Simmons et al., 2004; Adibhatla and Hatcher, 2008), the finding that 8-OHG in the DRG was reduced with meloxicam (Fig. 5) suggests that reducing peripheral oxidative stress by inhibiting COX-2 at the DRG, may be sufficient to prevent pain from nerve root compression. These findings begin to establish the contribution of oxidative stress in pain from neural trauma. However, since 8-OHG is only a marker of oxidative damage and ROS was not directly measured, further studies are needed to understand the spatiotemporal relationships between the peripherally- and centrally-derived oxidative stress responses and pain.

In addition to preventing neuropathic pain behaviors (Philips et al., 2017) (Fig. 1), systemic administration of meloxicam immediately prior to a painful nerve root compression prevents the spinal inflammation and peripheral oxidative stress that typically accompany that injury (Figs 2–5). Although additional studies are needed to further delineate the spatiotemporal mechanisms by which COX-2 inhibition alleviates pain following neuropathic injury, these findings suggest that a reduction in peripheral oxidative damage (Fig. 5) after selective COX-2 inhibition by meloxicam (Furst, 1997; Hawkey, 1999; Kimura and Kontani, 2009) may be due to a reduction in peripheral inflammation. In fact, decreased sPLA2 production and macrophage infiltration in the DRG has been found with this same meloxicam pre-treatment paradigm, along with prevention of the upregulation of spinal sPLA2 and activation of spinal glial cells that is typically observed after painful nerve root injury (Zhang et al., 2017). Together, these findings suggest that downregulation of sPLA2 in the spinal cord and DRG may contribute to the analgesic effects of meloxicam; further, the spinal changes may be due to regulation of neuroinflammation in the periphery. While COX-2 inhibitors have been recognized as alleviating pain by also reducing the robust neuroinflammatory response observed in neuropathic pain (Ma et al., 2002; Tzeng et al., 2005), this work also suggests COX-2 inhibition may alleviate pain by reducing inflammation-induced oxidative stress. Given that microglial activation (Winkelstein and DeLeo, 2002; Hubbard and Winkelstein, 2005; Rothman and Winkelstein, 2007; Rothman et al., 2009a) and oxidative stress tend to have more pronounced effects earlier after injury

(Goecks et al., 2012; Nayernia et al., 2014; Geis et al., 2017), further studies probing immune cell-mediated ROS production at earlier times are needed to understand the interconnected contributions of inflammation and oxidative stress in the establishment of neuropathic pain. Nevertheless, this work begins to identify possible mechanisms by which meloxicam and agents that utilize COX-2 inhibition can be used as promising interventions in the treatment of neuropathic pain.

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Abbreviations:

8-OHG	8-hydroxyguanosine
AA	arachidonic acid
COX-2	cyclooxygenase-2
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRG	dorsal root ganglion
GFAP	glial fibrillary acidic protein
IBA1	ionized calcium binding adaptor molecule 1
MAP2	microtubule associated protein 2
NADPH	nicotinamide adenine dinucleotide phosphate
NRC	nerve root compression
NSAID	nonsteroidal anti-inflammatory drug
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species

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Highlights

- Pre-treatment with meloxicam reduces microglial and astrocytic activation in the spinal cord at day 7
- Spinal sPLA2 upregulation is prevented by meloxicam, with reduced production in neurons, microglia and astrocytes
- 8-OHG colocalizes more in spinal neurons than glial cells and meloxicam reduces neuronal 8-OHG in the DRG

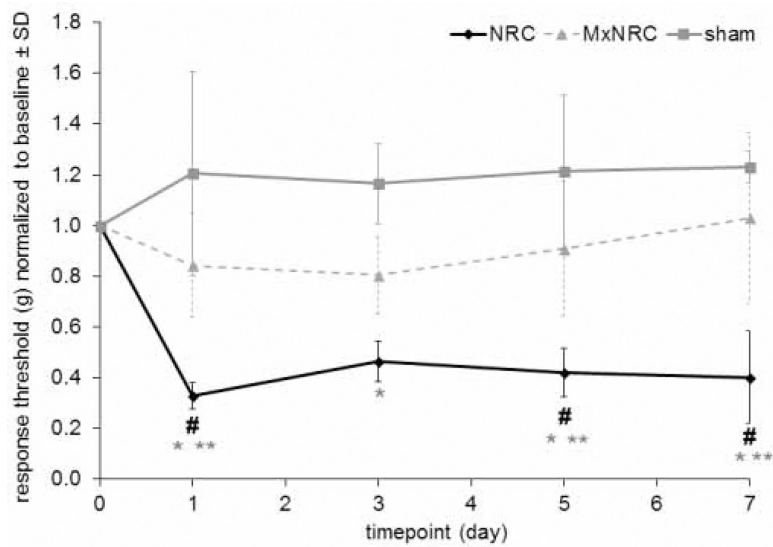


Figure 1.

Pretreatment with meloxicam (MxNRC) prevents the reduction in normalized forepaw response thresholds (mean±SD) at days 1, 5, and 7 compared to NRC (**p<0.008). After NRC, responses decrease from baseline on days 1, 5, and 7 (#p<0.04). Normalized thresholds decrease significantly for the NRC group relative to sham starting at day 1 and lasting through day 7 (*p<0.003). Responses for MxNRC and sham groups are not different on any day.

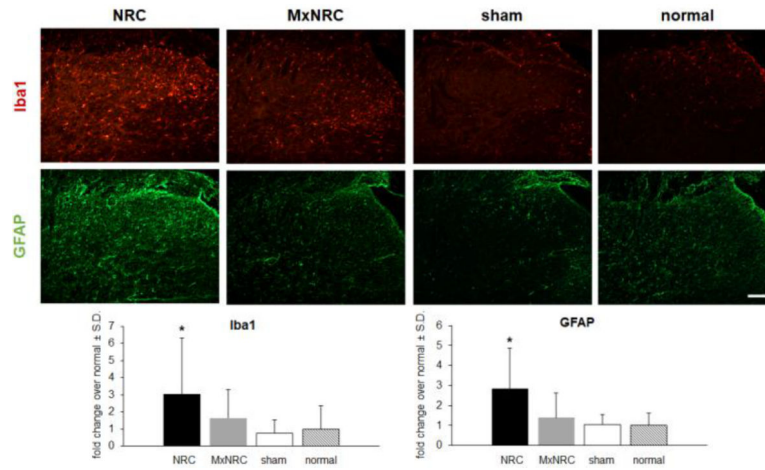


Figure 2.

Meloxicam injection prevents the microglial and astrocytic activation that is typically observed after NRC in the superficial dorsal horn at day 7. Representative images show Iba1 (red) and GFAP (green) labeling in the ipsilateral dorsal horn of the C7 spinal cord. In the MxNRC group, Iba1 labeling remains at sham and normal levels and is significantly lower than levels in the NRC group (* $p < 0.0456$). The increased GFAP in the superficial dorsal horn after NRC is prevented by meloxicam (* $p < 0.001$), with GFAP expression similar to sham and normal levels. Scale bar is $100\frac{1}{4}\mu\text{m}$ and applies to all panels.

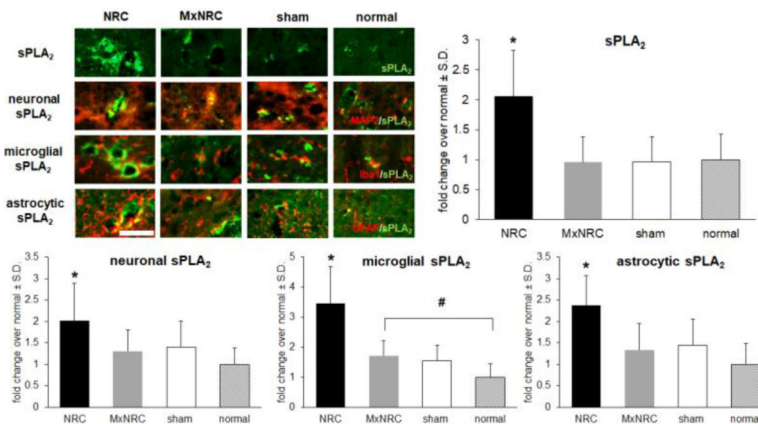


Figure 3.

Pre-treatment with meloxicam before NRC prevents upregulation of spinal sPLA2. Representative images in the spinal dorsal horn show less expression of sPLA2 (green) with meloxicam treatment than NRC, and similar to sham and normal levels. Similarly, there is less colocalization (yellow) evident between sPLA2 (green) and each of MAP2, Iba1 and GFAP (red) in the meloxicam treated (MxNRC), sham and normal groups compared to the NRC group. Spinal sPLA2 expression in the NRC group is significantly higher (* $p < 0.0012$) than levels in meloxicam-treated, sham and normal groups. The same is true of neuronal (* $p = 0.0007$) microglial (* $\#p = 0.0006$) and astrocytic sPLA2 (* $p = 0.002$) expression compared to NRC levels. Yet, microglial sPLA2 levels with meloxicam pre-treatment remain significantly higher ($\#p = 0.0074$) than normal levels. Scale bar is 200 μ m and applies to all panels.

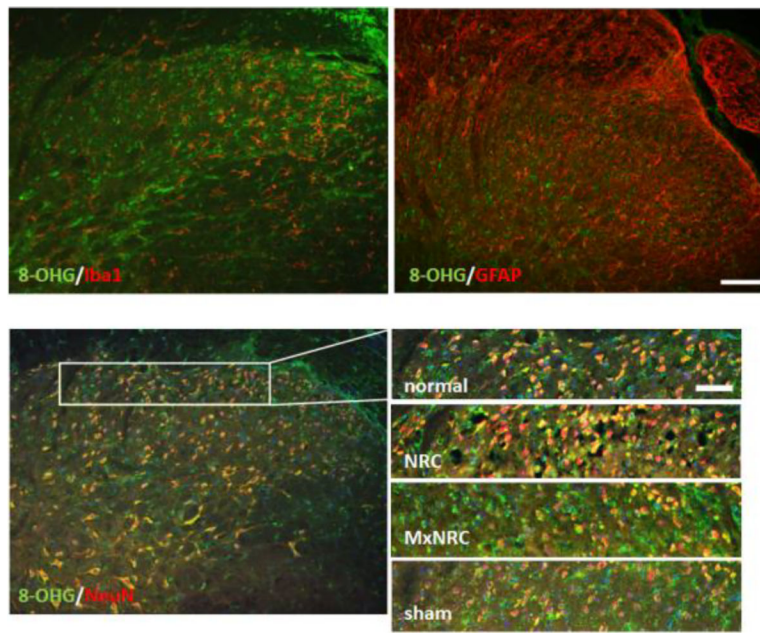


Figure 4. Representative images of the C7 spinal dorsal horn at day 7 showing that the oxidative stress marker 8-OHG does not colocalize with Iba1 or GFAP but does colocalize with the neuronal marker, NeuN. Enlarged images show colocalization of 8-OHG and NeuN in the superficial dorsal horn for each study group. Scale bar in the full size images is 100µm and is 50µm in the insets of the superficial dorsal horn.

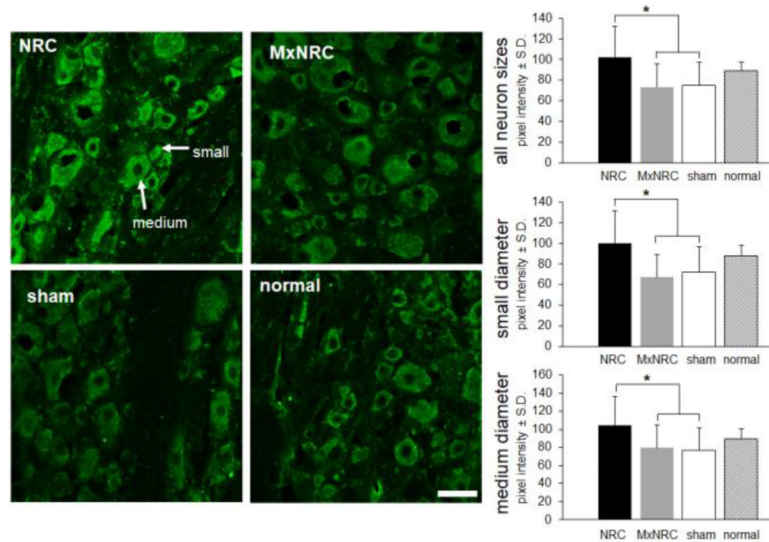


Figure 5. Representative images and quantification of 8-OHG in the C7 DRG ipsilateral to the injury at day 7. Increases in 8-OHG is evident in the NRC group relative to MxNRC and sham for all neuron sizes (* $p < 0.0146$). The same patterns of increases are observed in both the small- (* $p < 0.0160$) and medium-diameter (* $p < 0.0292$) neurons. Scale bar is 50 μ m and arrows show small and medium neurons.

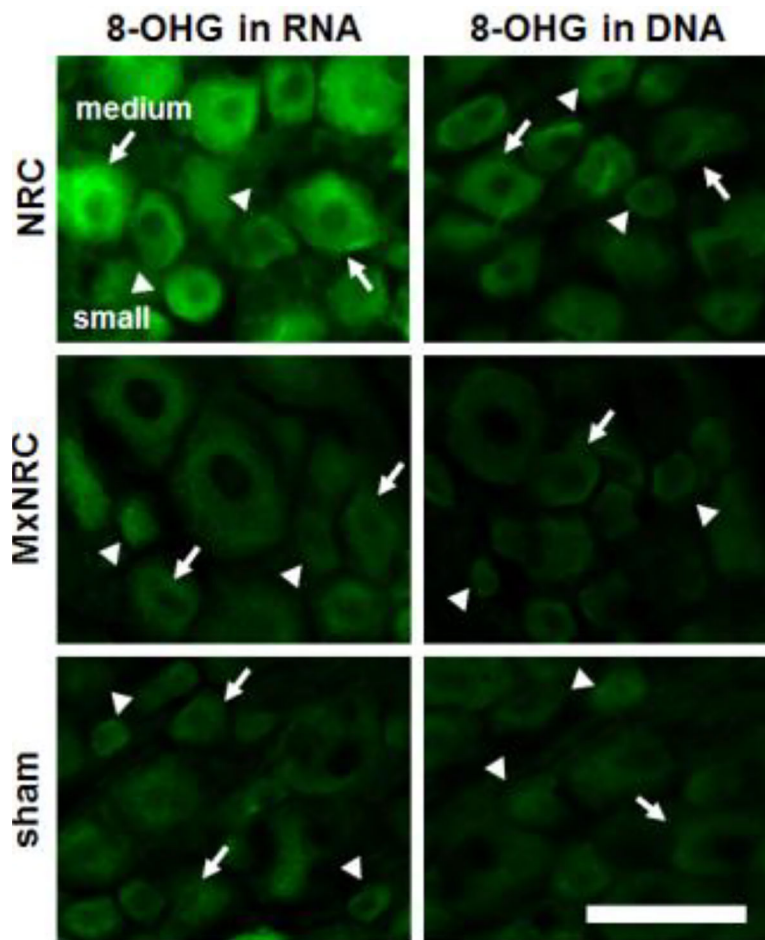


Figure 6. Representative images of 8-OHG expression in RNA and DNA in the C7 ipsilateral DRG neurons of all sizes at day 7. 8-OHG in both RNA and DNA are elevated in the NRC group, with more 8-OHG observed in RNA than DNA. Meloxicam treatment reduces 8-OHG in both RNA and DNA to sham levels in neurons of all sizes, including small-diameter (arrowhead) and medium diameter (arrow) neurons. Scale bar is 150 μ m and applies to all panels.