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Polyethylene glycol immediately repairs neuronal membranes and inhibits free radical production after acute spinal cord injury

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Abstract

Membrane disruption and the production of reactive oxygen species (ROS) are important factors causing immediate functional loss, progressive degeneration, and death in neurons and their processes after traumatic spinal cord injury. Using an *in vitro* guinea pig spinal cord injury model, we have shown that polyethylene glycol (PEG), a hydrophilic polymer, can significantly accelerate and enhance the membrane resealing process to restore membrane integrity following controlled compression. As a result of PEG treatment, injury-induced ROS elevation and lipid peroxidation (LPO) levels were significantly suppressed. We further show that PEG is not an effective free radical scavenger nor does it have the ability to suppress xanthine oxidase, a key enzyme in generating superoxide. These observations suggest that it is the PEG-mediated membrane repair that leads to ROS and LPO inhibition. Furthermore, our data also imply an important causal effect of membrane disruption in generating ROS in spinal cord injury, suggesting membrane repair to be an effective target in reducing ROS genesis.

Keywords: lipid peroxidation, membrane integrity, polyethylene glycol, reactive oxygen species, scavenger, spinal cord injury.

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Injury to neuronal membranes plays a pivotal role in initial tissue disruption and subsequent degeneration that leads to long-term functional deficits and disabilities in traumatic brain and spinal cord injury. Injured neurons have the ability to initiate the repair process to restore their membrane integrity (Meiri *et al*. 1981; Yawo and Kuno 1985; Xie and Barrett 1991; Krause *et al*. 1994; Shi and Pryor 2000; Shi *et al*. 2000). The effectiveness of this spontaneous repair, however, varies considerably between animal species and in response to different experimental conditions (Meiri *et al*. 1981; Yawo and Kuno 1985; Xie and Barrett 1991; Krause *et al*. 1994; Shi and Pryor 2000; Shi *et al*. 2000). It is still not clear to what extent endogenous repair can be achieved by CNS neuronal membranes following compression. The current study is designed to first examine the membrane repair process in a well-developed *in vitro* spinal cord preparation following controlled compression (Shi and Blight 1996). We chose to examine the membrane permeability based on the abilities of three molecules of different molecular weights that enter or leave the cell at various times post-injury.

Our previous studies, and those from other laboratories, suggest that enhancing membrane repair might be an effective intervention to promote functional recovery following mechanical insult (Xie and Barrett 1991; Shi *et al*. 1999, 2000; Shi and Borgens 2000; Shi and Pryor 2000). We have shown that polyethylene glycol (PEG), a hydrophilic polymer, can reduce membrane permeability. This was measured using a horseradish peroxidase (HRP)-exclusion test (Shi and Borgens 2000). Such anatomical restoration is associated with functional recovery in both *in vitro* and *in vivo* spinal cord injury (Shi *et al*. 1999; Borgens and Shi 2000; Shi and Borgens 2000; Borgens and Bohnert 2001; Donaldson *et al*. 2002). However, it is unknown whether PEG can seal membrane breaches smaller than those allowing HRP uptake. Said another way, we do not know the completeness of membrane repair induced by PEG

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Abbreviations used: DPPH, 1,1-diphenyl-2-picrylhydrazyl; EB, ethidium bromide; HE, hydroethidine; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LPO, lipid peroxidation; PEG, polyethylene glycol; ROS, reactive oxygen species; SCI, spinal cord injury; SOD, superoxide dismutase; XO, xanthine oxidase.

compared to spontaneous repair. This can be achieved by evaluating a label that is 100 times smaller than HRP as well as a molecule that is four times larger and comparing this to sealing induced by PEG in the same preparation.

The influx of calcium and sodium has been suggested as one of the mechanisms which generate reactive oxygen species (ROS) in various types of injury, including traumatic spinal cord injury (Young 1992; Lewen *et al*. 2000). However, there has not been a direct examination of the relationship between membrane disruption and the genesis of ROS secondary to mechanical damage of spinal cord. Previously, we have reported a significant increase in ROS and lipid peroxidation (LPO) after compression in an *in vitro* guinea pig spinal cord injury (Luo *et al*. 2000; Luo and Shi 2001). Here we examine the role of PEG in the possible reduction of ROS where the effectiveness of the membrane sealing, ROS levels, and LPO can be quantified in the same preparation.

Methods and materials

Spinal cord isolation, the injury model, and PEG treatment

The technique for extraction of the cord has been described previously (Shi and Blight 1996; Shi *et al*. 2000). Following extraction, the cord was separated into two halves by midline sagittal division (Fig. 1a). The spinal cords were then maintained in oxygenated Kreb's solution at 37°C for at least an hour to ensure the maximal recovery from dissection before experimentation. The composition of the Kreb's solution was described in our previous publications (Shi and Blight 1996).

The isolated spinal cords were randomly divided into three groups: control-uninjured, injured-untreated, and injured-treated. The compression injury was induced by a constant-displacement of 5-s compression of the spinal cord using a modified forceps possessing a spacer (Fig. 1; Shi and Blight 1996; Shi *et al*. 1999; Borgens and Shi 2000; Shi and Borgens 2000; Borgens and Bohnert 2001; Donaldson *et al*. 2002). In the injured-treated group, samples were incubated with PEG solution immediately after compression injury for 3 min. The PEG solution was made by dissolving 5 g PEG (MW 2000) in 5 mL distilled water (final concentration: 50%, w/v). Following PEG treatment, the spinal cord strips were thoroughly rinsed with Kreb's solution. A similar concentration of PEG and the duration of treatment were shown to be neuroprotective but not toxic in previous studies (Shi and Borgens 1999, 2000; Borgens and Shi 2000; Borgens *et al*. 2002). All the samples were bubbled with 95% O_2/5% CO_2 and kept at 37°C throughout the duration of the experiment.

Measurement of membrane permeability

Three molecules with different molecular weights were used to determine induced membrane permeability to labels introduced from the interstitial fluid, or leaking from the cytosol. HRP (MW 44 kDa) and ethidium bromide (EB, MW 400 Da) were added to the incubation solution and thus exposed to cord strips. The uptake of these two molecules through membrane breaches was used as an indicator of membrane damage. Lactate dehydrogenase (LDH, MW 140 kDa) is the largest of the three testing agents. Leakage of this enzyme to the extracellular space is indicative of membrane disruption (Koh and Choi 1987). As each of these three molecules has a distinct size (based on its molecular weight), the information obtained from all three molecules gives a more complete picture of the dynamic of plasma membrane disruption. All the anatomical determination of resealing was carried out by a person who had no prior knowledge of the treatments of the specimens.

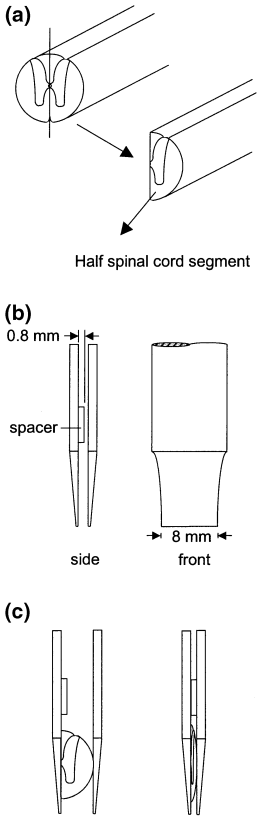

Fig. 1 Schematic drawing of the isolated spinal cord strips, the injury device, and its application. (a) After being isolated from the guinea pig, the spinal cord was split into two halves. (b) Front and side view of the end of the modified forceps, to show the tips ground to produce two parallel inner surfaces, 8 mm long, which rest 0.8 mm apart when the forceps are closed by finger pressure against the spacer. (c) Following the extraction, the tips of the forceps were placed on both sides of the strip. The forceps were then closed quickly, compressing the spinal cord, and were held against the spacer for 5 s.

Measurement of EB uptake

Ethidium bromide, a cell-impermeable fluorochrome, enters a cell only when the plasma membrane is damaged (Aeschbacher *et al*. 1986). Upon entrance, EB undergoes a 40-fold enhancement of fluorescence intensity after binding to nucleic acids. Therefore, quantification of fluorescence gives an indication of the intactness of the plasma membrane (Dey and Majumder 1988). In this study, we used EB as the smallest molecule of the three indicators of membrane damage to determine whether PEG would seal membranes sufficiently to exclude a molecule that is 100 times smaller

than HRP. The spinal cord strips were exposed to EB (1 μM) for 5 min in the dark. After being fixed with 3.7% formaldehyde for 2 h, the cord was then sectioned at a thickness of 40 μm. Observations of these sections were made with an Olympus Vanox fluorescent microscope (excitation filter: BP 545 nm; barrier filter: 0–590 nm; Olympus America Inc., Melville, NY, USA). The EB fluorescent images were captured through a 2× objective lens, using a high-resolution CCD camera (DEI-750, Optronic Eng., Goleta, CA, USA). The acquisition and analysis of data were done with an image analysis program, Sigmascan Pro (SPSS Science, Chicago, IL, USA). Camera exposure settings were constant for each experiment. To compensate for the variation in fluorescent intensity for individual images, all EB fluorescence values were corrected for background fluorescence. To rule out the possibility that EB attached only to the surface of cells, we used confocal microscopy (Bio-Rad confocal microscope, MRC-1024, Bio-Rad Laboratories, Hercules, CA, USA) to confirm that EB was indeed inside the cell subsequent to membrane damage (data not shown).

Measurement of HRP uptake

Horseradish peroxidase uptake was measured as described in previous studies (Asano *et al*. 1995; Shi and Borgens 2000; Shi and Pryor 2000; Shi *et al*. 2000). Segments of spinal cord strips were transferred at different times after injury to undergo HRP incubation, fixation, and staining with diaminobenzidine (DAB). Axons were counted and normalized by dividing this number by the unit area of the sample and expressed as a density (axons/mm^2).

Measurement of LDH release

The molecule of LDH is larger than HRP and EB, and offers unique information concerning the status of membrane integrity. At 15-min intervals from the time of the compression, the solution bathing the injured cord was collected and replaced with fresh Krebs. After the final incubation period (60-min post-injury), the cord strips were quickly homogenized with phosphate-buffered saline (PBS), centrifuged at 2000 *g* for 5 min and the supernatant was collected to assess the residual tissue LDH. LDH activity was determined using a LDH assay (*in vitro* toxicology assay kit, stock No. TOX-7, Sigma, St Louis, MO, USA). The amount of LDH released into the incubation solution was expressed as a percentage of the total LDH (including the LDH remaining in the tissue as well as that released into the solution).

Measurement of ROS and LPO

Measurement of superoxide

Superoxide (O_2^-) production was measured using hydroethidine (HE), a fluorescent dye oxidized to ethidium selectively by O_2^-. The oxidized HE (or ethidium) signifies the intracellular level of superoxide (Bindokas *et al*. 1996; Chan *et al*. 1998). The spinal cord samples were incubated with 1 mL of PBS with HE at final concentrations of 1 μM for 5 min at 37°C in the dark before fixation. Tissue fixation, sectioning, and image capturing and analysis were the same as those used in the measurement of ethidium bromide uptake.

Lipid peroxidation

Lipid peroxides (products of LPO and common indicators of lipid peroxidation by free radicals) were measured using a lipid hydroperoxide assay (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, the samples of spinal cord, 6 mm in length, were obtained from the center of the injury site, weighed, and homogenized. The homogenate was centrifuged at 2000 *g* for 5 min and the supernatant was collected. Lipid hydroperoxides were then immediately extracted from the sample into chloroform. The chromogenic reaction was conducted at 37°C for 5 min. The absorbance was read at 500 nm using a 96-well plate with a spectrophotometer (SLT spectra plate reader, SLT Labinstrument, Salzburg, Austria). 13-Hydroperoxy octadecadienoic acid (13-HpODE) was used as the standard. Tissue lipid peroxide was calculated and expressed as nanomoles per 100 mg of wet tissue (nmol/100 mg).

Antioxidant activity assays using cell-free systems

Quenching of DPPH (DPPH test)

The stable free radical scavenging capacity of PEG was tested as bleaching of 1,1-diphenyl-2-picrylhydrazyl (DPPH; Mellors and Tappel 1966) and compared to superoxide dismutase (SOD) and ascorbic acid as reference inhibitors. DPPH is a stable N-centered free radical. The DPPH scavenging ability of free radical scavengers can be determined by measuring the decrease in DPPH levels at 517 nm. DPPH was dissolved in 60% ethanol. The test compounds (SOD, ascorbic acid, PEG) were added to 100 μM DPPH for 30 min at room temperature. The absorbance was monitored spectrophotometrically at 517 nm.

Superoxide scavenging test

The superoxide scavenging ability of the test compounds (SOD, PEG) was determined by monitoring their competition with cytochrome c for O_2^- (Quick *et al*. 2000). The superoxide (O_2^-) was generated by xanthine/xanthine oxidase (XO; Fridovich 1970). The reaction mixture contained different concentrations of test compounds in a 50-mM potassium phosphate buffer (pH 7.4), 0.1 mM xanthine and 40 μM cytochrome c. The reaction was initiated by adding xanthine oxidase (0.02 U/mL). Using a spectrophotometer (SLT spectra plate reader, SLT Labinstrument, Salzburg, Austria), the absorbance at 550 nm was monitored automatically every 2 min during a 10-min period after mixing all of the reaction reagents. SOD (100 U/mL) was used as a reference inhibitor.

Xanthine oxidase inhibition assay

The XO activities with xanthine as the substrate were measured spectrophotometrically by monitoring uric acid production from the absorbance change at 295 nm (Noro *et al*. 1983; Sweeney *et al*. 2001). The assay mixture consisted of 0.5 mL of test solution, 1.3 mL of 1 : 15 M phosphate buffer (pH 7.5), and 0.2 mL of enzyme solution. The mixture was pre-incubated at 30°C for 10 min. The reaction was initiated by adding 1.5 mL of substrate solution. The assay mixture was incubated at 30°C and the absorbance (295 nm) was measured spectrophotometrically every 30 s for 10 min, using a UV : vis spectrophotometer (Lambda 3B, Perkin Elmer, Shelton, CT, USA) with a temperature control unit. All assays were triplicated. Allopurinol, a known inhibitor of XO, was used as a reference inhibitor at a final concentration of 10 μg/mL in the assay mixture. XO inhibitory activity was expressed as the

percentage inhibition of XO in the above assay mixture system, calculated as percentage inhibition = $[1\text{-}(\Delta A/\text{min}_{\text{test}})/(\Delta A/\text{min}_{\text{blank}})] \times 100\%$, where $\Delta A/\text{min}_{\text{test}}$ is the linear change in absorbance per minute of test material, and $\Delta A/\text{min}_{\text{blank}}$ is the linear change in absorbance per minute of blank.

Chemicals

LDH assay kit, HRP (MW 44 kDa, type VI), DAB, xanthine, xanthine oxidase, cytochrome c, ascorbic acid, SOD (product number: S 2515), allopurinol, and DPPH were purchased from Sigma Chemical Co. Ethidium bromide and HE were obtained from Molecular Probes (Eugene, OR, USA). Lipid peroxide assay kit was purchased from Cayman Chemical Company (Ann Arbour, MI, USA).

Statistical analysis

The data is expressed as a mean ± SD. The data were analyzed by one-way ANOVA using the statistics software package SPSS (version 11, SPSS, Chicago, IL, USA). Results showing overall significance were subjected to post-hoc Least-significance difference test; $p < 0.05$ was considered statistically significant.

Results

The dynamics of membrane damage following compression

Fifteen minutes after injury, membrane permeability increased significantly to EB, HRP, and LDH compared with the control-uninjured group (Figs 2c and i, 3c and i and 4a). Although the increase in permeability lasted at least 60 min post-injury for all three molecules, the time courses were somewhat different, as indicated by the measurements made at different time points post-injury. No significant difference was found in the EB fluorescence intensity at 1 min and 60 min post-injury (Figs 2c, f and i; $p > 0.05$). However, similar measurements for HRP and LDH were different. The membrane permeability to HRP peaked at 1 min post-injury, and then gradually decreased thereafter, although it still remained significantly higher than the control-uninjured group at 60 min post-injury (Fig. 3i; $p < 0.05$). The membrane permeability to HRP at 60 min

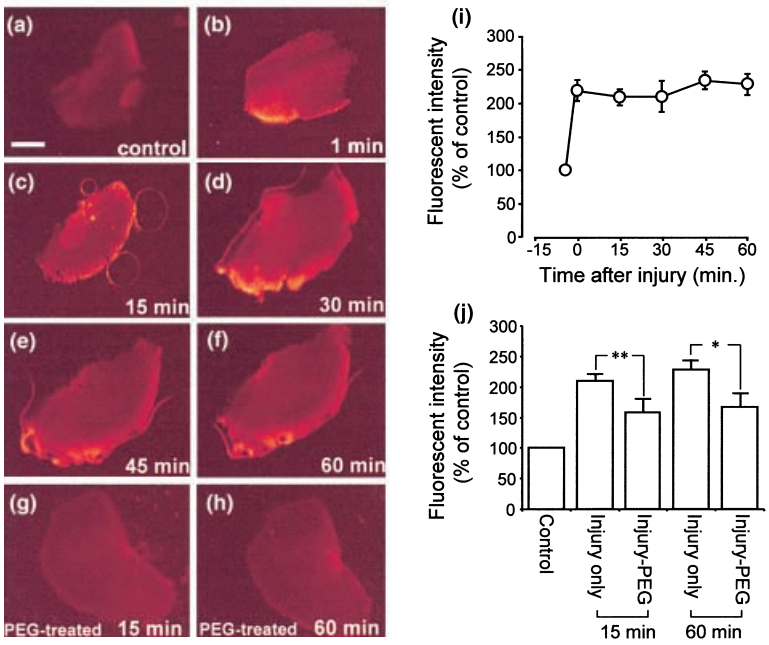

Fig. 2 Ethidium bromide (EB) fluorescence intensity following spinal cord compression under different conditions. All histological sections for this and subsequent figures were obtained from the center of the injury site. (a) Fluorescence intensity of a representative cross-section in the control-uninjured group. (The poor image is due to extremely low fluorescence.) (b–f) Representative micrographs showing the changes in ethidium bromide fluorescence intensity in the injured-untreated group at 1 min (b), 15 min (c), 30 min (d), 45 min (e), and 60 min (f) following compression injury. Note the stability of EB fluorescence intensity between 1 min and 60 min post-injury. (g,h) Fluorescence intensity in the injured- and PEG-treated group at 15 min (g) and 60 min (h) following injury and PEG treatment. (i) Quantification of EB fluorescence intensity changes as a function of time up to 1 h post-compression. The fluorescence intensity of the sample area was normalized by background subtraction (see Methods). Fluorescence intensity is expressed as a percentage of the control group. Note the compression injury caused significant increase of EB fluorescence intensity compared with the pre-injury value ($n = 6$). All values post-injury were significantly different compared to pre-injury values ($p < 0.01$). However, there is no significant difference of EB fluorescent intensity at 60 min after compression when compared with that at 1 min post-injury ($p > 0.05$). (j) Quantification of EB fluorescence intensity changes as a result of PEG treatment at 15 and 60 min following injury and treatment. The fluorescence intensities were quantified in a manner similar to that described in (i) of this figure ($n = 6$). $^{**}p < 0.01$, $^{*}p < 0.05$. The scale bar in (a) = 200 μm, for (a–h).

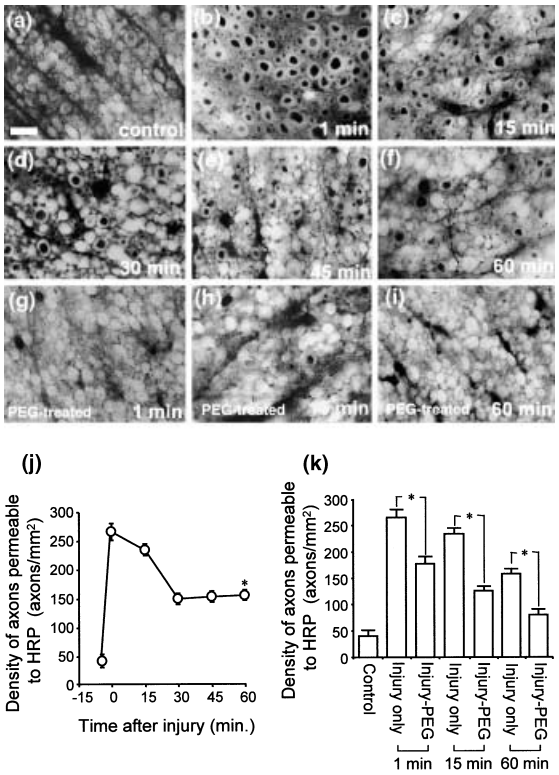

Fig. 3 Horseradish peroxidase (HRP) labeling following compression injury under different conditions. (a) HRP labeling of a representative cross-section of spinal cord in the control-uninjured group. Note the lack of axonal labeling. (b–f) Representative micrographs showing the changes in density of HRP labeling in the injured-untreated group at 1 min (b), 15 min (c), 30 min (d), 45 min (e), and 60 min (f) following compression injury. (g–i) HRP labeling in the injured-PEG-treated group at 1 min (g), 15 min (h) and 60 min (i) following injury and PEG treatment. Note the near elimination of HRP uptake. (j) The density of axons permeable to HRP (axons/mm^2) as a function of time post-injury. Note the compression injury caused significant increase in HRP labeling compared with that of pre-injury values ($n = 6$). Also note the peak of HRP labeling occurred at 1 min after injury, and then gradually decreased to reach a plateau by 30 min post-injury. All values post-injury were significantly different compared to pre-injury ($p < 0.01$). The asterisk indicates a significant difference of HRP labeling at 60 min after compression when compared with that at 1 min post-injury ($p < 0.05$). (k) The density of axons permeable to HRP (axons/mm^2) as a function of PEG treatment. PEG was applied for 3 min immediately following compression. Note that HRP labeling decreased significantly at 1 min, 15 min and 60 min post-injury ($n = 6$, $^*p < 0.01$). Scale bar in (a) = 10 μm, for (a–i).

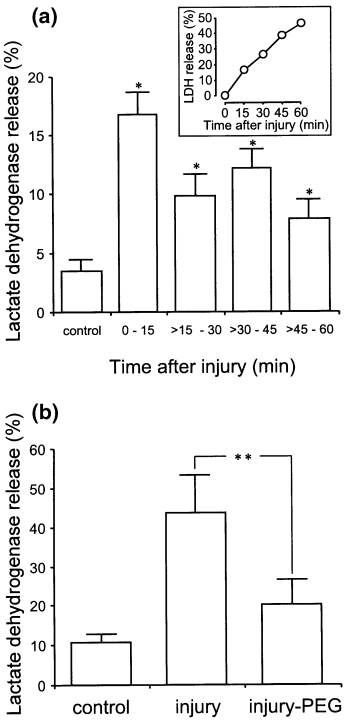

Fig. 4 LDH release after compression injury and its inhibition by PEG. (a) LDH release measured every 15 min post-compression. Specifically, at the end of each 15-min interval from the time of the compression, the solution bathing the injured cord was collected and replaced with fresh Krebs. The collected Krebs was assessed for LDH activity based on a lactate dehydrogenase assay. The data are displayed as the value in each 15-min period independently or accumulatively (inset). In the inset graph where the accumulative method was employed, the data at each time point are the sum of the values from the current interval and all the previous intervals from the time of injury. Note the peak of LDH release occurred at the first 15-min interval post-injury. Asterisks indicate significant difference of post-injury LDH release when compared with that of pre-injury ($n = 5$), $^*p < 0.05$. In addition, the LDH released in the last period (45–60 min) was significantly lower than that in the initial period (0–15 min), $p < 0.05$. (b) LDH release as a function of PEG treatment. The accumulative LDH release data at 60 min was used as the comparative time post-injury. PEG was applied immediately post-injury for 3 min. Note that PEG significantly decreased LDH release following compression ($n = 5$), $^{**}p < 0.01$.

was, however, significantly lower than that at 1 min ($p < 0.05$). Similarly, the membrane permeability to LDH peaked in the initial period (0–15 min) post-injury, and then decreased thereafter (Fig. 4a; $p < 0.05$), though the LDH released during 45–60 min post-injury was still significantly higher than that of the control-uninjured group (Fig. 4a; $p < 0.05$). The difference in membrane permeability to LDH between the initial period (0–15 min post-injury) and the period of 45–60 min post-injury was significant ($p < 0.05$).

PEG repairs membrane damage

When the spinal cord strips were exposed to PEG (50% w/v) for 3 min immediately after injury, the permeability to all molecules was decreased compared to the injured-untreated group. Specifically, exposure to PEG resulted in reductions of EB florescence at 15 min and 60 min post-compression by

47% and 48%, respectively, compared to the injured-untreated group ($p < 0.001$ and $p < 0.05$, respectively). The percent reduction in HRP permeability caused by exposure to PEG was 33% at 1 min ($p < 0.01$), 46% at 15 min ($p < 0.01$), and 46% at 60 min ($p < 0.01$). Similarly, the accumulation of LDH in extracellular fluid aliquots was also reduced by 45% at 60 min following compression and PEG treatment ($p < 0.01$, Fig. 4b).

Effect of PEG on ROS generation and lipid peroxidation

Because membrane damage has been implicated in ROS induction and lipid peroxidation, we determined whether ROS and LPO were reduced as a result of PEG application. As shown in Figs 5(a–d), superoxide generation, signified by the fluorescence of oxidized HE (ethidium), was increased by 118% (218% of control) at 1 h post-injury ($p < 0.01$). The application of PEG significantly reduced superoxide production at 1 h post-injury, as indicated by the reduction in fluorescence from 218% to 178% of the control-uninjured group ($n = 5$, $p < 0.05$).

The level of LPO was similarly decreased as a result of PEG treatment. As shown in Fig. 5(e), the LPO level increased significantly at 1 h post-compression in the injured-untreated group. Specifically, the concentration of lipid peroxides, a standard measurement of tissue LPO level, was 19.3 ± 1.7 nmol/100 mg post-injury, while the pre-injury level was 9.1 ± 0.41 nmol/100 mg (Fig. 5e). This represents a more than 100% increase ($p < 0.01$, $n = 5$). The application of PEG reduced this level of lipid peroxidation to 14.6 ± 1.2 nmol/100 mg, a 24.3% decrease ($p < 0.05$, $n = 5$).

Antioxidant activity of PEG

In light of PEG's ability to reduce ROS level and LPO post-injury, we decided to examine the possibility that PEG itself acts as a free radical scavenger in addition to its known ability to repair membrane disruption. This was first tested using a DPPH quenching test. SOD (100 U/mL) and ascorbic acid (100 mM), two known free radical scavengers, significantly decreased the absorbance of DPPH by 56% and 63%, respectively ($p < 0.01$, $n = 5$; Fig. 6a). However, PEG at two concentrations (50% and 5%, w/v) had almost no effect on the absorbance of DPPH ($p > 0.05$, $n = 5$; Fig. 6a). These results strongly suggest that PEG is not a free radical scavenger.

Because the application of PEG reduced the injury-induced O_2^- (Fig. 5d), we further tested whether PEG could directly scavenge O_2^-. This was performed using a cell free superoxide-scavenging test based on cytochrome c reduction by superoxide. As a reference inhibitor, the application of SOD resulted in almost complete inhibition of cytochrome c reduction. PEG at two concentrations (25% and 2.5%, w/v) failed to inhibit cytochrome c reduction (Fig. 6b), suggesting that PEG is not an O_2^- scavenger.

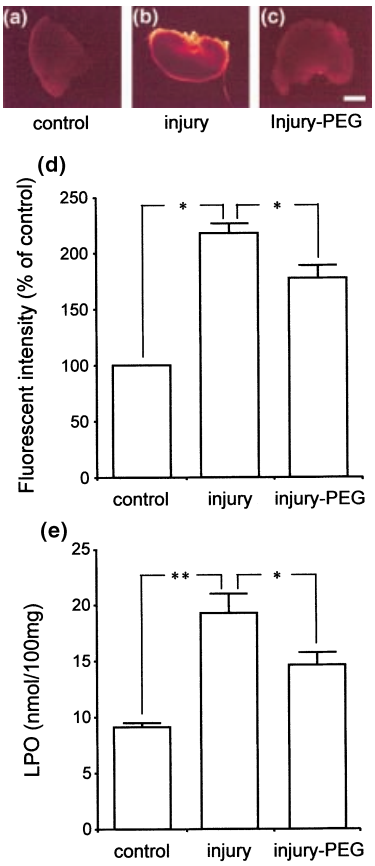

Fig. 5 ROS generation and lipid peroxidation after spinal cord compression injury and inhibition by PEG. (a–c) Representative micrographs showing oxidized hydroethidine (HE) fluorescence intensity in the control-uninjured (a), injured-untreated (b), and injured-PEG-treated cord groups (c). For (b) and (c) measurements were made 60 min post-injury. PEG was applied immediately following compression for 3 min. Note the significant increase of oxidized HE fluorescence following compression (b) and its significant reduction by PEG (c). (d) Quantification of oxidized HE fluorescence intensity in uninjured and in compression-injured cords at 1 h post-injury, with and without PEG treatment. As in previous figures, the fluorescence intensity of the sample area was normalized by background subtraction (see Methods). Values are expressed as percentages of the control groups. Note the compression injury caused a significant increase in oxidized HE fluorescence intensity ($n = 5$, $^*p < 0.01$). PEG significantly reduced oxidized HE fluorescence intensity ($n = 5$, $^*p < 0.05$). (e) PEG inhibits lipid peroxidation at 60 min following injury. LPO (nmol/100 mg) was expressed as mean ± SD. Note the compression injury caused a significant increase in the level of LPO ($n = 5$, $^{**}p < 0.01$). Again, a brief exposure of PEG significantly reduced LPO levels ($n = 5$, $^*p < 0.05$).

We next determined the effect of PEG on the activity of XO, one of the key enzymes responsible for superoxide generation. Allopurinol, a known XO inhibitor, was used as a reference inhibitor. As shown in Fig. 7, XO activity was almost completely inhibited by allopurinol (10 μg/mL, 97% inhibition). PEG at two concentrations (50% and 5%, w/v)

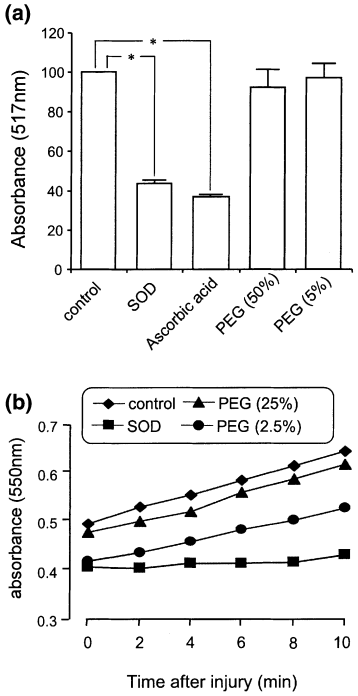

Fig. 6 ROS scavenging tests using cell-free systems. (a) The scavenging ability of PEG was examined using a DPPH quenching assay. The decrease in DPPH absorbance (i.e. a decrease in DPPH concentration) is expressed as percentage of controls where free radical scavengers were not included. Note that SOD (100 U/mL) and ascorbic acid (100 mM), two known free radical scavengers, significantly decreased the level of DPPH ($n = 5$, $^*p = 0.0005$), while PEG at two concentrations (50% and 5%, w/v) had no effect on the level of DPPH ($n = 5$, $p > 0.05$) when compared to control. (b) Superoxide quenching tests. In this assay, the level of superoxide was quantified as a reduction of cytochrome c – monitored by the absorbance of reduced cytochrome c at 550 nm. Note the steady increase of the reduced-form of cytochrome c level over a period of 10 min in the control (◆) group. SOD (■, 100 μ/mL) completely inhibited this increase in the reduced-form of cytochrome c. Again, PEG at two concentrations [25% (▲) and 2.5% (●), $n = 5$] failed to inhibit the reduction of cytochrome c.

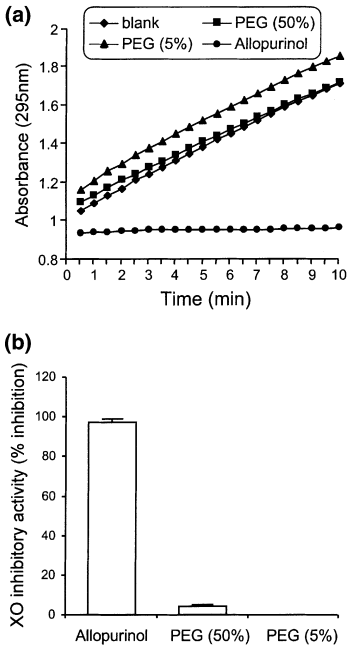

Fig. 7 PEG application and xanthine oxidase (XO) activity. The XO activity was measured spectrophotometrically using the xanthine/XO system. The increase of absorbance of uric acid at 295 nm reflects XO activity. (a) Data obtained from one experiment from a total of five. Note the linear increase in the absorbance at 295 nm in the blank (◆, control without free radical scavengers), reflecting the stable activity of XO over a period of 10 min Allopurinol (●, 10 μg/mL), a known XO inhibitor, completely inhibited this increase (shown as a function of absorbance). PEG at two different concentrations [50% (■) and 5% (▲)] did not affect this increase in absorbance. (b) Quantification of the inhibitory effect of allopurinol and PEG when measured at the end of a 10-min testing period ($n = 5$).

had little effect on the xanthine oxidase activity (4.5% and 0.05% inhibition, respectively), suggesting that PEG was not a XO inhibitor.

Discussion

Plasma membrane damage after spinal cord injury

Although it is well established that membrane damage can result from mechanical insults in both *in vitro* and *in vivo* CNS injury (Borgens *et al*. 1980; Emery *et al*. 1987; Xie and Barrett 1991; Pettus *et al*. 1994; Asano *et al*. 1995; Fitzpatrick *et al*. 1998; Shi and Borgens 1999, 2000; Shi *et al*. 2000), it is not entirely clear how battered neurons and/or their processes can effectively repair membrane disruption. In the current study, the neurons and axons of the spinal cord displayed evidence of spontaneous resealing within 60 min of compression. However, such spontaneous repair failed to completely seal the membrane disruption to even HRP uptake during this time. Previous study indicates that transected axons in a similar test paradigm can seal the cut end to HRP uptake within 60 min of injury under physiological conditions (37°C and 2 mM extracellular calcium; Shi and Pryor 2000; Shi *et al*. 2000). In this study, only 50% of compressed axons excluded HRP at 60 min after injury (Fig. 3). This paradoxically indicates that compressed axons may not spontaneously seal themselves as well as they do when completely severed. Furthermore, the poor membrane resealing secondary to compression injury was again revealed when EB, a molecule that is 100 times smaller than HRP, was used. Membrane permeability to EB increased immediately following compression and remained virtually the same for the entire 60-min period of monitoring (Fig. 2).

The possible mechanisms underlying this paradox are not entirely clear. One clue may be that transection produces a

clean, localized, transverse breach while compression produces a longitudinal membrane 'lesion' perhaps millimeters in extent. It is reasonable to suggest that compression may produce numerous membrane disruptions to even one axon within the area of damage. This notion of 'one injury' versus 'multiple injuries' should likely influence the dynamics of the membrane to reseal and repair the disruptions. It is worthwhile to remember that spinal cord injury in humans is mainly associated with compression rather than acute transection of axons. Thus, compression may produce the more intractable injury associated with prolonged axonal degeneration and functional loss seen in traumatic spinal cord injury in animal and man.

PEG repairs neuronal membrane after spinal cord injury

In a series of experiments, we have shown that even a brief application of PEG could repair membrane damage and improve functional recovery in both *in vitro* and *in vivo* guinea pig spinal cord injury (Shi and Borgens 1999; Shi *et al*. 1999; Borgens and Shi 2000; Borgens and Bohnert 2001; Borgens *et al*. 2002; Donaldson *et al*. 2002). Moreover, PEG reduced the membrane permeability to HRP when examined at 15 min after a standardized compression injury (Shi and Borgens 2000). In this report, PEG significantly reduced the permeability of membrane to HRP as early as 1 min following its application (Fig. 3). This further emphasized the effectiveness of PEG in repairing membrane disruption, consistent with our previous results where compressed spinal cord white matter improved compound action potential conductance within minutes of PEG application (Shi and Borgens 1999). Such functional recovery demonstrates that PEG-induced reduction of EB and HRP permeability is due to the improvement of membrane integrity by repairing membrane disruption, rather than non-specifically blocking the entry of EB and HRP. We now know that PEG application can significantly reduce the membrane permeability to EB, which has a MW 100 times smaller than that of HRP. This further underscores PEG's ability to seal and repair even minute 'holes' in the axolemma. Taken together, the current studies indicate that PEG not only can accelerate sealing against large molecules like HRP, but to much smaller molecules such as EB as well. Moreover, we have shown even larger holes in the membrane than that which permit HRP uptake can be closed – as demonstrated by the reduced extracellular accumulation of LDH lost from neurons and axons after damage and subsequent to PEG treatment.

Additionally, we measured the permeability of these three molecules as a function of time (up to 60 min) post-injury. This allowed an evaluation of the kinetics of PEG-induced membrane sealing under different conditions, as well as a comparison to endogenous membrane repair. There are two results from these experiments worthy of comment: (i) it took at least 15 min for the endogenous repair mechanism to significantly lower membrane permeability to HRP and LDH, while PEG-induced sealing to these same compounds was almost immediate; (ii) endogenous sealing mechanism(s) failed to significantly reduce the injury-induced intracellular elevation of EB by 1 h after injury. PEG application, however, induced a significant decrease in membrane permeability to EB as early as 15 min after injury. In summary, damaged axonal membranes sealed faster and more completely as a consequence of PEG treatment.

PEG inhibits ROS

Our data indicate that PEG not only repairs membrane disruption, a primary damage, but also strikingly reduces the ROS elevation, a secondary injury associated with mechanical injury. This is significant as ROS is a key component of secondary cascades in the pathogenesis of traumatic spinal cord and brain injury (Hall 1989, 1995; Hall *et al*. 1992). This is also consistent with previous data showing that PEG application can induce significant functional and behavioral recovery even when delayed for up to 8 h after injury in adult guinea pigs (Borgens and Shi 2000; Borgens *et al*. 2002). We have also shown that PEG has high affinity for only injured cord tissue independent of its route of administration (topical or injection into the blood), suggesting a particular application to any hemorrhagic neural injury (Borgens and Bohnert 2001; Donaldson *et al*. 2002).

It is probable that PEG-mediated ROS reduction is dependent on its capacity to seal the membrane, and not on any scavenging capacity intrinsic to the polymer. Clearly, increased membrane permeability has been linked to ROS generation (Lewen *et al*. 2000). Plasma membrane disruption results in an influx of extracellular calcium activating several free radical pathways, up regulating superoxide (O_2^-) and hydrogen peroxide (H_2O_2), in particular (Young 1992; Lewen *et al*. 2000). It has yet to be shown, but likely, that PEG repairs membrane damage, reducing calcium influx, and thereby inhibiting ROS and LPO. This explanation is also consistent with results where Poloxamer-188, a polyethylene–polypropylene–polyethylene co-polymer (triblock), restored membrane integrity and blocked lipid peroxidation (Marks *et al*. 2001). In addition, PEG has been shown to reduce lipid peroxidation in non-neuronal tissue, such as rat hepatocytes during cold storage (Mack *et al*. 1991) and rat kidney following cold storage and normothermic reperfusion (Hauet *et al*. 1998, 2001). Altogether these findings indicate that PEG has membrane protective effects in a variety of cells against many different types of insults.

Though PEG can indeed restore membrane integrity, it does not follow that this is the only mechanism by which PEG might reduce secondary injury. The alternatives include: (i) accelerating the degradation of ROS (enhanced scavenging) or (ii) directly inhibiting key enzymes in the generation of ROS. These data, however, do not support such speculations; quite the contrary, they are antithetical to them. Specifically, two sensitive cell-free scavenging tests revealed

PEG to have virtually no ability to scavenge DPPH, an N-centered stable free radical (Fig. 6a), or O_2^-, a major component of ROS (Fig. 6b). Further, PEG had little effect in suppressing XO, one of the key enzymes responsible for O_2^- generation (Fig. 7). In summary, based on the fact that PEG does not have the ability to scavenge superoxide and DPPH nor the ability to inhibit XO, we conclude that it is likely that PEG inhibits superoxide and LPO mainly through the restoration of membrane integrity and subsequent reduction of ROS genesis following compression injury.

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