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Depletion of Reduced Glutathione Enhances Motor Neuron Degeneration *in vitro* and *in vivo*

Liyang Chi¹, Yan Ke², Chun Luo¹, David Gozal², and Rugao Liu^{1,*}

¹ Department of Anatomy and Cell Biology, University of North Dakota School of Medicine, 501 N. Columbia Road, Grand Forks, ND 58202

² Kosair Childrens Hospital Research Institute, Department of Pediatrics, University of Louisville, 570 S. Preston St., Suite 204, Louisville, KY 40202

Abstract

The mechanism of selective and age-dependent motor neuron degeneration in human amyotrophic lateral sclerosis (ALS) has not been defined and the role of glutathione (GSH) in association with motor neuron death remains largely unknown. A motor neuron-like cell culture system and a transgenic mouse model were used to study the effect of cellular GSH alteration on motor neuron cell death. Exposure of NSC34 motor neuron-like cells to Ethacrynic Acid (EA) or L-Buthionine Sulfoximine (BSO) dramatically reduced the cellular GSH levels, and was accompanied by increased production of reactive oxygen species (ROS) measured by the DCF fluorescent oxidation assay. In addition, GSH depletion enhanced oxidative stress markers, AP-1 transcriptional activation, c-Jun, c-Fos and HO-1 expression in NSC34 cells analyzed by a luciferase reporter, western blotting and quantitative PCR assays respectively. Furthermore, depletion of GSH decreased mitochondrial function, facilitated apoptosis inducing factor (AIF) translocation, cytochrome c release, and caspase 3 activation, and consequently led to motor neuron-like cell apoptosis. In an ALS-like transgenic mouse model overexpressing mutant G93A-SOD1 gene, we showed that the reduction of GSH in the spinal cord and motor neuron cells is correlated with AIF translocation, caspase 3 activation, and motor neuron degeneration during ALS-like disease onset and progression. Taken together, the *in vitro* and *in vivo* data presented in the current report demonstrated that decreased GSH promotes multiple apoptotic pathways contributing, at least partially, to motor neuron degeneration in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that primarily affects motor neurons in brain cortex, brainstem and spinal cord (Williams and Windebank, 1991). The mechanisms underlying the selective and age-dependent motor neuron degeneration remain largely unidentified, and effective therapy for ALS is not yet available. Mutations of Cu,Zn-superoxide dismutase (SOD1) gene cause motor neuron degeneration and have linked to 2–5% of ALS cases (Rosen et al., 1994; Rosen et al., 1993). Several potential mechanisms of motor neuron degeneration in ALS have been proposed based on clinical studies, animal model and cell culture system analyses. Increased oxidative stress, glutamate toxicity, protein

*Corresponding author: Rugao Liu, Ph.D., Associate Professor, Department of Anatomy and Cell Biology, University of North Dakota School of Medicine, 501 N. Columbia Road, Grand Forks, ND 58202, Telephone: (701)-777-2559, Fax: (701)-777-2477, E-mail: rliu@medicine.nodak.edu

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aggregation and Cu/Zn cytotoxicity have all been suggested to contribute to motor neuron degeneration (Cleveland and Rothstein, 2001; Li et al., 2003; Liu et al., 2002; Shaw et al., 2001; Shaw and Eggett, 2000; Shibata et al., 2000). Of these, increased oxidative stress appears to be an early and sustained event in association with motor neuron death in ALS (Bogdanov et al., 1998; Liu et al., 1998), although the specific mechanism leading to oxidative damage on motor neurons remains to be defined. Oxidative stress can be potentially increased by enhanced production of reactive oxygen species (ROS), decreased antioxidants/antioxidant enzyme systems or a combination of both. Glutathione (GSH), a tripeptide of γ -glutamylcysteinylglycine, is one of the most abundant antioxidants in cells and tissues. Reduction of GSH enhances ROS production and promotes oxidative damage. A previous study demonstrated increased GSH binding in the spinal cords of patients with sporadic ALS (Lanius et al., 1993), suggesting that GSH may play a role in the pathogenesis of ALS. In a cell culture model, it has been shown that expression of mutant SOD1 gene decreased cellular levels of GSH, suggesting the reduction in GSH bioavailability may participate in the mutant SOD1-mediated motor neuron degeneration (Lee et al., 2001).

GSH is the most abundant and effective scavenger against ROS directly in mammalian cells. In addition, GSH is also a key substrate for antioxidant enzymes that detoxify hydrogen peroxide and lipid peroxide catalyzed by glutathione peroxidase. More recently, it has been demonstrated that GSH participates in cellular signal transduction pathways, and modulates ionotropic receptor function (Bains and Shaw, 1997; Grima et al., 2003; Janaky et al., 1993). GSH is synthesized in two sequential enzymatic reactions catalyzed by γ -glutamylcysteine synthetase (γ -GCS) and GSH synthetase. L-Buthionine Sulfoximine (BSO) is a selective inhibitor of γ -GCS. Exposure of cells to BSO inhibits GSH synthesis and decreases intracellular levels of GSH. Thus, BSO has been frequently used to study the role of GSH in association with oxidative stress-induced neuronal cell and other cell death. On the other hand, because BSO does not completely deplete mitochondrial and nuclear GSH, other agents, including ethacrynic acid (EA) have been used to effectively deplete cellular, mitochondrial and nuclear GSH (Keelan et al., 2001; Rizzardini et al., 2003). Alterations in GSH synthesis, or in GSH pools, have been associated with neuronal cell death and mimic a variety of human neurodegenerative diseases, such as Parkinson's disease (Bharath et al., 2002; Jha et al., 2000; Mytilineou et al., 2002; Paik et al., 2003), Alzheimer's disease (Adams, Jr. et al., 1991; Cecchi et al., 1999; Janaky et al., 1999; Karelson et al., 2002) and Schizophrenia (Do et al., 2000). Nevertheless, the role of GSH in the pathogenesis of motor neuron degeneration in ALS remained largely undefined. To this end, we have focused on a cell culture system and an ALS-like transgenic mouse model to study the effect of GSH on motor neuron cell death. We showed that reduction of intracellular GSH increases oxidative stress, decreases mitochondrial function, activates multiple apoptotic pathways, and consequently contributes to motor neuron degeneration *in vitro* and *in vivo*.

Materials and Methods

Chemicals and Reagents

Ethacrynic acid (EA), L-Buthionine-SR-Sulfoximine (BSO) and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Cell culture medium and other reagents were purchased from Invitrogen Inc. (Carlsbad, CA).

Animals

Transgenic mice overexpressing mutant G93A-SOD1 were initially purchased from Jackson Laboratory (Stock #002726, Bar Harbor, ME). Mice were maintained and bred in the Biomedical Research Center at the University of North Dakota School of Medicine. This transgenic mouse line expresses high copy number of mutant G93A-SOD1 and develops a

rapid disease onset and progression (Gurney et al., 1994). Based on pathological characterization and symptomatic manifestation, we divide the lifespan of mutant G93A-SOD1 transgenic mice into clinical disease free (before 60 days of age), disease onset (70–90 days of age), and disease progression (100–130 days of age) stages (Gurney et al, 1994;Liu et al., 1998). The spinal cord lumbar regions from these stages in mutant G93A-SOD1 and age-matched control mice were used for GSH, GSSG, and apoptotic assays. The experimental protocols for using laboratory animals were approved by the Institutional Animal Use and Care Committee and were in close agreement with the National Institutes of Health guideline for the care and use of laboratory animals. All efforts were made to minimize animal stress, discomfort, and number of animals used.

Cell culture and treatments

Mouse NSC34 motor neuron-like cells (Passages 15 to 40) (kindly provided by Dr. Neil Cashman, University of Toronto) were cultured in a humidified atmosphere of 95% air-5% CO₂ in a 37°C incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 g/ml streptomycin (DMEM complete medium) as previously described (Bishop et al., 1999;Cashman et al., 1992). Cell culture medium was replaced every 3–4 days. Once 80–90% confluence was reached, cells were disrupted with 0.15% trypsin and 1 mM EDTA for 3–5 minutes. Dissociated cells were spun down and replated in new culture dishes or flasks (Corning Life Sciences, NY).

GSH depletion and GSH assay

Two independent methods, EA depletion and BSO inhibition were used to reduce cellular GSH respectively (Lucas et al., 1998;Roychowdhury et al., 2003;Seyfried et al., 1999;Tukov et al., 2004). Briefly, for EA-mediated GSH depletion, NSC34 cells were incubated with EA concentration from 20 to 100 M for up to 12 hours. For BSO mediated inhibition of GSH synthesis, NSC34 cells were incubated with BSO concentration from 25 to 200 M for up to 48 hours. EA or BSO treated cells were harvested and processed for a variety of analyses described in the following sections.

Two independent methods were carried out to measure cellular GSH. For the colorimetric assay, control, EA or BSO treated cells were incubated with 40 M monobromobimane (MBM) for 30 minutes. Then, the fluorescent intensity was directly measured with a fluorometer (Molecular Device Inc., Sunnyvale, CA) (Svardal et al., 1990;Yan and Huxtable, 1995). An arbitrary unit of fluorescent intensity was used to express the relative levels of cellular GSH after EA or BSO treatments. For the biochemical assay, a Bioxytech GSH/GSSG-412 kit was used and the manufacturer's suggested procedures were followed (Oxis International, Inc., Foster City, CA). Briefly, 10×10⁶ NSC34 cells from control, EA or BSO treated experiments were homogenized completely in MES buffer [200 mM 2-(N-morpholino)ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0]. After centrifugation at 12,000g for 10 min at 4 °C, the supernatant was deproteinated and proceeded for GSH (or GSSG) assays with premixed cocktail buffer according to manufacturer's suggestions. The same biochemical assay was also used to determine the levels of GSH (or GSSG) in the spinal cord lumbar region of G93A-SOD1 transgenic mice and age-matched normal control mice at different stages corresponding to disease free, disease onset and disease progression (Gurney et al., 1994).

Assay of cellular production of reactive oxygen species (ROS)

A dichlorofluorescein (DCF) assay was used to determine cellular reactive oxygen species (ROS) generation in NSC34 cells. Briefly, 2×10⁵ control, EA or BSO treated cells/well cultured in a 96-well plate were incubated with 100 M of 6-Carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) for 1 hour in the dark. Then the cells were

measured for the oxidation of DCFH-DA in a fluorometer at the excitation and emission wavelengths of 485 nm and 530 nm respectively. Data analysis was performed as described by Wang and Joseph (Wang and Joseph, 1999). The fluorescent intensity measuring the oxidation of DCFH-DA by ROS represents the relative steady state of ROS generation in cells.

Transient transfection and luciferase activity assay

Transient transfection was carried out with an electroporator (BTX, San Diego, CA) described previously (Liu et al., 2002). Briefly, 10×10^6 cells were incubated with 50 g vector control and vector containing 2 $\times$ AP-Luciferase plasmid DNA in 0.4 ml Opti-MEM (Invitrogen) at room temperature for 10 minutes, then cells were electroporated using a low voltage mode at 240 V, 1 pulse, and 25 msec/V of pulse length. After electroporation, cells were kept at room temperature for 30 minutes and subsequently cultured in complete medium.

Adhesion assay

Twenty four-well plates were coated with fibronectin, collagen, or laminin overnight in 1 $\times$ phosphate buffer solution (PBS) respectively. After washing, 2×10^6 control or EA treated cells were cultured in each well of the plate for 12 hours. Cell culture medium was removed and the wells were briefly washed with 1 $\times$ PBS twice. Cells adhered to the substrate were dissociated with trypsin and counted. The percentage of adhesion was calculated by the number of cells adhered to each coated plate divided by the total number of cell seeded (n=4).

Quantitative real time PCR Analysis of Gene Expression

Vehicle control or EA treated cells were harvested and processed for RNA purification. The total RNA purified for quantitative Real-time PCR analysis was subjected to RNase free DNase treatment (Ambion, Austin, TX). After concentration measurement, one microgram total RNA from each sample was used to synthesize first-strand cDNA by using superscript first-strand synthesis kit (Invitrogen, Carlsbad, CA). Real time PCR was carried out with MX4000 system (Stratagene, San Diego, CA). The forward and reverse real-time PCR primers for amplification of HO-1 transcripts were 5'-CTCACTGGCAGGAAATCATCCC -3' and 5'-GAGAGGTCACCCAGGTAGCG, respectively. The probe was 5'-6-FAM (CACGCCAGCCACACAGCACTATGTAAAGC)BHQ-1-3'. Actin was used as endogenous control. The forward and reverse real-time PCR primers for amplification of actin transcripts were 5'-TACAATGAGCTGCGTGTGGC-3' and 5'-ATGGCTGGGGTGTGAAGGT-3' respectively. The probe was 5'-6-FAM (CACCTGTGCTGCTCACCGAGGC) BHQ-1 3'.

Subcellular fractionation

For subcellular fractionation between mitochondria and cytosol, a slightly modified method was adopted (Fujimura et al., 2000). Briefly, vehicle control or EA treated cells were harvested on ice. After two washes with ice cold 1 $\times$ PBS, cells were resuspended in lysis buffer (Fujimura et al., 2000) and homogenized with a Dounce homogenizer on ice for 15–20 strokes. After centrifugation at 2000 $\times$ g for 5 min, the supernatant was transferred to a new tube and the pellet was resuspended with 1/5 volume of original lysis buffer. After a second round of homogenization, the pellet was dissolved in new lysis buffer with 0.2% triton X-100 (mitochondrial fraction). The supernatants (Cytosolic fraction) were combined and stored in -20 °C freezer. After protein concentration measurement, western blotting was performed to analyze cytochrome c release. Similarly, a well established approach for subcellular fractionation between mitochondria and nuclei was adopted (Nur-E-Kamal et al., 2004;Saunders et al., 1997). Western analysis was performed to analyze cytochrome c release and AIF translocation.

Immunohistochemical analysis

For immunohistochemical staining, vehicle control and EA treated cells were harvested and washed twice in ice cold 1×PBS followed by fixation in 4% paraformaldehyde (PFA) for 1 hour on ice. After permeabilization with 0.2% triton X-100 in PBS and blocking with 10% goat serum, cells were incubated with specific antibodies overnight at 4 °C (AIF, cytochrome c and active caspase 3 antibodies were all at 1:300 dilution; Chemicon Inc.). Sections were washed 5 times with 0.2% triton X-100 in PBS for 5 minutes each and then incubated with specific secondary antibody conjugated with Fluorescein or Rhodamine for 2 hours at room temperature in the dark. After extensive washes, cells were incubated with 4'-6-Diamidino-2-phenylindole (DAPI) for nuclei staining and mounted with anti-fade medium. All images were collected and analyzed with a Nikon optical microscope (E800 model) equipped with the Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) and Photoshop software (Adobe Systems, San Jose, CA).

Western blotting analysis

Vehicle control or EA treated cells were scraped off the wells, and harvested in ice-cold 1× PBS. Cells were then centrifuged at 2500×g at 4 °C for 5 minutes. The supernatant was discarded, and the cell pellet was suspended in 400 l of ice cold lysis buffer (10mM K₂HPO₄, pH 7.2/1mM EDTA/5mM EGTA/10mM MgCl₂/50mM Glycerophosphate/1mM Na₃VO₄/2mM DTT/1% Triton X-100) with complete protease inhibitor (Roche Biochemicals, Indianapolis, IN). The cell lysate was spun down in a table centrifuge at 12,000×g at 4°C for 10 minutes, and the protein concentration in the supernatant was measured using BioRad protein assay kit (BioRad, Hercules, CA). The protein samples (20 g) were separated in a 12.5% SDS PAGE gel and transferred onto nitrocellulose membrane. The membrane was blocked with 5% dry-milk dissolved in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature on a shaker plate and then incubated with the specific antibody overnight at 4°C. The membrane was washed three times for 5 minutes each time with TBST at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (KPL, Gaithersburg, MD) for 1 hour at room temperature. The membrane was washed three times with TBST for 5 min each and visualized in ECL reagents (Amersham Bioscience, Piscataway, NJ).

Cell viability assay

Cell viability of NSC34 cells upon treatments was determined by trypan blue exclusion assay. Briefly, cells were incubated with 0.1% trypan blue dye for 10 min at room temperature and were counted on a hemacytometer with a microscope. Cell viability was expressed as the number of viable cells (dye-excluding) divided by total number of cells. In addition, cell viability was also measured by the LDH release assay based on the manufacturer's instructions (Sigma Chemicals, St. Louise, MO) to confirm the trypan blue exclusion assay.

Cell apoptosis assay

Apoptosis assays in control or EA treated cells were carried out according the manufacturer's instructions using an ELISA formatted assay that detects histone-associated DNA fragments (Roche Biochemicals, Indianapolis, IN). In addition, a terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was also performed to analyze apoptotic cell death. Two independent apoptotic assays generated very similar results.

Statistical analysis

Values were expressed as Mean ± SE. Specific comparisons between control and individual experiment were analyzed by Student t-tests with *p*-value less than 0.05 considered as statistical significance.

Results

Depletion of intracellular GSH in NSC34 motor neuron-like cells by chemical conjugation and biochemical inhibition approaches

A motor neuron-like cell culture model (Cashman et al., 1992; Bishop et al., 1999) and an ALS-like transgenic mouse model (Gurney et al., 1994) were employed to study the role of GSH in motor neuron death associated with ALS disease onset and progression. In the cell culture system, two different experimental approaches, chemical-mediated depletion and GSH synthesis inhibition, were used to decrease cellular levels of GSH. NSC34 cells exposed to EA and BSO had dose-dependent and time-dependent reduction of cellular GSH measured by colorimetric assay and biochemical assay respectively (Figure 1A–1G).

GSH depletion elevated cellular ROS production in NSC34 cells

The DCF assay was applied to measure the kinetics of ROS production in GSH depleted cells (Wang and Joseph, 1999). As shown in Figure 2, oxidation of DCF was dramatically increased as cellular GSH was depleted by exposure of cells to different doses of EA and BSO respectively. EA apparently reduced intracellular GSH more effectively (Figure 1) and promoted more ROS production (Figure 2A) than BSO (Figure 2B). Thus, EA chemical-mediated GSH depletion was subsequently used to study the role of GSH alteration in motor neuron-like cell death.

GSH reduction increased oxidative stress marker gene expression

We examined changes in early and secondary oxidative stress response genes to confirm the increased oxidative stress elicited by GSH depletion. EA treatment enhanced AP-1 transcriptional activation, as detected by a luciferase reporter gene assay (Figure 3A), c-Jun (Figure 3B) and c-Fos expression (data not shown) as detected by western analysis. Similarly, GSH depletion also increased HO-1 expression as detected by quantitative real-time PCR reaction and western blotting analyses, respectively (Figure 4).

GSH depletion decreased cell adhesion and increased cell death

Cell adhesion to substrate is critical to cell survival. Therefore, we tested the effects of GSH reduction on NSC34 cell adhesion to three major extracellular proteins, namely fibronectin, collagen, and laminin. As shown in Figure 5A, cells treated with EA showed a dose-dependent and time-dependent (data not shown) decrease in adhesion to all three extracellular proteins. Similarly, cells exposed to EA decreased mitochondrial function as measured by MTT assay (Figure 5B), and increased cell death detected by trypan blue exclusion assay (Figure 5C) and histone release (Apoptosis) assay (Figure 5D) respectively.

GSH depletion promoted AIF redistribution, cytochrome c release, caspase 3 activation and apoptotic cell death

A biochemical assay and an immunohistochemical analysis were used to identify the molecular pathways of cell apoptosis by GSH depletion. Mitochondrial and nuclear fractionations coupled with western analysis showed an increase of AIF translocation to nucleus following GSH depletion in NSC34 cells (Figure 6A). Immunohistochemical staining confirmed the increase of AIF translocation to the nucleus from the mitochondria upon GSH depletion (Figure 6B).

Similarly, mitochondrial and cytosolic fractionation coupled with western blotting analysis and immunohistochemistry showed that cytochrome C release was involved in NSC34 cell apoptosis caused by GSH depletion (Figure 6C, 6D). Activation of caspase activity contributes to apoptotic cell death in many systems including CNS (Parikh et al., 2003). GSH depletion

increased caspases-3 activation revealed by immunohistochemical staining with anti-active caspase-3 antibody (Figure 7A–7C). In addition, GSH reduction enhanced apoptosis as determined by histone release assay (Figure 5D) and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Figure 7D and 7E) in NSC34 cells.

Reduction of GSH and increase of GSSG in the G93A-SOD1 transgenic mouse spinal cords was associated with motor neuron death and ALS-like disease onset and progression

The well-established mutant G93A-SOD1 transgenic mouse model of ALS-like disease was used to analyze the levels of GSH in the course of ALS disease onset and progression. Compared to age-matched normal littermate controls, GSH levels in the lumbar region were slightly reduced at disease onset, and were significantly decreased during disease progression in G93A-SOD1 transgenic mice (Figure 8A and 8C). On the other hand, oxidized GSH (GSSG) levels were significantly increased during both ALS-like disease onset and progression (Figure 8B and 8D).

Compared to age-matched littermate controls, intracellular GSH levels in G93A-SOD1 transgenic mouse motor neuron cells were decreased at both ALS-like disease onset and progression (Figure 8E). Decreased GSH was associated with AIF nuclear translocation, caspase 3 activation, and motor neuron cell death (Figure 9) in ALS-like transgenic mice.

Discussion

Several lines of evidence suggest that increased oxidative stress plays an important role in motor neuron degeneration leading to ALS disease onset and progression (Hall et al., 1998; Tu et al., 1997). However, what causes the increased oxidative stress and how oxidative stress specifically contributes to motor neuron degeneration remains largely unknown. In addition, the relationships between alterations in the intracellular GSH levels and motor neuron death have not been determined. For this reason, the current study was designed to analyze the molecular processes of reduction of GSH-mediated motor neuron (motor neuron-like) cell death using a cell culture model and a mouse model recapitulating human ALS. The data presented in this study demonstrated three major findings: 1). Increased oxidative stress by alteration of intracellular levels of GSH was associated with motor neuron-like cell death (apoptosis) *in vitro* and *in vivo*; 2). Reduction in GSH was associated with redistribution of AIF from mitochondria to nuclei; 3). Cytochrome c-mediated caspase 3 activation, elicited by GSH reduction also contributed to motor neuron cell death (apoptosis). Thus, reduction of GSH emerges as an important factor associated with motor neuron degeneration in ALS by activation of multiple apoptotic pathways.

Cell culture and transgenic mouse models to study the role of altered GSH in motor neuron degeneration

A cell culture model was used as an initial step to test the role of GSH alteration in motor neuron-like cell death using both biochemical inhibition and chemical depletion approaches to reduce cellular availability of GSH (Figure 1). BSO is an inhibitor of γ -glutamylcysteine synthetase (γ -GCS) which blocks GSH synthesis. Exposure of cells to BSO leads to the reduction of cytosolic GSH, but not of mitochondrial GSH. EA, a thiol reactive molecule, conjugates directly to GSH and decreases intracellular and mitochondrial levels of GSH (Schafer and Buettner, 2001). For this reason, we focused the EA approach to deplete intracellular GSH and to study the effects of increased oxidative stress on motor neuron-like cell death.

We also employed a mutant G93A-SOD1 transgenic mouse model to analyze the role of GSH in motor neuron degeneration (Gurney et al., 1994). The lifespan of the ALS-like mice have

been characterized into three stages, namely, disease free (before 60 days old), disease onset (70–90 days old) and disease progression (100–130 day old) stages (Liu et al., 1998; Gurney et al., 1994). Based on the well-established pathological manifestations, we analyzed the levels of GSH in the lumbar region of mutant G93A-SOD1 transgenic mice at the different stages, and further compared to those of the age-matched normal littermate controls. In addition, we also applied an immunohistochemical method to detect GSH changes in motor neuron cells in the G93A-SOD1 transgenic mice and age-matched normal littermate controls. Thus, the combination of the cell culture model and the transgenic mouse model permitted delineation of the potential contributions of increased oxidative stress by reduction of intracellular GSH to motor neuron death (apoptosis) in ALS.

Reduction of GSH increased oxidative stress and induced motor neuron-like cell apoptosis

GSH is the most abundant and most effective cellular antioxidants, and alterations in GSH often result in redox changes. As shown in Figure 1 that exposure of NSC34 cells to BSO or EA reduced cellular GSH dramatically as measured by biochemical and colorimetric assays. GSH is biosynthesized from L-glutamate catalyzed by γ -GCS and glutathione synthetase (GS) in the cytosol. Several organelles contain their own GSH pools that are temporally independent of cytosolic GSH synthesis. For example, both the mitochondria and the nucleus have their own GSH pools, and BSO treatment does not deplete GSH from these pools, while EA does. Decreased GSH altered the redox status to the direction of more oxidized state in the cell culture experiments. As shown in Figure 2, GSH reduction dramatically increased ROS generation as measured by the DCF assay. In addition, GSH depletion increased the expression of the early oxidative stress markers, c-Jun, c-Fos (data not shown) and AP-1 activation (Figure 3), and secondary oxidative stress markers, such as HO-1 expression (Figure 4). Taken together, these experiments strongly suggest that decreased GSH availability leads to increased intracellular oxidative stress, and promotes oxidative stress propagation, for example, protein oxidation, lipid peroxidation and nucleic acid oxidation (data not shown).

The oxidized cellular environment induced by GSH depletion resulted in the inability of cells to adhere to extracellular matrix proteins (Figure 5A). Preserved ability of cells to adhere substrate proteins is essential for cell survival, and loss of cell adhesion may lead to subsequent cell death. In deed, GSH reduction caused mitochondrial dysfunction (Figure 5B) and cell death (Figure 5C and 5D). To identify the cell death mechanisms potentially induced by GSH depletion, we assessed AIF redistribution, and found that there was an increase in AIF translocation from mitochondria to nuclei upon GSH depletion (Figure 6A–6C). AIF is a flavoprotein residing in mitochondrial intermembrane (Cande et al., 2004; Lindholm et al., 2004; Vahsen et al., 2004). Translocation of AIF initiates cell apoptosis by cleavage internucleosomal DNA to relative large fragments. AIF nuclear translocation has been observed in a variety of cell culture systems and animal models subjected to a variety of stress conditions. The AIF redistribution detected in EA treated cells, and also observed in the mutant G93A-SOD1 mouse model is most likely elicited by increased oxidative stress associated with decreased GSH. The decreased GSH coupled with increased oxidative stress by mutant G93A-SOD1 that facilitates AIF nuclear translocation may underlie some of the events linked to ALS disease onset and progression.

The dramatic increase in cytochrome c release to cytosol detected by the fractionation assay and immunohistochemical analysis (Figure 6B and 6D) most likely reflects the increased activation of caspase 3, and consequently contributes to the markedly increased of apoptosis elicited through the reduction of GSH in NSC34 cells (Figure 7). Cytochrome c-mediated caspase activation has also been shown to lead motor neuron degeneration in ALS-like mouse and human ALS patients (Guegan et al., 2001; Li et al., 2000).

Decreased GSH is associated with motor neuron degeneration and ALS-like disease onset and progression in the G93A-SOD1 transgenic mouse model

More recently, alterations of glutathione S-transferase pi activity have been reported in ALS patients (Kuzma et al., 2006; Usarek et al., 2005). Nevertheless, the effects of GSH to motor neuron degeneration in ALS remains largely unknown, even though abnormalities in GSH are associated with pathophysiological mechanisms underlying neuronal losses in both Parkinson's disease and Alzheimer's disease (Paik et al., 2003; Jha et al., 2000; Karelson et al., 2002; Cecchi et al., 1999). To this end, we measured the GSH and GSSG levels in the spinal cord lumbar region of G93A-SOD1 transgenic mice at three defined stages, namely, disease free, disease onset and disease progression. A significant decrease of GSH and reciprocal increase of GSSG occurred in the ALS-like mice during the stages of disease progression. The decreased GSH and resultant cascade of oxidative reactions may therefore contribute to the age-dependent motor neuron cell death in this mouse model. To further substantiate this possibility, increased oxidative stress has been clearly associated with motor neuron degeneration during disease onset and progression (Hall et al., 1998; Liu et al., 1998; Bogdanov et al., 1998; Jung et al., 2001). More significantly, treatment of ALS-like transgenic mice with antioxidants, such as SOD and catalase mimetics (Jung et al., 2001), DMPO (Liu et al., 2002), iron porphyrin (Wu et al., 2003), or manganese porphyrin (Crow et al., 2005) delays disease onset and extends survival. Thus, reduction of GSH and increase of GSSG, most likely alter the intracellular redox environment, may play an active role in the cumulative damage incurred by motor neurons, and may therefore lead to ALS disease onset and progression.

It should be emphasized that increased oxidative stress is not the only consequence brought about by reduction of intracellular GSH. Decreases in GSH bioavailability may also lead to activation of glutamate receptors, and mobilization of intracellular calcium. Thus, reduction of GSH may trigger multiple signaling events, some of which may be implicated in induction of cell death (Figure 10). Notwithstanding, such considerations, increased oxidative stress induced by GSH reduction emerges as an early and sustained event that contributes to motor neuron degeneration both *in vitro* and *in vivo*.

In summary, we showed that increased oxidative stress is a frequent phenomenon occurring in the spinal cord of G93A-SOD1 ALS-like mice that is temporally associated with disease onset and progression. Alteration of GSH may play an important role in facilitation of motor neuron cell death via induction of pro-apoptotic pathways. These findings further lend support to the use of effective anti-oxidant therapeutic approaches targeting to mitochondria at early stage for delay ALS disease onset and progression.

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Abbreviation

AIF	Apoptosis Inducing Factor
ALS	Amyotrophic Lateral Sclerosis
BSO	L-Buthionine Sulfoximine
DCF	Dichlorofluorescein
DMEM	Dulbecco's Modified Eagle's Medium
EA	Ethacrynic Acid

FBS	Fetal Bovine Serum
γ-GCS	γ -Glutamylcysteine Synthetase
GSH	Glutathione
GSSG	Oxidized Glutathione
MBM	Monobromobimane
PFA	Paraformaldehyde
ROS	Reactive Oxygen Species
SOD1	Cu,Zn-Superoxide Dismutase
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling

Figure 1. Chemical depletion of GSH production and biochemical inhibition of GSH synthesis in NSC34 motor neuron-like cells

A. Representative images of control, EA and BSO treated cells demonstrating reduction of GSH analyzed by monobromobimane (MBM) assay. **B.** GSH depletion by EA for 8h in NSC34 cells causes a dose-dependent decrease of intracellular GSH detected with MBM assay. **C.** GSH depletion by EA at 60 M in NSC34 cells causes a time-dependent decrease of intracellular GSH detected with MBM assay. **D.** GSH synthesis inhibition by BSO for 24h in NSC34 cells causes a dose-dependent decrease of intracellular GSH detected with MBM assay. **E.** GSH synthesis inhibition by BSO at 75 M in NSC34 cells causes a time-dependent decrease of intracellular GSH detected with MBM assay. **F.** GSH depletion by EA in NSC34 cells causes a dose-dependent decrease of intracellular GSH detected with kinetic assay (refer to the detailed

assay in the Experimental Method Section). **G.** GSH synthesis inhibition by BSO in NSC34 cells causes a dose-dependent decrease of intracellular GSH detected with kinetic assay (refer to the detailed assay in the Experimental Method Section). All experiments were carried out with triplicate and three independent repeats.

Figure 2. GSH depletion enhances reactive oxygen species (ROS) production in NSC34 cells
A. GSH depletion by EA in NSC34 cells causes a dose-dependent increase of intracellular ROS production detected with DCF assay. **B.** GSH synthesis inhibition by BSO in NSC34 cells causes a dose-dependent increase of intracellular ROS production detected with DCF assay. All experiments were carried out with tetra-plicate and three independent repeats.

Figure 3. GSH depletion by EA increases early oxidative response gene expression

A. GSH depletion by EA in NSC34 cells enhances AP-1 transcriptional activation detected by a luciferase reporter assay. **B.** GSH depletion by EA in NSC34 cells increases a dose and time-dependent c-Jun expression detected by western blotting analysis.

Figure 4. GSH depletion by EA increases secondary oxidative response gene expression

A. GSH depletion by EA at 40 M in NSC34 cells dramatically increases a time-dependent HO-1 expression detected by western blotting analysis. **B.** GSH depletion by EA for 12h in NSC34 cells increases a dose-dependent HO-1 expression detected by western blotting analysis. **C.** GSH depletion by EA at 40 M for 4h and 8h in NSC34 cells dramatically increases HO-1 expression detected by quantitative real-time PCR analysis. **D.** Fold-increase of HO-1 expression in EA treated cells compared to vehicle control detected by quantitative real-time PCR analysis (n=3).

Figure 5. GSH depletion by EA increases NSC34 cell death

A. GSH depletion by EA in NSC34 cells decreases cell adhesion to fibronectin (FN), collagen (COL) and laminin (LA) in a dose-dependent manner. **B.** GSH depletion by EA in NSC34 cells increases mitochondrial dysfunction in a dose-dependent manner analyzed by MTT assay.

C. GSH depletion by EA in NSC34 cells increases cell death in a dose-dependent manner detected by trypan blue exclusion assay. **D.** GSH depletion by EA in NSC34 cells increases cell apoptosis in a time-dependent manner detected by histone protein release assay.

Figure 6. GSH depletion by EA promotes redistribution of pro-apoptotic factors in NSC34 cells

A. GSH depletion by EA in NSC34 cells enhances AIF nuclear translocation from mitochondria detected by western blotting analysis coupled with mitochondrial and nuclear fractionation.

B. GSH depletion by EA in NSC34 cells promotes cytochrome c release to cytosol from mitochondria detected by western blotting analysis coupled with mitochondrial and cytosolic fractionation.

C. GSH depletion by EA in NSC34 cells enhances AIF nuclear translocation from mitochondria detected by immunohistochemical staining.

D. GSH depletion by EA in NSC34 cells promotes cytochrome c release from mitochondria detected by immunohistochemical staining.

Figure 7. GSH depletion by EA promotes apoptosis in NSC34 cells

A. Vehicle control treated NSC34 cells are immunostained with active caspase 3, showing that most of cells are negative in active caspase 3 staining. **B.** GSH depletion by EA in NSC34 cells enhances caspase 3 activation and increases nuclear translocation detected by immunohistochemical staining. **C.** Percentage of active caspase 3 positive cells in EA treated NSC34 cells (n=3). **D.** GSH depletion by EA in NSC34 cells promotes a dose-dependent cell apoptosis detected by TUNEL assay. **E.** GSH depletion by EA in NSC34 cells promotes a time-dependent cell apoptosis detected by TUNEL assay.

Figure 8. GSH levels in mutant G93A-SOD1 transgenic mice at disease free (45 days of age), disease onset (80 days of age) and disease progression (110 days of age) compared to the age-matched normal mouse controls

Spinal cord lumbar region from mutant G93A-SOD1 transgenic mice and age-matched control mice were processed to determine the levels of GSH or GSSG. **A.** Representative kinetic assay of GSH (refer to the detailed assay in the Experimental Method Section) in the spinal cord lumbar region of mutant G93A-SOD1 transgenic mice compared to the age-matched normal mouse controls. **B.** Representative kinetic assay of GSSG (refer to the detailed assay in the Experimental Method Section) in the spinal cord lumbar region of mutant G93A-SOD1 transgenic mice compared to the age-matched normal mouse controls. **C.** The relative GSH levels in the spinal cord lumbar region of mutant G93A-SOD1 transgenic mice compared to the age-matched normal mouse controls. **D.** The relative GSSG levels in the spinal cord lumbar

region of mutant G93A-SOD1 transgenic mice compared to the age-matched normal mouse controls. E. GSH levels in motor neuron cells of mutant G93A-SOD1 transgenic mice compared to the age-matched normal mouse controls.

Figure 9. Redistribution of AIF and active caspase 3 in motor neuron cells of ALS-like mice Mutant G93A-SOD1 transgenic mice at disease progression stages (110 days of age) were processed to analyze the distribution of AIF and active caspase 3 in the spinal cord motor neurons. Compared with age-matched normal controls, AIF and active caspase 3 were translocated to the nuclei in the mutant G93A-SOD1 spinal cord motor neurons.

Figure 10.
The potential signal transduction pathways of GSH depletion-mediated motor neuron cell apoptosis.