Critical role of mitochondrial aldehyde dehydrogenase 2 in acrolein sequestering in rat spinal cord injury

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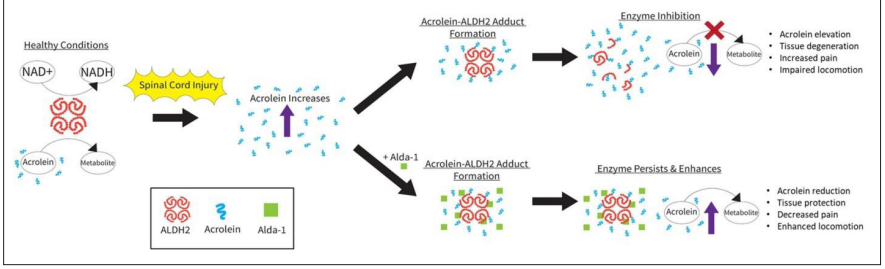
From the Contents

Graphical Abstract *Aldehyde dehydrogenase 2 in spinal cord injury*



Abstract

Lipid peroxidation-derived aldehydes, such as acrolein, the most reactive aldehyde, have emerged as key culprits in sustaining post-spinal cord injury (SCI) secondary pathologies leading to functional loss. Strong evidence suggests that mitochondrial aldehyde dehydrogenase-2 (ALDH2), a key oxidoreductase and powerful endogenous anti-aldehyde machinery, is likely important for protecting neurons from aldehydes-mediated degeneration. Using a rat model of spinal cord contusion injury and recently discovered ALDH2 activator (Alda-1), we planned to validate the aldehyde-clearing and neuroprotective role of ALDH2. Over an acute 2 day period post injury, we found that ALDH2 expression was significantly lowered post-SCI, but not so in rats given Alda-1. This lower enzymatic expression may be linked to heightened acrolein-ALDH2 adduction, which was revealed in co-immunoprecipitation experiments. We have also found that administration of Alda-1 to SCI rats significantly lowered acrolein in the spinal cord, and reduced cyst pathology. In addition, Alda-1 treatment also resulted in significant improvement of motor function and attenuated post-SCI mechanical hypersensitivity up to 28 days post-SCI. Finally, ALDH2 was found to play a critical role in *in vitro* protection of PC12 cells from acrolein exposure. It is expected that the outcome of this study will broaden and enhance anti-aldehyde strategies in combating post-SCI neurodegeneration and potentially bring treatment to millions of SCI victims. All animal work was approved by Purdue Animal Care and Use Committee (approval No. 1111000095) on January 1, 2021.

Key Words: acrolein; acrolein-lysine adduct; alda-1; enzymatic catalyst; lipid peroxidation; mitochondrial aldehyde dehydrogenase-2; neurotrauma; oxidative stress; reactive aldehydes; spinal cord contusion

Chinese Library Classification No. R453; R364; R741

Introduction

It is well-established that the most severe damage resulting from spinal cord injury (SCI) does not occur immediately after physical impact. Rather, mechanical insult induces a cascade of secondary biochemical reactions that exacerbate the initial effects of trauma and spread throughout the cord. Oxidative stress and lipid peroxidation-derived aldehydes have emerged as key culprits in sustaining such secondary injury, and contributing significantly to the pathological outcomes (Hamann et al., 2008a, b; Hamann and Shi, 2009; Due et al., 2014; Park et al., 2014). Aldehydes, represented by acrolein as the most reactive aldehyde, are capable of directly adducting proteins, lipids, and DNA, and are known to cause significant damage to key cellular organelles (Luo and Shi, 2005; Liu-Snyder et al., 2006; Shi et al., 2011b; Wang et al., 2017).

We have provided extensive evidence that acrolein plays a particularly damaging role in perpetuating oxidative stress, causing cellular degeneration and functional loss in SCI (Hamann et al., 2008a, b; Hamann and Shi, 2009; Due et al., 2014; Park et al., 2014). We have also shown that, as both a product and catalyst for lipid peroxidation, acrolein induces a vicious cycle of oxidative stress, amplifying and continuously propagating degeneration in SCI (Hamann et al., 2008a, b; Hamann and Shi, 2009; Due et al., 2014; Park et al., 2014). Further, while acrolein reduction can effectively mitigate neuronal damage, injecting acrolein produced pathologies reflecting those observed in SCI (Gianaris et al., 2016). These studies support the notion that acrolein is not only sufficient, but necessary to instigate neurodegeneration post-SCI. As such, lowering acrolein has emerged as a novel and effective therapeutic strategy in SCI (Hamann and Shi, 2009). However, while the application of exogenous aldehyde scavengers has led to significant benefits, many have unwanted side effects as they are FDA approved for other conditions (Park et al., 2014; Chen et al., 2016). Therefore, a need remains for exploring

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alternatives with minimal side effects, before further clinical application is considered.

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) is an oxidoreductase, effective at removing aldehydes in the brain and spinal cord (Chen et al., 2014; Zambelli et al., 2014; Liu et al., 2017). Despite its well-recognized role in aldehyde removal, ALDH2 remains largely under-studied in SCI. Relevantly, recently discovered ALDH2-selective activator, Alda-1, can catalyze ALDH2 enzymatic activities by multiple folds in rodents and *in vitro* (Chen et al., 2008; Perez-Miller et al., 2010). This offers a uniquely promising opportunity to test our hypothesis, solidifying this enzyme as a therapeutic target to suppress aldehydes while also further illuminating the role of ALDH2 in SCI.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 200–240 g and aged 7–8 weeks were purchased from Envigo (Indianapolis, IN, USA). A total of 55 rats were used in this study. Rats were grouped for use in either western blot, histology, or behavioral experiments. They were kept on standard 12-hour light/dark cycles and fed with chow diet. All animal work was approved by Purdue Animal Care and Use Committee (approval No. 1111000095) on January 1, 2021.

Rats were randomly divided into the control (normal control), SCI (SCI followed by DMSO and PEG only; vehicle control), SCI + Alda-1 groups (SCI followed by Alda-1 administration).

SCI models

Rats were fully anesthetized with intraperitoneal injection of 80 mg/kg of ketamine and 10 mg/kg xylazine (VETONE, Boise, ID, USA) before spinal cord surgery. For all the surgeries, a laminectomy was first conducted at T10. Following the exposure of spinal cord tissue, rats were given a contusion injury, using a New York University (NYU) weight drop impact device at a moderate setting (height of 25 mm and weight of 10 g). Rat health was carefully monitored post-surgery. The general procedures used for this study are well-established and detailed in our prior publications (Chen et al., 2016; Kish et al., 2021).

Treatments

Aged matched healthy rats were used as controls. Alda-1 was purchased from ApexBio (Houston, TX, USA) and dissolved in 50% DMSO and 50% PEG 400 (polyethylene glycol 400) as described by others (Zambelli et al., 2014). Injury rats were given either a vehicle with no Alda-1 (DMSO and PEG 400 only; vehicle control), or Alda-1 at 15 mg/kg, administrated by intraperitoneal injection. No injection volume exceeded 0.3 mL. Alda-1 has been shown to effectively enter the central nervous system through this administration technique (Guo et al., 2013; Stachowicz et al., 2016). Treatment was administered every 2 hours post-surgery for a total of 2 doses, twice on the following day (morning and evening), and once in the morning on day 2 and once an hour before sacrifice (48 hour acute time point, 6 total doses of Alda-1). For long-term behavioral tests, chronic twice daily intraperitoneal injections may cause complications, although Alda-1 was best administered routinely (2–3 times daily) due to relatively low bioavailability (Taneja et al., 2015). To address this, injury rats were administered a high dose of Alda-1 (60 mg/kg), or vehicle, once daily for 14 days, beginning 30 minutes after surgery. Rats were subjected to behavioral tests until sacrifice on 28 days post injury. All rats were sacrificed by transcardial perfusion with chilled phosphate-buffered saline (PBS) after deep anesthesia with ketamine and xylazine as mentioned above.

Co-immunoprecipitation

Immunoprecipitation of the spinal cord for each experimental group was done according to instructions from the Pierce Classic IP Kit (Cat# 26146; Thermo Fisher Scientific, Waltham, MA, USA), and as described previously by our group (Acosta et al., 2019). In short, 2 mg/mL of T10 spinal cord region protein lysate from each sample group was pre-cleared using the control agarose resin. The lysate (2 mg protein) was incubated with 1:1000 mouse anti-ALDH2 (Cat# J3017; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:1000 rabbit anti-acrolein-lysine (Cat# ab37110; Abcam, Cambridge, MA, USA) at 4°C overnight to form the immune complex. 20 µL of Pierce A/G Agarose (Cat# 20421; Thermo Fisher Scientific) was added into the mixture to capture the immune complex and incubated at 4°C for 1 hour. The elution of the immune complex was carried out using the 2× non-reducing lane marker, sample buffer, and dithiothreitol. The collection tubes were boiled in a water bath for 5 minutes and eluates were applied to 15% polyacrylamide gels for electrophoresis followed by immunoblotting with anti-ALDH2 and imaged using Azure c300 western blot imaging system (Azure Biosystems, Dublin, CA, USA).

Western blotting

After sacrifice, spinal cord tissue at the T10 region was dissected and frozen at –80°C. Tissue was homogenized with 1× radioimmunoprecipitation assay buffer and Protease inhibitor cocktail, and then centrifuged at 11,000 × *g* for 30 minutes. Supernatant was collected, and protein concentrations were measured using the Bicinochoninic Acid protein assay kit (Pierce, Rockford, IL, USA) and SPECTRAmax plate reader (Molecular Devices, Sunnyvale, CA, USA). Thirty micrograms of protein with 20% sodium dodecyl sulfate, β-mercaptoethanol, and 2× Laemmli buffer were loaded to a 15% Tris-HCl gels and electrophoresed at 80 V for 2–3 hours. Proteins were then transferred to a nitrocellulose membrane by electro blotting on ice at 70 V for 1 hour while submerged in cold 1× transfer buffer and 20% methanol (Tris-Glycine buffer from Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 1× casein (Cat# SP-5020, Vector, Burlingame, CA, USA) at room temperature for 10 minutes, and immunolabeled overnight at 4°C with one of these primary antibodies, 1:1000 mouse anti-acrolein-lysine (Cat# ab240918, Abcam), 1:1000 rabbit anti-ALDH2 (Cat# PA5-11483, Thermo Fisher Scientific), 1:1000 rabbit anti-beta-actin (Cat# 643801, BioLegend). After overnight incubation, the membranes were further incubated with either biotinylated anti-mouse or anti-rabbit secondary antibody (Cat# BA-9200 and BA-1000, respectively; Vector) at room temperature for 45 minutes, and signal amplified with an ABC-AmP kit for 10 minutes (Cat# AK-6000, Vector). All washes were done with 1× casein. The DuoLux substrate (Cat# SK-6605, Vector) immunodetection kit was used for chemiluminescent signal acquisition and the Azure c300 western blot imaging system (Azure Biosystems, Dublin, CA, USA) was used to image the membrane. The AlphaView software (Protein Simple, San Jose, CA, USA) was used to quantify the relative signal for each band. Resulting densitometry values were normalized with beta-actin.

Immunohistochemistry

Cord tissue was collected after perfusion and immediately fixed in cold 4% formaldehyde in 1× PBS, for a maximum of 48 hours. Tissue was then cryoprotected in 30% sucrose, and frozen in OTC using acetone and dry ice slurry and sliced into transverse sections with a cryotome (Leica, Wetzlar, Germany) at 25 µm thickness. Sections were stored in 0.01% sodium azide and PBS at 4°C, until staining of T10 epicenter tissue. Staining was done using separate techniques

for revealing acrolein, or tissue structure. For acrolein, sections were immunostained with mouse anti-acrolein-lysine (Cat# ab240918, Abcam) or rabbit anti-ALDH2 (Cat# ab108306, Abcam), followed by biotinylated anti-mouse or anti-rabbit secondary antibodies (Cat# BA-9200 and BA-1000, respectively; Vector), incubated in ABC avidin/biotin complex solution (Cat# 32020, Thermo Fisher Scientific) and developed using the DAB Peroxidase (HRP) Substrate Kit, 3,3′-diaminobenzidine (Pierce™ DAB Substrate Kit). Images were taken using a standard light microscope (OLYMPUS IX51, Tokyo, Japan). For revealing tissue structure, crystal violet (Nissl) and Eosin Y counterstains were done, as previously described by our group (Gianaris et al., 2016). Quantification was done using standard techniques for area measurement on ImageJ software (NIH, USA). Cyst is expressed as a percentage of total slice area.

Locomotor function

The recovery of locomotor function was assessed using the Basso, Beattie and Bresnahan (BBB) Locomotor Rating Scale described in our previous publications (Park et al., 2014). The score is based on the locomotor ability of SCI rodent models. Briefly, the BBB scale is a 22-point scale which ranges from 0, no observable movement of hindlimb, to 21, normal movement of hindlimb. Observations were done on days 0, 2, 7, 14, 21, and 28 post injury. On days 7–28, observations were done following mechanical allodynia recordings. Rats were observed in an open field for 5 minutes after they had gently adapted to the field. Left and right hindlimb were assessed separately and final scores averaged.

Mechanical allodynia

The foot withdrawal threshold to mechanical stimuli was tested as an indicator of mechanical hyper-reflexia, according to procedures described in our previous publication (Shi et al., 2021). The test was performed weekly after injury, on days 7, 14, 21, and 28. Briefly, rats were placed on a metal mesh floor, covered by a transparent plastic box, and allowed to acclimate separately for 20 minutes before testing. 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) was applied perpendicular to the plantar surface of the hindlimb with sufficient bending force for 3–5 seconds. A rapid withdrawal of the hind-limb with or without licking and biting was recorded as a positive response. When a positive response was observed, a lower-grade filament was then applied, and in the absence of a response, the next greater filament was presented (standard "up down" method). Rats were given at least 1 minute for rest between every two stimuli. Scores (gram of force) were averaged from both left and right hindlimbs and the 50% threshold acquired using a formula based on calculations for lethal dose (LD50's) (Deuis et al., 2017).

Cell viability assay

PC12 cells (Cat# CRL-1721, RRID: CVCL_0481) were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA, USA) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, and 1% penicillin. The incubator was set at 5% CO_2 at 37°C. Culture media were changed every other day, and cells were split every week. PC12 cells were not differentiated prior to experiments.

Acrolein (Sigma-Aldrich, St. Louis, MO, USA) was prepared at 50 mM in PBS as stock solution and diluted to the final concentration (100 μM) in medium upon use. Alda-1 was dissolved at 20 mM in DMSO as stock solutions and diluted to the desired concentration in medium. ALDH2 inhibitor disulfiram (MilliporeSigma, Burlington, MA, USA) (Mays et al., 1998) was dissolved in cell medium to predetermined concentrations. PC12 cells were plated in 96-well plates. After incubating for 24 hours, acrolein (500 μM) was added into each well of acrolein group and treatment groups (disulfiram or Alda-1). Equal volumes of DMEM were added into wells of the negative control group. After incubation for 15 minutes, Alda-1 (25 μM) or disulfiram (1 or 5 μM) was added at the desired final concentrations into wells and equal volume of medium was added into other wells. After incubating for 2 hours, cell viability was measured by Cell Counting Kit-8 (CCK-8) system according to the instruction provided by the manufacturer (Rockville, MD, USA). Briefly, CCK-8 solution (10 μL per 100 μL of medium in each well) was added, and the plates were then incubated at 37°C for 2 hours. The absorbance of each well was read at 450 nm using a microplate reader SPECTRAmax (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Tukey's *post hoc* and Student's *t*-test were used for statistical assessment where necessary (JMP 13 Software, SAS Institute Inc., Cary, NC, USA). The statistical significance threshold was set at $P < 0.05$.

Results

Expression and distribution of ALDH2 in rat spinal cord injury

We first tested the hypothesis that the protein expression level of ALDH2 is reduced in SCI, a situation known to have elevated levels of acrolein. As shown in **Figure 1**, western blot analysis showed a significant decrease in ALDH2 expression when examined 2 days following a contusive spinal cord injury (76% of control). Such reduction is significant when compared with control ($P < 0.05$, ANOVA). Treatment of Alda-1, an ALDH2 stimulator, resulted in a trend of recovery of ALDH2 (84% of control) compared with that in SCI (**Figure 1A**). In **Figure 1B**, in the control group, strong ALDH2 staining was and its organized pattern in both grey and white matter were observed. In both SCI and SCI + Alda-1 groups, ALDH2 appeared less organized with lighter staining.

Augmentation of ALDH2 and acrolein adduct formation in SCI

Aldehydes are highly reactive compounds and capable of adducting to proteins, such as ALDH2. In fact, at high concentrations, aldehydes are known to inhibit ALDH2, likely by this mechanism (Ferencz-Biro and Pietruszko, 1984; Mitchell and Petersen, 1988; Ren et al., 1999; Doorn et al., 2006; Yoval-Sanchez and Rodriguez-Zavala, 2012). As such, we then examined the possibility that the binding of acrolein to ALDH2, a likely step of acrolein-induced ALDH2 inhibition, was elevated in SCI, using a co-immunoprecipitation (co-IP) technique. As indicated in **Figure 2**, ALDH2 precipitated at 50 kDa as expected (ALDH2 IP lane). Arrows indicate that acrolein-bound ALDH2 is present in both injury and Alda-1 treated groups. Some background from beads was observed but did not interfere with the 50 kDa ALDH2 bands. Confirmation of successful co-immunoprecipitation of acrolein is further shown in **Additional Figure 1**.

Alda-1 administration lowers acrolein levels post-SCI

Since acrolein is known to increase in SCI, and ALDH2 can metabolize acrolein to lesser toxic metabolites (Yoval-Sanchez and Rodriguez-Zavala, 2012), we then tested the hypothesis that post-SCI elevated acrolein can be mitigated by the application of Alda-1 using both western blot and immunohistochemical analysis. In **Figure 3A**, western blot analysis demonstrates a significant increase in acrolein after SCI (152% of control) when compared to control ($P < 0.05$).

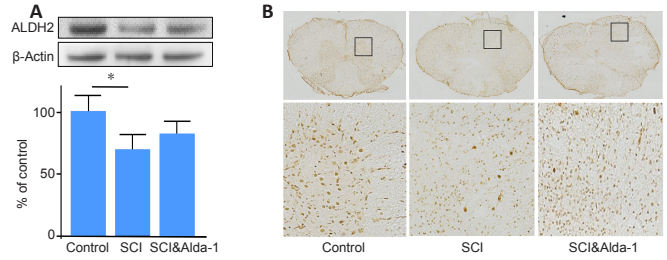

Figure 1 | **Reduction of ALDH2 levels post SCI and its influence by Alda-1.**
Acrolein levels were analyzed by western blot (A), with additional confirmation shown by IHC images (B). Groups were divided into "Control" for uninjured rats, "SCI" for injured rats given vehicle injection, or "SCI + Alda-1," injured rats given 15 mg/kg doses of Alda-1 until sacrifice at 48 hours acute timepoint (total 6 doses). (A) Top: representative ALDH2 bands (50 kDa) are shown above corresponding beta-actin bands (45 kDa). Bottom: Data is normalized to beta-actin and graphed below, showing change as a percent of control. Note the ALDH2 level was suppressed post-SCI (76% of control) which was significantly lower than control ($P < 0.05$). However, in the group of "SCI + Alda-1", the application of Alda-1 resulted in a trend of recovery of ALDH2 (84% of control) compared with that in SCI. Data are expressed as the mean ± SEM. *$P < 0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test). (B) Representative transverse spinal cord sections from each experimental group are immunostained for ALDH2 (brown color). IHC groups are as follows: Control, SCI, and SCI + Alda-1. Note the inconsistent staining after injury. Western blot and IHC: $n = 8$ and 3–4 for "Control", "SCI", and "SCI + Alda-1" groups, respectively. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; IHC: immunohistochemistry; SCI: spinal cord injury.

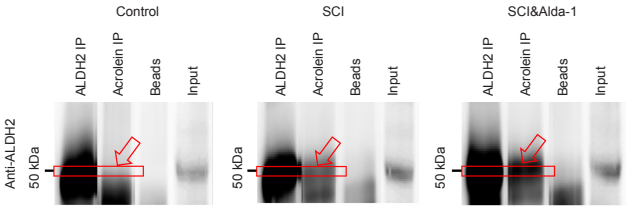

Figure 2 | **Co-immunoprecipitation indicating acrolein-bound-ALDH2 *in vivo* after SCI.**
Figure is imaged using anti-ALDH2 antibody. All lanes are consistent for sham "Control" uninjured rats, "SCI" for injured rats given vehicle injection, or "SCI + Alda-1," injured rats given 15 mg/kg Alda-1 until sacrifice at 48 hours acute time point (total 6 doses). "ALDH2 IP" lane shows successful precipitation of the enzyme in all conditions. "Acrolein IP" indicates whether or not ALDH2 positive staining is observed in the Acrolein IP pulldown using anti-acrolein-lysine adduct antibody. Staining indicates acrolein-bound-ALDH2 at 50 kDa, outlined by red boxes, with arrows highlighting ALDH2 staining in the acrolein pulldown lane, indicating binding of acrolein to ALDH2. Note that the anti-ALDH2 staining in acrolein IP lane is mild in control condition. However, such staining is intensified in both SCI and SCI & Alda-1 conditions, suggesting an augmented binding of acrolein and ALDH2 (arrows). "Beads" lane shows some background from IP beads, but at an irrelevant kDa. "Input" shows presence of ALDH2 in each sample, without immunoprecipitation. ALDH2: Mitochondrial aldehyde dehydrogenase-2; Alda-1: ALDH2-selective activator; IP: immunoprecipitation; SCI: spinal cord injury.

However, such elevated acrolein levels can be significantly suppressed to 118% of control when injured animals were treated with Alda-1, which was significant when compared to SCI only ($P < 0.05$). Consistent with western blot analysis, representative sections of spinal cord with DAB staining from Control, SCI, and SCI + Alda-1 groups reflects these findings. Specifically, acrolein level (brown color) was elevated following SCI, while Alda-1 could suppress post-SCI acrolein elevation. As such, the findings from both western blot and immunohistochemical staining suggest that ALDH2 enhancement could lower the post-SCI acrolein surge, indicative of a critical role of ALDH2 in post-SCI acrolein elevation (**Figure 3A–C**).

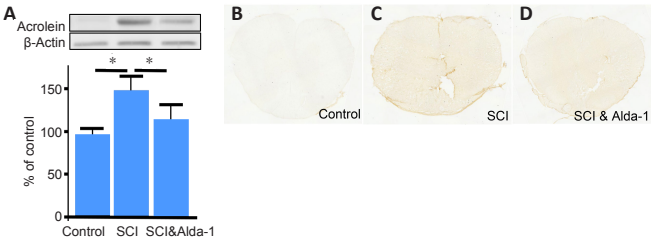

Figure 3 | **Analysis of acrolein levels post-SCI.**
Acrolein levels were analyzed by Western blot (A), with additional confirmation shown by IHC images (B–D). Groups were divided into "Control" for uninjured rats, "SCI" for injured rats given vehicle injection, or "SCI + Alda-1," injured rats given 15 mg/kg doses of Alda-1 until sacrifice at 48 hours acute time point (total 6 doses). (A) Top: Acrolein bands (50kDa) are shown above corresponding beta-actin bands (45 kDa). Bottom: Data is normalized to beta-actin and graphed, showing change as a percent of control. Compared to control, the level of acrolein-lysine adducts is significantly increased in the SCI group (100% *vs*. 152%, $P < 0.05$). However, the application of Alda-1 significantly mitigated such increase shown in SCI & Alda-1 group when compared to SCI only group (118% *vs*. 152%, $P < 0.05$). Data are expressed as the mean ± SEM. *$P < 0.05$ (one-way analysis of variance followed by Tukey's *post hoc* test). (B–D) IHC images are from representative transverse spinal cord sections, using anti-acrolein-lysine antibody and diaminobenzidine (DAB) staining shows acrolein as a brown color. IHC groups are as follows: Control (B), SCI (C), and SCI + Alda-1 (D). Note the modest staining in control (B), while more acrolein staining was observed in SCI (C). Consistent with western blot analysis, Alda-1 treatment resulted in lighter staining than that in the SCI-induced elevation of acrolein (D *vs*. C). Western blot and IHC: $n = 8$ and 3–4 for "Control", "SCI", and "SCI + Alda-1" groups, respectively. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; IHC: immunohistochemistry; IP: immunoprecipitation; SCI: spinal cord injury.

Alda-1 administration reduces cyst pathology

Acrolein is an established secondary injury mechanism known to cause severe structure damages. Furthermore, lowering the acrolein using acrolein scavengers could reduce spinal cord cyst, a common pathology observed after contusion injury, resulting from widespread cell death, and a key indication of post-SCI tissue destruction (Park et al., 2014; Gianaris et al., 2016). Therefore, we predicted that more effective ALDH2 could reduce acrolein and lead to reduced cyst area. As indicated in **Figure 4**, no clear cyst is visible in control cord (**Figure 4A**). However, SCI has resulted in conspicuous cyst formation in the injury site (**Figure 4B**). Again, the application of Alda-1 markedly reduced the size of the cyst (**Figure 4C**). Quantitative analysis revealed a significant decrease in cyst size with Alda-1 treatment compared with SCI (**Figure 4D**) ($P < 0.05$).

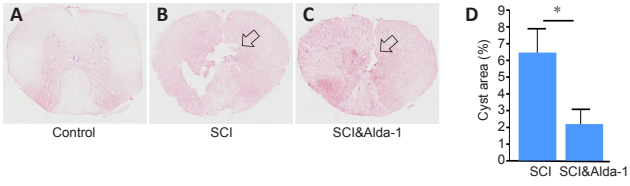

Figure 4 | **Alda-1 treatment reduces cyst formation in the spinal cord post-injury.**
(A–C) Representative images of crystal violate and eosin co-staining of spinal cord tissue, with transverse sections indicating cyst formation (arrows), 48 hours after injury, with or without Alda-1 treatment (total 6 doses). Control sections show no cyst formation (A), while large cysts could be discerned after injury (B). However, the treatment of Alda-1 in injured animals significantly reduced the size of the cyst (C). (D) When the cyst size is expressed as the % of the total area of the transverse section, it is revealed that the cyst in SCI was significantly reduced with the treatment of Alda-1 (D) ($P < 0.05$). $n = 3$–4 for "Control", "SCI", and "SCI + Alda-1". Data are expressed as the mean ± SEM. *$P < 0.05$ (Student's *t*-test). Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; SCI: spinal cord injury.

Alda-1 treatment improves post-SCI locomotor function

In prior studies by our group and others (Park et al., 2014; Gianaris et al., 2016; Burcham, 2017), the removal of acrolein and reactive aldehydes promoted functional recovery, although none had used Alda-1 for removal of reactive aldehydes. We assessed this here using the classic Basso, Beattie and Bresnahan (BBB) hindlimb locomotor rating scale, following a 2 week daily administration of a high dose of Alda-1. In **Figure 5**, SCI rats show significant decrease in locomotor function at all time points, when compared with control ($P < 0.001$). However, in the SCI + Alda-1 group, a trend of improved locomotor function was observed at 2, 7, and 14 days post injury compared with SCI rats. On days 21 and 28, Alda-1 rats showed a significant improvement in locomotion, when compared with SCI rats ($P < 0.05$ and $P < 0.01$, respectively).

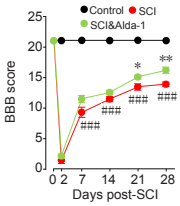

Figure 5 | Alda-1 treatment improves post-injury motor behavior.
In a 28 day post injury study, rats were assessed for motor function using the classic Basso, Beattie, Bresnahan (BBB) rating scale, which ranges from 0 (no hindlimb movement) to 21 (No observable changes in hindlimb movement). Graph shows the average BBB results for each group at each testing timepoint. Black line: healthy control group rats show no changes in locomotor scale over the 28 day study. Red line: Spinal cord injury (SCI) rats show a significant reduction of motor function, when compared to control, at every timepoint post injury. Green line: rats treated daily with a high dose of Alda-1 (60 mg/kg) for 2 weeks beginning 30 minutes after surgery (SCI & Alda-1). Rats in this group show a slight trend of increased BBB scores at 2, 7, and 14 days, and significant improvement at 21 and 28 days post injury, when compared to the SCI group. $n = 4$ for "Control", and 7–9 for "SCI", and "SCI & Alda-1". Data are expressed as the mean ± SEM. ###$P < 0.001$, *vs.* control; *$P < 0.05$,**$P < 0.01$, *vs.* SCI (one-way analysis of variance followed by Tukey's *post hoc* test). Alda-1: ALDH2-selective activator. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; SCI: spinal cord injury.

Alda-1 reduces pain-like behavior in SCI

Similarly, the same group of rats was assessed for pain-like behaviors using Von Frey filaments in **Figure 6**. The SCI rats show significant increase in sensitivity to smaller size filaments, when compared with control (days 7–28, $P < 0.001$). When administered a high dose of Alda-1 daily for 2 weeks, the SCI + Alda-1 group show a trend of decreasing sensitivity to filament size at 7, 14, and 21 days post injury when compared to the SCI group, although not significant. However, at 28 days, the SCI + Alda-1 group showed a significant improvement in mechanical hypersensitivity compared with SCI ($P < 0.01$).

No long term negative effects of Alda-1 were observed

Finally, as an assessment of general health, we recorded the rat weights at 2, 7, 14, 21, and 28 days post injury. In the Alda-1 + SCI group, less variability in weight gains and losses was observed when compared with the SCI group, however, this was not significant. Although not significant, this data indicates that administration of a high dose of Alda-1 for 2 weeks did not have an impact on weight (data not shown).

In vitro evidence of a critical protective role of ALDH2 against acrolein-mediated cell death

In order to further test the role of ALDH2 in influencing acrolein-mediated cell death, we used PC-12 cell culture system to test the hypothesis that enhancing or suppressing ALDH2 activity is accompanied by intensification or reduction of cell survival in the presence of acrolein. As indicated in **Figure 7**, while acrolein (500 μM) led to a significant reduction of cell viability (44% of control, $P < 0.01$), Alda-1 (25 μM) could significantly mitigate acrolein-mediated reduction of cell survival (65% of control, $P < 0.01$, *vs.* acrolein only). Interestingly, while Disulfram, an ALDH2 inhibitor, had no significant effect on acrolein-mediate cell death at 1 μM, it significantly worsened acrolein-mediated reduction of survival at 5 μM (10%) compared with acrolein only ($P < 0.01$).

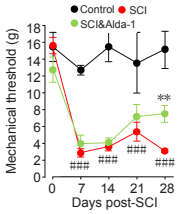

Figure 6 | Von Frey test for mechanical nociception reveals less sensitivity after Alda-1 treatment.
During a 28 day post injury study, rats were assessed for mechanical nociception using the Von Frey filament reflex test. The graphs y-axis (g = gram of force) shows the average positive reflexes to a given filament size for each group of rats at a given time point with lower scores indicating higher sensitivity. Black line: healthy control group rats show consistently high scores during the 28-day study. Red line: Spinal cord injury (SCI) rats show a significantly increased response to low filament scores for each timepoint post injury, when compared to control rats. Green line: rats treated daily with a high dose of Alda-1 (60 mg/kg) for 2 weeks beginning 30 minutes after surgery (SCI & Alda-1). Rats in this group show a slight trend in response to higher filament scores at 2, 7, 14, and 21 days, and significant improvement at 28 days post-injury, compared with the SCI group. $n = 4$ for "Control", and 7–9 for "SCI", and "SCI & Alda-1". Data are expressed as the mean ± SEM. ###$P < 0.001$, *vs.* control; **$P < 0.01$, *vs.* SCI (one-way analysis of variance followed by Tukey's *post hoc* test). Alda-1: ALDH2-selective activator. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; SCI: spinal cord injury.

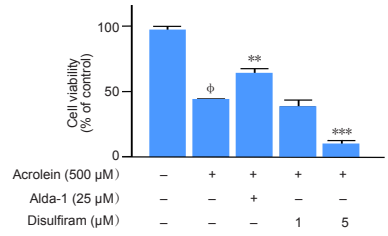

Figure 7 | Role of ALDH2 in protecting PC12 cells from acrolein toxicity *in vitro*.
Bar graph illustrates the cell viability of PC-12 cells in various conditions related to acrolein toxicity and its influence by the function of ALDH2. Cell viability is expressed as the % of the control. Note acrolein (500 μM) induced a significant reduction of cell viability (44%) compared with control (ϕ$P < 0.001$). However, the application of Alda-1 (25 μM), an ALDH2 stimulator, resulted in an increase of cell viability in SCI to 65%, a significant increase from SCI only (**$P < 0.01$). Interestingly, disulfiram, an ALDH2 inhibitor, when applied at 5 μM, but not at 1 μM, significantly decreased the cell viability (10%), which was significant compared with SCI only (***$P < 0.001$). Cell viability was determined 2 hours after incubation with various treatment conditions, involved with acrolein, disulfiram, or Alda-1. While Acrolein was applied in the beginning of the experiment, Alda-1 or disulfiram was administered 15 minutes after the start of acrolein incubation. Data are expressed as the mean ± SEM. one-way analysis of variance followed by Tukey's *post hoc* test was used. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; Disulfiram: ALDH2 inhibitor; SCI: spinal cord injury.

Discussion

There is strong evidence that acrolein, both a product and an instigator of oxidative stress, plays a critical pathogenic role in spinal cord injury (Hamann et al., 2008a, b; Hamann and Shi, 2009; Due et al., 2014; Park et al., 2014). Furthermore, anti-acrolein measures have been demonstrated as effective neuroprotective strategies to benefit both motor and sensory functional recovery following SCI. While the utilization of acrolein scavengers as means to reduce acrolein and mitigate related pathologies have been investigated in great details, other alternative anti-acrolein approaches have not attracted much attention in SCI (Chen et al., 2014). Aiming to broaden the spectrum of the acrolein sequestering strategy, and improve the effectives and potentially mitigate the inherent short falls of the existing acrolein scavengers, we have initiated an effort in searching for other methods of reducing acrolein. Specifically, in this investigation we have presented the initial evidence that ALDH2, an endogenous mitochondrial aldehyde metabolizing enzyme, is a critical part of acrolein-mediated pathology and more importantly, a potential target for anti-acrolein pharmaceutical intervention in SCI.

We have shown that, while acrolein is elevated significantly, Alda-1, an enhancer of ALDH2, can significantly reduce acrolein accumulation post-SCI. As Alda-1 is not known to scavenge acrolein directly, but known to catalyze ALDH2 enzymatic activities, the most likely explanation for its acrolein reduction effect is through increased ALDH2 detoxification activity (Che-Hong Chen et al., 2008; Perez-Miller et al., 2010). This finding not only underscores the importance of ALDH2 in acrolein removal, but suggests ALDH2 as an effective target to reduce acrolein. To further support such supposition, Alda-1-instigated acrolein reduction is associated with spinal cord damage reduction and tissue sparing post-SCI. These findings mirror the neuroprotective effects resulting from acrolein sequestering through externally applied aldehyde scavengers, such as hydralazine and phenelzine (Liu-Snyder et al., 2006; Hamann et al., 2008a; Park et al., 2014; Chen et al., 2016).

It is well documented that acrolein is elevated significantly following SCI. So far, such elevation has mainly been attributed to the production of acrolein due to injury-induced oxidative stress and lipid peroxidation (Uchida et al., 1998; Shi et al., 2011a). In the current study, however, we have also found that the expression of ALDH2 is significantly reduced in SCI. It is known that ALDH2 plays a critical role in preventing aldehyde accumulation and disease onset (Chen et al., 2014). Therefore, a significant reduction of ALDH2 will inevitably lead to elevation of acrolein in SCI (Liu et al., 2017). Thereby, our finding suggests that in the event of SCI and acrolein elevation, the degradation of ALDH2 further contributes to the accumulation of acrolein.

It is known that acrolein is capable of inhibiting ALDH2, particularly when acrolein concentration is high (Ferencz-Biro and Pietruszko, 1984; Mitchell and Petersen, 1988; Ren et al., 1999; Doorn et al., 2006; Yoval-Sanchez and Rodriguez-Zavala, 2012). Although we did not examine the catalytic activity of ALDH2 in the current study, the likelihood of ALDH2 inhibition by acrolein post-SCI is high, as acrolein is known to surge overwhelmingly post-SCI. It is possible that such inhibition also leads to the reduction of ALDH2 expression seen in this study, resulting from heightened degradation of ALDH2. This is based on the evidence that Alda-1, known to disinhibit ALDH2, induced a tendency of augmenting expression of ALDH2 in the presence of acrolein. Taken together, we propose that the reduction of ALDH2 expression, likely accompanied by the functional inhibition as well, contributes to post SCI acrolein surge. The Alda-1 mediated suppression of acrolein elevation and neuroprotection, is likely through the potentiation of the function of ALDH2 by directly enhancing its enzymatic capability, and by protecting the enzyme from acrolein inhibition.

The exact mechanism of acrolein-mediated ALDH2 inhibition is not clear. However, it is generally believed that the inhibitory site (through acrolein binding) and activation side (through Alda-1 binding) are two separate, yet related structures of ALDH2. Interestingly, structural analysis revealed that the binding of Alda-1 to the active site not only facilitates the aldehyde detoxifying, but also hinders aldehydes' access to the inhibitory site and protects ALDH2 from adduct formation (Perez-Miller et al., 2010).

Our immunoprecipitation experimentation demonstrates the presence of protein binding between ALDH2 and acrolein in SCI. Interestingly, it also revealed an elevated level of acrolein binding to the lysine residue of ALDH2 when Alda-1 was introduced. The mechanism of such phenomenon remains to be examined. Regardless, our IP finding demonstrates the binding of ALDH2 and acrolein post-SCI which is consistent with the known property of ALDH2 to interact and detoxify acrolein.

In addition to *in vivo* examination, we also tested the ability of ALDH2 to mitigate acrolein-mediated cell death directly in PC-12 cells which are known to express ALDH2 (Kong and Kotraiah, 2012). Specifically, we demonstrated the anti-acrolein ability of ALDH2 in PC12 cells by turning up or turning down the activity of ALDH2 and observing the subsequent cell viability changes in the presence of acrolein. As expected, enhancement of ALDH2 by Alda-1 mitigated cell death while suppressing ALDH2 worsened cell death in the presence of acrolein. This finding is consistent with a previous study where a genetically induced deficiency in ALDH2 in PC-12 cells increased the vulnerability to 4-hydroxy-2-nonenal (HNE), a reactive aldehyde similar to acrolein. These *in vitro* data from ours and others have provided strong evidence which supports and complements the *in vivo* date to indicate that ALDH2 functions as a protector against aldehydes and oxidative stress (Ohsawa et al., 2003).

In line with these findings, with a separate group of rats, we measured if daily administration of a high dose of Alda-1 for 2 weeks could improve functional outcomes. First, we assessed locomotor function and show Alda-1 provides significant improvements at later timepoints. This result is likely due to a decrease in acrolein-mediated neuronal cell death in the rats receiving Alda-1. In humans, issues with locomotion frequently follow a SCI, as well as the development of neuropathic pain (Shiao and Lee-Kubli, 2018). Thus, we assessed pain-like behavior in our rats and found that rats receiving Alda-1 had a significant reduction of pain at later timepoints. In prior work, we demonstrated that acrolein is capable of upregulating and activating pro-algesic transient receptor protein ankyrin 1 in rat models of SCI and Parkinson's, and that acrolein scavengers reduce such hyperalgesia (Park et al., 2015; Shi et al., 2021). Furthermore, one study using a mouse model of inflammatory pain showed Alda-1 administration significantly lessened pain and expression of pro-algesic receptor Early Growth Response 1 (Zambelli et al., 2014). Although the mechanism of Alda-1 lowering pain was not investigated in detail here, it likely involves lessening of acrolein mediated activation and upregulation of pro-algesic receptors. Taken together, in addition to serving as a critical agent to demonstrate the key role of ALDH2 in acrolein removal, Alda-1 may also be a strong candidate for translational purposes by improving behavioral outcomes of SCI. Furthermore, Alda-1 is readily bioavailable when given systemically, and is capable of crossing the blood brain barrier with no obvious side effects, as observed in animal studies (Guo et al., 2013; Fu et al., 2014; Ikeda et al., 2015; Stachowicz et al., 2016; Zhu et al., 2017; Rivera-Meza et al., 2019). Therefore, Alda-1 may have the potential to be developed for clinical usage.

However, despite the probable effectiveness and translational potential of Alda-1, the primary purpose and significance of our finding is to demonstrate the utility of ALDH2 activator and protector as a new and effective strategy to combat

acrolein toxicity. This would establish a novel pharmacophore, guiding the development of a new class of ALDH2-focused anti-acrolein drugs for treating SCI with high efficacy. Once established, ALDH2-targated anti-acrolein therapies may not just be treating SCI victims, but could also benefit patients with other conditions where acrolein is implicated, such as traumatic brain injury, Parkinson's diseases, Alzheimer's diseases, multiple sclerosis, Neuropathic pain, cardiovascular diseases, and even cancer (Chen et al., 2014; Ambaw et al., 2018; Acosta et al., 2019). Acrolein has also been linked to effects of aging, pollution, and smoking, which further expands the potential value of anti-acrolein therapies (Chen et al., 2013; Burcham, 2017).

Limitations

This study has several limitations. First, this study focused only on male rats and 2 day or 30 day time points. For more clinical relevance, future studies should consider the use of females, and intermediate timepoints for biochemistry (7 or 14 days). Furthermore, efforts to directly measure the activity of ALDH2 in the T10 spinal cord were unsuccessful (data not shown). This could be due to lower concentrations of ALDH2 in spinal tissue, when compared with others such as the liver where ALDH2 activity assays are more successful. Similarly, other studies have not been able to detect activity, but still found direct benefits of Alda-1 on tissue pathology (Lu et al., 2017). Finally, limitations exist in both the solubility and bioavailability of Alda-1 (Taneja et al., 2015). Ideally, studies should administer Alda-1 orally to enhance the exposure time and avoid the negative effects of IP injections with DMSO.

Conclusion

Enzyme ALDH2 is important for the protection of spinal tissue and for cell viability after spinal cord injury. Here we add to the growing evidence that ALDH2 expression is lowered after SCI, and can be damaged by surging levels of aldehydes. Thus, a need remains for boosting ALDH2. Enhancing the activity of ALDH2 with Alda-1 resulted in significant acrolein reduction, tissue protection, and improvement of motor and sensory function. It is expected that ALDH2-targeted acrolein sequestration may have broad benefits in combating post-SCI neurodegeneration and potentially an extensive impact on human health.

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Additional file:
Additional Figure 1: *Confirmation of ALDH2 precipitation and IgG.*

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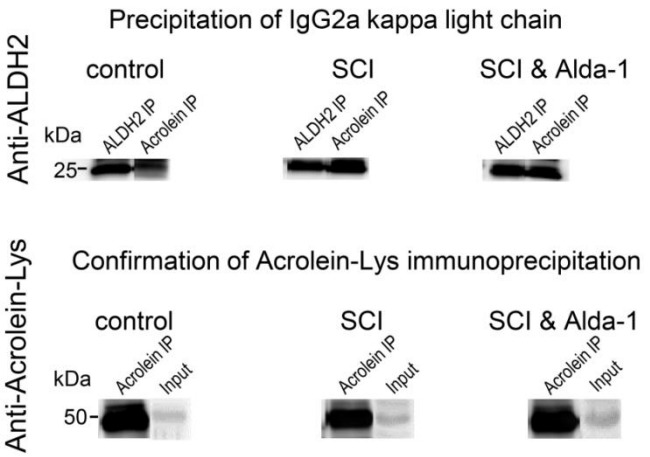

Additional Figure 1 **Confirmation of ALDH2 precipitation and IgG.**

To confirm the precipitated primary anti-ALDH2 or anti-acrolein-lysine antibodies are not interfering with the staining, kappa light chain primary antibodies were used. As expected, secondary antibodies stained the light chain of the primary antibodies, showing presence in both anti-ALDH2 and anti-acrolein-lysine pulldown lanes at 25 kDa (top portion of figure). In the bottom half of the figure, confirmation of successful acrolein precipitation is shown, using anti-acrolein-lysine to stain the acrolein immunoprecipitation; lane, with relevant input lanes also included. Compared to input, heavy staining was shown, indicating successful precipitation of acrolein at 50 kDa. Alda-1: ALDH2-selective activator; ALDH2: mitochondrial aldehyde dehydrogenase-2; SCI: spinal cord injury.