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Research Report

Polyethylene glycol inhibits apoptotic cell death following traumatic spinal cord injury

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ABSTRACT

We have previously shown that local administration of polyethylene glycol (PEG, MW: 2000 Da, 50% by weight), a known membrane repair agent, immediately after trauma in guinea pig spinal cord repairs neuronal membrane disruptions and reduces oxidative injury. Here we report that a similar application of PEG resulted in marked decreases in apoptotic cell death and caspase-3 activity. We suggest that PEG may suppress apoptosis through interactions with mitochondria. This is based on our current findings that in isolated mitochondria, PEG improves mitochondrial function and reduces the release of cytochrome *c*, a pro-apoptotic cell death factor. This hypothesis is further supported by our previous observation that PEG enters injured cells after spinal cord injury, placing PEG in a position to directly interact with mitochondria. In summary, we conclude that PEG reduces both necrosis and apoptosis through two distinct yet synergistic pathways: repair of disrupted plasma membranes and protection of mitochondria through direct interaction.

1. Introduction

We have reported that polyethylene glycol (PEG), a hydrophilic polymer, immediately repairs neuronal membranes and restores electrical impulse conduction when applied after mechanical insults (Luo et al., 2002; Shi and Borgens, 1999). PEG also partially restores, possibly through direct interaction, anatomical, and functional integrity of mitochondria in mechanically injured mammalian spinal cord tissues (Luo et al., 2004). Since synaptic terminals and cell bodies can have greater densities of mitochondria than axons, these results suggest that PEG might also have beneficial effects on cell bodies in addition to well-established protection of axons. Furthermore, PEG application following spinal cord trauma *in vivo* enhanced behavioral and neurological functions (Borgens and Shi, 2000; Borgens et al., 2002). These PEG-mediated beneficial effects are evident minutes to hours after PEG application. PEG reduces necrosis, dominant type of cell fatality in the acute stage of injury (Luo et al., 2002, 2004).

However, it is unknown whether PEG inhibits apoptotic cell death, another major factor in cell death in the sub-acute CNS trauma (Beattie et al., 2000; Eldadah and Faden, 2000; Emery et al., 1998; Keane et al., 2001; Raghupathi et al., 2000). Apoptotic cell death likely contributes to the expansion of the lesion and

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Abbreviations: PEG, polyethylene glycol; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end labeling; Ac-DECD-AFC, N-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin; GSH, glutathione; CsA, cyclosporin A; Apaf-1, apoptotic protease-activating factor 1; DATP, deoxy-ATP; PTP, mitochondrial permeability transition pore

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subsequent progressive neurological deficits (Beattie et al., 2000; Eldadah and Faden, 2000; Lu et al., 2000). Hence, it is important to determine whether PEG also reduces apoptosis, in addition to necrosis, in order to enhance long-term functional recovery in spinal cord injury. The significance of this is highlighted by the previous suggestion that there may be a balance of necrosis and apoptosis, and even if necrosis is reduced, the cells may still die later by apoptosis (Zipfel et al., 2000).

Recently, we have shown that in addition to its well-established ability to ameliorate primary injury through plasma membrane repair, PEG also significantly reduces oxidative stress after traumatic spinal cord injury (Luo et al., 2002; Luo and Shi, 2004), a major component of so-called secondary injury mechanisms. This effect has been shown to be at least partially attributed to the protection of mitochondria (Luo et al., 2004). Since oxidative stress plays a pivotal role in regulating apoptosis (Carmody and Cotter, 2001; Fleury et al., 2002; Raha and Robinson, 2001; Wood and Youle, 1995), it is tempting to speculate that PEG-mediated mitochondrial protection can also result in suppression of apoptosis.

The objective of this study was to test whether PEG reduces apoptotic cell death, and to determine the possible mechanisms in a well-developed *in vivo* guinea pig spinal cord injury model. Such experiments were designed to further clarify the role of PEG as a neuroprotective agent, not only in reducing necrosis in the acute stage but also apoptosis in the sub-acute and chronic stages. We hypothesized that PEG could inhibit apoptosis by inhibiting cytochrome *c* release, a known factor leading to apoptosis, from mitochondria. This hypothesis was based on our previous observations that PEG can enter injured cells after spinal cord injury and directly inhibit mitochondrial permeability transition in isolated mitochondria (Luo et al., 2004). This hypothesis was also based on the knowledge that the formation of mitochondrial permeability transition could lead to mitochondrial membrane rupture and cytochrome *c* release (Gorman et al., 2000; Green and Reed, 1998; Halestrap et al., 2000).

2. Results

2.1. *PEG inhibits apoptosis and caspase activity after SCI*

Apoptosis is a major source of cell loss and has been suggested to contribute to neurological deficits after spinal cord injury (SCI) (Beattie et al., 2000; Eldadah and Faden, 2000; Lu et al., 2000). To investigate whether treatment with PEG inhibited apoptosis, we studied apoptotic cell death using the TUNEL staining technique. Apoptotic cell death at T10–11, the epicenter of the injury, remained at a very low level during a period of 7 days after surgery in both the sham–vehicle (Figs. 1A, D) and sham–PEG groups (data not shown). There was no significant difference between these two groups ($P > 0.05$). Therefore only the sham–vehicle group was included for further statistical analysis, and one-way unpaired ANOVA and post hoc test were used. Compression injury induced a significant increase in apoptosis at 24 h and 7 days post injury ($P < 0.01$, injured–vehicle vs. sham–vehicle; Figs. 1B–D), with a higher value at 24 h after injury. Treatment with PEG immediately after injury significantly reduced apoptosis at both 1 day and 7 days after injury. As shown in Fig. 1D, the number of TUNEL-positive cells of the

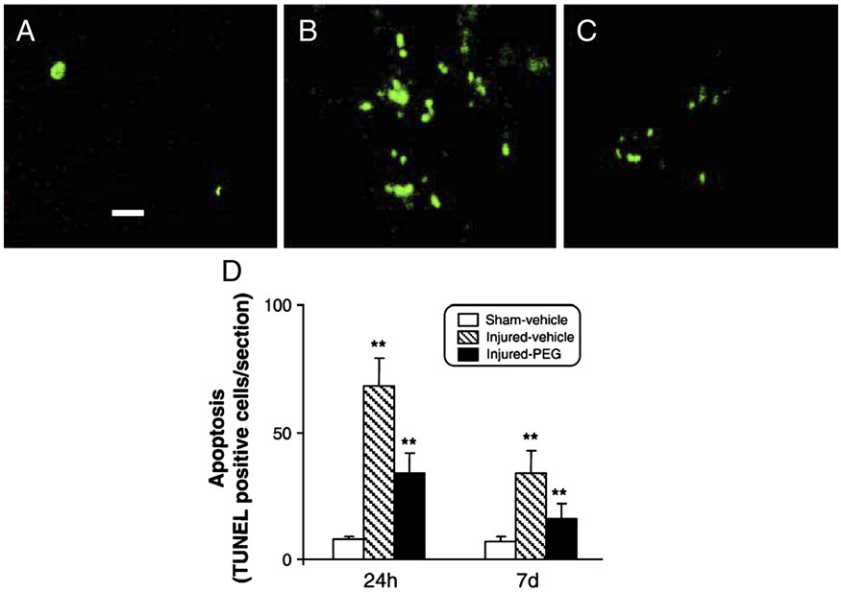

Fig. 1 – Apoptosis after SCI and the effects of PEG treatment. Apoptosis was determined by TUNEL staining and quantified by counting TUNEL-positive cells. (A–C) Representative photographs showing the TUNEL staining in samples from sham–vehicle (A), injured–vehicle (B), and injured–PEG (C) animals at 24 h after injury. Scale bar = 20 μM. (D) Quantification of TUNEL-positive cells from different groups. The results were expressed as number of TUNEL-positive cells per section ($n = 3–5$ per group per time point). $^{**}P < 0.01$ between sham–vehicle and injured–vehicle or injured–vehicle and injured–PEG groups.

injured–PEG group was significantly lower than that of the injured–vehicle group at both 1 day and 7 days after injury ($P < 0.01$ for comparisons between injured–vehicle and injured–PEG groups).

It has been demonstrated that caspase-3 activation plays an important role and serves as a marker for apoptotic cell death in both neurons and glia following spinal cord injury (Beattie et al., 2000; Eldadah and Faden, 2000; Emery et al., 1998; Keane et al., 2001; Raghupathi et al., 2000). In the current study, biochemical analysis of caspase-3 activity showed similar results to the above TUNEL staining observations (Fig. 2). In the injured–vehicle group, caspase-3 activity was significantly increased at 1 day and 7 days after injury ($P < 0.01$, compared with sham–vehicle). Similarly, PEG treatment resulted in decreased caspase-3 activity, with a significant reduction observed at 1 day ($P < 0.01$, injured–PEG vs. injured–vehicle).

2.2. *PEG reduces mitochondrial GSH loss after calcium stimulation*

During apoptotic cell death, mitochondria release cytochrome *c* and other pro-apoptotic cofactors necessary for activation of effector caspases. To investigate whether PEG can exert a direct protective effect on mitochondria, we isolated non-synaptic mitochondria from the spinal cord tissue and stimulated them with Ca^{2+}. Incubation with Ca^{2+} resulted in significantly lower levels of mitochondrial GSH, while treatment with PEG and CsA significantly attenuated GSH loss. Specifically, incubation of 100 μM Ca^{2+} decreased mitochondrial GSH from 4.89 ± 0.23 nmol/mg protein to 2.99 ± 0.31 nmol/mg protein (Fig. 3A, $P < 0.01$). In PEG- and CsA-treated groups, the GSH levels were 3.89 ± 0.35 nmol/mg protein and 4.49 ± 0.21 nmol/mg protein, respectively; both were significantly higher than the group treated with Ca^{2+} only (Fig. 3A, $P < 0.01$). The Ca^{2+} concentration used in these experiments is clinically relevant since it falls into the range of typical extracellular Ca^{2+} concentration after SCI (Young et al., 1982). Since GSH is important in maintaining mitochondrial function and integrity (Reed, 1990), these results suggest that PEG has a direct beneficial effect on mitochondrial function and are consistent with our previous *in vivo* findings that PEG improves synaptosomal mitochondrial function following spinal cord injury (Luo et al., 2004).

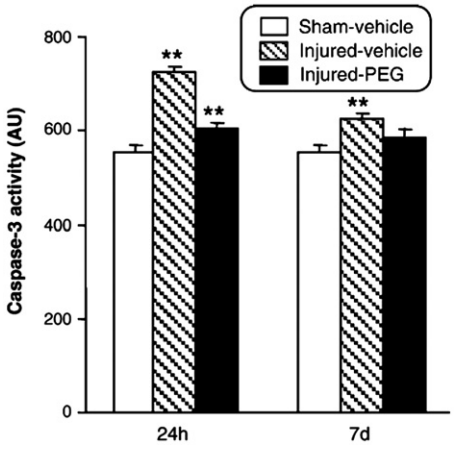

Fig. 2 – Caspase-3 activity changes at 24 h and 7 days after SCI. Caspase-3 activity was determined by caspase-3-specific substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DECD-AFC) and expressed as arbitrary units of fluorescence intensity. **$P < 0.01$ between sham–vehicle and injured–vehicle or injured–vehicle and njured–PEG groups, $n = 3$–5 animals per group.

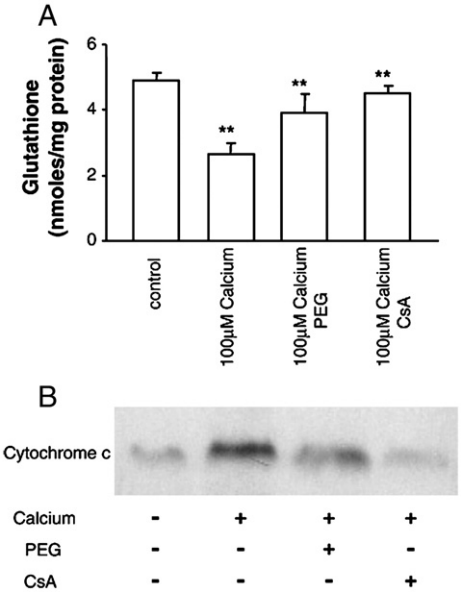

Fig. 3 – Effect of PEG on mitochondrial GSH and cytochrome *c*. Non-synaptic mitochondria were incubated at 0.5 mg of protein/ml in a medium containing (in mM) 125 KCl, 3 KH_2PO_4, 0.5 mM MgCl, 0.1% BSA, and 10 HEPES (pH 7.4). Stock 300-mosM solution of PEG (MW: 2000 Da) (102 mM) was made in 3 mM HEPES buffer (pH 7.4) (Pfeiffer et al., 1995). When used, PEG (final concentration: 10.2 mM) was added 5 min prior to the addition of Ca^{2+} and was present throughout the experiment. Mitochondria were then incubated with 100 μM Ca^{2+} for 30 min. (A) Mitochondrial GSH loss after Ca^{2+} stimulation. Preincubation with PEG significantly reduced GSH loss. **$P < 0.01$ between control and Ca^{2+} or between Ca^{2+} and Ca^{2+} plus PEG ($n = 3$). (B) PEG inhibited cytochrome *c* release in isolated mitochondria. After incubation, mixtures were centrifuged at 12,000×*g* at 4 °C and the supernatant was evaluated by Western blotting. A representative image from three independent experiments was shown.

2.3. *PEG inhibits cytochrome c release in vitro*

One prominently observed event during apoptosis is the permeability transition leading to the release of molecules such as cytochrome *c* from the intermembrane space. Since PEG has previously been shown to inhibit mitochondrial permeability transition (Luo et al., 2004), we investigated whether PEG reduces cytochrome *c* release from isolated, non-synaptic spinal cord mitochondria. As expected, Ca^{2+} induced significant cytochrome *c* release. Preincubation of PEG with mitochondria significantly inhibited Ca^{2+}-induced cytochrome *c* release (Fig. 3B). Cyclosporin A, a known inhibitor

of mitochondrial permeability transition (positive control), similarly inhibited cytochrome c release from isolated mitochondria treated with Ca^{2+}. These findings indicate that PEG-mediated reduction of cytochrome c release from isolated mitochondria is a likely mechanism by which it inhibits apoptotic cell death.

3. Discussion

3.1. PEG reduces cell death after SCI

We have previously demonstrated that application of PEG after spinal cord injury immediately reduced spinal cord tissue damage and promoted functional recovery (Shi and Borgens, 1999; Shi et al., 1999; Shi and Borgens, 2000). It was suggested that such acute stage neuroprotection offered by PEG is mainly through reduction of acute cell death or necrosis by immediate repair of membrane disruption. In this study, we have demonstrated that a similar exposure of PEG is effective in suppressing apoptotic cell death, a form of cell death with a later onset time than necrosis. This is consistent with our findings that PEG could reduce oxidative stress, a known factor causing apoptosis, after traumatic spinal cord injury (Luo et al., 2002, 2004; Luo and Shi, 2004). The fact that the apoptotic cell death was significantly lower in the injured–PEG group at 7 days after injury suggests that an early application of PEG is capable of effectively reducing not only necrosis but also apoptotic cell death. Said another way, the cells that were rescued by PEG at the acute stage were mostly still alive at a later stage and were not dying later due to apoptosis. Since continuous cellular destruction through apoptotic cell death may play a critical role in the pathophysiology of SCI, the current results are significant in that they emphasize the importance of early intervention in modifying the long-term biochemical and functional recovery after SCI. The current study has also further established PEG as an effective intervention in traumatic spinal cord injury, and perhaps in traumatic brain injury as well (Koob et al., 2005; Koob and Borgens, 2006). Moreover, previous studies showed that delayed treatment of PEG through intravenous or subcutaneous application had similar protective effects on CNS trauma (Borgens et al., 2002; Koob and Borgens, 2006). Whether such various PEG applications would inhibit apoptotic cell death needs further investigation. However, given the facts that all these variations of PEG application had protective effects, we speculate that they might inhibit apoptosis as well.

3.2. PEG reduces mitochondrial glutathione loss in vitro

Based on our study, we suggest that PEG-mediated inhibition of apoptosis is due in part to suppression of oxidative stress. Glutathione (GSH) is an important antioxidant in mitochondria. Mitochondrial GSH is the only defense available to metabolize hydrogen peroxide (Fernandez-Checa et al., 1998). Mitochondrial GSH is important for maintaining intramitochondrial protein thiol groups in the reduced states and preserving the integrity of mitochondrial membrane (Reed, 1990). The mechanism by which PEG attenuates GSH depletion is not clear. It could be related to the ability of PEG to inhibit mitochondrial membrane permeability transition, since previous studies suggested that GSH could be released through the mitochondrial membrane permeability transition pore (Savage and Reed, 1994). Nevertheless, by maintaining the mitochondrial pool of GSH, PEG not only reduces mitochondrial oxidative stress but also facilitates mitochondrial function, both of which could lead to the reduction of apoptosis.

3.3. PEG inhibits cytochrome c release in vitro

Further evidence linking mitochondria to PEG-mediated reduction of apoptosis is the direct inhibition of cytochrome c release by PEG. In this study, we have demonstrated that incubation of isolated mitochondria with PEG significantly attenuated the Ca^{2+}-induced cytochrome c release. Mitochondria play a central role in apoptotic cell death by controlling cellular energetics, increasing the production of reactive oxygen species, and releasing pro-apoptotic factors into the cytosol (Bernardi et al., 1999; Cai et al., 1998; Fiskum, 2000; Green and Reed, 1998; Kroemer et al., 1998; Raha and Robinson, 2001; Zamzami and Kroemer, 2001). The most prominent pro-apoptotic factor released from mitochondria is cytochrome c (Green and Reed, 1998; Zamzami and Kroemer, 2001). It is electrostatically bound to the outer surface of the inner mitochondrial membrane. After its release into the cytosol, cytochrome c is capable of binding to the apoptotic protease-activating factor 1 (Apaf-1). This complex activates procaspase-9 in the presence of deoxy-ATP (dATP), resulting in the activation of the caspase cascade (Green and Reed, 1998; Kroemer and Reed, 2000; Li et al., 1997). Therefore, inhibition of cytochrome c release is likely an important mechanism by which PEG reduces apoptosis and inhibits caspase activity *in vivo*. Furthermore, PEG-mediated inhibition of cytochrome c release is probably due to its already described ability to inhibit formation of mitochondrial membrane permeability transition pore (Luo et al., 2004).

3.4. The mechanisms of PEG-mediated neuroprotection

We have previously suggested that the possible mechanisms of PEG-mediated neuroprotection include repairing cell membranes (Borgens, 2001; Luo et al., 2002; Shi and Borgens, 1999) and direct interaction with mitochondria (Luo et al., 2004). This hypothesis is further supported by the results of the current study that PEG attenuated mitochondrial GSH loss and cytochrome c release. PEG has been shown to suppress the swelling of mitochondria or induce contraction of such swollen mitochondria in various preparations (Brustovetsky and Dubinsky, 2000; Halestrap et al., 1997; Pfeiffer et al., 1995). It is well established that mitochondrial swelling is associated with mitochondrial functional deficits, oxidative stress, and cytochrome c release (Halestrap, 1999; Zamzami and Kroemer, 2001). Therefore, the results of this study are consistent with previous reports that PEG alleviates mitochondrial swelling. Since synaptic terminals and cell bodies have higher densities of mitochondria than axons, and apoptotic cell death detected by TUNEL staining will not reflect axonal damage but only cell bodies, the current results suggest that PEG might also have protective effects on cell bodies, in addition to repairing axonal membrane.

For PEG to act directly on mitochondria, it needs to be able to reach an effective intracellular concentration. For example, for PEG at a MW of 2000 Da, we found the concentration that inhibited mitochondrial swelling *in vitro* to be about 2.0–10.2 mM (current study and unpublished observations). Since this is only 0.5–2.5% of the concentration of PEG (about 400 mM) applied to spinal cord *in vivo*, we believe that this concentration is achievable *in vivo*. Further experiments will be needed to determine if PEG could reach an effective concentration when administered *in vivo*.

In summary, based on our findings, we propose a hypothetical model for dual neuroprotective action of PEG in the spinal cord following traumatic injury (Fig. 4). A traumatic injury to the spinal cord causes breaches in neuronal membrane, allowing extracellular solutes (such as calcium) to enter the injured cells, leading to rapid cytosolic organelle damage (such as mitochondrial swelling as shown in Fig. 4A) and possible acute cell death. As a result of PEG application, a layer of PEG film is formed around the membrane that promotes membrane resealing (Figs. 4A–C). During this process, some PEG molecules enter the cell through the membrane breaches. PEG within the cytosol reaches mitochondria, protecting mitochondria from swelling (Figs. 4A–C). Specifically, by blocking the mitochondrial permeability transition pore, PEG inhibits cytochrome *c* release and subsequent activation of caspases and ultimately apoptotic cell death. PEG-mediated GSH protection further protects mitochondria from oxidative injury.

4. Experimental procedures

4.1. *Animal models and experimental groups*

A standard guinea pig spinal cord injury model described previously (Borgens and Shi, 2000; Borgens et al., 2002; Luo et al., 2004) was used in this study. Briefly, after anesthesia, a compression injury at T10–T11 was induced by a constant–displacement, 15-s compression of the spinal cord, using a modified forceps possessing a spacer. Within 5 min of injury, an aqueous solution of PEG (MW: 2000 Da, 50% by weight) was applied with a pipette to the exposed injury site for 2 min and then removed by aspiration (Borgens and Shi, 2000; Borgens et al., 2002). The site of the PEG application was then immediately lavaged with isotonic Krebs' solution (124 mM NaCl, 2 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM dextrose, 26 mM $NaHCO_3$) and any excess PEG and/or Krebs' solution was removed by aspiration. The wounds were closed and the animals were kept warm until awaking with heat lamps. Guinea pigs were housed individually and fed ad libitum.

The animals were randomly divided into four groups: sham–vehicle, injured–vehicle, injured–PEG, and sham–PEG. The animals in the sham–vehicle group received only dorsal laminectomy, spinal cord exposure, and Krebs' solution lavage. In the injured–vehicle animals, the injury site was exposed to normal saline solution and lavaged with Krebs' solution, which was subsequently removed by aspiration. The injured–PEG group received PEG application as described above. The sham–PEG group was designed to determine if PEG has any non-specific effects on the assays themselves. Animals in this group received PEG as described in the injured–PEG group, following dorsal laminectomy and spinal cord exposure. All of the solutions used in this section were prepared and used at room temperature (25 °C).

4.2. *TUNEL staining*

The animals in each treatment group were euthanized at 1 day and 7 days for anatomical studies by use of halothane anesthesia followed by transcardial perfusion with 6% paraformaldehyde in isotonic phosphate buffer saline at pH 7.4. The spinal cord encompassing the injury site was further postfixed with the same paraformaldehyde solution, and segments of the spinal cord were then embedded in paraffin and 10-μm sections were cut transversely from the center of the impact site. Apoptosis was determined using the terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) methodology (Gavrieli et al., 1992; Kaufmann et al.,

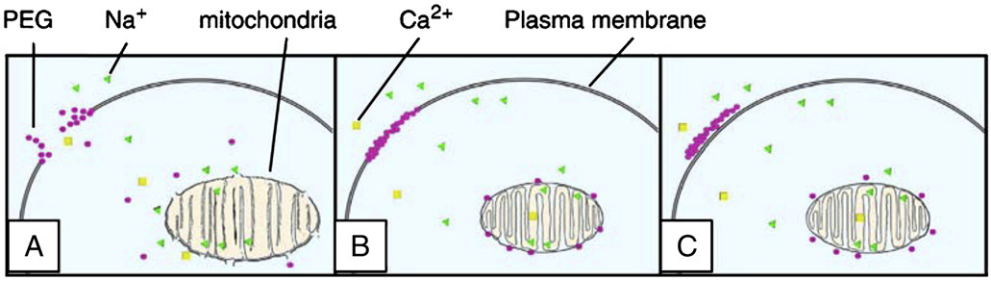

Fig. 4 – Hypothesis of possible mechanisms for PEG-mediated neuroprotection. (A) A traumatic injury to the spinal cord causes breaches in neuronal membrane and damages to cytosolic organelles such as mitochondria. Extracellular molecules may enter the injured cell through the breaches. When PEG is applied and reaches the injured cells, some of the PEG molecules work as membrane repairers, while at the same time some PEG molecules enter the cytosol. (B) PEG around the breach forms a protective film spanning the membrane breach, dehydrates the local membrane areas, and facilitates the movement of components of the membrane adjacent to the breach. This also prevents the entry of extracellular molecules. PEG within the cytosol reaches mitochondria, protecting mitochondria from swelling. (C) The components of the membrane adjacent to the breach flow into one another and membrane breach is sealed. Mitochondrial swelling is reduced. Circle dots: PEG molecules; Triangle dots: Sodium ions; Square dots: calcium ions.

2000) using *In Situ* Cell Death Detection kit (Roche Diagnostics). Briefly, sections were deparaffinized and then treated with proteinase-K (20 μg/ml in 10 mM Tris–Cl, pH 7.6, for 15 min at 37 °C), permeabilized for 8 min in 0.1% Triton X-100/sodium citrate, and treated with TUNEL reaction mixture according to the manufacturer's protocol. Positive neurons were determined by fluorescent microscopy after dehydration and mounting under the coverslip. Terminal transferase was omitted as a negative control. The total number of TUNEL-positive cells was determined by an observer blinded to the treatment.

4.3. *Caspase-3 activity assay*

Caspase-3 activity was determined using caspase-3-specific substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DECD-AFC) (Gurtu et al., 1997; Namura et al., 1998). The spinal cord tissue containing the injury site was homogenized in the homogenization buffer with anti-proteinase cocktail. After centrifuging twice at 2000×*g*, the supernatant was further centrifuged at 12,000×*g* for 30 min. The supernatant was again collected and diluted to 50 μg protein/50 μl with homogenization buffer. After the addition of 225 μl assay buffer (25 mM HEPES (pH 7.5), 0.1% (w/v) CHAPS, 10 mM DTT, 100 units/ml aprotinin, 1 mM PMSF) containing 100 μM of substrate Ac-DECD-AFC, the reaction mixture was incubated for 2 h at 37 °C and reactions were stopped by addition of 1.225 ml of ice-cold assay buffer. Fluorescence was measured immediately with a fluorometer using an excitation wavelength of 360 nm and an emission wavelength of 480 nm. Reaction blanks containing 50 μl of homogenization buffer and 225 μl of assay buffer were incubated at 37 °C for 2 h and then diluted with 1.225 ml of ice-cold assay buffer.

4.4. *In vitro experiments of non-synaptic mitochondria from normal spinal cord tissue*

Non-synaptic mitochondria were purified using a standard method (Lai and Clark, 1979) from normal spinal cord tissue. The mitochondria were suspended in respiration buffer containing (in mM) 125 KCl, 3 KH_2PO_4, 0.5 mM MgCl, 0.1% BSA, and 10 HEPES (pH 7.4) at 0.5 mg of protein/ml. After incubation, mitochondria were harvested by centrifugation, and used to detect mitochondrial GSH content. To detect *in vitro* cytochrome *c* release, an aliquot of spinal cord mitochondria was preincubated with PEG or cyclosporin A (CsA, 1 μM) for 5 min in respiration buffer before calcium was added. Mitochondria were then incubated with 100 μM calcium for 30 min. Mixtures were centrifuged at 12,000×*g* at 4 °C and the supernatant was evaluated by Western blotting. For Western blotting analysis, the samples were electrophoresed on a 15% SDS–polyacrylamide gel. Gels were blotted to nitrocellulose membrane (0.2 μM). The primary antibody was sheep anti-cytochrome *c* antiserum (Sigma Chemical Co., St. Louis, MO). Bands were visualized by using enhanced chemiluminescent substrate (SuperSignal WestPico; Pierce).

For the experiments with PEG, the mitochondria were preincubated with PEG 5 min before adding Ca^{2+}. Stock 300-mosM solution of PEG 2000 (102 mM) was made in 3 mM HEPES buffer (pH 7.4) (Pfeiffer et al., 1995). When used, PEG (MW: 2000 Da) (final concentration: 10.2 mM) was added prior to the 5-min preincubation period and was present throughout the experiment. When isosmotic PEG solution is mixed with any other isosmotic solutions, the resultant osmotic pressure remains constant (Pfeiffer et al., 1995).

4.5. *Chemicals*

All chemicals including polyethylene glycol (PEG) (MW is ~2000), CsA, and anti-cytochrome *c* antiserum were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

4.6. *Statistical analysis*

The data are expressed as a mean±SD. The data were analyzed by one-way ANOVA using the statistics software package SPSS (version 12, SPSS, Chicago, IL, USA). Results showing overall significance were subjected to post hoc least-significance difference test; $p<0.05$ was considered statistically significant.

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