



# Acrolein Contributes to the Neuropathic Pain and Neuron Damage after Ischemic–Reperfusion Spinal Cord Injury

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Abstract—Besides physical insult, spinal cord injury (SCI) can also result from transient ischemia, such as ischemia-reperfusion SCI (I/R SCI) as a postoperative complication. Increasing evidence has suggested that oxidative stress and related reactive aldehyde species are key contributors to cellular injury after SCI. Previous work in spinal cord contusion injury has demonstrated that acrolein, both a key product and an instigator of oxidative stress, contributes to post-traumatic hyperalgesia. It has been shown that acrolein is involved in post-SCI hyperalgesia through elevated activation, upregulating, and sensitizing transient receptor potential ankyrin 1 (TRPA1) in sensory neurons in dorsal root ganglia. In the current study, we have provided evidence that acrolein likely plays a similar role in hypersensitivity following I/R SCI. Specifically, we have documented a post-I/R SCI hypersensitivity, with parallel elevation of acrolein locally (spinal cord tissue) and systemically (urine), which was also accompanied by augmented TRPA1 mRNA in DRGs. Interestingly, known aldehyde scavenger phenelzine can significantly alleviate post-I/R SCI hypersensitivity, reduce acrolein, suppress TPRA1 upregulation, and improve motor neuron survival. Taken together, these results support the causal role of acrolein in inducing hyperalgesia after I/R SCI via activation and upregulation of TRPA1 channels. Furthermore, endogenously produced acrolein resulting from metabolic abnormality in the absence of mechanical insults appears to be capable of heightening pain sensitivity after SCI. Our data also further supports the notion of acrolein scavenging as an effective analgesic as well neuroprotective strategy in conditions where oxidative stress and aldehyde toxicity is implicated. © 2018 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: oxidative stress, 3-HPMA, aldehyde, inflammation, lipid peroxidation.

### INTRODUCTION

Although spinal cord injury (SCI) usually is a consequence of destructive physical force, it can also result from transient ischemia, seen mostly as a surgical complication following thoracoabdominal aortic

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aneurysm repair. In fact, the rate of delayed paralysis from ischemia–reperfusion SCI (I/R SCI) as a postoperative complication has been reported to be as high as 11.4% (Cambria et al., 2002). Although it is suggested that oxidative stress is one of the key pathological mechanisms of neuronal damage derived from I/R SCI, the exact mechanism of injury is not clear. Many methods, such as systemic hypothermia and cerebrospinal fluid drainage, have been examined to mitigate this injury, yet the results have been unsatisfactory and none of these approaches have reliably and effectively prevented this complication (Svensson, 2005). Consequently, no therapy has been established to effectively deter functional loss in ischemic SCI.

Neuropathic pain is a proven sensory abnormality in human patients following I/R SCI (Beauchesne et al., 2000). Neuropathic pain has been shown to be a significant symptom for patients: 72% of the patients with SCI

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DRG, dorsal root ganglia; I/R SCI, ischemia-reperfusion SCI; LC, liquid chromatography; LPO, lipid peroxidation; MDA, malondialdehyde; MS, mass spectrometer; PBS, phosphate buffered saline; PFA, paraformaldehyde; ROS, reactive oxygen species; SCI, spinal cord injury; TRPA1, transient receptor potential ankyrin 1; 3-HPMA, 3-hydroxypropyl mercapturic acid.

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complained of neuropathic pain as the major symptom affecting their quality of life sometimes to a greater extent than motor deficits (Werhagen et al., 2004). Therefore, ameliorating neuropathic pain is of great importance. Neuropathic pain following I/R SCI has also been observed in animal models (Yu et al., 2014), greatly facilitating the efforts to understand the mechanism of neuropathic pain post-I/R SCI.

Although the lack of oxygen and nutrients can devastate biochemical activity and lead to the cellular dysfunction during ischemia, excessive byproducts of reactive oxygen species (ROS) and lipid peroxidation (LPO), for example, malondialdehyde (MDA) and nitric oxide, are believed to be more detrimental and critical after I/R SCI (Lukacova et al., 1996; Liu et al., 2015; Gokce et al., 2016). Acrolein, another product of LPO, has been shown to have greater reactivity and neurotoxicity than MDA (Pizzimenti et al., 2013). As a strong electrophile, it is capable of modifying proteins, nucleic acids, and lipids resulting in impairment of mitochondrial function or damage of cellular membrane in neural cells (Shi et al., 2011a).

In our previous studies, acrolein has been validated as a critical factor for neural tissue damage and functional loss in contusive SCI (Shi et al., 2002, 2011a, 2015; Hamann and Shi, 2009; Park et al., 2014a, 2015). In particular, the role of acrolein in post-traumatic hyperreflexia has previously been demonstrated in a rat contusive spinal cord iniury (SCI) models. In such a mechanical SCI, acrolein elevation was shown to coincide with post-SCI hyperalgesia, and acrolein-sequestering using acrolein scavengers attenuated pain behavior in rats (Due et al., 2014; Park et al., 2014a,b; Park et al., 2015; Chen et al., 2016; Butler et al., 2017). Furthermore, injection of relevant concentrations of acrolein to the rat spinal cord incited pain-related behavior and diffusive inflammation, closely mirroring post-SCI hypersensitivity (Due et al., 2014; Park et al., 2014b; Gianaris et al., 2016). Aldehydes are known to directly excite nociceptive neurons via the transient receptor potential ankyrin 1 (TRPA1) channel present in dorsal root ganglia (DRG) and spinal dorsal horn (Bautista et al., 2006; Macpherson et al., 2007; McNamara et al., 2007; Trevisani et al., 2007). While TRPA1 mRNA is elevated in contusive SCI, acrolein suppression treatment can partially reverse such elevation (Due et al., 2014; Park et al., 2015). In addition, injection of acrolein can also elevate TRPA1 mRNA (Due et al., 2014; Park et al., 2015). Therefore, acrolein is both critical and sufficient in causing post-SCI hypersensitivity, likely through direct binding and activation of TRPA1, but also by augmenting TRPA1 expression. Furthermore, acrolein scavengers have also been show to offer neuroprotection to improve motor function (Park et al., 2014b; Chen et al., 2016). As such, there is strong evidence suggesting a pathological role of acrolein in sensory and motor deficits in mechanical SCI.

Therefore, the primary goal of this study was to ascertain the role of acrolein in the pathology of I/R SCI and the correlation between acrolein and neuropathic pain and pathologies related to motor function. In addition, the neuroprotective role of acrolein-scavenger phenelzine was tested in I/R SCI models. To our knowledge, this is the first study to explore the pathological role of acrolein in I/R SCI.

#### EXPERIMENTAL PROCEDURES

#### Animals

Male Sprague–Dawley rats, weighing around 200–250 g, were included in this study. All animal studies were approved under the Purdue Institutional Protocol number 111100095. Animals were handled and housed strictly following the Purdue University Animal Care and Use Committee Guidelines and ARRIVE guidelines. They were housed at least one week before the surgery to allow for acclimation to the housing facility.

## Ischemic–reperfusion spinal cord injury (I/R SCI) models

A mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) was administered for anesthetization via intraperitoneal (IP) injection. We followed the method described by Zivin and DeGirolami (1980) to induce lumbar ischemic-reperfusion spinal cord injury. Briefly, hair of the abdomen was shaved and the skin was sterilized twice with povidone iodine. A midline incision was made and the abdominal organs were pushed aside gently to expose the abdominal aorta. To block the bloodstream, a microaneurysm-clip (S&T vascular clamp, Fine Science Tools Inc. CA. USA) was placed on the aorta just caudal to the left renal artery but without damaging the blood supply of left renal artery. Then, the incision was temporarily closed. During the surgery, a half dosage of anesthetic mixture (40 mg/kg ketamine + 5 mg/kg xylazine) was administered via IP injection to maintain anesthesia when the rats showed any signs of awakening. After 45-min or 90-min ischemic injury, the micro-aneurysm-clip was unlocked from the aorta and the incision was closed laver by laver.

During the surgery, the rats were placed on the heating pad to maintain a normothermic condition around 37 °C and monitored with a sterilized rectal temperature probe. After the surgery, bladder exercise was performed to stimulate the autonomic urinary reflex.

#### Application of phenelzine

Phenelzine sulfate salt (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) and sterilized with a 0.45-µm filter. Our previous work has shown phenelzine treatment at a dosage of 15 mg/ kg to be effective and safe for rats (Chen et al., 2016). For treatment of neuropathic pain, phenelzine solution was administrated via IP injection immediately after releasing of the micro-aneurysm-clip and continued daily for two weeks. For detection of the changes of acrolein–protein adducts, phenelzine was applied immediately after the injury and 2 h before sacrifice. For the assessment of neuronal survival following injury and its influence by phenelzine treatment, phenelzine was administered immediately after injury and continued daily for two weeks.

#### Mechanical hyperreflexia assessment

The hind paw withdrawal threshold to mechanical stimuli was analyzed to quantify the mechanical neuropathic pain after I/R SCI. Following our previous method (Due et al., 2014; Park et al., 2014b, 2015; Chen et al., 2016), animals were placed inside a transparent plastic box on the top of a metal mesh floor in a quiet circumstance. Prior to the testing, they were kept alone for at least 10 min to acclimate to the experimental apparatus. Subsequently, a series of calibrated Von Frey filaments (range: 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0 and 15.0 g, Stoelting, Wood Dale, IL, USA) were applied perpendicular to the plantar surface of the hind-limb with sufficient bending force for 3-5 s. When the rats showed brisk movement with or without lipping or biting, this testing result was defined as positive reaction. Otherwise, it was considered as negative reactive at this stimulation. Following a positive reaction, a sequential lower stimulus (the next smaller filament) was applied. In the event of negative response, a sequential greater stimulus (the next bigger filament) was used. Rats were given at least 1 min for rest between every two stimuli. The up-down method proposed by Chaplan et al. (1994) was used to calculate the threshold of mechanical pain and the average of two hind-limb' scores was calculated as the final score. Mechanical sensitivity was evaluated every other day for two weeks.

## Isolation of spinal cord and immunoblotting for acrolein–protein adducts

After deep anesthesia, animals were perfused with oxygenated Krebs solution (all in mM: 124 NaCl, 2 KCl, 1.24 KH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 ascorbic acid, 1.3 MgSO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, and 10 glucose) via trans-cardiac routine. Then, the spinal cord from L2 to L4 was isolated to quantify the concentration of acrolein–protein adducts.

Consistent with our previous studies (Zheng et al., 2013; Park et al., 2014b, 2015; Chen et al., 2016), the extracted spinal cord segments were incubated with a 1% Triton solution with the Protease Inhibitor Cocktails (Sigma-Aldrich, St. Louis, MO, USA) and it was homogenized using a sonicator. Then, the samples were incubated on ice for 1 h and centrifuged at 13,500g for 30 min at 4 °C. The BCA protein assay was used to ensure equal loading for all samples. Samples were transferred to a nitrocellulose membrane using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). Following transfer, the membrane was blocked using blocking buffer (0.2% casein with 0.1% Tween-20 in phosphatebuffered saline) for 1 h and incubated with a primary rabbit anti-acrolein antibody (1:1000, Abcam, catalog Number: ab37110, Cambridge, UK) for at least 18 h at 4 °C. Subsequently, the membrane was washed with  $3 \times 15$  min and then incubated with anti-rabbit IgG HRP-linked secondary antibody (1:1000, Cell Signaling Technology, MA, USA) for 1 h at room temperature. After final washing with  $3 \times 15$  min. the membrane was exposed to substrate and visualized by chemiluminescence (Clarity Western ECL Substrate, BiO-RAD, Hercules, California, USA) according to the manual instructions. The software of

Image J (NIH, Bethesda, MD, USA) was used to measure the band density and the concentration of acrolein–protein adducts was normalized by the concentration of GAPDH (1:1000, Santa Cruz Biotechnology, Texas, TX, USA).

#### TRPA1 gene expression analysis with real-time PCR

The analysis of TRPA1 gene expression was similar to our previous studies (Due et al., 2014; Park et al., 2015; Chen et al., 2016). In brief, dorsal root ganglia (DRGs) from L2 to L6 were collected one week after I/R SCI. All samples were homogenized using a sonicator in Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA was extracted following with chloroform extraction and isopropanol precipitation. Then, the concentration of isolated RNA was measured using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesized with an iScript<sup>™</sup> cDNA Synthesis kit (BIO-RAD, Hercules, California, USA). Primers for TRPA1 channel and  $\beta$ -actin were designed as the following (Nozawa et al., 2009): TRPA 1 forward: 5'-TCCTATACTGGAAG CAGCGA-3'; reverse: 5'-CTCCTGATTGCCATCGACT-3'; **B-actin** forward:5'-GCGCTCGTCGTCGACAACGG-3'; reverse: 5'-GTGTGGTGCCAAATCTTCTCC-3'.

The accumulation of PCR products were detected by the level of iQ<sup>TM</sup> SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) fluorescence following the manual guide. The target gene expression level was normalized by the expression level of  $\beta$ -actin using the method of  $2^{-\triangle\triangle Ct}$ . All data then were normalized to the average of the control group.

# Urine collection and quantification of acrolein metabolite, 3-hydroxypropyl mercapturic acid (3-HPMA)

Following our previous study (Zheng et al., 2013; Park et al., 2014b; Chen et al., 2016), the rats were placed inside a standard metabolic cage for urine collection. To induce urination, 0.3 cc of saline was administered via IP injection. Water and food sources were carefully separated to prevent urine dilution and contamination.

Before LC/MS/MS analysis of 3-HPMA, ENV + cartridges (Biotage, Charlotte, NC, USA) were used to prepare solid phase extraction. Briefly, each cartridge was conditioned with 1 mL of methanol, followed by 1 mL of water, and then 1 mL of 0.1% formic acid in water. A volume of 500 µL of urine was spiked with 200 ng of deuterated 3-HPMA (d3-3-HPMA) (Toronto Research Chemicals Inc, Toronto, Canada) and mixed with 500 uL of 50 mM ammonium formate and 10 uL of undiluted formic acid. Subsequently, 1 mL of 0.1% formic acid was used to wash each cartridge twice and then 1 mL of 10% methanol/90% of 0.1% formic acid in water was used for washing. All cartridges were completely dried under nitrogen gas and eluted with 600 µL methanol plus 2% formic acid three times. Following that, the eluates were dried in an evaporation centrifuge and then reconstituted in  $100 \,\mu\text{L}$  of 0.1%formic acid. To analyze the concentration of 3-HPMA, an Agilent 1200 Rapid Resolution liquid chromatography

(LC) system coupled with an Agilent 6460 series QQQ mass spectrometer (MS) was used according to the manual instructions.

#### Urinary creatinine assay

Urinary creatinine level was measured using a urine creatinine assay kit (Cayman Chemical Company, MI, USA) to normalize the 3-HPMA concentration. Creatinine standards and diluted urine samples (12× and 24×) were incubated with the alkaline picrate solution for 20 min in 96-well plates. The standard curve was constructed following the manufacturer's manual. The absorbance was measured at 490–500 nm with a standard spectrophotometer as an initial reading then, 5  $\mu$ L of acid solution was added to each solution and incubated on a shaker for 20 min. Again, absorbance at 490–500 nm was used as the final reading and the difference between the initial and final value was used for quantitative analysis.

#### Immunofluorescence imaging

Twenty-four hours after the 90-min I/R SCI, rats were sacrificed and the spinal cord from L3 to L4 was harvested following 4% paraformaldehyde (PFA) transcardiac perfusion. The harvested tissue was fixed in 4% PFA for 24 h and then cryoprotected with a 15% sucrose solution for 24 h. followed by 24 h with a 30% sucrose solution. Subsequently, all tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek USA Inc, CA, USA) and frozen at -80 °C immediately. Then, the tissues were transversally sectioned at 10 µm and mounted on gelatin coated slides. Sections were incubated with 0.25% Trition-X100 in phosphate-buffered saline (PBS) for 15 min and then blocked using blocking agent (10% Goat serum mixed with 0.3 M glycine in 1% BSA/PBST) for 45 min. After washing with PBS for  $3 \times 15$  min, all tissues were incubated with primary rabbit anti-acrolein-conjugated antibody (1:200, Abcam, catalog Number: ab37110, Cambridge, UK) overnight at 4 °C. After washing with PBS for  $4 \times 15$  min, the slides were incubated with goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor ® 488 (life technologies, Massachusetts, USA) for 1 h at room temperature and then washed with PBS for  $4 \times 15$  min. Nuclei were then stained with 4',6-dia midino-2-phenylindole (DAPI) for 10 min and all slides were observed with a fluorescence microscopy under  $400 \times$  magnification.

#### Nissl staining and histological evaluation

Two weeks after I/R SCI, all rats were sacrificed and perfused with Krebs solution and then 4% formaldehyde via trans-cardiac approach. The lumbar spinal cord at L4 segment was harvested and fixed with 4% PFA for 24 h. Subsequently, tissues were processed with routine paraffin embedding and transversally sectioned at 5  $\mu$ m. Nissl body was stained with toluidine blue for 30 min and then washed with 95% ethanol for 30 s.

All digital images were captured using a same microscope (Axio, Carl Zeiss, Oberkochen, Germany) and a previous reported method was used to observe and calculate the motor neurons (Wang et al., 2014). In brief, the whole images of spinal cord were captured under  $4 \times$  magnification at first and then divided into four quadrants: a line was drawn between anterior median fissure and posterior median sulcus as the longitudinal axis: then, the transverse axis was drawn through the median of the central canal and being perpendicular to the longitudinal axis. The left ventral (anterior) guadrant and right ventral (anterior) guadrant were focused to count the numbers of normal neurons. For normal neurons, the cells are abundant with Nissl granules and the cell nucleus was round with clearly visible nucleoli. By contrast, damaged neurons present as shrunken cellular bodies and the disappearance or lack of Nissl granules, as well as nuclear condensation with invisible nucleolus (Saito et al., 2011; Wang et al., 2014).

One slide was selected randomly for each samples and the numbers of normal neurons was counted by two authors who are blind to the groups independently using Image J software. Average of the numbers of normal neurons in left and right Ventral Horns was calculated and compared between each group.

#### **Statistical methods**

All the data were expressed as Mean  $\pm$  SEM. A one-way or two-way ANOVA was used for comparison among three groups and then Post-hoc comparison was made between each group. P < 0.05 was used as statistical significance.

#### RESULTS

### Mechanical hyperreflexia after 45 min- or 90-min I/R SCI and analgesic effect of phenelzine treatment

A significant mechanical tactile hyperreflexia was observed in 90-min I/R SCI group and 45-min I/R SCI group. In 90-min I/R SCI group, the significance hyperreflexia started at the second day following injury, when the mechanical paw withdraw threshold was 11.80  $5 \pm 2.057$  g, which is significantly lower when compared to the value of  $15.0 \pm 0.0$  g in sham-surgery group at this time point (P < 0.01). Additionally, the mechanical withdraw threshold was significantly different between 45-min I/R SCI group and sham-surgery group starting at the sixth day post-injury, with a value of 5.461  $\pm$  1.16 7 g for the injured group (p < 0.01 when compared to sham-surgery group). There is no significant difference between 90-min I/R SCI and 45-min I/R SCI at any time points. Both groups sustained the mechanical pain-like behavior until the end of the study (14 days post injury) (Fig. 1).

The mechanical hyperreflexia could be significantly attenuated by phenelzine treatment starting six days post injury and treatment initiation, with a value of withdrawal thresholds at  $9.017 \pm 1.017$  g, which is significantly higher than that in 90-min I/R SCI group,  $3.687 \pm 0.681$  g (p < 0.05). The analgesic effect of



Fig. 1. Mechanical hyperreflexia after 45-min or 90-min I/R SCI and analgesic effect of phenelzine treatment. Beginning at the second day after injury, the 90-min I/R SCI group showed significantly increased mechanical hyperreflexia compared to the sham-surgery group. Similarly, the 45-min I/R SCI group also showed significant mechanical hypersensitivity began at the sixth day post injury. The mechanical hyperreflexia following 90-min I/R SCI could be significantly attenuated by phenelzine at 15 mg/kg via daily IP injection for two weeks.  ${}^{\#}P < 0.05$ ,  ${}^{\#\#}P < 0.01$  when compared between 90-min I/R SCI and sham-surgery groups; \*P < 0.01 when compared between 45-min I/R SCI group and sham-surgery group,  $^{++}P < 0.01$  when compared between 90-min I/R SCI and 90-min I/R SCI + phenelzine groups. <sup>&&</sup>P < 0.01 when compared between sham-surgery and 90min I/R SCI + phenelzine groups. (N = 5 for each groups and all data were expressed as Mean ± SEM. A two-way ANOVA and Bonferroni's test were used for statistics comparisons).

phenelzine treatment remained until 14 days post-injury (Fig. 1). However, the value of withdrawal thresholds in 90-min I/R SCI + phenelzine group is still significantly lower than those in sham-surgery group, starting six days post injury (P < 0.01).

# Elevation of acrolein–lysine adducts in lumbar spinal cord following I/R SCI

Acrolein antibodies that were designed to bind acroleinlysine adducts are capable of recognizing and therefore quantifying any protein with lysine residues that have reacted with and formed an adduct with acrolein (Uchida et al., 1998; Hamann and Shi, 2009; Shi et al., 2011a; Tully et al., 2014). Detection of acrolein-adduct proteins utilizing these antibodies and immunoblotting thus enables the assessment of proteins that are affected by acrolein and permitting the estimation of the level of acrolein that reacts with proteins. Such methods of acrolein quantification has been used in our original study to demonstrate the elevation of acrolein in mechanically injured SCI (Hamann et al., 2008; Park et al., 2014b; Chen et al., 2016; Tian and Shi, 2017). As such, this method was used to detect the changes of acrolein in spinal cord tissue in I/R SCI in the current study. Twenty-four hours after 90-min I/R SCI, the concentration of acrolein-lysine adducts were significantly elevated  $(1.174 \pm 0.033)$  when compared to the sham-surgery group (0.279  $\pm$  0.031, relative concentration normalized by GAPDH, p < 0.05). However, phenelzine at 15 mg/kg, administrated twice, immediately after injury, and 2 h before sacrifice effectively reduced the concentration of acrolein–lysine adducts. Specifically, phenelzine reduced the acrolein–lysine adduct to  $0.389 \pm 0.049$  which is significant lower than injury only,  $1.174 \pm 0.033$  (p < 0.05, Fig. 2). In addition, there is no difference between sham-surgery group and I/R and phenelzine-treated group (P > 0.05).

# Increased concentration of 3-HPMA in urine after I/R SCI

3-HPMA is a stable acrolein–glutathione metabolite in urine. As depicted in Fig. 3, the concentration of 3-HPMA had increased significantly to  $3.380 \pm 0.477 \mu$ g/mg creatinine at 24 h after 90-min I/R SCI from its own pre-injury baseline of  $2.029 \pm 0.202 \mu$ g/mg creatinine (p < 0.05). However, acrolein increase was effectively suppressed when phenelzine was applied at a dosage of 15 mg/kg 3 times post injury (immediately, 24 h, and 48 post-injury). Specifically, the comparisons of 3-HPMA level between 90-min I/R SCI group and 90-min I/R SCI + phenelzine group at 24 h and 48 h are  $3.380 \pm 0.477 \ vs \ 1.645 \pm 0.138 \mu$ g/mg creatinine (p < 0.01) and  $2.194 \pm 0.262 \ vs \ 1.323 \pm 0.115 \mu$ g/mg creatinine (p < 0.05) respectively.

#### Augmented TRPA1 mRNA expression level in the DRGs after I/R SCI

To further explore the molecular mechanism of neuropathic pain after I/R SCI, expression of TRPA1 channels in DRGs was examined one week after the injury. As shown in Fig. 4, TRPA1 mRNA level had a 3.418  $\pm$  0.576 fold increase in expression in 90-min I/R SCI group compared to the sham-surgery group. However, phenelzine treatment significantly decreased the expression of TRPA1 mRNA to 1.351  $\pm$  0.293 fold in 90-min I/R SCI + phenelzine group (*P* < 0.05).

# Elevated acrolein–lysine adducts in neurons, the reduction in neurons, and their reversal by phenelzine in spinal cord in I/R SCI rats

In order to assess the change of acrolein–protein adducts and its association with neurons in the spinal cord tissue following I/R SCI, we have performed structural analysis using immunofluorescence imaging. As shown in Fig. 5, while little labeling of acrolein–lysine adduct could be perceived in sham-surgery group, abundant acrolein– lysine adducts were detected inside neurons in Ventral Horn at 24 h following 90-min I/R SCI. Furthermore, phenelzine treatment led to the conspicuous reduction in the acrolein–lysine adduct elevation in I/R SCI rats.

In addition to the change of the level of acroleinprotein adducts, we also assessed the status of the number of neurons in 90-min I/R SCI group two weeks after the injury, and its possible influence by acrolein scavenging. As indicated in Fig. 6, a 90-min I/R SCI resulted in a significant reduction of neurons, labeled by Nissl staining, when compared to the sham-surgery



**Fig. 2.** Increased level of acrolein–lysine adducts at spinal cord L2– L4 segment after 90-min I/R SCI. Bar graph displaying the significant elevation of acrolein–lysine adducts at 24 h after the I/R-90 min injury when compared to the sham-surgery group. Phenelzine at a dosage of 15 mg/kg was administrated immediately after the surgery and 2 h before the sacrifice via IP injection, which resulted in significant reduction in elevated acrolein–lysine adducts. No statistically significant difference was detected between sham-surgery group and I/R and phenelzine-treated group. Photographic images (top) show representative blots for each experimental condition. (\*P < 0.01when compared between each group, N = 6 for each group, all data were expressed as Mean  $\pm$  SEM, a one-way ANOVA and Tukey's test).



**Fig. 3.** Elevation of 3-HPMA after 90-min I/R SCI and its suppression by phenelzine treatment. Twenty-four hours following 90-min I/R injury, the concentration of 3-HMPA increased significantly in urine when compared to the pre-SCI baseline (\*P < 0.05). However, phenelzine at 15 mg/kg significantly reduced the concentration of 3-HPMA (\*P < 0.01). Similar effects of phenelzine treatment could be detected forty-eight hours after the injury. (\*P < 0.05). (N = 5 for all groups and all data were expressed as Mean ± SEM, a one-way ANOVA and Tukey's test).



**Fig. 4.** Elevated level of TRPA1 mRNA after 90-min I/R SCI at lumbar DRGs. Gene expression was normalized by the expression of  $\beta$ -actin and compared to the cycle threshold (Ct) value of  $\beta$ -actin of normal spinal tissues. The difference of gene expression was shown as the fold ratio and calculated using the method of  $2^{-\Delta\Delta Ct}$ . (\**P* < 0.01 when comparisons were made between 90-min I/R SCI group and 90-min I/R SCI + phenelzine group). (*N* = 6 for each groups, all data were expressed as Mean ± SEM, a one-way ANOVA and Tukey's test).

group (19,00  $\pm$  0.78 and 9.625  $\pm$  0.617, p < 0.005). A continuous daily treatment of phenelzine for two weeks at a dosage of 15 mg/kg significantly prevented the loss of neurons (14.313  $\pm$  0.40, p < 0.05 when compared to injury only). However, the number of motor neurons in 90-min I/R SCI and phenelzine treatment group was still lower than that of sham-surgery group (p < 0.05).

### DISCUSSION

In this study, we have shown that acrolein appears to play a similar role in the pathology related to sensory and abnormalities in ischemic SCI. We have motor demonstrated that acrolein was elevated both locally in spinal cord tissue and systemically in urine after I/R SCI which was accompanied by post-I/R SCI hyperalgesia, elevation of TRPA1 mRNA, and loss of motor neurons. Furthermore, treatment with the acrolein scavenger phenelzine significantly suppressed post-I/R SCI acrolein accumulation in both spinal cord tissue and in urine, reduced TRPA1 mRNA in DRGs, attenuated mechanical hyperreflexia, and reduced motor neuronal loss in ventral spinal cord following I/R SCI. Although a pathological contribution of acrolein in brain ischemic injury has been demonstrated before (Wood et al., 2006; Saiki et al., 2011), this is the first study suggesting a role of acrolein in ischemic injury in spinal cord.

It is interesting to note that there are multiple similarities related to the magnitude of acrolein elevation post injury between mechanical and ischemic SCI. When examined at 24 h following injury, the increase in



**Fig. 5.** Augmented acrolein–lysine adducts labeling in ventral horn of spinal cord 24 h after 90-min I/R SCI. Representative images indicate the elevation of acrolein–lysine adduct labeling in relation to neurons and its mitigation by phenelzine. N = 5 in each of the three groups. (A–C) No obvious acrolein–lysine adduct labeling was noted in sham-surgery group. (D–F) Abundant labeling of acrolein–lysine adducts was found inside ventral horn neurons at 24 h after 90-min I/R SCI (arrows). (G–I) Phenelzine treatment at a dosage of 15 mg/kg reduced the labeling of acrolein–lysine adducts. Bar=20  $\mu$ m (A–I).

acrolein-protein adduct in spinal cord tissue is 240% in mechanical injured (moderate) SCI, and 320% folds in I/ R SCI (Fig. 2) (Park et al., 2014b). Furthermore, the increase in urine 3-HPMA is 70% in I/R SCI and 80% in mechanical SCI (Fig. 3) (Zheng et al., 2013; Chen et al., 2016). In addition, in both situations, phenelzine, an acrolein scavenger, at a dosage of 15 mg/kg body weight can largely eliminate the post-injury elevation of acrolein-protein adducts locally in spinal cord tissue, and reduce acrolein metabolite (3-HPMA) by 50% systemically in urine (Figs. 2 and 3) (Chen et al., 2016). Interestingly, in both situations, phenelzine can alleviate pain like behavior by 50% when treatment started immediately following injury. These data suggest that both types of SCI share a common pathological role of acrolein, and may respond to acrolein scavenger with similar effectiveness.

In this initial study, we have examined the ischemiarelated pathologies in spinal cord up to two weeks post ischemic insult. While we have discovered both sensory- and motor-related abnormalities in the acute and subacute stage, a longer period of observation is necessary to provide more complete knowledge of the dynamics of acrolein-related secondary injuries in I/R SCI. Such knowledge will help to better understand the pathological mechanism, devise effective treatment, and determine the critical window for therapeutic intervention.

Taken together, acrolein-related pathology appears to be involved in both mechanical and non-mechanical ischemic injury of spinal cord. These results support the notion that acrolein-mediated pathology is a secondary injury mechanism common to both mechanical and ischemic SCI (Wood et al., 2006; Shi et al., 2011a; Park et al., 2014a,b; Chen et al., 2016). These findings have several points of significance. First, it supports the hypothesis that acrolein-mediated pathology is a common injury mechanism in multiple types of CNS injury, including both mechanical, such as mechanical TBI and SCI, and non-mechanical insults, such as ischemic SCI and stoke. Secondary, since the secondary injury of CNS trauma shares many similar iniury mechanisms with chronic neurodegenerative disease, particularly oxidative stress and inflammations. it supports the notion that aldehyderelated injuries is also an important factors in many chronic neurodegenerative diseases, such as Alzheimer's diseases (Calingasan et al., 1999; Lovell et al., 2001), Parkinson's diseases (Shamoto-Nagai et al., 2007; Wang et al., 2017; Ambaw et al., 2018), and multiple sclerosis (Leung et al., 2011). In light of the ample existing evidence, it seems to signify a wide-spread involvement of aldehvde in CNS trauma and diseases (Stevens and Maier, 2008; Hamann and Shi, 2009; Shi et al., 2011a,b;

Tully and Shi, 2013; Park et al., 2014a; Shi et al., 2015; Yan et al., 2016; Ambaw et al., 2018). Thirdly, because of the commonality of acrolein involvement, anti-acrolein treatment may offer beneficial effect for many other CNS disorders. As such, acrolein-suppressing strategy may not only benefit SCI patients (Hamann and Shi, 2009; Park et al., 2014a), but will likely be applied to treat other disorders where aldehyde has been be implicated, such as traumatic brain injury (Walls et al., 2016), diabetic nerve damage (Daimon et al., 2003; Feroe et al., 2016), multiple sclerosis (Leung et al., 2011), Alzheimer's and Parkinson's disease (Calingasan et al., 1999; Lovell et al., 2001; Ambaw et al., 2018). Therefore, reducing acrolein-mediated neuronal abnormality could have a broad impact on human health.

In the current study, acrolein suppression not only reduced neuropathic pain-like behavior, but also led to the reduction in neuronal loss in spinal cord following I/ R SCI (Figs. 1 and 6). This is in good agreement with reported studies from others and our own lab to attribute acrolein as a neuronal cytotoxic compound (Uchida et al., 1998; Shi et al., 2002, 2011b; Luo and Shi, 2004, 2005; Luo et al., 2005; Liu-Snyder et al., 2006a,b; Wood et al., 2006; Tian and Shi, 2017). For example, incubation of PC12 cells with acrolein *in vitro* induced significant cell death (Luo et al., 2005; Tian and Shi, 2017). In addition, treatment with nucleophilic acrolein scavenging drugs improved cell viability following acrolein incubation (Luo et al., 2005; Tian and Shi, 2017). This has been further corroborated by increased



Fig. 6. A decreased number of motor neurons two weeks after 90min I/R SCI and reduction in motor neuron loss in phenelzine-treated group. (A) Representative image from sham-surgery group; normal motor neurons had abundant Nissl granules and the cell nucleus was round with clearly visible nucleoli. (B) 90-min I/R SCI group and (C) 90-min I/R SCI + phenelzine group: normal motor neurons were rare after I/R SCI but phenelzine treatment significantly mitigated the loss of motor neurons. (D) Quantification analysis indicated the total number of motor neurons was reduced in 90-min I/R SCI when compared to control. In addition, phenelzine treatment resulted in a higher number of normal motor neurons than in the 90-min I/R SCI group. However, the number of motor neurons in 90-min I/R SCI and phenelzine treatment group was still lower than that of sham-surgery group. (\*\*P < 0.01, \*\*\*P < 0.005), Bar = 20 µm (A–C). All data were expressed as Mean  $\pm$  SEM and N = 4 for each group. A one-way ANOVA and Tukey's test.

axolemmal permeability in *ex vivo* spinal cord tissue following acrolein exposure (Luo and Shi, 2004) and by functional improvement and reduction in protein carbonylation in scavenger-treated animals following contusive SCI (Park et al., 2014b; Chen et al., 2016). Since acrolein and other reactive aldehydes can enter a selfregenerating cycle when interacting with the polyunsaturated fatty acids of plasma membranes (Esterbauer et al., 1991) and known to directly damage mitochondria (Luo and Shi, 2005), cellular membrane disruption (Shi et al., 2002) and mitochondrial impairment (Luo and Shi, 2004; Hill et al., 2017) may be the primary mechanisms of acrolein induced cell death in I/R SCI.

Due to well-established neuronal cytotoxicity of acrolein, it is perhaps not surprising that anti-acrolein treatment could confer neuroprotection in addition to its analgesic effect, seen in this and other studies. Specifically, in the current study, we have found that the treatment of acrolein scavengers not only reduced acrolein levels (Figs. 2, 3, and 5), but also partially prevented the loss of motor neurons following I/R SCI (Fig. 6). These findings build on our previous studies by confirming that a metabolic insult alone is adequate to induce a level of acrolein production which is not only capable of inducing hypersensitivity, but also causes

motor neuron death. This result is consistent with our previous report where injection of acrolein into healthy rats caused the reduction in motor neurons in rats (Due et al., 2014; Gianaris et al., 2016). In addition, multiple prior studies also demonstrated that anti-acrolein therapy could enhance motor recovery in SCI (Park et al., 2014a, b; Chen et al., 2016). Taken together, acrolein toxicity involves both sensory and motor function and acrolein scavengers could offer symptom relief, and preserve neuronal tissue in both sensory and motor system.

Consistent with multiple prior studies, the current study further strengthens the notion that targeting aldehydes is perhaps a more effective strategy to battle oxidative stress than solely scavenging reactive oxygen species (ROS) (Hamann and Shi, 2009; Shi et al., 2011a). For example, while oxidative stress has been suggested to be a significant contributor to cellular damage following both contusive and ischemic injury (Lukacova et al., 1996; Wood et al., 2006; Hamann and Shi, 2009; Park et al., 2014b); directly targeting reactive oxygen species remains challenging due to extremely limited half-lives of ROS (Uchida et al., 1998; Shi et al., 2011a). Secondary products of oxidative stress such as acrolein appear to be more suitable therapeutic targets due to more extended lives (Esterbauer et al., 1991).

While acrolein is likely an effective therapeutic target to reduce oxidative stress and acrolein scavengers an effective method of reducing acrolein, the practical utility of acrolein scavenger remains to be an active research area (Hamann and Shi, 2009; Shi et al., 2011a; Park et al., 2014a). The use of FDA approved pharmaceuticals such as hydralazine and phenelzine not only provides initial proof of principle data on the benefit of anti-acrolein therapy in animal research, but also possesses translational power due to established pharmacokinetic and toxicological data based on decades of clinical usage (Baker et al., 1992; Burcham et al., 2000, 2002; Kaminskas et al., 2004; Hamann and Shi, 2009; Shi et al., 2011a; Park et al., 2014a,b; Chen et al., 2016). In the current study, we have shown that treatment of phenelzine attenuated the elevation of acrolein, TRPA1 expression, motor neuron death, and hyperalgesia following I/R SCI. Phenelzine, a hydrazine-based monoamine oxidase inhibitor, acts as an acrolein scavenger via formation of an imine adduct (Wood et al., 2006). In our previous studies, we have shown that phenelzine effectively preserved nervous tissue, promoted motor recovery, and mitigated pain-like behaviors after contusive SCI (Chen et al., 2016). We have also demonstrated that hydralazine, another hydrazide pharmaceutical, can provide similar neuroprotection and analgesia after contusive SCI (Due et al., 2014; Park et al., 2014b). Given that the presence of nucleophilic carbonyl scavenging groups is the primary commonality between these two compounds, we consider acrolein scavenging to be a significant contributor to their neuroprotective and analgesic effects. Because of the combination of neuroprotection and analgesia afforded by acrolein scavenging therapies, we expect that acrolein scavenging may be a promising therapeutic or preventative strategy for I/R SCI-related complications following thoracoabdominal aortic surgery.

However, despite the effectiveness of the existing acrolein scavenging in animal studies, both phenelzine and hydralazine can present significant clinical challenges in certain medical conditions. For example, hydralazine has antihypertensive effects which may limit its application in patients whose blood pressure needs to be maintained at a certain level (Khan, 1953; Pandit, 1984). However, such hypotensive effect of hydralazine may be unlikely when scavenging acrolein, since it has been demonstrated that hydralazine at the level that can effectively lower the acrolein, caused no significant change in blood pressure in rodents (Leung et al., 2011; Zheng et al., 2013). Phenelzine, on the other hand, inhibits hepatic metabolism of tyramine which may result in hypertensive crisis (Baker et al., 1992; Yu, 1994). Therefore, close monitoring of the blood pressure for SCI animals that receive these two scavengers may still be warranted as a result of possible variations in responding to hydralazine or phenelzine treatment. Taken together, while both scavengers are effective and safe to reduce acrolein in rats, its clinical value as a safe effective acrolein scavenger remained to be confirmed in future clinical trials. As such, identification of effective acrolein scavenging agents with minimal side effects will be critical to translate this strategy from a laboratory discovery to clinical usage.

Although we have provided strong evidence supporting a significant pathological role of acrolein in I/ R SCI, aldehyde toxicity is likely not the only key factor in the post I/R SCI secondary injury cascades. This conjecture is consistent with the findings from the current and other studies. For example, while the accumulation of acrolein-lysine adducts in the spinal cord tissue was largely suppressed by phenelzine, the mitigation of pain like behavior and the loss of motor neurons are only partial (Figs. 1, 2 and 6). One of the possible explanations is that other factors are responsible for motor neuron death and hypersensitivity post I/R SCI. This is in agreement with the commonly believed notion that a variety of interconnected factors are involved in the post-SCI secondary injuries. For instance, there is strong evidence suggesting that peroxynitrite, a potent reactive nitrogen species, is one of the key culprits in oxidative stress, and plays a critical role in triggering secondary cell death and inflammation after SCI (Xiong et al., 2007; Xiong and Hall, 2009; Yu et al., 2009). Furthermore, peroxynitrite has also been specifically linked to post-SCI aldehyde generation and hypersensitivity (Bao and Liu, 2003; Carrico et al., 2009; Janes et al., 2012; Little et al., 2012). Given the complexities that surround the cell survival and function dysfunction in SCI, it is likely that future successful pharmacological intervention may need to take multiple factors into consideration. As such, a combination therapy is likely to be more successful than any single strategy to enhance function recovery to overcome secondary degenerative cascades.

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### DISCLOSURE STATEMENT

Riyi Shi is the co-founder of Neuro Vigor, a start-up company with business interests of developing effective therapies for CNS neurodegenerative diseases and trauma.

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