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Exogenous Acrolein intensifies sensory hypersensitivity after spinal cord injury in rat



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ABSTRACT

Acrolein, an $\alpha_{4}\beta$ -unsaturated aldehyde associated with oxidative stress, is also a major toxic component of tobacco cigarette smoke, which has been reported in the clinic to coincide with the exacerbation of neuropathic pain after SCI. Previous reports have shown that acrolein involvement in spinal cord injury (SCI) is crucial to the development and persistence of neuropathic pain. Through the activation and upregulation of the transient receptor protein ankyrin-1 (TRPA1) cation channel, acrolein is capable of sensitizing the central nervous system in the acute and chronic stages of SCI. Here, we report that the acute or delayed nasal exposure of acrolein, apart from cigarette smoke but at concentrations similar to that found in cigarette smoke, resulted in increased neuropathic pain behaviors in a rat model of contusion SCI. We also found that this hyperalgesia occurred concurrently with an augmentation in systemic acrolein, detected by an acrolein-glutathione metabolite in the urine. The application of an acrolein scavenger, phenelzine, was shown to reduce the hyperalgesic effect of acrolein inhalation. The previously determined ability of acrolein to bind to and activate the TRPA1 channel and elicit algesic responses may be a mechanism of the phenomenon seen in this study. Upon the exposure to actual cigarette smoke after SCI, intensified neuropathic pain behaviors were also observed and persisted for at least 1 week after the cessation of the exposure period. Taken together, these results indicate that cigarette smoke, through mechanisms involving acrolein, poses a threat to the vulnerable CNS after SCI and can contribute to neuropathic pain. This investigation also provides further evidence for the potential utility of acrolein scavengers as a therapeutic strategy in SCI-resultant neuropathic pain.

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Introduction

Chronic neuropathic pain (NP), which can persist for decades postinjury, drastically impairs the quality of life for spinal cord injury (SCI) victims beyond paralysis, frequently resulting in depression or even suicide [1]. NP is defined as pain due to a lesion or disease of the nervous system, which activates peripheral or central mechanisms of pain [2]. In practice, NP can be exhibited as hyperalgesia, an increased response to a noxious stimulus, or allodynia, a painful response to a normally non-painful stimulus. NP can develop at any level (above-, below- or at-level) relative to the SCI and can increase in intensity over time [3].

A myriad of biochemical changes, termed secondary injury, result in the complex cellular alterations that are observed after SCI. Cellular membrane disruption and resulting ionic concentration changes trigger the activation of inflammation pathways and the release of chemokines and cytokines [4–8]. The accumulation of reactive oxygen species facilitates demyelination through lipid peroxidation, the byproducts of which further perpetuate the toxic production of the overabundant ROS and reactive aldehydes [7–10]. In turn, the regulation of many neurotransmitters, neurotrophic factors, ion channels, and receptors is altered, resulting in both motor and sensory dysfunction [11–17]. The multifarious nature of this sequalae of SCI is the major reason that there has been difficulty in developing treatment strategies for post-SCI NP.

Despite decades of effort, this devastating condition remains one of most challenging medical problems with no satisfactory treatment available [18]. Some targeting strategies include inhibiting pain-transmitting neurons [10,19,20], activating inhibiting interneurons [4,12,19], or reducing the toxic inflammatory environment of injury [10,15,16,21], but few of these types of studies show satisfactory results when translated from animal models into clinical trials. Antiepileptics (pregabalin/gabapentin) have been widely used in preclinical and clinical studies to combat post-SCI NP [22–27]. These drugs have had moderate success in reducing NP compared to a placebo [26,27], but they also come with significant adverse side effects [3,27]. Another group of drugs, antidepressants (amitriptyline and lithium) have also been utilized in reducing NP, but these come with significant adverse side effects that have been well documented in the mental health treatment

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community [26,28,29]. The administration of opioids has also been investigated for NP treatment, resulting in limited success and the concern of drug dependency and the development of resistance to the drugs [30,31]. Transcutaneous electrical nerve stimulation (TENS) has been investigated as a way to activate inhibitory interneurons to diminish the transmission of pain signals, resulting in conflicting outcomes [32,33]. While these individual efforts have not resulted in a "one-size fits all" treatment modality for those suffering from post-SCI NP, the results have indicated that it is a complex arena for the identification safe and effective treatment development [17].

A fundamental goal of NP research in general is to identify molecular pathways that contribute to sensitization or excitation of nociceptive neurons and ultimately elucidate the mechanisms of neuropathic pain syndromes which can steer and spur effective analgesic therapies and preventive measures. While the exact etiology of NP remains unknown and much of the attention has been centered on endogenous causes, growing evidence indicates that environmental components may also be important pain-triggering factors, offering novel opportunities to elucidate the mechanisms of NP and devise possible measures to treat or even prevent post-SCI pain [34].

Recent evidence has suggested the role of unsaturated aldehydes, especially acrolein, in sensory dysfunction after SCI. Acrolein, an α , β -unsaturated aldehyde associated with oxidative stress, has been identified as both a product and initiator of lipid peroxidation, and therefore a key perpetuator of oxidative stress, a hallmark of post-SCI secondary injury [7-9,15,35-39]. Relevant to this study, acrolein has been shown as a significant contributor of sensory hypersensitivity post-SCI [14-16,21]. Acrolein is known to directly bind to and activate the transient receptor potential ankyrin-1 (TRPA1) ion channel, eliciting calcitonin-gene related peptide (CGRP)-dependent pathways leading to pain [40-42]. In addition, acrolein alone, in the absence of injury, has been shown to be able to upregulate the expression of TRPA1, which has also been demonstrated following SCI [14,15,21]. Furthermore, as a pro-inflammatory neurotoxin, acrolein may further intensify pain by stimulating the production of pro-nociceptive cytokines [43-47]. Therefore, these synergistic effects may enable acrolein to act as an important algesic factor post-SCI. Such supposition is further ascertained by the following experimental findings: acrolein is elevated post-SCI, lowering acrolein leads to a reduced level of post-SCI pain, and the injection of acrolein to rat spinal cord mimics elevated pain-like behavior seen post-SCI [14-16.21.48.49].

Tobacco cigarette smoke, an environmental pollutant with exposure occurring through primary and secondary means, is suspected of triggering post-SCI NP as evidenced by a recent clinical observation [50]. Yet the critical component linking these two events remains elusive. Interestingly, acrolein has been identified as a major emission product of tobacco smoke which can accumulate in smokers [51-53]. Recently, we have shown that acrolein concentrations are elevated significantly in both spinal cord and in urine in a rodent model following two weeks of inhalation of acrolein at a concentration similar to that in cigarette smoke [54]. Therefore, it is possible that acrolein is a key culprit mediating smoke-triggered pain, a hypothesis we intended to test in this study. Using a well-established rat contusion SCI model, coupled with an existing acrolein and cigarette inhalation system, we have found that acrolein inhalation was indeed capable of exacerbating post-SCI pain-like behavior accompanied by a corresponding increase of acrolein in urine, which was mimicked by cigarette smoke. Furthermore, acrolein inhalation-induced sensory hypersensitivity can be partially mitigated by the systemic application of phenelzine, an acrolein scavenger known to reduce acrolein levels in rats [15]. Our data suggests that exogenous acrolein, when absorbed through nasal exposure such as in the event of cigarette smoking, could play a crucial role in triggering or worsening neuropathic pain-like behavior in rats with contusion SCI. This study contributes further to the growing body of work regarding the complex mechanisms behind the development of neuropathic pain.

Methods and Materials.

Animals.

Male Sprague-Dawley rats, 200-250 g (Harlan Labs, Indianapolis, IN, USA), were housed and cared for in accordance with the Purdue University Animal Care and Use Committee Guidelines (protocol: 1,111,000,095). Animals were given one week for acclimation before any procedures occurred.

Spinal cord contusion injury.

A moderate spinal cord contusion injury was performed aseptically and as previously described [16,21]. Briefly, after animals were anesthetized using a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) via an intraperitoneal injection, a laminectomy was performed at the T-10 level, exposing the spinal cord with the dura matter still intact. The contusion injury was induced using a New York University (NYU)-style impactor, with a 10 g weight. Moderate injuries were performed with a weight drop from 25 mm. Animals were allowed to recover on a heating pad and were given subcutaneous injections of saline (3 mL) to prevent dehydration. No analgesic treatment was administered in the acute phase of the injury. If a rat showed signs of excessive pain, warranting the use of an analgesic, it was excluded from the study. Manual bladder expression was performed twice daily until normal bladder function was regained by the animal. All rats regained a bladder reflex by the 4th day after the injury.

Mechanical paw withdrawal threshold.

Mechanical hyperalgesia was quantified using von Frey filaments with a 50% threshold determined by the up-down method [55]. In short, animals were placed in a transparent box on top of a metal mesh and were allowed 10 min to acclimate prior to testing. Von Frey filaments of varying sizes (0.06–15.0 g, Stoelting, Wood Dale, IL, USA) were applied with sufficient bending force to the plantar surface of each hind paw for a period of 3–5 s. Withdrawing, licking, or biting were considered positive responses for pain. The animals were allowed at least 60 s between each stimuli and were not tested until 14 days after the contusion SCI to allow for enough motor function recovery to identify paw withdrawals. Percent changes in mechanical paw withdrawal levels for a specific day were calculated by taking the difference in the score of that day from the average of 3 baseline (days 14–18, post-SCI) pain scores and dividing it by that average baseline. Percent changes were calculated for each animal and then the mean was taken.

Urine collection and analysis.

Levels of the acrolein-glutathione metabolite, 3-hydroxypropyl mercapturic acid (3-HPMA), were detected in the urine of animals exposed to acrolein or an air-only control before or after SCI. The urine of animals was collected before inhalation and at one week intervals during and after the inhalation period using standard metabolic cages. Food and water were supplied during the collection period, but were carefully separated to prevent any dilution of the urine. The urine was analyzed by previously described methods using liquid chromatography and tandem mass spectrometry after solid-phase extraction [56]. The levels of 3-HPMA were normalized to creatinine concentrations in each urine sample.

Inhalation models.

The inhalation of acrolein was facilitated by the mixing of compressed air with compressed acrolein in a ratio to form a final concentration of 1.5 ppm acrolein [57]. This mixture was fed directly to a custom-built translucent $6"\times12"\times24"$ air-tight chamber, where the animals were placed, as shown in Fig. 1. A maximum of 8 rats were placed in the chamber at one time. A charcoal filter was fitted to the outlet of the chamber and the chamber was housed in a fume hood. Air-only inhalation was used as a control, utilizing only the compressed air at the same total volumetric flow rate as the acrolein inhalation model.

For tobacco cigarette smoke inhalation, reference cigarettes (3R4F, University of Kentucky Reference Cigarette Program, KY, USA) were kept frozen until use. The cigarettes were conditioned at room temperature for 30 min prior to ignition. The cigarettes were coupled to a vacuum pump that was in line with the same air-tight chamber that was



Fig. 1. Inhalation System. (A) Acrolein inhalation setup. Animals were placed in an air tight chamber inside a fume hood. A mixture of acrolein and air, forming a concentration of 1.5 ppm, was fed into the chamber. Control experiments were done using air only. (B) Tobacco cigarette smoke inhalation setup. A pump was used to draw air through a lit cigarette and the smoke was directed into the air tight chamber at the same flow rate that the acrolein mixture was pumped into the chamber.

used for acrolein and air inhalation. Cigarette smoke was pumped using the same total volumetric flow rate as acrolein inhalation. Cigarette smoke inhalation occurred at the same frequency as acrolein inhalation.

Inhalation sessions occurred twice daily for 30 min over the course of 14 days. Inhalation sessions began on the day of injury or 22 days after injury, allowing for a baseline mechanical paw withdrawal threshold to be established beginning 14 days after injury.

Phenelzine treatment.

Phenelzine sulfate (Sigma, St. Louis, MO, USA) was dissolved in phosphate buffer solution before being sterilized and filtered. For animals in the phenelzine treatment group, dosages of 15 mg/kg were administered via an intraperitoneal injection once daily beginning 22 days after injury, on the first day of acrolein inhalation, and continued until the final day of inhalation. The dose of 15 mg/kg was determined to be a safe yet effective dose to reduce neuropathic pain-like behavior in previous work [15]. Injections occurred before the first session of inhalation.

Dorsal root ganglia and spinal cord isolation.

After deep anesthesia, using a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture, animals were perfused with oxygenated Kreb's solution (124 mM NaCl, 2 mM KCl, 1.24 mM KH₂PO₄. 26 mM NaHCO₃,10 mM ascorbic acid, 1.3 mM MgSO₄, 1.2 mM CaCl₂, and 10 mM glucose). The vertebral column was removed and the spinal cord was carefully separated from the vertebrae using a dorsal laminectomy. The dorsal root ganglia (DRG) from L1-L6 were carefully removed and preserved for biochemical investigation.

RT-PCR for TRPA1 quantification.

The previously collected DRG and spinal cord tissue was homogenized with Trizol (Sigma, St. Louis, MO). Chloroform, isopropyl alcohol, and linear polyacrylamide were used to extract and precipitate the RNA. Thirty microliters of RNAase free water was added to each sample and the RNA concentration was quantified using a NanoDrop 2000c (Thermo Scientific, Waltham, MA). A mass of 1.25 μ g of RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio—Rad, Hercules, CA). The TRPA1 primers were 5'-TCCTATACT GGAAGCAGCGA-3', and 5'-CTCCTGATTGCCATCGACT-3'. Primers for 18S were used as an internal control: 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'. The magnitude of fluorescence of iQ-SYBR Green Supermix (Bio-rad, Hercules, CA) was used to determine the PCR products and was normalized by the fluorescence of 18S. The $\Delta\Delta$ CT method was used to calculate the final relative gene expression of TRPA1 [58].

Statistics

Data is presented as the mean +/- the standard error of the mean. Student's t-tests and one-way ANOVA's with post-hoc analysis were utilized to examine statistical significance of the data. For all data, a *p*-value <0.05 was considered significant.

Results

Moderate contusion SCI results in neuropathic pain behavior.

Previously, it has been shown that moderate contusion SCI induces neuropathic pain behavior measured using von Frey filaments [21]. Here it is again confirmed, in Fig. 2, that moderate contusion injury, using an NYU-style impactor, results in a significant reduction in mechanical paw withdrawal thresholds. Specifically, thresholds dropped from 12.65 \pm 0.94 g to 2.57 \pm 0.79 g two weeks after injury, indicating an augmented neuropathic pain behavior.

Short term inhalation of acrolein prior to injury has no effect on postinjury mechanical pain thresholds.

In order to examine if an acute pre-disposition of acrolein inhalation could affect neuropathic pain after SCI, animals were exposed to acrolein or air for 14 days prior to the induction of the SCI. The paw withdrawal threshold was assessed on day 7 of the inhalation period and no significant changes were found compared to baseline or pre-inhalation (Fig. 3A). Also, there was no significant difference in the change in paw withdrawal threshold stimulated by von Frey filaments between the air inhalation and acrolein inhalation groups when examined 2 weeks post-SCI.

Decrease in paw withdrawal thresholds after acute post-SCI acrolein inhalation.

In this group of experiments, immediately after SCI, animals were subject to acrolein inhalation for 14 days to investigate any effects of increased acrolein exposure in the acute stage of SCI. Acrolein inhalation resulted in the significant decrease in paw withdrawal threshold 14 days after the injury compared to a group which did not receive acrolein inhalation immediately after injury, as seen in Fig. 3B. The postinjury day 14 paw withdrawal threshold for the acute acrolein



Fig. 2. Mechanical hyperalgesia induced by contusion SCI. Post-injury paw withdrawal thresholds were significantly lowered two weeks after a moderate contusion SCI (* P < 0.0001). Data is represented as mean +/- SEM. Student's *t*-test was used for statistical analysis. N = 9 for both groups.



Fig. 3. Changes in mechanical paw withdrawal threshold upon exposure to acrolein or air prior to, or acutely after SCL A). Paw withdrawal thresholds did not significantly change from baseline levels (pre-inhalation) on day 7 of the inhalation period before injury. There was also no difference in the percent change of mechanical paw withdrawal thresholds two weeks post-SCI between the air and acrolein inhalation groups. However, the percent decrease in paw withdrawal threshold after SCI compared to baseline levels is significant for both acrolein and air inhalation groups (* P < 0.005). All data are represented as mean +/- SEM. A multivariate test was used for statistical analysis. N = 4 for all data. B). Upon inhalation exposure to acrolein over the first 14 days following SCI, mechanical paw withdrawal thresholds were significantly lower than that of those which were not exposed to acrolein when examined at post-SCI days 14 (last day of inhalation), 16, and 18 (* P < 0.0001). All data are represented as mean +/- SEM. A one-way ANOVA was used for statistical analysis. N = 5-9 for the acrolein inhalation group.

inhalation group was 0.29 ± 0.07 g while the injury only group had paw withdrawal thresholds of 2.25 ± 1.00 g. Similar significant differences in paw withdrawal threshold were seen between the two groups on post-injury days 16 and 18.

Decrease in paw withdrawal thresholds after delayed post-SCI acrolein inhalation.

The exposure to acrolein through inhalation beginning three weeks after spinal cord injury also resulted in observable decreases in paw withdrawal threshold. Fig. 4 shows the percent change in threshold compared to the post-injury baseline threshold. Significant decreases in threshold were seen on day 7 and 13 of inhalation (day 29 and day 35 post-SCI). Specifically, the decrease in paw withdrawal threshold was $-35.5\% \pm 13.3$ on day 7 and $-51.1\% \pm 7.1$ on day 13. By day 19, 6 days after the end of the inhalation period, the difference was no longer significant. Upon exposure to only air, there were no significant changes in paw withdrawal thresholds over the course of the inhalation period.

Phenelzine treatment reduces the reduction of paw withdrawal threshold during delayed post-SCI acrolein inhalation.

In previous studies, it has been shown that acrolein scavengers are capable of attenuating mechanical hypersensitivity after SCI [14–16,21]. To this end, a known effective acrolein scavenger, phenelzine, was administered during the inhalation period to determine its effect on hyperreflexia behaviors during acrolein inhalation after SCI [15]. Fig. 4 shows the significant difference in the change in paw withdrawal threshold from the baseline during the inhalation period. Specifically, percent changes in threshold with phenelzine treatment during acrolein inhalation were $-19.1\% \pm 6.7$ on day 1, $-14.4\% \pm 9.9$ on day 7, and -16.6 ± 11.5 on day 13. There was no difference



Fig. 4. Changes in paw withdrawal threshold over the course of a 14 day delayed inhalation period and its attenuation with the administration of the acrolein scavenger, phenelzine. Inhalation began 22 days after SCI. Inhalation exposure to acrolein resulted in significant changes from baseline mechanical paw withdrawal thresholds on days 7 and 13 of the inhalation period (* P < 0.05). There was no difference in the baseline mechanical paw withdrawal thresholds (day 0) among three groups: air inhalation, acrolein inhalation + phenelzine. Differences in the percent change of paw withdrawal threshold between acrolein inhalation and air inhalation were significant on Day 1, 7, and 13 of the inhalation period, and differences between the acrolein inhalation and the phenelzine treated group were significant on day 13 of inhalation (# P < 0.05). All data are represented as the mean +/- SEM. A one-way ANOVA was used for statistical analysis using an LSD post hoc analysis. N = 4-10 for all groups.

between the acrolein inhalation and the acrolein inhalation plus phenelzine group on days 1 and 7. However, the phenelzine-treated group significantly attenuated the reduction of paw withdrawal threshold at day 13, indicating that phenelzine treatment is capable of mitigating acrolein inhalation-induced exacerbation of post-SCI sensory hypersensitivity.

Increase in 3-HPMA due to acrolein inhalation.

The acrolein-glutathione metabolite, 3-HPMA, has been quantified in urine in recent studies to estimate endogenous systemic acrolein levels [14,15,56]. It has been shown that endogenous acrolein levels are increased in the days to weeks after contusion SCI [14,15,56]. We were interested in whether acrolein inhalation would further elevate the post-SCI systemic acrolein levels by monitoring urine 3-HPMA. Fig. 5 depicts the 3-HPMA/creatinine levels, normalized to 14 day post-SCI level (week 0, prior to inhalation). During week 1 of inhalation, the level of 3-HPMA was significantly increased, with a normalized value of 1.7 ± 0.3 . Fig. 5 also shows that air inhalation after SCI resulted



Fig. 5. Quantification of the acrolein-metabolite, 3-HPMA, in the urine of animals during delayed post-SCI inhalation of air or acrolein, normalized to post-SCI (week 0, pre-inhalation) baseline. After one week of acrolein inhalation, 3-HPMA levels were significantly increased (# P < 0.05, comparison to pre-inhalation; * P < 0.05, comparison to air inhalation group). All data are expressed mean +/- SEM. A one way ANOVA was performed with an LSD post-hoc. N = 8 for all groups.

in no statistical change of the 3-HPMA levels over the course of inhalation.

Cigarette smoke inhalation exacerbates sensory hypersensitivity after SCI.

The inhalation of actual tobacco cigarette smoke over the course of 14 days (delayed application) resulted in significant decreases in paw withdrawal thresholds when compared to air inhalation on day 1, 7, and 13 of the inhalation period, as shown in Fig. 6. The magnitude of these decreases were $-58.2 \pm 12.1\%$, $-63.8 \pm 4.8\%$, and $-75.0 \pm 2.4\%$, respectively which is comparable to that caused by acrolein inhalation on days 7 and 13. Note that acrolein inhalation did not cause significant paw withdraw threshold changes on day 1 of inhalation (Fig. 4). Further, unlike acrolein inhalation, the decrease in paw withdraw threshold resulting from cigarette smoke inhalation did not recover by day 19, six days after the termination of inhalation.

Discussion

In our previous reports, we have shown that acrolein, when generated endogenously, is critically involved in the neurodegeneration and functional deficits after SCI [7,8,14–16,21]. Specifically, we have shown that acrolein plays an important role in post-SCI sensory hypersensitivity [14,15,21]. In this study, we demonstrate that when either acrolein alone or cigarette smoke, which is known to contain acrolein, were inhaled post-SCI, rats displayed further exacerbation of hypersensitivity. Such nasal intake of acrolein can further increase pain sensation beyond the already elevated post-SCI pain-like behavior following both the acute and delayed exposure. Therefore, acrolein is capable of being a significant contributor to post-SCI sensory hypersensitivity through both endogenous and exogenous sources. To our knowledge, this is also the first report of influences of exogenous acrolein on post-SCI sensory hypersensitivity in an animal model.

Previously, it was reported that people with SCI-induced chronic neuropathic pain have experienced heightened pain sensitivity when smoking tobacco cigarettes, and less pain following the termination of smoking [50,59]. However, the molecular mechanisms of smoke-



Fig. 6. Changes in paw withdrawal threshold over the course of a 14 day delayed inhalation period with exposure to air, acrolein, or cigarette smoke. Inhalation began 22 days after SCI. Inhalation exposure to acrolein resulted in significant changes from baseline mechanical paw withdrawal thresholds on days 7 and 13 of the inhalation period (* P < 0.05). In addition, exposure to cigarette smoke resulted in significant changes in paw withdrawal thresholds on days 1, 7, and 13 of the inhalation period (* P < 0.05). No changes from baseline mechanical paw withdrawal thresholds were observed upon exposure to air. Changes in paw withdrawal levels remained significantly lower than baseline on day 19 (6 days following the cessation of inhalation) for the cigarette smoke inhalation group. Differences in the percent change of paw withdrawal threshold between the acrolein and air inhalation groups and between cigarette smoke and air inhalation groups were significant on Day 1, 7, and 13 of the inhalation period (# P < 0.05). Id data are represented as the mean +/- SEM. A one-way ANOVA was def for statistical analysis using an LSD post hoc analysis. Data for air or acrolein inhalation are the same as those used in Fig. 5, which are shown here for comparison. N = 5-10 for all groups.

induced hypersensitivity is not yet clear. We show in the current study that the inhalation of acrolein alone, apart from cigarette smoke, at a concentration similar to that emitted from cigarettes, can produce significant increases in pain-like behaviors after SCI (Figs. 3B and 4). Such acrolein-induced pain-exacerbating effects can be mimicked by the inhalation of cigarette smoke, which is known to contain a significant amount of acrolein (Fig. 6). In addition, the exacerbation of painlike behavior due to acrolein inhalation was accompanied by a further elevation of urine 3-HPMA beyond the already increased level post-SCI (Fig. 5). Furthermore, hypersensitivity resulting from acrolein inhalation can be partially mitigated by phenelzine, an effective acrolein scavenger known to be able to reduce acrolein through systemic application in rats (Fig. 6) [15]. Taken together, these data suggest that exogenous acrolein is capable of further intensifying post-SCI hypersensitivity in rat. Furthermore, acrolein is likely, at least in part, responsible for heightening sensory hypersensitivity resulting from cigarette smoke inhaled in rats post-SCI.

Despite the strong evidence that acrolein inhalation initiated post-SCI can further intensify pain-like behavior, similar treatment prior to injury did not affect the post-SCI sensory hypersensitivity (Fig. 3A). This was attempted to examine the possibility that an existing smoker could have a more severe pain-like behavior after suffering an SCI. Given that no difference in paw withdrawal threshold was observed between the acrolein and air inhalation groups when examined two weeks after injury, we concluded that a short term predisposition to acrolein inhalation alone would not adversely affect the resulting neuropathic pain behaviors after a SCI. One possible explanation for this phenomenon could be that it may require a longer period of exposure to acrolein, either alone and through cigarette smoke, to exert an influence on the post-SCI neuropathic pain.

The exposure to acrolein beginning immediately after injury was intended to assess the susceptibility of the CNS in the acute stage of the injury (Fig. 3B). Given that the acute stage of recovery for a SCI victim is often spent in the hospital where smoking cigarettes is usually not permitted, this model of inhalation may not be very clinically relevant. However, it does give an opportunity to examine if further elevation of acrolein acutely would have a longer lasting algesic effect compared to delayed acrolein inhalation, a hypothesis which remains to be tested.

In the case of the delayed post-SCI inhalation, a more clinical relevant scenario than acute post-SCI inhalation, both acrolein and cigarette inhalation produced a significant increase of mechanical hypersensitivity (Figs. 4, 6). However, there are noticeable differences among these two groups. For example, in the acrolein inhalation group, the significant elevation of pain-like behavior was observed at 7 and 13 days of inhalation period, but not 1 day after commencement. In addition, the mechanical hypersensitivity levels returned to the pre-inhalation baseline when examined 6 days following the cessation of inhalation. In the case of cigarette smoke inhalation, however, the pain-like behavior was elevated after just 1 day of inhalation and remained elevated throughout the inhalation period (Fig. 6). Furthermore, the mechanical hypersensitivity levels remained heightened for at least 6 days following the termination of inhalation. This indicates that short term nasal exposure of acrolein alone may not have lasting algesic effects. This also seems to suggest a very likely phenomenon that, in addition to acrolein, other factors within cigarette smoke may contribute synergistically to elicit pain, leading to shorter latency, and longer lasting augmentation of pain-like behavior post SCI in rats when comparing to exposure to acrolein alone. In fact, there are actually >4700 toxicants from different chemical classes existing in cigarette smoke [60,61]. It is therefore likely that other pro-algesic factors exist within the cigarette smoke. Nevertheless, the fact that acrolein is indeed sufficient to incite pain seems to suggest that acrolein is a possible major contributing factor to hyperalgesia in cigarette smoke, but may not be solely responsible for the increased pain associated with cigarette smoke.

It is interesting to note that the administration of the acrolein scavenger, phenelzine, an FDA-approved anti-depressant, provided attenuation of the exacerbated pain-like behavior elicited by post-SCI acrolein inhalation (Fig. 4). Phenelzine has been used in previous studies to combat the damage caused by acrolein in the weeks following SCI [15]. Specifically, phenelzine application could effectively lower acrolein levels both locally in the spinal cord, and systemically in urine post-SCI, and provided analgesic effects [15]. Therefore, the most likely cause of phenelzine-mediated analgesic effect in exogenous acrolein-mediated pain is through its ability to scavenge acrolein. These results further support the notion that acrolein is a key algesic factor in SCI though both endogenous and exogenous mechanisms. Despite its acrolein scavenging capability, some possible systemic effects of phenelzine need to be taken into consideration with in vivo application. For example, in addition to its ability to act as a monoamine oxidase inhibitor (MAOI), phenelzine is also known for causing severe hypertensive crises in some special circumstances [62,63]. Therefore, close monitoring of the blood pressure in animal post-SCI and human patients that receive phenelzine is warranted due to possible variations in responding to phenelzine treatment. In addition to phenelzine, other known acrolein scavengers, such as hydralazine, which possesses a similar functional group to phenelzine, may provide similar analgesic effects in cigarette smoke-induced pain through acrolein scavenging as demonstrated in previous studies [14,16,21].

Taken together, the effectiveness of phenelzine in preventing exogenous acrolein-mediated hypersensitivity further underscores the crucial role of acrolein in instigating and exacerbating post-SCI chronic neuropathic pain. But perhaps equally important, these data also suggest anti-acrolein and related aldehydes as novel strategic targets for pain treatment in post-SCI neuropathic pain. As such, this study may galvanize the interests and effort to search other effective scavengers to provide an analgesic with higher effectiveness and lower side effects.

In previous reports, it was found the TRPA1 mRNA expression was significantly increased for at least two weeks after SCI [14,15,21]. In this investigation, there were no further significant increases in TRPA1 mRNA expression during the inhalation period post-SCI (data not shown). However, nasal exposure of acrolein is capable of producing hypersensitivity after SCI. One plausible explanation for this is that the resultant elevation of pain-like behavior following acrolein exposure is mainly due to the direct binding and activation of TRPA1 by acrolein, which is a known to directly activate the TRPA1 channel. It is unknown why the expression of TRPA1 was not further augmented upon acrolein inhalation. One possibility is that it is not a linear relationship between acrolein levels and TRPA1 upregulation. Perhaps the level of TRPA1 expression is already saturated following SCI which prevented further significant augmentation upon acrolein exposure [14]. Another possibility is that there may be changes in the post-translational mechanisms of TRPA1 that induce discrepancies between the mRNA and protein levels [64]. Nevertheless, the evident elevation of TRPA1 expression post-SCI explains, at least in part, why short term acrolein exposure could significantly exacerbate post-SCI pain-like behavior, when exposure was made post-injury, but not pre-injury. It is interesting to note that TRPA1 channel upregulation has been linked to transition of acute to chronic pain stage [65]. Therefore, we suggest that short term cigarette smoke may likely cause only temporally heightened pain-like behavior. This is consistent with prior clinical observations that short term smoking-induced pain augmentation in SCI victims could subside when smoking is ceased [50]. However, it is likely, although remained to be verified, that longer cigarette smoking durations could lengthen the time for the smoking-related pain to diminish following the cessation of smoke.

Conclusions

The results of this work further solidify, beyond current understanding, the ability of acrolein to cause post-SCI sensory hypersensitivity from both endogenous and exogenous sources. Further, this investigation also demonstrated for the first time, that the inhalation of actual cigarette smoke leads to intensified neuropathic pain behaviors in an animal investigation, a phenomenon mirrored by clinical observations. Finally, this report has also demonstrated that phenelzine, an acrolein scavenger, can attenuate the augmented pain behavior by acrolein-inhalation, suggesting anti-acrolein and related aldehydes to be a novel strategy to combat post-SCI neuropathic pain, a devastating condition with no satisfactory treatment. Since acrolein is also a known pro-inflammatory aldehyde, it is possible that anti-acrolein treatment could also lead to the attenuation of inflammation, a phenomenon could be examined through immunohistochemical analysis in the future follow up study. This study adds to the growing body of work on the mechanisms of development of post-SCI NP, which could help spur novel treatment modalities that utilize a multifaceted approach. Additionally, since acrolein-mediated neuropathic pain could potentially play a role in many illnesses where acrolein and neuropathic pain have been implicated, the knowledge generated from the current investigation may have wide applications for many other pathologies conditions includes visceral pain [66], venous pain [67], migraine [68], pain in multiple sclerosis [69–71], and pain in diabetes [72,73]. Therefore, this study may have a significant impact on the understanding of the mechanisms and management of various pain-related conditions far beyond SCI.

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Conflict of interest disclosure.

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

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