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Hydralazine inhibits compression and acrolein-mediated injuries in *ex vivo* spinal cord

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Abstract

We have previously shown that acrolein, a lipid peroxidation byproduct, is significantly increased following spinal cord injury *in vivo*, and that exposure to neuronal cells results in oxidative stress, mitochondrial dysfunction, increased membrane permeability, impaired axonal conductivity, and eventually cell death. Acrolein thus may be a key player in the pathogenesis of spinal cord injury, where lipid peroxidation is known to be involved. The current study demonstrates that the acrolein scavenger hydralazine protects against not only acrolein-mediated injury, but also compression in guinea pig spinal cord *ex vivo*. Specifically, hydralazine (500 μmol/L to 1 mmol/L) can significantly alleviate acrolein (100–500 μmol/L)-induced superoxide production, glutathione depletion, mitochondrial dysfunction, loss of membrane integrity, and reduced compound action potential conduction. Additionally, 500 μmol/L hydralazine significantly attenuated compression-mediated membrane disruptions at 2 and 3 h following injury. This was consistent with our findings that acrolein-lys adducts were increased following compression injury *ex vivo*, an effect that was prevented by hydralazine treatment. These findings provide further evidence for the role of acrolein in spinal cord injury, and suggest that acrolein-scavenging drugs such as hydralazine may represent a novel therapy to effectively reduce oxidative stress in disorders such as spinal cord injury and neurodegenerative diseases, where oxidative stress is known to play a role.

Keywords: acrolein, aldehyde, hydralazine, lipid peroxidation, oxidative, spinal cord.
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Reactive oxygen species (ROS) and lipid peroxidation (LPO) have been associated with numerous diseases that few other pathological factors can match, including aging, neoplasia, trauma, and ischemia-reperfusion injury (Halliwell and Gutteridge 1999). The mechanism of involvement of LPO has been an area of intense research aiming to prevent, slow down, and even reverse the development of various diseases. In the case of spinal cord injury, it is well established that LPO plays an important role in neuronal degeneration, cell death, and overall functional deficits (Hall 1989, 1991; Hall and Braughler 1993). This is believed due in part to that fact that neuronal cells contain a relatively large proportion of polyunsaturated fatty acids and are rich in mitochondria, both of which are a potential target and source of free radicals. Because of these unique features, the CNS is particularly vulnerable to oxidative injury. In spite of strong evidence suggesting that post-trauma oxidative stress plays a critical role in the pathogenesis of spinal cord injury, conventional strategies aiming to scavenge free radicals have largely failed to produce any effective treatment that can curtail oxidative injury. Hence, further understanding of the mechanisms of oxidative stress and identification of a novel and more effective target is highly warranted and desirable.

In addition to the much studied ROS, highly reactive α,β-unsaturated aldehydes, including malondialdehyde, 4-hydroxynonenal (HNE), and acrolein, are produced as a byproduct of LPO (Witz 1989; Esterbauer *et al.* 1991; Uchida 1999; O'Brien *et al.* 2005). Among them, acrolein has been shown to be by far the most reactive with various biomolecules including proteins, DNA, and glutathione, and reacts 110–150 times faster with glutathione than HNE or

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Abbreviations used: AD, Alzheimer's disease; CAP, compound action potential conduction; DTNB, 5,5′-Dithiobis (2-nitrobenzoic acid); HE, dihydroethidium; HNE, 4-hydroxynonenal; LDH, lactate dehydrogenase; LPO, lipid peroxidation; PB, phosphate buffer; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TMR, tetramethyl rhodamine dextran.

crotonal (Witz 1989; Esterbauer *et al.* 1991; Ghilarducci and Tjeerdema 1995; Uchida 1999; Kehrer and Biswal 2000). In addition, acrolein stimulates the generation of ROS and subsequent LPO, which may perpetuate oxidative stress through self-reinforcing positive feedback (Adams and Klaidman 1993). Furthermore, acrolein's half-life, on the order of hours to days (Ghilarducci and Tjeerdema 1995), is many orders of magnitude longer than the transient ROS. Acrolein readily forms conjugates with proteins and glutathione that likely have significantly longer half-lives than free acrolein, and have themselves been demonstrated to be highly reactive (Adams and Klaidman 1993; Burcham *et al.* 2004; Kaminskas *et al.* 2004b; Burcham and Pyke 2006).

Acrolein has been measured by a variety of methods in its free or protein-bound state, and has been demonstrated to be significantly increased following spinal cord injury in guinea pigs (Luo *et al.* 2005a), in the brains of individuals with Alzheimer's disease (AD) (Lovell *et al.* 2001), and in neurofibrillary tangles (Calingasan *et al.* 1999). Furthermore, *in vitro* evidence is readily available demonstrating the toxicity of acrolein in the CNS (Lovell *et al.* 2001; Shi *et al.* 2002; Peasley and Shi 2003; Luo and Shi 2004, 2005; Luo *et al.* 2005a,b). Therefore, we hypothesize that acrolein's long-lived potential to perpetuate oxidative stress provides one possible explanation for the ineffectiveness of ROS scavengers in clinical trials, and it thus represents a novel and potentially more effective target for reducing oxidative stress.

The antihypertensive drug hydralazine has been shown to bind to and neutralize acrolein (Burcham *et al.* 2000, 2002; Kaminskas *et al.* 2004a) and acrolein–protein adducts (Burcham *et al.* 2004; Kaminskas *et al.* 2004b; Burcham and Pyke 2006). Hydralazine also prevented allyl alcohol-induced hepatotoxicity, which is mediated by acrolein, in cultured hepatocytes (Burcham *et al.* 2000, 2004) and in mice *in vivo* (Kaminskas *et al.* 2004b). In addition, hydralazine prevented acrolein-mediated injuries in PC12 cells (Liu-Snyder *et al.* 2006a). The purpose of this study is to continue this line of investigation and evaluate the protective effects of hydralazine against acrolein-mediated injury as well as compression injury in *ex vivo* guinea pig spinal cord.

Experimental procedures

Isolation of spinal cord

The experimental protocols have been reviewed and approved by the Purdue University Animal Care and Use Committee. Guinea pigs were housed and handled in accordance with Purdue University Animal Care and Use Committee guidelines. All efforts were made to minimize the number of animals used and their discomfort. Guinea pigs were pre-anesthetized with an i.p. or i.m. injection of acepromazine (0.6 mg/kg). Anesthesia was induced by i.m. injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Once animals were very deeply anesthetized (assessed by pinching the toe and/or abdominal musculature), they were perfused transcardially with approximately 500 mL of cold, oxygenated Kreb's solution (124 mmol/L NaCl, 2 mmol/L KCl, 1.24 mmol/L KH_2PO_4, 1.3 mmol/L $MgSO_4$, 1.2 mmol/L $CaCl_2$, 10 mmol/L glucose, 26 mmol/L $NaHCO_3$, and 10 mmol/L ascorbic acid), prepared fresh daily. The vertebral column was then rapidly removed and a complete dorsal laminectomy performed along the length of the vertebral column, exposing the spinal cord. The spinal cord was carefully removed and divided into 1 cm segments for each experiment. For electrophysiology measurements, 4 cm segments of ventral white matter were isolated as previously described (Shi and Blight 1996; Shi and Borgens 1999; Shi and Whitebone 2006). Modified Kreb's solution (same as above with ascorbic acid omitted and warmed to 37°C) was used for preparing all other solutions unless otherwise specified, as it has been found previously that exogenous ascorbic acid prevents acrolein-mediated injury *in vitro* (Logan *et al.* 2005). Controls were incubated in modified Kreb's solution only. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Exclusion of tetramethyl rhodamine dextran

Membrane permeability was measured by exclusion of the hydrophilic dye tetramethyl rhodamine dextran (TMR, 10 kD) (Invitrogen, Carlsbad, CA, USA). Briefly, segments of spinal cord were incubated for 4 h at 37°C in one of the following groups: control, 100 μmol/L acrolein, 100 μmol/L acrolein plus 500 μmol/L hydralazine (hydralazine application delayed by 15 min following the start of acrolein incubation), or 500 μmol/L hydralazine only (to determine the effects of hydralazine on healthy spinal cord). At the end of 4 h, spinal cord segments were transferred to 0.01% lysine fixable TMR and incubated for 15 min. Spinal cord segments were then fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB), prepared fresh daily, for 2 h. Segments were then imbedded in Tissue-Tek OCT compound (VWR, Batavia, IL, USA), frozen in liquid nitrogen, and stored at −80°C for up to 1 month. Sections were cut at 50 μm on a cryostat and cover-slipped with Immu-Mount (Thermo Electron, Waltham, MA, USA). Sections were visualized by epi-fluorescence on an Olympus BX61 microscope with a standard rhodamine cube (excitation filter: BP545, emission filter: LP590, Olympus, Center Valley, PA, USA), fluorescence quantified using Image J (NIH, Bethesda, MD, USA), and averaged for five sections randomly selected from the center of each spinal cord segment.

Lactate dehydrogenase assay

Membrane permeability was also measured by lactate dehydrogenase (LDH, 140 kD) release. LDH is an intracellular enzyme that is normally present at low concentrations in the extracellular fluid and is only released from cells whose membranes have been injured. LDH release from spinal cord was measured similar to previously described techniques (Luo *et al.* 2002a; Luo and Shi 2004). Briefly, segments of spinal cord were removed from each animal and incubated in Kreb's solution for at least 1 h to allow axonal membranes to repair from injury that occurred as a result of tissue isolation (Shi and Blight 1996; Shi and Pryor 2000). Segments were then incubated for 1 h at 37°C in one of the following groups: control, 500 μmol/L acrolein, or 500 μmol/L acrolein plus 500 μmol/L hydralazine (hydralazine application delayed by 5 min). Samples were then rinsed three times and incubated for

one additional hour at 37°C in modified Kreb's solution to allow LDH to leak out of any membrane breaches that were produced in each of the treatment groups. Two hundred microliter samples of the solution bathing the segments were then removed. Levels of LDH were assayed using the TOX-7 kit (Sigma-Aldrich).

Superoxide production

Superoxide production was detected by dihydroethidium (HE) (Invitrogen) (Bindokas *et al.* 1996; Tarpey *et al.* 2004). Segments of spinal cord were incubated for 4 h at 37°C in one of the following groups: control, acrolein (100 or 500 μmol/L), or acrolein (100 or 500 μmol/L) plus 500 μmol/L hydralazine (hydralazine application delayed by 15 min). After 4 h, spinal cord segments were incubated in 50 μmol/L HE (prepared fresh daily in modified Kreb's from 5 mmol/L stock in dimethyl sulfoxide) for 30 min. Spinal cord segments were then fixed for 2 h in 4% paraformaldehyde in PB, prepared fresh daily. Segments were then imbedded in Tissue-Tek OCT compound (VWR), frozen in liquid nitrogen, and stored at −80°C for up to 1 week. Sections were cut at 50 μm on a cryostat and cover-slipped with Immu-Mount (Thermo Electron). Sections were visualized by epi-fluorescence on an Olympus BX61 microscope with a standard rhodamine cube (excitation filter: BP545, emission filter: LP590, Olympus), fluorescence quantified using Image J (NIH), and averaged for five sections randomly selected from the center of each spinal cord segment.

The superoxide scavenging ability of hydralazine was evaluated according to previously described methods (Quick *et al.* 2000). Briefly, superoxide was generated in a cell-free system by reaction of 6 mU/mL xanthine oxidase with 100 μmol/L hypoxanthine. Superoxide levels were assessed by loss of 46 μmol/L cytochrome *c*, measured by rate of change in absorbance at 550 nm. Catalase was added to prevent 'suicide loss' of cytochrome *c*. Superoxide dismutase (SOD, 575 U/mL) was used as positive control.

Glutathione concentration

Concentrations of total glutathione and GSSG were quantified using 2-vinylpyridine and 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) recycling, similar to previously described methods (Griffith 1980; Anderson 1985; Eyer and Podhradsky 1986). Thoracic spinal cord was used, as it has been previously reported that there is a statistically significant difference in the glutathione concentration in different regions of spinal cord (Honegger *et al.* 1989; Lucas *et al.* 2002). Segments of spinal cord were incubated for 4 h at 37°C in one of the following groups: control, 100 μmol/L acrolein, or 100 μmol/L acrolein plus 500 μmol/L hydralazine (hydralazine application delayed by 15 min). Tissue was then weighed and homogenized in 5% sulfosalicylic acid (3 mL/100 mg tissue). Homogenate was centrifuged for 10 min at 10 000 *g* and 4°C. Supernatants were frozen at −20°C for up to 2 weeks, and then assayed for glutathione concentration. GSSG was used as a standard.

MTT assay

Segments of cervical spinal cord were used for these experiments, as different regions of the spinal cord were found to have significantly different results in the MTT assay (unpublished observations). Spinal cord segments were incubated for 4 h at 37°C in one of the following groups: control, 100 μmol/L acrolein, or 100 μmol/L acrolein plus 500 μmol/L hydralazine (hydralazine application delayed for 15 min). At the end of 4 h, synaptosomal mitochondria were isolated by methods previously described (Luo and Shi 2004). Briefly, spinal cord segments were homogenized in 3 mL isolation buffer (0.25 mol/L sucrose, 0.5 mmol/L dipotassium EDTA, 10 mmol/L Tris–HCl), centrifuged twice for 3 min at 4°C and 2000 *g*. The supernatant was then centrifuged for 10 min at 4°C and 16 000 *g*. The crude mitochondrial pellet was resuspended in 1 mL phosphate-buffered saline (PBS) (0.9 mmol/L $CaCl_2$, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 0.5 mmol/L $MgCl_2$, 137 mmol/L NaCl, 6.5 mmol/L Na_2HPO_4, pH 7.2–7.4). This procedure yielded a suspension containing approximately 8–10 mg/mL protein as determined by the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA) (data not shown). Hundred microliter of MTT solution (100 mmol/L pyruvate, 12 mmol/L MTT) was then added to the mitochondrial suspension and incubated for 1 h at 37°C. Formazan crystals were pelleted by centrifugation and dissolved in 1 mL of a 50/50 (v/v) solution of ethanol and dimethyl sulfoxide. The absorbance of 200 μL was read at 550 nm minus the background at 660 nm. The ethanol/dimethyl sulfoxide solution was used as a blank.

Electrophysiology

The isolated strips of ventral white matter were placed in a sucrose-gap recording chamber. The construction of the recording chamber has been described in our previous publications (Shi and Blight 1996, 1997; Shi and Borgens 1999; Jensen and Shi 2003; Shi and Whitebone 2006). The central compartment of the chamber was continuously perfused with oxygenated Kreb's solution (2 mL/min). The ends of the tissue were drawn through the sucrose gap channels and ended inside the compartments filled with isotonic potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels, using fragments of plastic cover-slip and a small amount of silicone grease. Isotonic sucrose solution was continuously run through the gap channels at a rate of 1 mL/min. The temperature of the chamber was maintained at 37°C. For membrane potential studies, white matter strips were transected (resulting in depolarization), and 60 min following transection the membrane potential was recorded. For compound action potential (CAP) recordings, white matter strips were exposed to 500 μmol/L acrolein (with or without 1 mmol/L hydralazine) for 60 min and then washed for 60 min with Kreb's solution. For the length of the experiment, axons were stimulated in the form of constant-current unipolar pulses of 0.1 ms at intensities corresponding to maximal CAP response, and CAPs recorded by silver–silver chloride wire electrodes. Subsequent analysis was performed using custom Labview® software (National Instruments™, Delaware Water Gap, PA, USA) on a Dell PC™ (Austin, TX, USA).

Compression injury

One centimeter segments were isolated as described and incubated in Kreb's for at least 1 h to allow for recovery of injury resulting from tissue isolation (Shi and Blight 1996; Shi and Pryor 2000). Compression injury was produced by 70% strain at a constant rate of 5 mm/s. Applied force and vertical displacement were simultaneously measured to ensure a uniform, standardized, and repeatable injury. Segments were placed in 5 mL of modified Kreb's solution for 3 h at 37°C in one of the following treatment groups: control (no

injury), 500 μmol/L hydralazine (no injury), compression, and compression plus 500 μmol/L hydralazine (applied 15 min after compression). 50 μL of the fluid bathing the tissue was collected every hour and assayed for LDH by the TOX-7 kit (Sigma-Aldrich).

Detection of acrolein-lys by immunohistochemistry

Acrolein-lys adducts were detected by immunohistochemistry, similar to previously described methods (Calingasan *et al.* 1999; Luo *et al.* 2005a; Shen *et al.* 2005). Briefly, spinal cord segments were incubated at 37°C in one of the following treatment groups: control (no injury), 500 μmol/L hydralazine (no injury), compression, and compression plus 500 μmol/L hydralazine (applied 15 min after compression). Segments of spinal cord were then fixed for 18 h in 4% paraformaldehyde in PB, prepared fresh daily, imbedded in Tissue-Tek OCT compound (VWR), frozen in liquid nitrogen, and stored at −80°C for up to 1 month. Sections were cut at 15 μm on a cryostat and then post-fixed in a 50/50 (v/v) solution of methanol/acetone at −20°C for 15 min. Slides were rinsed in PBS and incubated in 0.3% Triton X-100 (in PBS) for 30 min. Antigen retrieval was performed by incubating slides in citrate buffer (10 mmol/L trisodium citrate, 0.05% Tween 20, pH 6.0) heated to 95°C for 20 min. Slides were allowed to cool for 10 min at 22°C, washed in PBS, and blocked in 5% goat serum (in PBS) for 1 h. Slides were then incubated for 18 h at 4°C in 1 : 250 polyclonal rabbit anti-acrolein (in PBS with 2% goat serum, 0.1% sodium azide) (Novus Biologicals, Littleton, CO, USA). Slides were washed in PBS and incubated for 2 h in 1 : 200 Alexa Fluor 488 goat anti-rabbit IgG, highly cross-adsorbed (in PBS) (Invitrogen). Sections were washed in PBS and visualized by epi-fluorescence on an Olympus BX61 microscope with a standard fluorescein cube (excitation filter: BP495, emission filter: SP515, Olympus), fluorescence quantified using Image J (NIH), and averaged for three sections randomly selected from the center of each spinal cord segment. For negative controls, anti-acrolein antibody was omitted (data not shown).

Statistical analysis

Unless otherwise specified, unpaired Student's *t*-test (for comparison of two groups) or one-way ANOVA and *post hoc* Newman Keul's test (for more than two groups) were used for statistical analyses (InStat, San Diego, CA, USA). Normality was tested for by Shapiro–Wilk test (STATA, College Station, TX, USA). Equal variances were tested by the method of Barlett for $n \geq 5$ (InStat), and by less than two-fold difference in SD for $n < 5$. Results are expressed as mean ± SD. A p-value of < 0.05 was considered statistically significant.

Results

Membrane permeability

Hydralazine alone did not have a significant effect on injury to spinal cord segments in the TMR dye exclusion assay (Fig. 1b). Specifically, fluorescence intensity was 94.0 ± 40.8% of control values ($p = 0.78$, $n = 4$). Acrolein treatment did result in a significant increase in permeability to the hydrophilic TMR dye (Fig. 1) and the intracellular enzyme LDH (Fig. 2), an effect which was attenuated by 500 μmol/L hydralazine. Specifically, 100 μmol/L acrolein increased permeability to TMR from 100 ± 22.9% (controls) to 133.5 ± 27.4% of control values ($p < 0.05$). TMR permeability in tissue treated with acrolein and hydralazine was reduced to 93.7 ± 16.8% of control values ($p < 0.05$ com-

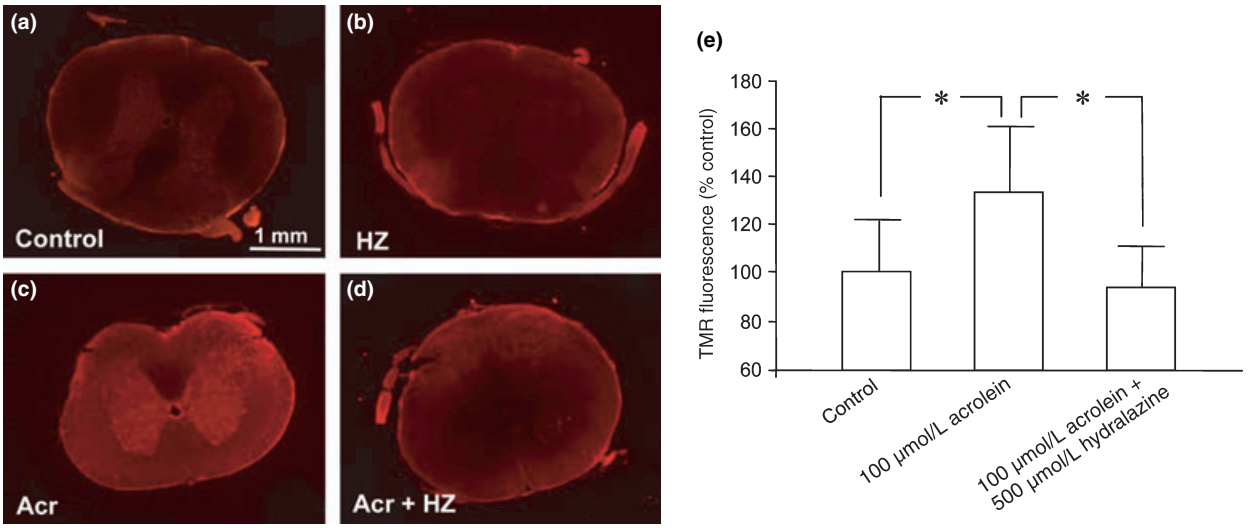

Fig. 1 TMR staining following acrolein and hydralazine exposure. Membrane integrity was assessed using TMR (10 kD), a hydrophilic dye that is excluded from cells with an intact membrane. Spinal cord segments were incubated for 4 h in one of the following treatment groups: (a) control, (b) 500 μmol/L hydralazine (HZ), (c) 100 μmol/L acrolein (Acr), or (d) 100 μmol/L acrolein and 500 μmol/L hydralazine (Acr + HZ). Notice the increase in fluorescence intensity in tissue treated with acrolein (c), especially in the white matter around the edges of the tissue. This increased intensity is reduced by treatment with hydralazine. (e) Fluorescence intensity was quantified using Image J (NIH) and is expressed as percent control values ± SD ($n = 6$). Treatment with 100 μmol/L acrolein resulted in increased permeability to TMR dye compared to controls. 500 μmol/L hydralazine attenuated acrolein-mediated membrane damage. *$p < 0.05$.

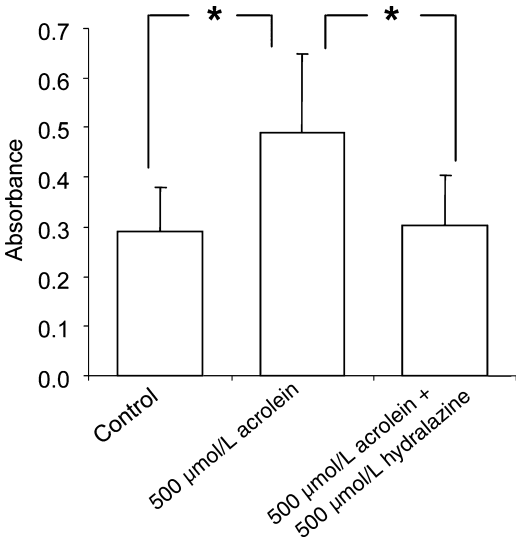

Fig. 2 LDH release following acrolein and hydralazine exposure. Membrane integrity was assessed by release of LDH (140 kD), an intracellular enzyme that leaks out of injured cells. The level of LDH released from neuronal cells was determined following 1 h incubation in 500 μmol/L acrolein with or without 500 μmol/L hydralazine. Results are expressed as mean absorbance ± SD ($n = 6$). Acrolein treatment resulted in a significant increase in membrane permeability to LDH, an effect which was attenuated by hydralazine. One-way paired ANOVA and *post hoc* Newman Keul's test were used for statistical analysis. *$p < 0.01$.

pared to acrolein alone), which is not significantly different from controls ($p > 0.05$). In addition, 500 μmol/L acrolein significantly increased LDH release from spinal cord compared to control values ($p < 0.01$). Hydralazine treatment in acrolein-injured cord significantly reduced LDH release ($p < 0.01$ compared to acrolein alone) to a value that is similar to controls ($p > 0.05$).

Oxidative stress

Hydralazine significantly attenuated oxidative stress in spinal cord exposed to acrolein, as assessed by superoxide production and glutathione concentration. Acrolein treatment resulted in a trend toward a concentration-dependent increase in superoxide production, measured by HE fluorescence (Fig. 3). The increased fluorescence is especially prominent in the gray matter (Fig. 3b and d). This effect was attenuated by hydralazine treatment, and segments treated with acrolein and hydralazine appeared characteristically devoid of fluorescence in the gray matter (Fig. 3c and e). Acrolein-mediated increases in superoxide production were statistically significant at concentrations of 500 μmol/L (167.1 ± 14.5% of control values, $p < 0.01$), but not 100 μmol/L (113.2 ± 19.1%, $p > 0.05$), when compared to controls (100.0 ± 18.7%) (Fig. 3f). The acrolein-induced increase in superoxide production was attenuated by treatment with hydralazine (104.4 ± 26.5% of control values, $p < 0.01$ compared to 500 μmol/L acrolein alone) to the extent that tissue treated with 500 μmol/L acrolein and 500 μmol/L hydralazine did not differ from controls ($p > 0.05$).

The superoxide scavenging assay was performed using a cell-free system to evaluate the intrinsic superoxide scavenging abilities of hydralazine, with SOD as positive control (Fig. 3g). Loss of cytochrome *c* by superoxide production was almost completely prevented by the scavenger SOD (6.2 ± 1.0% of control values, $p < 0.001$). In contrast, hydralazine did not have significant superoxide scavenging abilities at a concentration of 500 μmol/L (95.5 ± 7.6%) or 1 mmol/L (93.3 ± 2.5%) ($p > 0.05$). This suggests that hydralazine, at the concentrations used in this study, is not an efficient scavenger of superoxide.

Total glutathione was significantly decreased from 917.2 ± 82.7 (control) to 712.3 ± 79.0 nmol/mg tissue following exposure to 100 μmol/L acrolein ($p < 0.05$) (Fig. 4a). However, glutathione concentration in tissue injured by acrolein in the presence of hydralazine was 944.1 ± 69.1 nmol/mg tissue, which was significantly greater than tissue treated with acrolein alone ($p < 0.05$), and was not different than controls ($p > 0.05$). In spite of changes in the total glutathione concentration, the proportion of GSSG was not significantly different in any treatment groups ($p > 0.05$) (Fig. 4b), although there was a slight trend toward an increase in GSSG following acrolein treatment (1.35 ± 0.18% of total glutathione) compared to controls (1.09 ± 0.14%).

MTT test

Hydralazine treatment also alleviated acrolein-mediated mitochondrial injury (Fig. 5). Specifically, in the MTT assay of synaptosomal mitochondria, acrolein treatment resulted in a reduction of absorbance to 35.0 ± 17.2% of control values (100 ± 13.9%, $p < 0.001$). In tissue treated with acrolein and hydralazine, this reduction was only 79.8 ± 26.6% of controls ($p < 0.01$ compared to acrolein alone), which is not significantly different when compared to controls ($p > 0.05$).

Electrophysiology

Acrolein-mediated loss of axonal conductivity was also significantly attenuated by hydralazine (Fig. 6). In the absence of acrolein, ventral white matter strips did not exhibit significant decline in amplitude of CAPs for at least 120 min (data not shown), consistent with our previous studies (Luo *et al.* 2002b). However, 60 min exposure to 500 μmol/L acrolein followed by a 60 min wash resulted in reduction of CAP to 25.0 ± 18.6% of pre-injury values (Fig. 6c). Addition of 1 mmol/L hydralazine to acrolein solutions significantly attenuated acrolein-mediated CAP reduction. Specifically, hydralazine application significantly attenuated reduction of CAP amplitude to 63 ± 24.8% of pre-injury values, ($p < 0.05$ compared to acrolein alone).

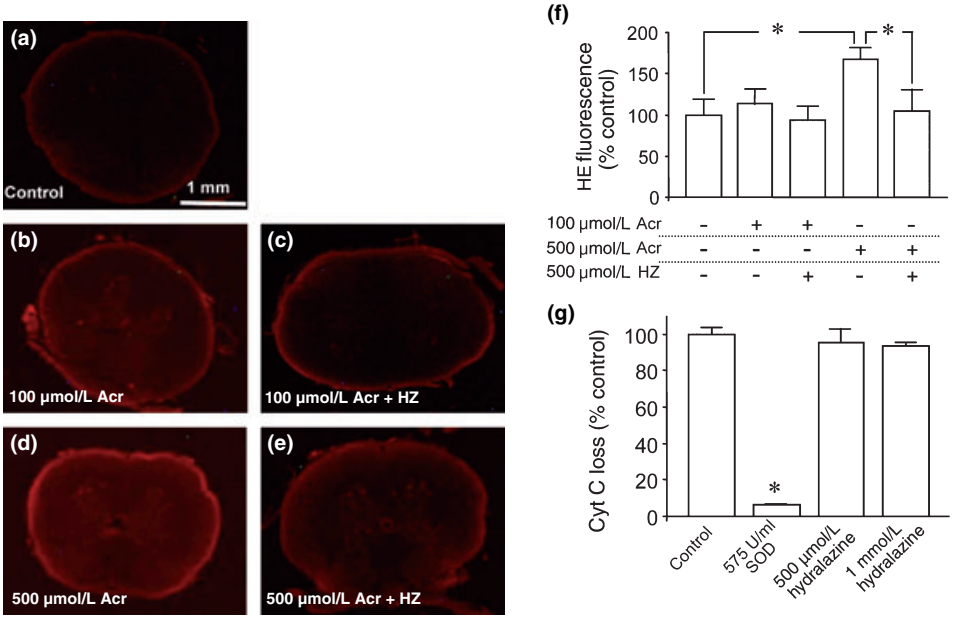

Fig. 3 Effects of acrolein and hydralazine on superoxide. Superoxide production is detected by increased fluorescence in HE-stained spinal cord. Representative images are shown for (a) control, (b) 100 µmol/L acrolein (Acr), (c) 100 µmol/L acrolein and 500 µmol/L hydralazine (Acr + HZ), (d) 500 µmol/L acrolein, or (e) 100 µmol/L acrolein and 500 µmol/L hydralazine. Notice the slight increase in fluorescence intensity in spinal cord treated with 100 µmol/L acrolein (b) and the even greater intensity with 500 µmol/L acrolein (d). This effect is most obvious in the gray matter, and is reduced by treatment with hydralazine. Subpial fluorescence can probably be attributed to glial cells as well as artifactual oxidation of HE. (f) Fluorescence was quantified using Image J (NIH), and is expressed as percent control values ± SD ($n = 3$). The increase in fluorescence intensity induced by 100 µmol/L acrolein was slight and statistically insignificant. 500 µmol/L acrolein, however, produced a significant increase in fluorescence. This effect was reduced by adding 500 µmol/L hydralazine to the acrolein solution. *$p < 0.01$. (g) Superoxide was generated by reaction of hypoxanthine with xanthine oxidase, and levels were assessed by loss of cytochrome *c*. The rate of change in absorbance of cytochrome *c* was monitored at 550 nm. Values are expressed as percent control ± SD ($n = 5$). 575 U/mL SOD, used as positive control, demonstrated significant scavenging of superoxide. Hydralazine at a concentration of 500 µmol/L or 1 mmol/L did not result in significant scavenging of superoxide. *$p < 0.001$ (compared to control).

In order to determine whether hydralazine has any direct effect on membrane repair (in addition to its acrolein-scavenging abilities), the change of membrane potential in response to transection was also evaluated. As described in our previous studies, transection resulted in an immediate depolarization followed by a slow repolarization (Shi and Borgens 2000; Shi and Pryor 2000; Shi *et al.* 2000, 2001). Membrane depolarization for each white matter strip is normalized for the post-transection value (% of max). Sixty minutes following transection, membrane depolarization in white matter strips treated with 500 µmol/L hydralazine was reduced to 3.3 ± 5.8%, which is not significantly different than control values of 2.7 ± 2.5% ($p = 0.82$, $n = 3$) (Mann–Whitney test, STATA). This suggests that hydralazine does not directly influence membrane repair because of mechanical injury.

Compression injury

Compression injury resulted in a time-dependent trend of LDH release (Fig. 7). LDH release was significantly increased following compression (compared to controls) at all time points ($p < 0.05$ at 1 h; $p < 0.001$ at 2 and 3 h). One hour following injury, hydralazine did not have a significant effect on LDH release in compressed cords (compared to compression only, $p > 0.05$). However, hydralazine treatment resulted in significantly less LDH released at 2 and 3 h following compression injury (compared to compression only, $p < 0.05$). Hydralazine treatment did not have a significant effect on uninjured cords at any time point (compared to controls, $p > 0.05$). In addition, compression injury increased immunostaining for acrolein-lys adducts to from 100 ± 36.0% to 126.0 ± 44.4% of control values ($p < 0.001$), an effect that was reduced to 96.7 ± 37.1% of controls by hydralazine treatment ($p < 0.001$) (Fig. 8). Immunostaining in uninjured cords treated with hydralazine tended to be slightly lower than controls (95.1 ± 35.4% of controls), an effect that was not stastically significant ($p > 0.05$).

Discussion

Consistent with previous studies, we show here that acrolein, in the absence of other injuries, is capable of producing

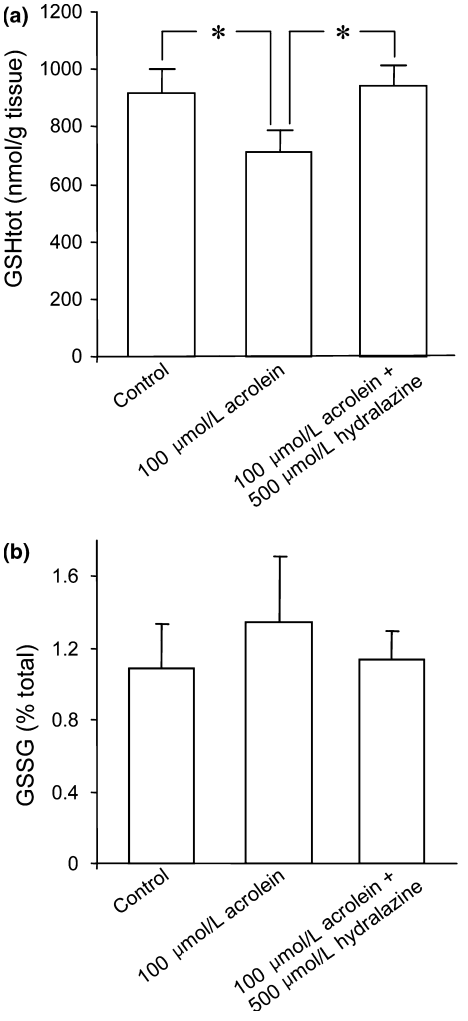

Fig. 4 Effects of acrolein and hydralazine on glutathione. (a) Concentration of total GSH, measured by the DTNB recycling method, is expressed as nmol/g tissue ± SD ($n = 3$). Incubating spinal cord segments for 4 h in 100 μmol/L acrolein resulted in depletion of endogenous glutathione. This effect was completely inhibited by 500 μmol/L hydralazine. *$p < 0.05$. (b) The concentration of GSSG was measured using 2-vinylpyridine and the DTNB recycling method, and is expressed as the percent of total glutathione ± SD ($n = 3$). There is a trend toward increased oxidation of glutathione following 4 h incubation with 100 μmol/L acrolein, although this effect is not statistically significant ($p > 0.05$). GSSG levels in spinal cord treated with acrolein and hydralazine are nearly identical to controls.

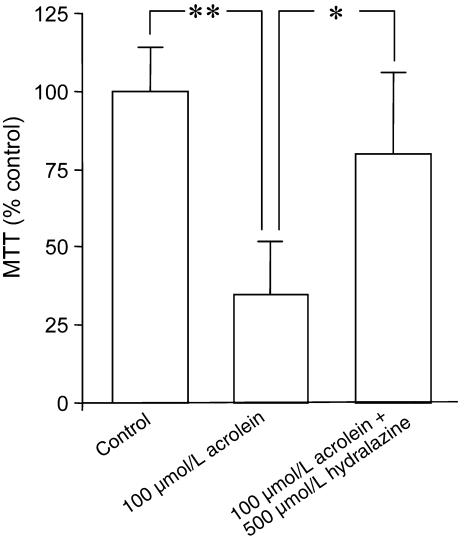

Fig. 5 Effect of acrolein and hydralazine on MTT assay. The MTT assay was performed on synaptosomal mitochondria isolated from spinal cord segments treated with acrolein and hydralazine. This assay is positively correlated with mitochondrial function. Results are expressed as percent control values ± SD ($n = 5$). Incubation for 4 h in 100 μmol/L acrolein reduced the results of the MTT assay, an effect which was significantly attenuated by 500 μmol/L hydralazine. *$p < 0.01$, **$p < 0.001$.

oxidative stress and inflicting anatomical and functional damage (Shi *et al.* 2002; Luo and Shi 2004, 2005; Luo *et al.* 2005b; Liu-Snyder *et al.* 2006b). We further demonstrate that hydralazine, even when applied 15 min after acrolein, is capable of inhibiting acrolein-mediated injuries in *ex vivo* guinea pig spinal cord. Perhaps the most significant finding of this study, however, is that hydralazine attenuates membrane damage following compression injury in *ex vivo* spinal cord, which is consistent with our findings that acrolein-lys adducts are increased following compression injury *ex vivo*, an effect that is prevented by hydralazine treatment. We also report that hydralazine is not an efficient scavenger of superoxide, one of the most abundant ROS, at the concentrations used in this study and has no direct effect on recovery of membrane potential following transection. Hydralazine's ability to inhibit acrolein-induced and compression injuries can thus most likely be attributed to its acrolein-trapping capabilities, and not superoxide scavenging or direct repair of damaged membranes.

It is noteworthy that a delayed application of hydralazine resulted in not only inhibition, but also reversal of acrolein-mediated injuries. For instance, we have previously shown that acrolein can inflict significant membrane damage beginning as early as 15 min following exposure (Luo and Shi 2004). However, when applied 15 min following the onset of the exposure of acrolein, hydralazine not only prevented additional acrolein-induced membrane damage, but also resulted in a restoration of membrane integrity to a level that is similar to uninjured cords. This indicates that hydralazine not only prevents further damage of the membrane, but also allows the repair of already damaged membranes. This is consistent with the notion that neuronal membranes have an intrinsic ability to repair disruption (Shi and Pryor 2000). We hypothesize that such repair mechanisms can be either masked or overwhelmed under conditions of oxidative stress, and removal of toxic LPO byproducts such as acrolein restores the balance to favor intrinsic repair mechanisms.

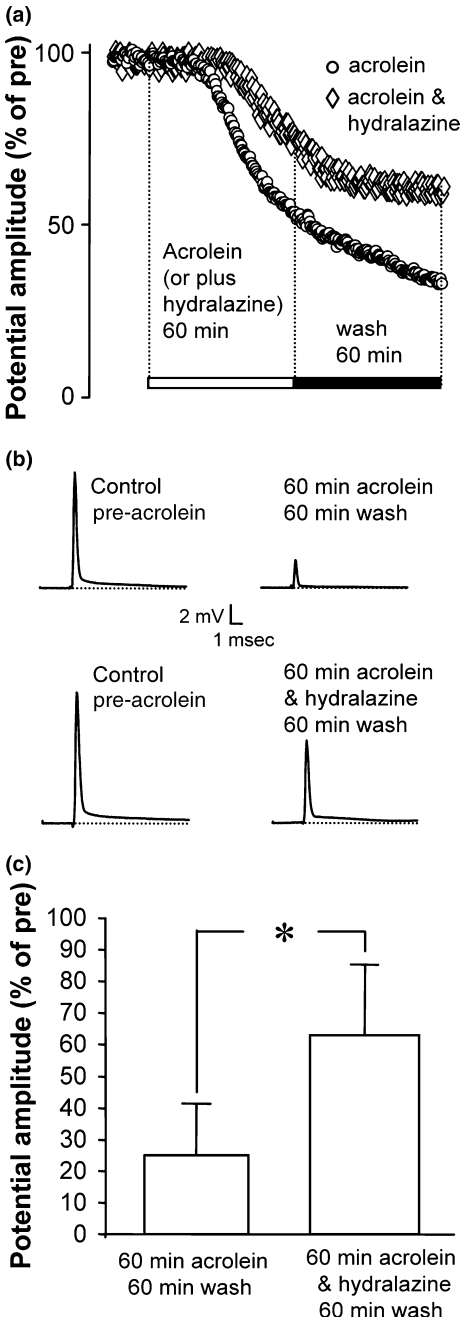

Fig. 6 Effects of acrolein and hydralazine on CAP. Axonal function was assessed by CAP following treatment with acrolein and hydralazine. Ventral white matter strips were exposed to acrolein at a concentration of 500 μmol/L with (n = 4) or without (n = 5) 1 mmol/L hydralazine for 60 min. White matter strips were then washed with Kreb's solution and CAPs recorded for an additional 60 min. Figure (a) is a plot of a representative recording of the amplitude of CAPs from a white matter sample. Figure (b) shows representative waves of individual CAPs before and after acrolein exposure, with or without hydralazine. CAPs were normalized for the pre-injury value, expressed in (c) as percent of pre ± SD. Note that the acrolein-mediated reduction of CAP amplitude was significantly attenuated by hydralazine. *p < 0.05.

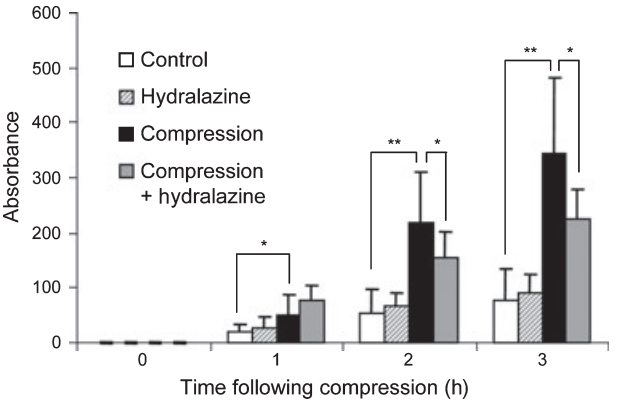

Fig. 7 LDH release following compression. Membrane integrity was assessed by release of LDH (140 kD), an intracellular enzyme that leaks out of injured cells. LDH release from spinal cord was assayed at 1-h intervals following compression injury as a measure of membrane damage, and is expressed as mean absorbance ± SD (n = 5). Compression injury resulted in increased LDH release from spinal cord at all time points. Hydralazine significantly attenuated LDH release at 2 and 3 h following compression, but not at 1 h. Hydralazine had no significant effect on uninjured spinal cord at any time point. One-way paired ANOVA and *post hoc* Newman Keul's test were used for statistical analysis. *p < 0.05, **p < 0.001.

It is well established that glutathione is one of the body's primary defense mechanisms against oxidative injury, and furthermore that acrolein readily forms conjugates with glutathione that are subsequently excreted from the body. However, depletion of glutathione is also an important mechanism by which acrolein shifts the balance of pro- and antioxidant systems toward oxidative stress. In the current study, we found that total glutathione was significantly decreased in the presence of acrolein, while GSSG remained relatively unchanged. This scenario is potentially more detrimental than if glutathione were merely recycled between GSH and GSSG, as restoration of the antioxidant defense system requires synthesis of glutathione rather than reduction of GSSG by glutathione reductase. The loss of total glutathione with little or no increase in GSSG is similar to what is seen in brain and spinal cord injury *in vivo* (Cooper *et al.* 1980; Lucas *et al.* 2002), and supports the hypothesis that toxic aldehydes such as acrolein overwhelm the endogenous antioxidant system and play an important role in oxidative stress following CNS trauma.

One unique feature of this line of investigation is the parallel examination of electrical impulse conduction, one of the most important functions of nervous system, along with other anatomical and biochemical assessments. It is noteworthy that the acrolein-mediated decline in CAP amplitude was not restored even 60 min after acrolein was washed off, but rather CAP tended to continue to decline (Fig. 6a). This suggests that the injury processes that are induced by acrolein are continued even after it is removed, which is consistent

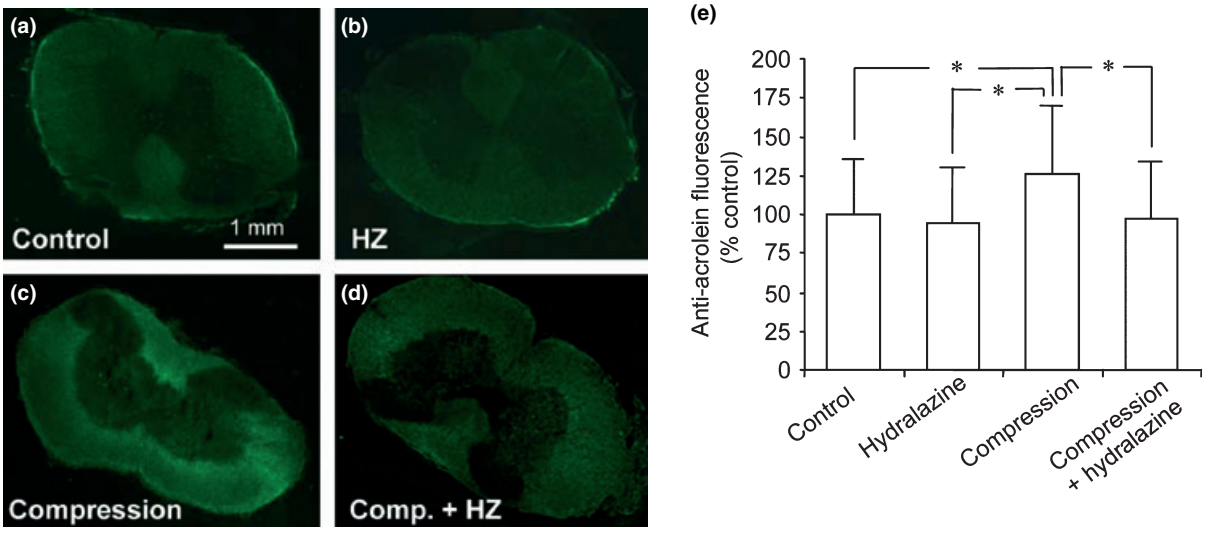

Fig. 8 Detection of acrolein-lys by IHC following compression. Acrolein-lys adducts were detected by immunohistochemistry. Representative images are shown for: (a) control, (b) 500 μmol/L hydralazine (HZ), (c) compression, and (d) compression plus 500 μmol/L hydralazine. (e) Fluorescence intensity was quantified using Image J (NIH) and is expressed as percent control values ± SD (n = 5). Compression injury resulted in a significant increase in acrolein-lys immunoreactivity, an effect that was prevented by hydralazine treatment. Note that fluorescence intensity is greatest in the white matter. One-way paired ANOVA and *post hoc* Newman Keul's test were used for statistical analysis. *$p < 0.001$.

with the hypothesis that acrolein formation is a bioamplification step in oxidative injury. In tissue injured by acrolein with hydralazine added, the decline of CAP amplitude was significantly attenuated. Thus, hydralazine combats not only acrolein-mediated toxicity, but also the perpetuation of the injury processes that are set into play by acrolein.

Perhaps the most clinically relevant finding of this study is that protection by hydralazine was also seen following compression injury without application of exogenous acrolein, which further implicates the role of acrolein in mechanical spinal cord injury. This protection by hydralazine was only seen at 2 and 3 h following injury; hydralazine treatment did not attenuate compression-mediated membrane damage at 1 h (consistent with resting membrane potential recordings 1 h following transection), which suggests that significant acrolein-mediated injury begins 1–2 h following mechanical injury *ex vivo*. The increase in membrane permeability at 1 h can thus be largely attributed to the effects of the primary mechanical injury, while at 2 and 3 h secondary injury processes are probably providing an additional contribution to membrane damage. This is consistent with our findings that protein-bound acrolein is significantly increased following compression injury *ex vivo*, and furthermore that this effect is prevented by hydralazine. In summary, we have demonstrated that hydralazine prevents compression-mediated increases in protein-bound acrolein, attenuates acrolein and compression-mediated injuries, and that these effects are not likely mediated by direct repair of mechanical injury to membranes or scavenging of superoxide. Thus, hydralazine's ability to improve membrane integrity following compression is most likely because of its acrolein-trapping abilities. As there was no exogenous acrolein added in this model of compression injury, this further supports the hypothesis that endogenous acrolein plays a significant role in the pathogenesis of spinal cord injury and represents a novel and effective target for inhibiting secondary injury mechanisms. Additional studies are underway to further evaluate hydralazine-mediated protection following compression injury.

Although the concentration of acrolein in spinal cord is unknown, concentrations have been evaluated in other tissue types. Specifically, acrolein concentrations are estimated to reach 80 μmol/L in a model for respiratory tract lining fluids of a smoker (Eiserich *et al.* 1995). In the plasma of patients with renal failure, protein-bound acrolein reaches 180 μmol/L, a six-fold increase (Sakata *et al.* 2003). In the normal and AD brain, acrolein is increased from 0.9 (controls) to 2.5 nmol/mg protein (AD brains) in the amygdala, and from 0.7 to 5 nmol/mg protein in the parahippocampal gyrus (Lovell *et al.* 2001). We estimated the protein concentration of guinea pig spinal cord to be 9.6 ± 2.5 g/100 g wet tissue (n = 3) (Bicinchoninic Acid Protein Assay, Pierce; spinal cord homogenate permeabilized with 3% Triton X-100). Using a specific gravity of 1 for spinal cord (estimated by volume displacement), the concentrations of acrolein used in this study, 100–500 μmol/L, correspond to 1.0–5.2 nmol/mg protein, which is likely in the range of what could be found following spinal cord injury *in vivo*. Furthermore, acrolein concentrations have been demonstrated to be increased 7 days following injury (Luo *et al.* 2005a), and could remain

increased even longer. While this model uses exposure times of 4 h or less (because of the limited survival time of *ex vivo* tissue), acrolein exposure *in vivo* is likely much longer, and concentrations even lower than 100 μmol/L may have significant toxicity *in vivo*. In addition, acrolein could potentially reach locally higher concentrations at certain intracellular locations, such as near the plasma membrane where it is formed.

The concentration of hydralazine used in this study, 500 μmol/L or 1 mmol/L, was selected based on previous studies in PC12 cells that used concentrations ranging from 25 μmol/L to 1 mmol/L (Liu-Snyder *et al.* 2006a). Furthermore, previous studies identified by ^{1}H NMR the primary reaction product to be (1*E*)-acrylaldehyde phthalazin-1-ylhydrazone, a product of 1 : 1 acrolein-trapping by hydralazine (Kaminskas *et al.* 2004a). They further demonstrated that, in a cell-free system, equimolar concentrations of hydralazine and acrolein resulted in nearly complete loss of acrolein. In this study, we have used up to a five-fold molar excess of hydralazine to ensure effective trapping in tissue. Higher concentrations were not used because of previous reports of the toxic effects of concentrations in excess of 1 mmol/L *in vitro* (Williams *et al.* 1980; Weglarz and Bartosz 1991; Runge-Morris *et al.* 1994). Although these concentrations could not be achieved in serum *in vivo*, as the concentration following i.v. administration at the antihypertensive dose is estimated to peak at 0.5–1.0 μmol/L (Reece 1981), this study is important proof-of-principle that acrolein-trapping is an effective means of inhibiting secondary injury processes in spinal cord. While topical application of hydralazine is being evaluated as potential treatment, we are also currently working to design a more effective acrolein-trapping agent for systemic treatment. For example, a potentially more effective agent may be able to trap many molecules of acrolein, or be specifically targeted to the injury site, and yet still be able to enter the cell.

Oxidative stress plays an important role in the pathogenesis of not only spinal cord trauma, but many other diseases as well, including AD, Parkinson's disease, ischemia-reperfusion injury, trauma, inflammation, and neoplasia. Thus, acrolein could potentially play an important role in these diseases as well. Indeed, acrolein has already been found to be significantly increased in the AD brain (Lovell *et al.* 2001). Significant increases in HNE, another aldehydic LPO byproduct, have also been detected in Parkinson's disease brains (Yoritaka *et al.* 1996). In addition, acrolein is a component of cigarette smoke that has been implicated in lung cancer (Feng *et al.* 2006) and is a known carcinogen (Esterbauer *et al.* 1991; Feron *et al.* 1991; Cohen *et al.* 1992; Kehrer and Biswal 2000). As a result of its well-established toxicity, relatively long half-life, and role in perpetuating oxidative injury, acrolein represents a novel target for preventing oxidative injury and potential treatment for not only spinal cord injury, but many other diseases as well.

Acknowledgements

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