The Endogenous Antioxidant Glutathione as a Factor in the Survival of Physically Injured Mammalian Spinal Cord Neurons

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Abstract. Glutathione is part of the system of cellular defenses against lipid peroxidation and other free radical-mediated damage. An established in vitro trauma model was utilized to evaluate whether glutathione is a factor in the survival of mammalian spinal cord neurons following physical injury. Cultured murine spinal neurons were subjected to a standard lesion: transection of a primary dendrite 100 μm from the perikaryon. Prior reduction of glutathione with ethacrynic acid or buthionine sulfoximine caused a dose-dependent decrease in neuronal survival 24 hours after dendrotoomy. Prior glutathione augmentation with γ-glutamylcysteine or L-2-oxo-4-thiazolidine carboxylic acid significantly increased survival, but N-acetyl-cysteine was not protective. Gamma glutamylcysteine effected the most rapid increase in glutathione (peak at 10 min), and survival was 72% ± 10 when 0.2 mM γ-glutamylcysteine was added immediately after dendrotoomy compared with 38% ± 4 in the control group (p < 0.0001). These results indicate that the level of glutathione is a factor in spinal cord neuron survival after physical trauma, and that glutathione augmentation may be an effective acute phase spinal cord injury (SCI) intervention strategy.

Key Words: Anatomy; CNS trauma; Cell death: Free radicals; Lipid peroxidation; Spinal cord injury; Tissue culture.

INTRODUCTION

There is substantial evidence that the tissue deterioration that underlies the devastating loss of function following traumatic injury to the spinal cord (SC) is primarily the result of free radical, especially lipid peroxidation (LPO), damage to SC tissue (for reviews see 1, 2). Cell membrane and vascular disruption with consequent ion gradient deregulation and extravasation of blood cells are the immediate effects of trauma to the cord (for reviews see 3, 4). These events are believed to precipitate injury cascades leading to the formation of reactive oxygen species (ROS) (1, 2). LPO is initiated when a free radical such as the hydroxy radical or peroxynitrite extracts a hydrogen from a fatty acid to form an alkyl radical. The alkyl radical then rearranges and combines with molecular oxygen to form a peroxy radical. In the propagation stage the peroxy radical steals a hydrogen from a neighboring fatty acid becoming a lipid peroxide (for reviews 2, 5, 6). The lipid peroxide will rearrange and fragment. The neighboring fatty acid (now an alkyl radical) will repeat the process causing further damage. Once begun, LPO occurs rapidly. Significant increases in LPO products have been detected in SC tissue as early as 5–60 minutes (min) after trauma (2, 5, 6, 7–9).

Experimental and clinical studies suggest that bolstering SC cells’ defenses against free radical attack is a promising therapeutic strategy. For example, in vivo studies found that pretreatment with α-tocopherol and selenium reduced LPO, attenuated ischemia, and improved functional outcome in cats subjected to spinal cord injury (SCI) (10–12). Conversely, prior reduction of α-tocopherol resulted in more LPO, ischemia, and neuropathology (13). Alpha-tocopherol removes hydroxy radicals and other ROS, and terminates LPO by reduction of alkoxy and peroxy radicals. Other studies found that prior administration of superoxide dismutase reduced ischemia, hypotension, edema, and mortality in rats after SCI, and improved functional outcome (14, 15). Superoxide radicals keep iron in the ferrous state which favors the formation of hydroxy radicals (5, 16, 17), and superoxide dismutase may keep hydroxyl radical levels low by converting superoxide to hydrogen peroxide.

Even more compelling are the results of studies of methylprednisolone (MP). Demopolous et al (18) hypothesized that steroid compounds with lipophilic properties might protect cell membranes from free radical attack and attenuate LPO. Early studies of MP showed that it had powerful antioxidant properties (19–21). In vivo experiments provided the initial evidence that high dose MP therapy could improve outcome after SCI (for reviews see 1, 2). The NASCIS 2 clinical trial involving 487 patients confirmed a modest but significant protection in human victims of SCI treated with MP within 8 hours (h) of the traumatic event (22). As a result, MP has become the acute phase therapy of choice for SCI, and an effort is underway to develop treatments that are even more effective than MP against LPO and other free radical-mediated injuries (14, 23–25).

Glutamylcysteinylglycine or glutathione (GSH) is the most abundant intracellular thiol and a component of a system of endogenous defenses including ascorbic acid, α-tocopherol and catalase that protects cells against damage by free radicals (5, 17, 27). GSH interferes with the
initiation phase of LPO by reducing the levels of ROS including hydrogen peroxide and hydroxy radicals. It limits the propagation of LPO injury by serving as a cofactor for glutathione peroxidase(s). GSH also inhibits the initiation and propagation phases through its roles in the regeneration of ascorbate and, indirectly, of α-tocopherol. Indeed, several studies have suggested that cytotoxicity as indicated by LPO does not occur until levels of GSH fall below 10–15% of their original values (28, 29). In addition, GSH functions in DNA synthesis and repair, and in the reduction of disulfide bonds of proteins to confer or restore thiol-dependent enzyme activity (5).

In view of its multi-level roles in the protection of cells against oxygen toxicity, GSH has been described as “...the most important cellular antioxidant ...” (30). This statement is particularly true of the nervous system which has low levels of catalase compared with other organs (31) and, therefore, is more dependent on GSH and glutathione peroxidase(s) for H2O2 removal. However, despite their critical roles in the prevention of oxidative damage to CNS tissues, there have been few studies of the effects of SC trauma on GSH and its related enzymes, or of the relationship between the GSH content/enzyme activity of CNS cells and tissues and their susceptibility to damage by mechanical trauma. The present study represents a first attempt to determine the contribution of cellular GSH levels to the SC’s responses to acute injury. We employed a well-characterized in vitro injury model developed by our laboratory (32, 33) to evaluate the role of GSH in the deterioration and death of mammalian SC neurons subjected to a standard, physical (membrane disruption) lesion: transection of a primary dendrite 100 μm from the perikaryon (Fig. 1). The cellular lesions were created using techniques of UV laser microbeam cell surgery that were also developed in this laboratory (34). A recent study conducted with this model demonstrated a significant increase in the survival of lesioned neurons treated with 30 μg/ml MP; a concentration close to the maximum plasma concentration measured in laboratory animals given the 30 mg/kg dose used clinically (35). The MP study suggested that damage by free radicals may be a major factor in neuronal loss after physical injury even when many of the environmental perturbations believed to contribute to their formation (ischemia, bleeding, excitatory amino acid release, etc.) are not present. The studies of GSH also indicate that nerve cell deterioration and death after mechanical trauma is a free radical-driven process. The results of these studies are presented herein.

MATERIALS AND METHODS

Culture Substrate

For the studies of lesioned neuron survival and the monoclobimane tests, SC cells were grown on glass coverslips (Carolina Biological) that had been glued to culture dishes with silicon sealant (Dow-Corning). Prior to gluing the coverslips were flame with a butane torch (36) and coated with poly-D-lysine and laminin to enhance adhesion (Boehringer Mannheim). Cultures were grown in 60 mm Lux permanox culture dishes for the studies in which SC neurons were observed with

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Fig. 1. Example of standard dendritic lesion. Scale bars = 30 μm. A. An SC neuron prior to dendrite transection. The arrow indicates the target point which is 100 μm from the edge of the perikaryon. This is the standard lesion distance used for studies of neuronal survival after physical injury. Survival of SC neurons lesioned at this distance under normal culture conditions ranges from 40–60% (32, 35, 78, 79). Surgery is performed on primary dendrites (i.e. no major branches between the edge of the perikaryon and the target point). B. Same neuron 5 min after dendrite transection. Note that a small neurite very close to the target area (arrow) was not affected.
transmission electron microscopy (TEM). A 15 mm diameter area in each dish was treated with polylysine and laminin to enhance adhesion. Cultures utilized to test the effects of GSH-augmenting/depleting agents on GSH were grown in polylysine-coated 60 mm culture dishes.

Culture Protocol

The SC from 14 day mouse embryos were dissociated as previously described (35). Three tenths of a milliliter of the cell suspension containing 4.2 x 10⁶ cells was placed on each coverslip in the dishes for the lesioned cell survival and monochorobibane studies. For the cultures used for TEM, 0.3 ml containing 1.9 x 10⁶ cells was placed in the center of each dish. For the GSH assay cultures, 1.2 x 10⁶ cells in 2.0 ml was placed in each dish. All cultures were incubated at 37°C in 10% CO₂ in air. Cultures for the survival, GSH assay and TEM studies were maintained as previously described (35). To obtain purified neuronal cultures for the monochorobibane studies, the seeding medium was removed on the fourth day and replaced with serum-free N2 medium containing 13 μg/ml fluoroxyuridine (FUDU), and 33 mg/ml uridine (U). N2 selectively supports neurons while FUDU + U retards proliferation of non-neuronal cells (37). Every 3–4 days one third of the medium on these cultures was replaced with fresh N2 medium. Cultures were used for the various studies 21–28 days after seeding.

Preparation of GSH-Augmenting/Depleting Agents

The GSH-depleting agents ethacrynic acid (EA) and buthionine sulfoximine (BSO) were purchased from Sigma Chemical Company and Aldrich Chemical Company respectively. The GSH-augmenting agents N-acetyl-cysteine (NAC), L-2-oxo-4-thiazolidine-carboxylic acid (LOT) and gamma-glutamylcysteine (GC) were purchased from Sigma Chemical Company. Stock solutions of NAC, GC and BSO were prepared in sterile, distilled, deionized water. Stock solutions of LOTC and EA were prepared in ethanol.

Determination of SC Cell Viability in Toxicity and Lesioned Neuron Survival Studies

After cell surgery or exposure to the GSH-augmenting/depleting agents, previously selected SC cells were evaluated initially for signs of stress and viability with phase contrast light microscopy (PCLM). Viability was then tested by erythrosin B dye exclusion (EB) according to the method of Phillips (38) with modifications described by Emery and co-workers (39).

Toxicity Tests of GSH-Augmenting/Depleting Agents

An initial study was performed to determine the maximum nontoxic concentration of each GSH-depleting/augmenting agent. The maximum nontoxic concentration is defined as the maximum concentration of an agent that causes no death or visible stress of SC neurons or glia during a 24 h exposure. At least 2 cultures were exposed to each concentration of an agent. Control cultures for each study received only water, in the case of LOTC or EA, ethanol. Prior to treatment, 3 areas in each culture were selected and photographed. Each area contained 5–15 neurons. Neurons were distinguished from glia and other non-neuronal cell types as described previously (35). After addition of an agent, the cultures were incubated at 37°C for 24 h. At the end of the incubation period, the selected areas were relocated and evaluated with PCLM for signs of stress. Neuronal viability in the selected areas was then determined with EB.

Measurements of Total GSH Levels in SC Cultures

The methods for harvesting cells and for measuring total GSH levels in the cells have previously been described by Malicky et al (40). The method for measuring GSH was based on that of Eyer and Podhradsky (41). GSH was measured using a SLM-AMINCO DW 2RC Midan II Kinetic Processor dual beam spectrophotometer with monochromator 1 set at 412 nm, monochromator 2 set at 550 nm and a Slt setting of 030. A standard curve was constructed from the absorbance readings of standards containing known concentrations of GSH. GSH in treated and untreated SC cultures was measured by comparing the absorbance readings of samples taken from disrupted cells to the standard curve. The concentration of GSH in each sample was divided by the amount of protein in the sample and reported as nmol/mg. Protein was determined by the Lowry method (42). Bovine gamma globulins were used as the standard protein.

Laser Microbeam System and Arrangements for Cell Surgery

The new generation laser microbeam system was custom constructed by Carl Zeiss, Inc., and consists of a pulsed nitrogen laser (Laser Sciences, Inc., Newton, Mass.) optically coupled to a Zeiss Axiospkop. Figure 2 shows the system and describes its performance features. The arrangements for maintaining medium temperature (35–37°C), pH (7.3–7.4), osmolarity (330 mOsmols) and sterility during laser cell surgery have been described previously (35).

Neuronal Selection for Laser Cell Surgery

The criteria for identification of SC cells as neurons, selection of neurons for surgery or to be unoperated controls, and for distinguishing dendritic from axonal processes are described in detail in a previous publication (35).

Laser Cell Surgery Protocol

Experiments utilized sets of cultures from the same seeding date. Twenty-four h prior to surgery, 10 neurons in each culture were selected for dendrotoamy and 10 were selected to serve as unoperated controls. Laser marks were made below the surface of the coverslip near selected cells as reference marks to assist relocation. The laser marks did not disturb the overlying cells. Maps were drawn of the selected neurons’ locations. Photographs were made of the selected neurons to assist relocation and identification. Neurons selected for surgery were subjected to a standard lesion: transection of a primary dendrite 100 μm from the soma (Fig. 1). Transection was achieved by firing multiple laser pulses at a frequency of 20 Hz and an energy density of 1–3 μJ/μm² into the dendrite. The multiple-shot, low energy method of laser surgery creates precise (±1.0 μm positioning error) transections without pressure waves (34). The mechanism that underlies neurite transection with this method has been described previously (34).
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LASER MICROBEAM SYSTEM

VIDEO CAMERA

FOCUSING LENS

NITROGEN LASER

CROSSLINE GENERATOR

AXIOSKOP

VIDEO MONITOR

Performance features of laser system:
337 nm wavelength
85 kW maximum output
3 nsec pulse length
0-20 Hz firing frequency
< 1.0 mm minimum focal diameter with 4X objective

Fig. 2. The new generation laser microbeam system was custom constructed by Carl Zeiss Inc. and consists of a pulsed nitrogen laser (Laser Sciences Inc., Mass.) that is optically coupled to a Zeiss Axioskop. The laser emits a beam at 337 nm and has a maximum output of 85 kW, a 3 nsec pulse length, and a variable (0–20 Hz) firing frequency. The pathway of the laser beam is indicated by the dashed lines. A culture dish is shown on the microscope stage as it would be during laser cell surgery. A 40× Zeiss Achroplan objective is used for experiments in which cell surgery is performed. The diameter of the focused laser beam at the target point is less than 1.0 μm. A video camera (Hamamatsu Japan) attached to the microscope projects the microscope image onto a monitor (Javalin Torrance, Calif.). A crossline generator (Boekeler, Ariz.) projects “cross hairs” onto the monitor screen which are aligned with the laser focus. The cross hairs provide a visible reference that allows positioning of target cells in the laser focus. A Semprex K mechanical stage is used to move the culture dish when scanning cultures and positioning cells for surgery.

Fluorescent Indication of GSH in Neuronal Cells with Monochlorobimane

Cultures of SC neurons were incubated at 37°C with 5 μM monochlorobimane for 10 min. The cultures were then rinsed twice with phosphate buffered saline and 3.0 ml ice-cold methanol was added to each dish. The dishes were placed in the freezer at −20°C. After 5 min the methanol was poured off. The coverslips containing the cells were removed from the dishes, air dried and mounted on microscope slides with non-fluorescing medium. The presence of GSH in neurons was indicated by blue fluorescence when they were viewed with a fluorescence microscope at an excitation wavelength of 380 nm and using a 450 nm emission filter.

Transmission Electron Microscopy

As described above, laser marks, photographs and maps were made to assist relocation and identification of the selected areas. Cultures were exposed to varying concentrations of EA or BSO and fixed after 1–4 h or 2–7 days respectively for TEM observation. Descriptions of the protocols for fixation, embedding, sectioning and staining of cells for TEM can be found in a previous paper (43). Cell sections were viewed with a JEOL JEM 100 CXII electron microscope.

Statistical Comparisons (SAS release 6.1)

A one way ANOVA test was used for statistical comparisons of the results of the studies of lesioned neuron survival. Posthoc comparisons were performed in the pretreatment studies with the Scheffe F test. The level of significance for each test was p < 0.05.

RESULTS

Effects of GSH Depletion with BSO or EA on Cultured SC Neurons and Glia

BSO and EA were selected for the studies of the effects of GSH depletion. BSO interferes with the initial rate-limiting reaction of the GSH synthetic pathway that is catalyzed by gamma-glutamylcysteine synthetase (44–46). BSO rapidly reduces GSH in the cytosol but does not affect mitochondrial levels for many days (44–46). EA directly and rapidly reduces both cytosolic and mitochondrial GSH by glutathione S-transferase catalyzed conjugation (47, 48).

Toxicity of BSO or EA to SC Cells

Toxicity studies were performed to determine the maximum nontoxic concentrations of BSO and EA for uninjured SC neurons and glia. The maximum nontoxic concentration of an agent is defined as the maximum concentration at which after a 24 h exposure there are no signs of stress (increased phase brightness, swelling, loss of adhesion, blebs, vacuoles, etc.) detectable with PCLM, and no SC cell death as determined by EB dye exclusion. This concentration becomes a point of reference for subsequent studies of the effects of an agent. Figure 3 shows the kinds of changes that may be observed with PCLM in SC cells exposed to the maximum nontoxic and higher concentrations of an agent (in this case, EA).

EA was added to SC cultures at concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM. In cultures treated with 0.01 mM EA for 24 h there was no cell death or obvious signs of stress (Fig. 3A, B). There was also no cell death in cultures exposed to 0.05 mM EA for 24 h; however, there was moderate glial retraction and neurons were more phase bright with some vacuolization. Some areas of cultures exposed to 0.1 mM EA for 24 h had loss of cell adhesion and neuronal death (Fig. 3C, D). These changes became more widespread at higher concentrations. The 0.01 mM concentration was selected as the maximum nontoxic concentration.

BSO was added to SC cultures at concentrations of 0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, 2.0 mM and 5.0 mM. A 24 h exposure of SC cultures to 0.2–5.0 mM BSO decreased total GSH by 65–75% (Fig.
4B2). However, despite this effect on GSH, signs of stress and SC cell death were not observed in cultures exposed to this range of concentrations of BSO for 24 h. The period of exposure to BSO was extended and signs of stress were observed in all groups at 72–96 h after BSO application (data not shown) at which time GSH levels were reduced by more than 95% (Fig. 4B2). The lower toxicity of BSO compared with EA probably reflects its slower depletion of mitochondrial GSH (44–48).

Effects of BSO or EA on GSH of SC Cultures

Figure 4 shows the effects of EA and BSO on total GSH. For clarity, the data are shown as both the concentrations of GSH and the percent change values relative to untreated controls. EA was applied at concentrations of 0.01 mM (the maximum nontoxic concentration) and 0.1 mM (significant stress after 24 h). By 2 h after EA application total GSH in the treated groups was 0.4 nmol/mg ± 0.01 and 0.3 nmol/mg ± 0.04 respectively compared with 1.9 nmol/mg ± 0.3 in the control group (Fig. 4A1). This represented an approximately 80% decrease in GSH in each group (Fig. 4A2). Interestingly, by 24 h GSH increased in the treated groups (Fig. 4A1). The increases ranged from 500–800% of GSH in the control cultures (Fig 4A2). Because the magnitude of the early GSH depletion was similar in both treated groups, the differences in the toxicity of these concentrations of EA (Fig. 3) may have been the result of differences in their impact on critical mitochondrial levels rather than on GSH in general. The rebound of GSH at 24 h probably
Fig. 4. Effects of EA and BSO on GSH in SC cultures. A1. In SC cultures treated with 0.01 mM or 0.1 mM EA for 2 h levels of GSH were below 0.5 nmol/mg compared with 1.9 nmol/mg ± 0.3 in the control group (no EA). However, by 24 h GSH levels in the EA-treated groups were in excess of 3.5 nmol/mg. The GSH rebound observed at 24 h probably represents an effort to rescue the cells by stimulating GSH synthesis. Mbamba et al (80) reported a similar increase of GSH in senescent fibroblasts just prior to death. Each point represents the mean of GSH measurements from 3 cultures (± SD). A2. Changes in GSH in cultures treated with 0.01 mM and 0.1 mM EA shown as percentages of untreated control values. The lower GSH in the EA-treated cultures at 2 h represented a reduction of approximately 80% from control levels. GSH in EA-treated cultures at 24 h were 500–800% of control (no EA) levels. B1. During the first 2 days after addition of 0.2–5.0 mM BSO, GSH in SC cultures was generally within the range of 0.5–2.0 nmol/mg compared with levels of 4.5 nmol/mg in the control (no BSO) group. The levels in the treated cultures were below 0.2 nmol/mg on days 4 and 5. Interestingly, GSH levels also declined in the control cultures and ranged from 2.7–2.9 nmol/mg on days 3–5. The decrease in GSH in the untreated cultures after day 2 probably represents a natural decline due to increasing culture age (50). The GSH content of 3–4-week-old primary SC cultures was usually in the range of 0.5–2.0 nmol/mg. The reason for the unusually high levels of GSH in this study is unknown; however, astrocytes are the principal GSH-containing compartment of the CNS (55) and the high levels of GSH in the BSO control group may reflect an unusually high proportion of astrocytes in these cultures. Each point represents the mean of GSH measurements from 3 cultures (± SD). B2. Changes in GSH in cultures treated with BSO shown as percentages of untreated control values. On days 1 and 2 GSH in the treated cultures decreased by 60–87%. By days 4 and 5 the decreases in all groups were 95% or greater.
represents an effort by the cells to counteract EA by increasing GSH synthesis. The fact that cells in the 0.1 mM group still showed stress and died (Fig. 3) indicates that it did not occur fast enough to prevent lethal injury. GSH has been observed to increase in other cell types following a serious stress (49) and may represent a kind of “last ditch” rescue effort.

The effect of BSO on GSH has been described as biphasic consisting of a large and rapid initial decrease that reflects reduction of cytosolic levels followed by a gradual depletion over several days of mitochondrial stores (44–46). A similar pattern was observed in SC cultures treated with BSO. After a 24 h exposure the concentrations of total GSH in SC cultures treated with BSO were substantially below levels in control (no BSO) cultures (Fig. 4B1); reductions of 71%, 65%, and 74% respectively were observed in the groups treated with 0.2 mM, 1.0 mM or 5.0 mM BSO (Fig. 4B2). By 4 days GSH had decreased by more than 95% in all of the BSO treatment groups at which time the levels were below 0.5 nmol/mg. Because BSO treatment did not cause cell stress until 72–96 h after application, these results suggest that inhibition of GSH synthesis is not deleterious until total GSH is decreased to less than 0.5 nmol/mg.

In unmanipulated SC cultures total GSH is usually in the range of 0.5–2.0 nmol/mg. However, GSH in the control cultures of the BSO study were above 4.5 nmol/mg on days 1 and 2 and ranged from 2.7–2.9 nmol/mg on days 3–5. The reason for the high GSH levels in the BSO control group is not known. However, it is likely that the difference reflects some heterogeneity of the SC culture cell populations. The decline of GSH in the control cultures on days 3–5 was probably a consequence of increasing culture age. Sagara et al (50) also observed that GSH in cultured brain neurons decreased more than 50% over a 4-day period.

Ultrastructure of Unlesioned SC Neurons Treated with EA or BSO

Because of the difference in the toxicity of EA and BSO, a study was undertaken to compare the ultrastructural changes in SC neurons exposed with concentrations of these 2 agents that cause comparable reductions of GSH. A total of 28 SC neurons treated with 0.01–0.1 mM EA for 1–4 h and 31 SC neurons treated with 5.0 mM BSO for 2–7 days were examined. Figure 5 shows the changes that were consistently observed in the EA and BSO-treated cells. The first panel (Fig. 5A) is an example of the somal ultrastructure of an untreated SC neuron. Although this cell contained a few small vacuoles, there were no signs of serious ultrastructural disturbance. In contrast, an SC neuron treated with 0.01 mM EA did show some mitochondrial swelling by 2 h (Fig. 5B), and most of the mitochondria in a neuron exposed to 0.1 mM EA for 2 h were grossly dilated (Fig. 5C). High amplitude swelling is a sign of irreversible damage to mitochondria which, if widespread, is considered indicative of lethal cell injury (51). Thus, the swollen mitochondria observed in neurons exposed to 0.1 mM EA are consistent with the severe stress of some SC cells observed with PCLM after a 24 h exposure to that concentration (see Fig. 3C, D). In contrast to the EA-treated neurons, there were no obvious changes in the ultrastructure of neurons treated with 5.0 mM BSO until 4–6 days after application at which time the mitochondria in some neurons became slightly dilated (Fig. 5D). This corresponds to the time when stress was first detectable by PCLM. The differences in the effects of the 2 GSH-depleting agents on the ultrastructure of SC neurons are consistent with their respective mechanisms of action (44–48), and help to explain the differences in their toxicity to SC cells at concentrations that reduce total GSH to a similar degree. A full report of the ultrastructural changes in SC neurons exposed to the GSH-reducing/augmenting agents is in preparation.

 Effects of GSH Reduction on Survival of Lesioned SC Neurons

To determine the influence of GSH on lesioned SC neuron survival SC cultures were treated with EA or BSO prior to surgery. EA was added to SC cultures 2 h prior to cell surgery at concentrations of 0.005 mM, 0.01 mM (the maximum nontoxic concentration) or 0.05 mM (some SC cell stress at 24 h). Survival at 24 h was 38% ± 5.32% ± 5 and 25% ± 6 in the 0.005 mM, 0.01 mM and 0.05 mM groups respectively compared with 47% ± 5 in the untreated control group (Fig. 6A; one factor ANOVA, p < 0.0001). Post-hoc comparisons with the Scheffe F test found that the difference between the control and the 0.05 mM group was statistically significant (p < 0.0001). No other significant differences were found. Similarly, survival of lesioned SC neurons pre-treated for 24 h with 0.5 mM and 5.0 mM BSO was 42% ± 8 and 22% ± 8 respectively compared with 46% ± 6 in the untreated control group (Fig. 6B; one factor ANOVA, p < 0.0006). Post hoc comparisons found that survival in the 5.0 mM group was significantly different from survival in the control and the 0.5 mM groups (Scheffe F test, p < 0.0006). These data indicate that a prior reduction of GSH decreases the likelihood of SC neuron survival after physical injury.

 Effects of GSH Augmentation with GC, LOTC or NAC on Cultured SC Neurons and Glia

The ability of cells to take up GSH itself is limited (52). However, GC, LOTC and NAC are readily transported into cells and are frequently utilized experimentally to increase GSH (52, 53). Accordingly, these 3 agents were selected for the studies of the effects of GSH augmentation on SC neurons and glia. LOTC and NAC
Fig. 5. Ultrastructural changes in SC neurons exposed to concentrations of EA that cause a significant and rapid depletion of GSH (see Fig. 4). Scale bars = 10 μm. A. Somal ultrastructure of an untreated (no EA) SC neuron. The mitochondria (arrowheads) and Golgi bodies (G) are not dilated (see inset) and the cytoplasm is not vacuolated. B, Somal ultrastructure of cultured SC neuron exposed for 2 h to 0.01 mM EA. This treatment causes an 80% decrease in GSH (see Fig. 4A2). The mitochondria are starting to show dilation of the intracisternal spaces and general swelling. These changes can be seen quite clearly by comparing the mitochondria (M) in the inset to those in A (note that the inset magnification is half that of inset in A). The
are cysteine pro-drugs that are used for augmentation of intracellular cysteine which is a substrate for the first, rate-limiting step of the GSH synthetic pathway that is catalyzed by γ-glutamylcysteine synthetase. GC is utilized by glutathione synthetase which catalyzes the second and final step in the GSH synthetic pathway.

**Toxicity of GC, LOTC or NAC to SC Cells**

SC cultures were exposed for 24 h to GC at concentrations 1.0 mM, 2.0 mM, 5.0 mM or 10.0 mM. The highest concentration of GC that caused no visible stress or death of SC neurons and glia after a 24 h exposure was 5.0 mM. Observations of SC cultures exposed for 24 h to LOTC at concentrations 1.0 mM, 2.0 mM, 5.0 mM or 10.0 mM established 5.0 mM as the maximum nontoxic concentration. There have been reports that protection of cells from oxidative stress by NAC requires treatment with very high (60 mM) concentrations of this agent (54). Accordingly, SC cultures were exposed to concentrations of NAC ranging from 0.1 mM–60.0 mM. In cultures treated for 24 h with NAC at concentrations up to 10.0 mM there was no death of neurons or glia and no obvious signs of cell stress. Although there was no cell death at the 30 mM concentration, there were signs of neuronal stress (vacuoles in the somata and ragged neurites) and a 15–20% loss of carpet adhesion. At the 60 mM concentration of NAC there was an estimated 50% loss of glial adhesion and almost 30% of the neurons selected for observation were dead or had disappeared. Thus, 10 mM was established as the maximum nontoxic concentration of NAC.

**Effects of GC, LOTC and NAC on GSH of SC Cultures**

SC cultures were treated with a range of concentrations of each GSH-augmenting agent, and changes in GSH were monitored during the early period after application (10 min, 1 h, 2 h and, in the case of LOTC, 4 h) and at 24 h. Although all 3 agents demonstrated an ability to augment GSH, interpretation of the results of these studies was complicated by the fact that during the initial 1–2 h period GSH also increased in all of the untreated control groups. This change was particularly evident in the GC and LOTC studies where GSH in the control groups reached levels of 8–10 nmol/mg compared with the 0.5–2 nmol/mg levels typical of undisturbed SC cultures. This effect was presumably a response of the SC cells to the physical stress of the medium manipulation which was performed in each of the control groups to mimic the addition of an agent. For clarity, data from each study are presented both as the actual GSH measurements (mean values ± SD) and as the percent changes from the untreated control levels.

The changes in GSH in each of the studies seemed to follow a common pattern. Within 1–2 h there was a pronounced increase in GSH in most of the treated groups that generally preceded the increase in their respective untreated control group. By 2–4 h GSH in both the control and treated groups had started to decline. By 24 h GSH in the control groups had returned to levels that were within the 0.5–2.0 nmol/mg range that is normal for undisturbed SC cultures. In the treated groups the decrease was more gradual and GSH was still in the 2–7 nmol/mg range at 24 h.

The most rapid increases in GSH were observed in the cultures treated with GC. By 10 min after application of 0.1 mM, 1.0 mM or 5.0 mM GC GSH reached levels of 3.0 ± 0.2 nmol/mg, 6.1 ± 1.8 nmol/mg, and 10.4 ± 1.5 nmol/mg respectively (Fig. 7A1). These represented increases of 200–900% above the levels in control cultures (Fig. 7A2). The levels in the GC-treated cultures peaked at 1 h when GSH in the control cultures also showed a sharp increase, and by 2 h GSH in both the control and GC-treated cultures had started to decline (Fig. 7A1). At 24 h GSH in the GC-treated cultures was 200–400% above control levels (Fig. 7A2).

In the cultures treated with LOTC the changes in GSH at 10 min were much more modest than in the GC-treated cultures (Fig. 7B1), and represented increases that were less than 100% above control levels (Fig. 7B2). However, by 1 h GSH in cultures treated with 0.1 mM, 1.0 mM and 5.0 mM LOTC measured 2.0 nmol/mg ± 0.5, 3.4 nmol/mg ± 1.2, and 6.0 nmol/mg ± 1.1 respectively (Fig. 7B1). These levels represented increases of 200–900% above GSH in the untreated control group (Fig. 7B2). The levels in the LOTC-treated cultures peaked at 2 h when GSH in the control cultures also showed a sharp increase. By 4 h GSH in both the control and LOTC-treated cultures had started to decline (Fig. 7B1). At 24

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Golgi bodies (G) are not dilated and there are no vacuoles in the cytoplasm. C. Somai ultrastructure of cultured SC neuron exposed to 0.1 mM EA for 2 h. All of the mitochondria (M) were enlarged and some displayed high amplitude swelling with loss of cristae. Cells with widespread high amplitude mitochondrial swelling are considered to be at the "point of no return" in the process of necrosis (51). These changes are consistent with the results of the EA toxicity study which found that exposure of SC cultures to this concentration of EA for 24 h caused loss of cell adhesion and neuronal death in many areas (see Fig. 3C, D). D. Somai ultrastructure of cultured SC neuron exposed to 5.0 mM BSO for 4 days. Despite the 95% reduction in GSH caused by this treatment (Fig. 4B2), the only ultrastructural change at this time was a slight dilation of the mitochondria (M inset). This observation is consistent with the results of the BSO toxicity study which found that there were no visible signs of stress or death until 3–5 days after BSO was added. No changes in the Golgi (G), nucleus (N), or other structures were observed.
Efforts of GSH-Augmenting Agents on GSH in SC Neurons

In cultures of mixed SC cells the neurons stratify on top of the glia and tend to be dispersed. This is an advantage for laser cell surgery because one can be certain that a target neurite belongs to a particular cell (Fig. 1). In contrast, neurons in purified cultures tend to cluster, and it is often difficult to determine to which cell a neurite belongs. GSH in neurons is low compared with the levels in glia (55). We were, therefore, uncertain whether elevation of GSH in mixed cultures after treatment reflected increases in neuronal as well as glial levels. To test this purified cultures of SC neurons were treated with GC or NAC and incubated with monochlorobimane, an indicator that becomes fluorescent after enzymatic conjugation to GSH (56). The fluorescence intensity in both the GC- and NAC-treated neuronal cultures was greater than that of untreated cultures (observations of 2 cultures/group). Figure 8 compares a group of neurons in a control culture (Fig. 8A) with a group of neurons in a culture treated with 5 mM GC for 10 min (Fig. 8B). In contrast, neurons in cultures exposed to 5 mM BSO for 5 days showed reduced fluorescence (data not shown). LOTC and EA could not be evaluated because stocks are made in ethanol which also causes monochlorobimane to fluoresce. These results indicated that the changes in GSH in SC cultures treated with the various augmenting/depleting agents represented changes in neuronal as well as glial levels of this antioxidant.

Effect of Pre-Treatment with GSH-Augmenting Agents on SC Neuron Survival After Dendroty

When GC was applied 2 h prior to cell surgery at concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM or 5.0 mM, lesioned neuron survival at 24 h was 53% ± 6, 77% ± 6, 74% ± 9, 76% ± 5, 72% ± 4 and 70% ± 7 respectively compared with 48% ± 10 survival in the control (no GC) group (Fig. 9A; one factor ANOVA, p < 0.0001). Post hoc comparisons with the Scheffe F test found that, with the exception of the 0.05 mM GC group, survival in all of the treated groups was significantly different from survival in the control group (p < 0.0001).

In the case of LOTC, an initial study had found that there was no significant difference in the survival of con-
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trol (no LOTC) lesioned neurons and lesioned neurons that were pretreated with 5.0 mM LOTC (data not shown) which was the maximum nontoxic concentration. However, when LOTC was applied 4 h before surgery at concentrations of 0.1 mM, 0.5 mM, 1.0 mM or 2.0 mM, survival of lesioned neurons at 24 h was 68% ± 5, 72% ± 8, 72% ± 8, and 68% ± 5 respectively compared with 52% ± 8 survival in the control group (Fig. 9B; one factor ANOVA, p < 0.0001). Post hoc comparisons found that survival in each treated group was significantly different from survival in the control group (Scheffe F test, p < 0.0018). These findings suggested that the 5.0 mM concentration may have caused a subtle stress that was not detectable with PCLM, and which offset the otherwise beneficial effects of GSH augmentation by LOTC.

In contrast to GC and LOTC, treatment of SC cultures with 0.5–30 mM NAC 2 h before surgery did not increase survival of SC neurons 24 h after dendrotox, and survival in the 30 mM NAC group was actually substantially lower than survival in the control (no NAC) group (18% ± 5 versus 58% ± 8 respectively; Fig. 9C; one factor ANOVA, p < 0.0001). Post hoc comparisons found that survival in the 30 mM group was significantly different from the other groups, and that survival in the 1.0 mM group was significantly different from survival in the control group (Scheffe F test, p < 0.0001). No other significant differences were found.

The higher survival in the groups of lesioned neurons pretreated with GC and LOTC and the lack of protection by NAC presumably reflect the large increases in GSH effected by the first 2 agents (Fig. 7A1, B2) and the relatively modest increases effected by NAC (Fig. 7C1, C2). Although 30 mM NAC caused some SC cell stress (see above), a 30 mM group was included in the study of its effects on lesioned neuron survival because of reports that very high concentrations are required for protection (54). The fact that lesioned cell survival was actually much lower in the 30 mM NAC group than in the control group indicates that application of such high concentrations may, in fact, have a detrimental effect on physically injured SC neurons.

Effects of Post-Traumatic Application of GSH-Augmenting Agents on SC Neuron Survival After Dendrotox

Studies were undertaken to determine whether GSH augmentation after dendrotox can increase the survival of lesioned SC neurons. GC and LOTC were the agents utilized for these experiments. Because the fate of SC neurons subjected to dendrotox transection close to perikaryon is generally decided within 2 h (32, 39), the GSH-augmenting agents were applied immediately after completion of the cell surgery for each experiment. In the group of lesioned neurons treated with 0.2 mM GC survival at 24 h was 72% ± 10 compared with 38% ± 4 in the control group (Fig. 10A; one factor ANOVA, p < 0.0001). However, in the group of lesioned neurons treated with 2.0 mM LOTC, 24 h survival was not significantly different from survival in the control group (Fig. 10B). As described above, the peak percent increases in GSH effected by these 2 agents were comparable (Fig. 7A1, B2). However, in GC-treated cultures the rise of GSH was much more rapid. Thus, the ability of GC to confer protection when applied to SC neurons after lesioning may reflect the rapidity as well as the degree of its effect on GSH.

DISCUSSION

The present study demonstrated that survival of SC neurons subjected to transection of a primary dendrite 100 μm from the perikaryon: (1) is decreased by agents that decrease GSH, and (2) is increased by agents that augment GSH. These results are the first evidence that GSH is a factor in the survival of SC neurons after physical injury. Like the earlier study of MP (35), these in vitro observations indicate that ROS play an important role in neuronal death after physical injury even in the absence of environmental disturbances that generate them directly (e.g., ischemia, elevated excitatory amino acids, release of iron from hemoglobin, etc.). In addition, the evidence that posttraumatic treatment with GC can effect a significant increase in survival of lesioned SC neurons suggests that GSH augmentation may have potential as a treatment strategy for acute phase SCI. These and other results of this investigation are discussed more fully below.

GSH in SC Cultures

Primary SC cultures contain a mixed cell population including neurons, oligodendrocytes, astrocytes, microglia, and fibroblasts. GSH in undisturbed SC cultures generally ranged from 0.5–2.0 nmol/mg. The levels were somewhat higher than those reported for cultures of purified cerebral cortical neurons (≤ 1 nmol/mg); however, they were substantially lower than those measured in cultures of purified astrocytes (16–25 nmol/mg) which are believed to be the principal GSH-containing compartment of the CNS (55). Cell population heterogeneity was undoubtedly a factor in the variability of GSH levels in SC cultures. For example, the cultures used to study the effects of BSO on GSH may have had an unusually large proportion of astrocytes (see Fig. 4B1).

Effect of GSH Reduction and Augmentation on Uninjured SC Cells

Stress was detectable by light microscopy in primary cultures of mixed SC neurons and glia that were treated with EA or BSO at concentrations and exposure times sufficient to cause a decrease of GSH to levels below 0.5 nmol/mg (Fig. 3; Fig. 4). Preliminary TEM examinations

Fig. 7. Effects of GC, LOTC and NAC on GSH in cultures of SC neurons and glia. A1. GSH was 3–10 nmol/mg in SC cultures treated with 0.1–5.0 mM GC for 10 min compared with 1.0 nmol/mg ± 0.1 in the control group (no GC). GSH reached maximum levels of 9–13 nmol/mg in the GC-treated cultures at 1 h; however, GSH in the control cultures had also increased to 9.6 nmol/mg ± 2.2 at that time. After 2 h GSH in both the treated and control cultures had started to decline. By 24 h GSH in the control cultures was 0.6 nmol/mg ± 0.8 which is within the normal range for undisturbed SC cultures. GSH in the treated...
of GSH-depleted cells revealed swollen mitochondria but no other obvious ultrastructural changes (Fig. 5). Treatment with BSO has also been reported to cause swelling of mitochondria in the cerebral cortex of newborn rats (46, 57). GSH is the principal antioxidant in mitochondria which lack catalase (46), and the selective damage to these organelles is consistent with their dependence on this antioxidant. Given the role of GSH in prevention of LPO, it is likely that the swelling was in part the result of peroxidative damage to mitochondrial membranes. Curiously, there was a large increase in the GSH content of SC cultures exposed to EA for 24 h (Fig. 4A1, A2). This increase was observed even in cultures treated with the 0.1 mM concentration which caused significant stress (Fig. 3). Elevation of GSH after a stress has been observed in other cell types (49) and may represent an attempt to rescue the cells by stimulating GSH synthesis.

GC and LOTC were the most effective of the GSH-augmenting agents. By 10 min and 1 h respectively after application of 5 mM GC or LOTC, GSH levels exceeded the levels in untreated cultures by 900% (Fig. 7A2, B2) and ranged from 6–10 nmol/mg (Fig. 7A1, B1). However, within the first h after application of 30 mM NAC the maximum increase was only 52% (Fig. 7C2), and at no time did GSH exceed 4 nmol/mg (Fig. 7C1). Tests with monochlorobimane on purified neuronal cultures confirmed that GC and NAC affected GSH levels in SC neurons (Fig. 8A, B). Mixed SC cultures exposed for 24 h to concentrations of GC or LOTC above 5 mM showed stress suggesting that further elevation of GSH is detrimental. High GSH may stress cells by damaging mitochondria. Exposure of isolated mitochondria to an excess of GSH causes glutathione peroxidase to detach from the membranes resulting in lipid peroxide formation and swelling (58, 59). Thus, it is likely that very high as well as very low GSH can cause LPO injury to these organelles. TEM studies are in progress to determine whether mitochondrial swelling occurs in neurons exposed to concentrations of GC or LOTC above 5 mM.

A transient increase in GSH was also observed in the untreated control cultures of the GC and LOTC studies that reached levels in excess of 8 nmol/mg (Fig. 7A1, B1). The increase of GSH in the control cultures was first observed 1–2 h after the beginning of the experimental period and occurred after the elevation of GSH in the treated cultures. The elevation of GSH in the control cultures was probably the result of the medium manipulation which was performed to mimic the application of the GSH-augmenting agents. Some cell swelling is usually observed when medium is removed or added and is indicative of culture stress; however, to the best of our knowledge, there are no reports of the effects of this treatment on GSH. The changes in the levels of GSH in the GC or LOTC control cultures after medium manipulation suggest that physical stress to SC cells causes a transient increase in GSH synthesis. In contrast, the increase of GSH in the NAC study control group was modest (Fig. 7C1) suggesting that the physical stress of medium manipulation in this group of cultures was not as great.

Effect of GSH Augmentation on Lesioned SC Neuron Survival

GC and LOTC effected large increases in GSH within 1 h after application (Fig. 7A2, B2), and both agents increased survival of lesioned SC neurons significantly when applied 2–4 h before cell surgery (Fig. 9A, B).
Fig. 8. Effect of GC on GSH of murine SC neurons (purified cultures) as indicated by monochlorobimane fluorescence. Cultures were from the same seeding date. Scale bar (A) = 40 μm. A. Group of untreated neurons in culture incubated for 10 min with 5 μM monochlorobimane. The pale blue fluorescence indicates that neuronal cells in this culture contained some GSH. B. Group of SC neurons in culture treated with 5 mM GC for 10 min. The intense blue fluorescence indicates that neuronal GSH was elevated by this treatment (compare cell indicated by arrow with neuron indicated by arrow in A). This finding is also consistent with the previous study that showed that this treatment caused a 92.1% increase in GSH in cultures of mixed SC neurons and glia (see Fig. 7A2).

Survival of lesioned neurons treated with concentrations of GC or LOTC that increased GSH by 900% was not very different from survival of lesioned neurons treated with concentrations of GC or LOTC that increased GSH by only 200% (Fig. 9A, B). NAC effected much more modest increases in GSH (Fig. 7C2; maximum differences from controls < 100%), and prior application of NAC did not increase survival (Fig. 9C). Although there was also a substantial increase in the GSH of untreated control group for each study, this occurred after the initial increase in the experimental groups and was transient. Thus, in the pretreatment studies protection of lesioned neurons occurred when GSH was initially increased to at least 4.0 nmol/mg (approximately 200% above maximum levels in control cultures).

Application of 0.2 mM GC after cell surgery also effected a significant increase in lesioned SC neuron survival (Fig. 10A; 72% ± 10 vs 38% ± 4 in the control group, p < 0.0001). However, application of LOTC after surgery had no effect (Fig. 10B). The ability of GC to rescue lesioned neurons when applied posttraumatically was probably the result of its much more rapid effect on GSH (200–900% increase by 10 min after application; Fig. 7A2) compared with LOTC (< 100% increase at 10 min; Fig. 7B2). This in turn probably reflected the fact that GC acts at the second reaction of the 2-step GSH synthetic pathway whereas LOTC is utilized at the first, rate-limiting step (52, 53). Rapid action is a necessity for an experimental strategy to be effective in this injury model because the fate of neurons subjected to dendrotyomy within 200 μm of their perikarya is generally decided within 2 h after lesioning (32).

LOTc and NAC are cysteine pro-drugs that have been used extensively as GSH-augmenting agents (for reviews see 52, 53). LOTC is a 5-oxoprolin analog that is converted by 5-oxoproline to cysteine. The mechanism for deacetylation of NAC to cysteine is less well understood. The reason for the greater effect of LOTC on SC culture
Fig. 9. Effects of GC, LOTC, and NAC on lesioned neuron survival. Each bar in the graphs represents the mean percent survival (± SD) 24 h after cell surgery in 5–9 experiments (50–90 lesioned neurons/group). Survival of preselected unlesioned control neurons in each group was 100% (50–90 neurons/group). * = Scheffe F comparison showed statistically significant differences from control group. A. An increase in lesioned neuron survival was observed when GC was applied to SC cultures 2 h before cell surgery at concentrations 0.1–5.0 mM. Survival was highest in the 0.1–0.5 mM GC groups (74–77%) compared with 48% ± 10 in the control (no GC) group. A one factor ANOVA comparison found that the differences in survival among the groups were statistically significant (p < 0.0001). Post hoc comparisons with the Scheffe F test found that the differences in survival between the control group and the 0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM, and the 5.0 mM groups were significant (p < 0.0001). No other significant differences were found. B. Survival of lesioned neurons 24 h after dementatomy was also higher when 0.1–2.0 mM LOTC was added to the SC cultures 4 h prior to cell surgery (one factor ANOVA, p < 0.0012). Survival in the treated groups ranged from 68% ± 5 to 72% ± 8 compared with 52% ± 8 in the control group. Post hoc comparisons with the Scheffe F test found that the differences in survival between the control group and all of the treated groups were significant (p < 0.0018). No other significant differences were found. C. In contrast to the effects of GC and LOTC, neuronal survival 24 h after dementatomy was not increased by exposure to 0.5 mM, 1.0 mM, 5.0 mM, 10.0 mM, or 30 mM NAC 2 h prior to cell surgery. In fact, survival in the 30 mM NAC group (18% ± 5) was considerably lower than survival in the control (58% ± 8) and other treated groups. An ANOVA comparison found a significant difference in survival among the groups (one factor ANOVA, p < 0.0001). Post hoc comparisons with the Scheffe F test found that the differences in survival between the 30 mM group and all other groups were significant (p < 0.0001). Survival in the 1.0 mM group was also significantly different from survival in the control group (p < 0.0001). The lack of protection by NAC compared with GC or LOTC may be explained by the fact that its effect on GSH is much more modest (see Fig. 7).

levels of GSH compared with NAC is not known. However, LOTC has also been shown to be nearly twice as effective as NAC for augmentation of liver GSH in mice treated with acetaminophen (52, 60). The difference between LOTC and NAC may reflect dissimilarities in their rates of uptake. Another possibility is that there are differences in the oxoprolinase and deacetylation activities of SC neurons.
L-oxo-thiazolidine carboxylate (mM)

**Fig. 10.** Effect of posttraumatic application of LOTC or GC on lesioned SC neuron survival. * = statistically significant difference from control group. A. Survival of lesioned SC neurons treated with 0.2 mM GC immediately after completion of cell surgery was 72% ± 10 compared with 38% ± 4 in the control (no GC) group. The difference in the 24 h survival was statistically significant (one factor ANOVA, p < 0.0001). Each bar represents the mean survival (± SD) in 10 experiments (100 lesioned neurons/group). Survival of preselected unlesioned control neurons was 100% (10 neurons/experiment or a total of 100 neurons/group). B. Prior application of LOTC at concentrations of 0.1–2.0 mM increased survival of SC neurons 24 h after dendrotoxy (Fig. 9B). However, when 2.0 mM LOTC was applied immediately after cell surgery there was no significant difference in 24 h survival of lesioned neurons in the LOTC-treated and control (no LOTC) groups (one factor ANOVA, p < 0.7598). Each bar represents the mean percent survival in 5 experiments (10 lesioned neurons/experiment or a total of 50 lesioned neurons/group). Survival of preselected unlesioned control neurons was 100% (10 neurons/experiment or a total of 50 neurons/group).

**Mechanism of Protection of Lesioned SC Neurons by GSH Augmentation**

Protection of lesioned neurons by GC and LOTC may have been the result of direct antioxidant effects of these agents rather than of their ability to increase GSH. However, although NAC has considerable antioxidant activity (61, 62), it did not increase lesioned neuron survival. Given that GC and LOTC effected much larger increases in GSH than NAC, it is our opinion that the protection conferred by these agents was the result of their ability to augment GSH, and that the level of GSH is a factor in the survival of physically injured SC neurons. This conclusion is supported by the EA and BSO studies which showed that GSH reduction decreased survival.

GSH is an important cellular defense against LPO injury (5, 17, 27), and experiments are in progress to compare the amount of LPO in GC-treated and control lesioned SC neurons. However, GSH may also affect cell responses to injury at the DNA level. The concentration of GSH has been shown to influence expression of the c-jun proto-oncogene (63, 64). In the nervous system increased c-jun expression has been linked to diverse events including developmental neuronal death (65, 66) and regeneration after axotomy (67, 68). In SC neurons subjected to dendrotoxy elevation of c-jun and junB proteins appears to correlate with mortality (69, 70), and an initial study has shown that prior application of GC delays jun protein elevation in lesioned cells (71). Whether GSH's effects on immediate early gene expression are related to its ability to inhibit LPO, or whether these constitute separate responses is presently unknown.

**Significance of the Results to SCI**

GSH acts to inhibit both the initiation and the propagation phases of LPO (see Introduction), and the evidence that the level of GSH influences neuronal survival after physical injury is consistent with the LPO theory of SCI. The high polyunsaturated fatty acid content of nervous tissue has long been considered an important factor in the susceptibility of the brain and SC to LPO damage (1, 2, 18). GSH may be another factor that contributes to the particular vulnerability of the nervous system to LPO. Comparisons of GSH levels in various tissues of mice and rats have shown that GSH in the brain was in the moderate or high range (5, 72). However, in vivo and in vitro studies indicate that most of the GSH is in the glial compartment (55, 56). The relatively low GSH in CNS neurons may render them especially vulnerable to LPO, and may explain the protection of lesioned SC neurons by GSH-augmenting agents.

Protection of lesioned neurons by posttraumatic application of GC indicates that GSH augmentation may have potential as an experimental strategy for the treatment for acute phase SCI. However, whether GSH can be rapidly and efficiently elevated in the SC and brain is a matter of debate. For example, although Secchi and colleagues (73) have reported that administration of GSH ameliorated the symptoms of Parkinsonism, both the ability of GSH to cross the adult blood brain barrier and its uptake by cells appear to be very limited (52, 74). Similarly, attempts to augment brain GSH using LOTC have met...
with mixed success (75–77). More promising were the results of a study of GC which found that intracerebroventricular injection of this agent resulted in a significant elevation of GSH in the substantia nigra and brain stem (75).

Concluding Remarks

These in vitro studies have provided evidence that GSH is a factor in the survival of SC neurons after physical injury, and that augmentation of the GSH content of the SC may have potential as an acute phase SC trauma treatment strategy. Future studies will focus on the subcellular mechanisms whereby GSH augmentation protects physically injured SC neurons.

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