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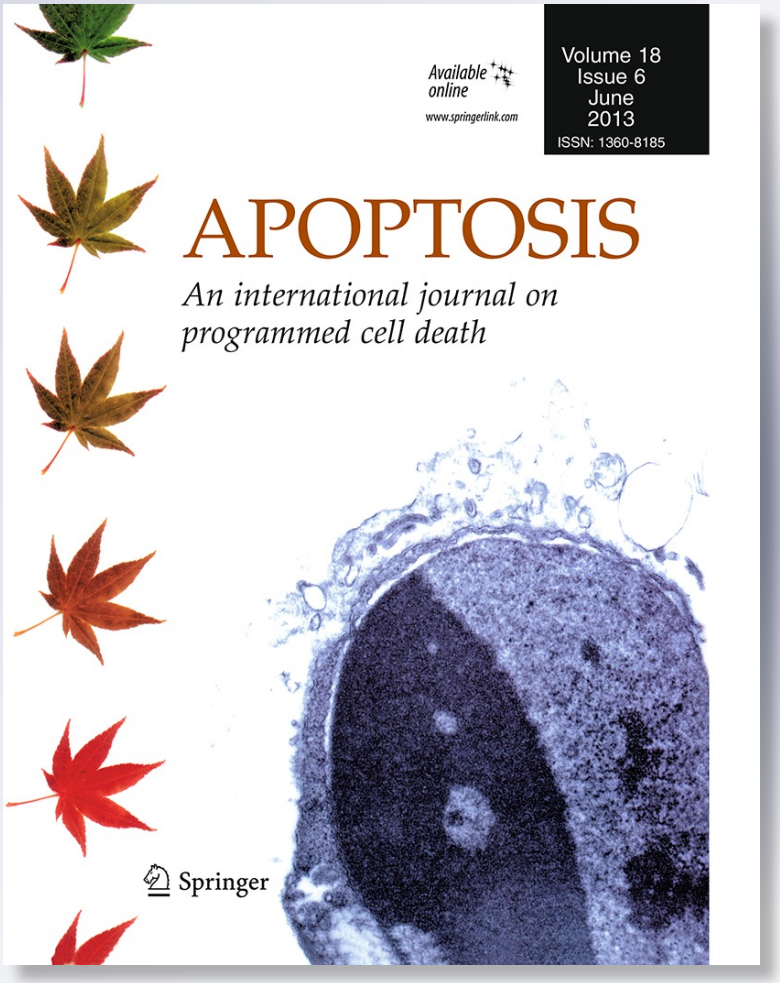

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ORIGINAL PAPER

Neuroprotection against neuroblastoma cell death induced by depletion of mitochondrial glutathione

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Abstract Mitochondrial glutathione pool is vital in protecting cells against oxidative stress as the majority of the cellular reactive oxygen species are generated in mitochondria. Oxidative stress is implicated as a causative factor in neuronal death in neurodegenerative disorders. We hypothesized that depletion of mitochondrial glutathione leads to mitochondrial dysfunction and apoptotic death of SK-N-SH (human neuroblastoma) cells and investigated the neuroprotective strategies against GSH depletion. SK-N-SH cells were treated with two distinct inhibitors of glutathione metabolism: L-buthionine-(S, R)-sulfoximine (BSO) and ethacrynic acid (EA). EA treatment caused depletion of both the total and mitochondrial glutathione (while BSO had no effect on mitochondrial glutathione), enhanced rotenone-induced ROS production, and reduced the viability of SK-N-SH cells. Glutathione depletion by BSO or EA demonstrated positive features of mitochondria-mediated apoptosis in neuroblastoma cell death. Prevention of apoptosis by Bcl2 overexpression or use of antioxidant ebselen did not confer neuroprotection. Co-culture with U-87 (human glioblastoma) cells protected SK-N-SH cells from the cell death. Our data suggest that depletion of mitochondrial glutathione leads to mitochondrial dysfunction and apoptosis. The study indicates that preventing mitochondrial glutathione depletion could become a novel strategy for the development of neuroprotective therapeutics in neurodegenerative disorders.

Keywords Glutathione · Glutathione depletion · Mitochondrial glutathione · Apoptosis · Oxidative stress · Neuroprotection

Abbreviations

BSO	L-Buthionine-(S,R)-sulfoximine
EA	Ethacrynic acid
ETC	Electron transport chain
GSH	Glutathione
PI	Propidium iodide
ROS	Reactive oxygen species
SK-N-SH	A human neuroblastoma cell line
U-87	A human glioblastoma cell line

Introduction

Glutathione (GSH) is a tripeptide (γ-glutamyl cysteinyl glycine) antioxidant synthesized in cells. GSH is depleted

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in the brains of individuals suffering from neurodegenerative diseases, such as Parkinson's disease (PD) [1]. GSH functions as the intracellular reservoir of cysteine and is one of the most important antioxidant moieties as it neutralizes reactive oxygen species (ROS), peroxides and reduces oxidized biomolecules [2, 3].

Cellular levels of glutathione can be pharmacologically altered. L-Buthionine-(S, R)-sulfoximine (BSO) is a potent inhibitor of γ-glutamyl cysteine synthetase (γ-GCS), a rate-limiting enzyme essential for glutathione biosynthesis [4]. As most of the γ-GCS is present in the cytoplasm, BSO depletes cytoplasmic GSH effectively [4, 5]. When cytoplasmic GSH is depleted, transport of GSH from the cytosol into the mitochondria decreases and hence the mitochondrial GSH (mtGSH) pool is decreased after prolonged BSO administration. Ethacrynic acid (EA), prescribed for its loop diuretic effect, is a potent inhibitor of glutathione-S-transferases [6]. In addition, EA depletes the mtGSH pool to a greater extent than the cytoplasmic pool in astrocytes [7, 8]. We re-tested and used this action of EA to selectively deplete mtGSH and we used 24 h as time point for our experiments since BSO takes longer time than that to have effect on mtGSH. Since mtGSH is very important in cellular antioxidant defense against ROS, its depletion could lead to cell death [9, 10].

GSH is compartmented in brain [11, 12]. Astrocytes have higher levels of GSH than neurons and export GSH intermediates at a higher rate than neurons [13, 14]. Furthermore, astrocytes can use larger number of substrates such as glutamate and cysteine or cystine as building blocks for GSH biosynthesis whereas neurons depend on glutamine and cysteine [15]. Intracellularly, highest amount of GSH is present in the cytoplasm where it is synthesized by the action of enzymes γ-glutamyl cysteine synthetase and glutathione synthetase in two ATP-dependant steps [16]. Smaller pool of mtGSH is formed by its transport into the organelle by GSH, dicarboxylate, and oxoglutarate carriers [17, 18].

GSH depletion-induced cell death mechanism is still incompletely understood. In addition, there is an ongoing search for novel approaches to enhance neuroprotection in neurodegenerative diseases. In this study, we hypothesized that glutathione depletion induces mitochondria-mediated apoptosis in SK-N-SH cells and used BSO and EA to study the role of mtGSH in it. We also aimed to test the putative protective strategies to rescue SK-N-SH cells from the effects of mtGSH depletion. We employed co-culture of SK-N-SH cells with U-87 glioblastoma cells, overexpression of anti-apoptotic bcl-2 or treatment with ebselen, which is a scavenger of hydrogen peroxide and hydroperoxides [19].

Materials and methods

All chemicals were from Sigma-Aldrich unless mentioned otherwise.

Cell culture

SK-N-SH cells (human neuroblastoma, no N-*myc* amplification, from ATCC) in modified Eagle's medium with 10 % FBS were used to study GSH depletion-mediated cell death mechanisms. All treatments were carried out 16 h after subculture of SK-N-SH cells at about 70 % confluency in 24-, 48-, or 96-well cell culture plates from Corning. U-87 (human glioblastoma) cells (ATCC) were employed in co-culture with SK-N-SH cells to determine their neuroprotective potential.

Cell viability determination

Cell viability was determined using MTT dye (3-(4,5-dimethylthia-zol,2-yl)-2,5-diphenyltetrazolium bromide). After incubating SK-N-SH cells with various concentrations of either L-buthionine-(S, R)-sulfoximine (BSO) or ethacrynic acid (EA) for 24 h, MTT (final conc. 0.05 % w/v) was added and kept for 4 h at 37 °C. The formazan crystals formed by intact cells were dissolved in DMSO and their absorbance was measured at 567 nm using a microplate reader (Biotek Synergy HT).

Cytosolic and mitochondrial fractionation

Mitochondrial and cytosolic fractions derived from SK-N-SH cells were obtained using differential centrifugation method as described previously [20]. SK-N-SH cells were homogenized in a buffer consisting 0.32 M sucrose, 5 mM HEPES with protease inhibitor cocktail tablet from Roche and pH adjusted to 7.4 with Trizma™ base. Homogenates were centrifuged at 2,000×*g* and 4 °C for 1 min to separate nuclear pellet and the resultant supernatant was subjected to centrifugation at 12,000 g and 4 °C for 10 min to obtain the mitochondrial pellet and cytosolic supernatant. Assays for citrate synthase (a mitochondrial enzyme) and lactate dehydrogenase (a cytosolic enzyme) were used to assess the relative purity of the isolated fractions [21].

Determination of GSH content

Total GSH content in SK-N-SH cells was determined by the method of Griffith [22]. Briefly, SK-N-SH cells were homogenized in a buffer containing sulphosalicylic acid (4.31 % w/v) and 0.25 mM EDTA. Total (reduced +

oxidized) GSH in cell homogenates was then determined by chemical reaction between the GSH and Ellmann's reagent and measuring the absorbance of the reaction product at 412 nm using a microplate reader.

Induction and assessment of ROS generation

Production of ROS by SK-N-SH cells was induced by treatment with rotenone (1 μM, 24 h) [23]. Carboxy H_2DCFDA (2′,7′-dihydrodichlorofluorescein diacetate) (Molecular probes) was used as an indicator of ROS. SK-N-SH cells were incubated with 10 μM of this dye (dissolved in DMSO and diluted with PBS) for 45 min and then the medium was replaced with sterile PBS. The fluorescence of the oxidized form of the dye was measured at excitation of 492 nm and emission of 521 nm using a microplate reader.

Induction of apoptosis and caspase-3 assay

Apoptosis was induced in SK-N-SH cells by treatment with etoposide (40 μM) [24]. Caspase-3 activity was measured using a commercially available caspase-3 fluorescence detection kit (Sigma-Aldrich). After 24 h treatment, cell pellet was lysed in a buffer consisting of 50 mM HEPES, pH 7.4, 5 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 5 mM DTT and was centrifuged at 600×*g* for 5 min. The resultant supernatant was tested for activity of cleaving caspase-3 substrate to form 7-amino-4-methylcoumarin that was measured using excitation of 360 nm and emission of 460 nm. Caspase-3 antibody (8G10) utilized was from Cell Signaling.

Assessment of necrosis and apoptosis by flow cytometry

A commercial kit from Southern Biotech was employed to carry out flow cytometric determination of annexin V and propidium iodide (PI) labeling using BD FACS calibur. Briefly, at the end of 24 h of treatment, SK-N-SH cells were centrifuged at 400×*g* for 5 min at room temperature and were washed once with PBS. Annexin V was then added in the presence of the binding buffer and kept on ice for 20 min. PI was then added and flow cytometric analysis was carried out. Analysis of data was carried out using Flowjo software (version 7.5).

Complex-I activity of mitochondrial electron-transport chain

Complex-I activity was determined using the method of Walker [25]. SK-N-SH cells were homogenized in a buffer consisting 50 mM Tris–HCl, pH 7.4, 1 mM EDTA and 2 % cholate and sonicated to clarity and then were assayed in a buffer containing 50 mM potassium phosphate, pH 7.4, and soyabean phospholipids. NADH oxidation was then monitored at 340 nm using a UV spectrophotometer.

Immunoblotting of apoptotic markers (bcl-2, bax and cytochrome c)

A mouse monoclonal bcl-2 antibody (Dako), polyclonal rabbit bax and cytochrome c antibodies (Santa Cruz Biotechnology) were employed to estimate levels of these proteins in SK-N-SH cells exposed to glutathione depletion by BSO and EA and to confirm bcl-2 overexpression. SK-N-SH cells were homogenized and total protein content was estimated by BCA protein assay. The cellular proteins were separated on Tris–HCl gels, transferred to PVDF membrane and the expression of the protein of interest was determined using enhanced chemiluminescence detection. Bands were quantified using ImageJ software.

Tests of protection against depletion of mitochondrial GSH

Co-culture of SK-N-SH and U-87 cells

SK-N-SH cells were plated on Corning Costar well plates (6-well, 12-well, or 24-well). U-87 cells were seeded on Millipore hanging culture inserts (pore size 0.4 μm) on separate plates and introduced to SK-N-SH plates after 4 h to achieve co-culture condition. SK-N-SH and U-87 cells were in 1:1 ratio in co-culture. Co-culture of SK-N-SH cells with U-87 was tested for neuroprotection using cell viability assay, ROS generation measurement, GSH content and caspase-3 activity.

Overexpression of bcl-2 in SK-N-SH cells

Plasmid (pBMN-Z-I-Neo) containing D34A caspase-resistant bcl-2 (a generous gift from Dr. Nolan, Stanford University) was introduced in a retrovirus vector as described [26]. High titer of bcl-2 was achieved by infecting PA-317 packaging cells with plasmid containing retroviruses. Repeated serial infections of SK-N-SH cells by viral supernatant of PA-317 cells were carried out. Antibiotic G-418 was used to select stable SK-N-SH cells. Bcl-2 overexpressing or empty vector expressing SK-N-SH cells along with wild type cells were used to study neuroprotection against glutathione depletion using cell viability assay and also evaluated for total GSH content and caspase-3 (full-length and cleaved form) expression.

Ebselen treatment

SK-N-SH cells were treated with ebselen (1 or 3 μM), BSO (5 mM), EA (50 μM), ebselen plus BSO or ebselen plus

EA. After 24 h, cellular viability was determined using MTT dye as described earlier.

Statistical analysis

All reported values are mean ± standard error of the mean (SEM). Experiments were analyzed using either one-way ANOVA or two-way ANOVA (SPSS software version 13). Differences between the means were considered statistically significant if $*p < 0.05$ and $**p < 0.01$.

Results

Effect of GSH depletion on cell viability, GSH content, and ROS generation

Treatment of SK-N-SH cells with BSO or EA produced dose- and time-dependant reduction in their viability (Fig. 1a, b). The effect of decreasing SK-N-SH cell viability was more potent for treatment with EA than with BSO. Based on these data, doses of BSO (1, 2.5, and 5 mM) and those of EA (5, 25, and 75 μM) were selected and 24 h was used as the treatment time for all subsequent experiments. To ascertain that BSO- or EA-induced cell death was due to GSH depletion, we utilized 24 h co-treatment with GSH ethyl ester (1 mM) and BSO or EA. GSH ethyl ester treatment reversed BSO- or EA-induced decrease in cell viability (Fig. 1c). We carried out subcellular fractionation of SK-N-SH cells to study the effects of BSO and EA on GSH subcellular compartmentation. Citrate synthase activity was enriched in the mitochondrial fraction and lactate dehydrogenase activity was enriched in the cytosolic fraction (Supplemental Fig. 1a, b), both pointing to the relative purity of the isolated subcellular fractions. Treatment of SK-N-SH cells with EA decreased both their cytosolic and mitochondrial pools of GSH with highly significant reduction with the dose of 75 μM whereas BSO decreased only cytosolic GSH pool with no significant impact on the mitochondrial GSH (mtGSH) (Fig. 2a, b). This differential effect of BSO and EA on mtGSH allowed us to analyze the role of mtGSH using these two inhibitors. Also, as expected, treatment with GSH ethyl ester (1 mM) for 24 h increased total GSH content and also reversed the decrease in GSH content of SK-N-SH cells by BSO or EA (Fig. 2c).

Next, we performed ROS assay using rotenone as a positive control. BSO treatment did not increase ROS generation, at the time-point and concentrations employed, (Fig. 2d) but EA treatment at 75 μM increased cellular ROS generation (Fig. 2e). Thus, our results indicate the importance of mtGSH in neutralizing ROS.

GSH depletion leads to mitochondria-mediated apoptosis in SK-N-SH cells

Caspase-3 and annexin V/propidium iodide (PI) were used as apoptotic markers to assess the effect of glutathione depletion on apoptosis in SK-N-SH cells. BSO (2.5 mM)

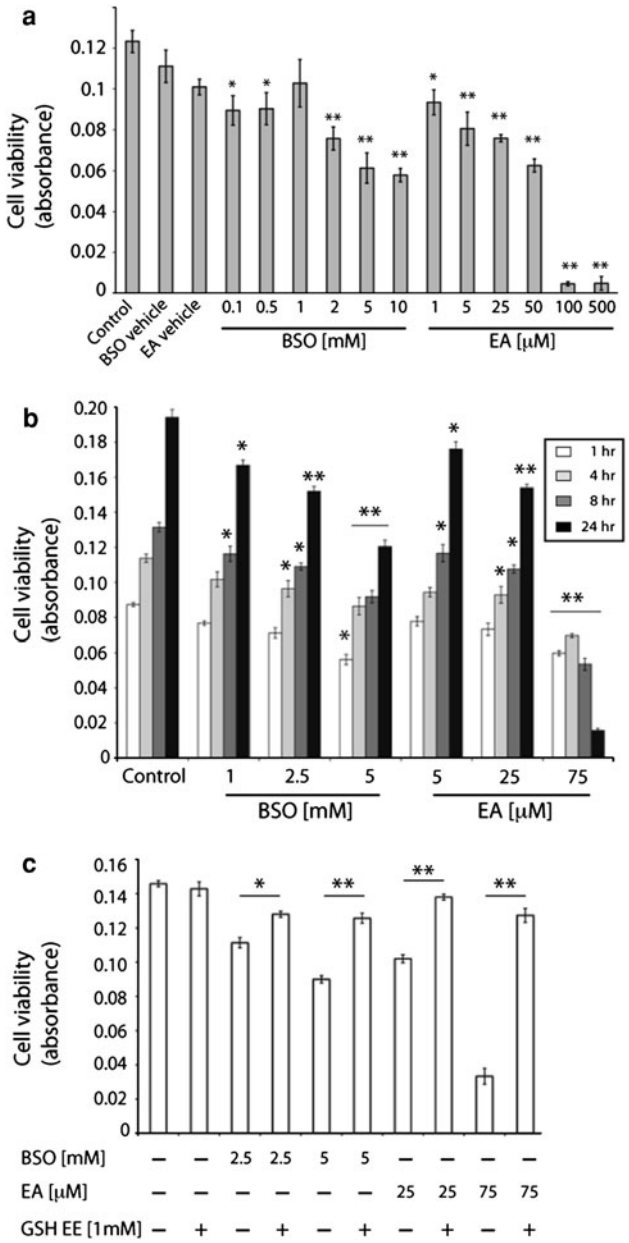
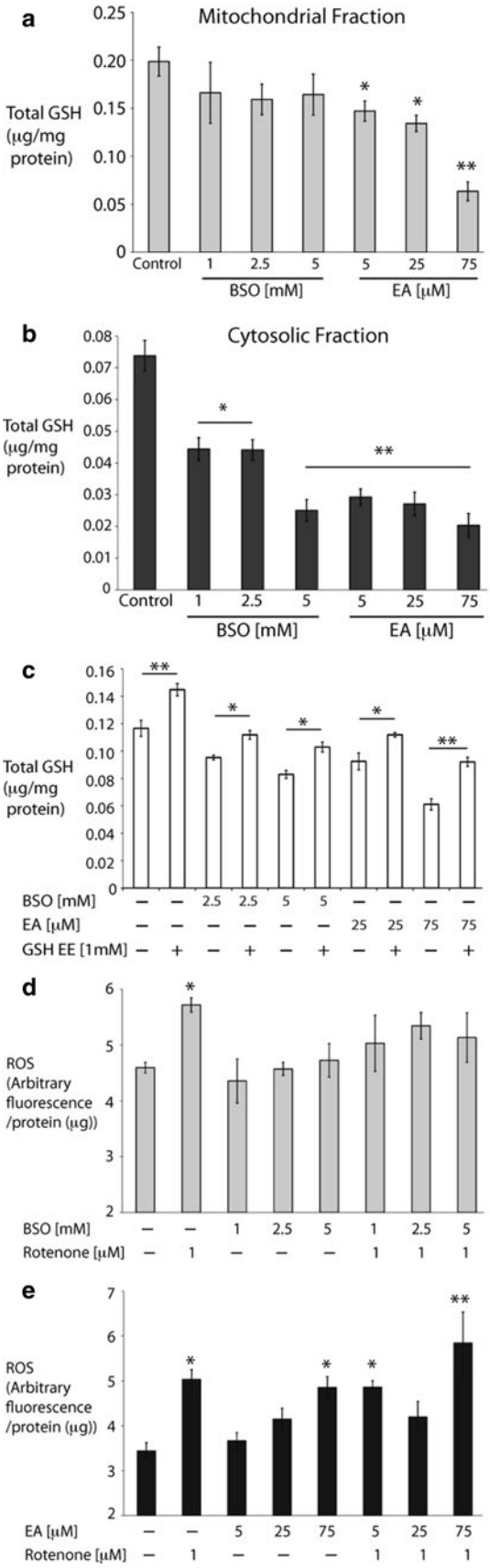


Fig. 1 Reduction in viability of SK-N-SH cells by BSO and EA **a** Viability of SK-N-SH cells against treatment with various concentrations of BSO and EA for 24 h, **b** Cell viability was measured at 1, 4, 8, and 24 as absorbance of the metabolized MTT dye after treatment with BSO and EA. **c** Cell viability was measured after 24 h treatment with GSH ethyl ester or BSO or EA or their combinations. All values are mean ± SEM of at least three different experiments ($*p < 0.05$, $**p < 0.01$) analyzed by one-way ANOVA followed by Dunnett's post hoc test



◀**Fig. 2** Effect of BSO and EA treatment on mitochondrial and cytoplasmic GSH content and ROS generation Total GSH content in the mitochondrial (**a**), and the cytoplasmic (**b**) fractions of SK-N-SH cells treated with various doses of BSO or EA. **c** Total GSH content in SK-N-SH cells treated with GSH ethyl ester or BSO or EA or their combinations was measured. SK-N-SH cells were treated with BSO (**d**) or EA (**e**) in the presence or absence of rotenone for 24 h and their effects on ROS generation was studied using carboxy derivative of H2DCF-DA. Rotenone was used as a positive control. All values are mean ± SEM of at least three different experiments (*$p < 0.05$, **$p < 0.01$) analyzed by one-way ANOVA followed by Dunnett's post hoc test

and EA (25 μM) treatment increased caspase-3 activity (Fig. 3a). We observed a no increase and a significant decrease in caspase-3 activity, respectively, with BSO 5 mM and EA 75 μM treatment. It may be that the active caspase-3 could have already acted on its substrates and thus no longer active at the time-point since we observed a highly significant decrease in cell viability with these treatments. EA 25 μM treatment induced apoptosis despite no increase in ROS generation and this effect could be due to its effect on glutathione levels (Fig. 2a, b) and complex-I activity (Fig. 3b). FITC-conjugated annexin V was used as a marker of apoptosis and propidium iodide (PI) was used as marker of necrosis. BSO 5 mM treatment increased PI-positive cells to 5.2 % (top left quadrant) from 2.6 % for control. EA treatment at 75 μM resulted in significant increase in the number of PI- positive cells (as many as 21 % of the total population, top right quadrant) and annexin V-positive cells (as many as 18 % of the total population, bottom right) compared with those for control (Fig. 3c). Thus, EA treatment at 75 μM induced mixed apoptototic-necrotic cell death. This demonstrates that mtGSH depletion leads to apoptosis. BSO 1 mM, BSO 2.5 mM, EA 5 μM or EA 25 μM treatment did not increase annexin V/PI marked cells (data not shown).

To distinguish between the effects of BSO and EA on mitochondrial proteins, we measured complex-I activity of electron transport chain (ETC). We utilized rotenone as an ETC complex-I inhibitor. We used combination of BSO or EA and rotenone to see whether there is synergy between ROS generation by GSH depletion and rotenone. Complex-I activity was decreased by treatment of SK-N-SH cells with 75 μM EA similar to the decrease in complex-I activity induced by 1 μM rotenone (Fig. 3b). BSO 5 mM treatment increased complex-I activity (Fig. 3b) and decreased ROS generation by 1 μM rotenone treatment (Fig. 2d). Since inhibition of complex-I is known to generate ROS, these data taken together explains why BSO did not increase ROS generation.

To analyze mitochondria-mediated apoptosis, we compared the effects of BSO and EA on bcl-2, bax, and cytochrome c in mitochondrial versus cytosolic fractions of

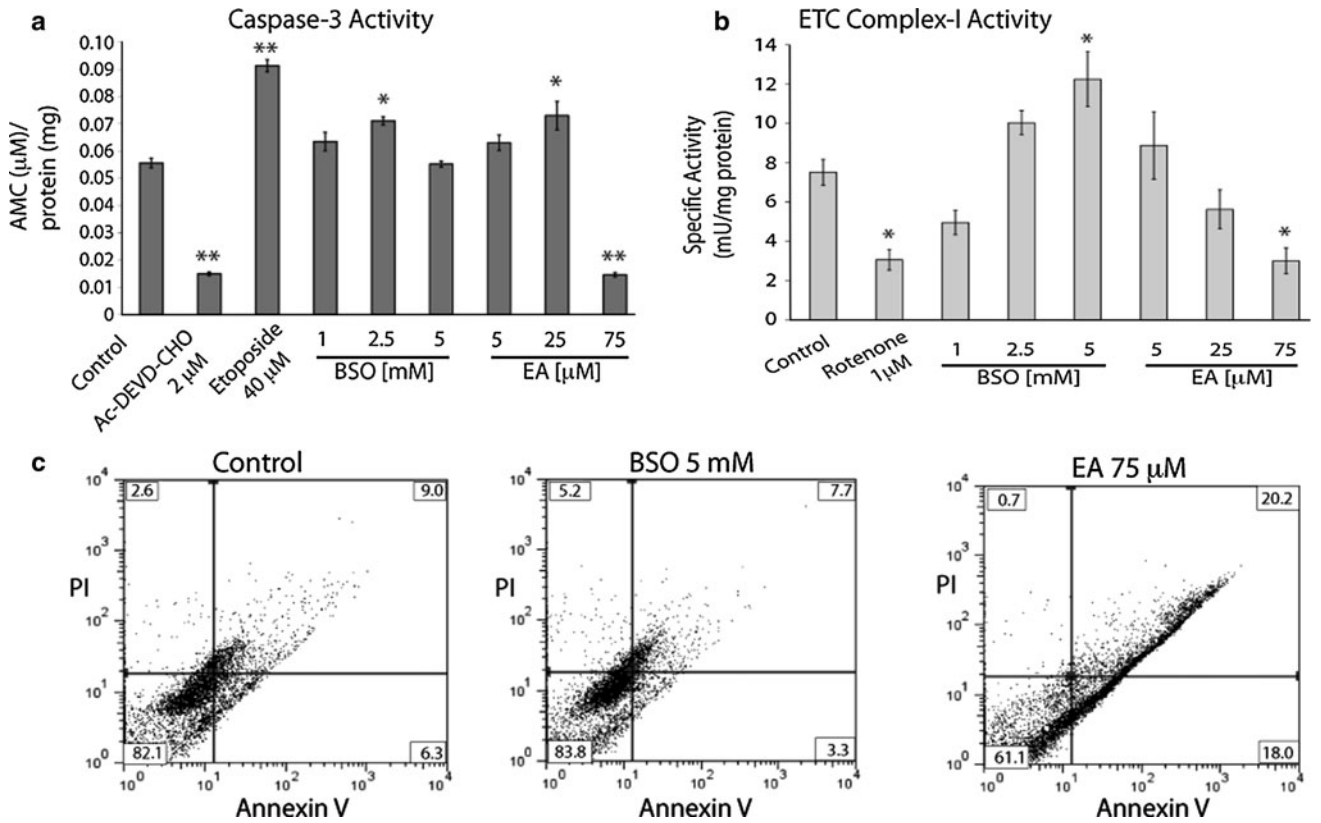


Fig. 3 Effect of BSO and EA treatment on apoptosis using indicators caspase-3, complex-I activity and annexin V/PI **a** Caspase-3 activity was measured in SK-N-SH cells after 24 h treatments with BSO or EA. Etoposide was used as positive control and Ac-DEVD-CHO is a caspase-3 inhibitor, **b** activity of complex-I was measured using rotenone as positive control. All values are mean ± SEM of at least three different experiments ($*p < 0.05$, $**p < 0.01$) analyzed by one-way ANOVA followed by Dunnett's post hoc test, **c** annexin V and propidium iodide (PI) staining of SK-N-SH cells, as marker of apoptosis and necrosis respectively, was determined using flow cytometry. *Top left* quadrant: PI positive cells, *top right* quadrant: annexin V and PI positive cells, *bottom left* quadrant: annexin V and PI negative cells, and *bottom right* quadrant: annexin V positive cells

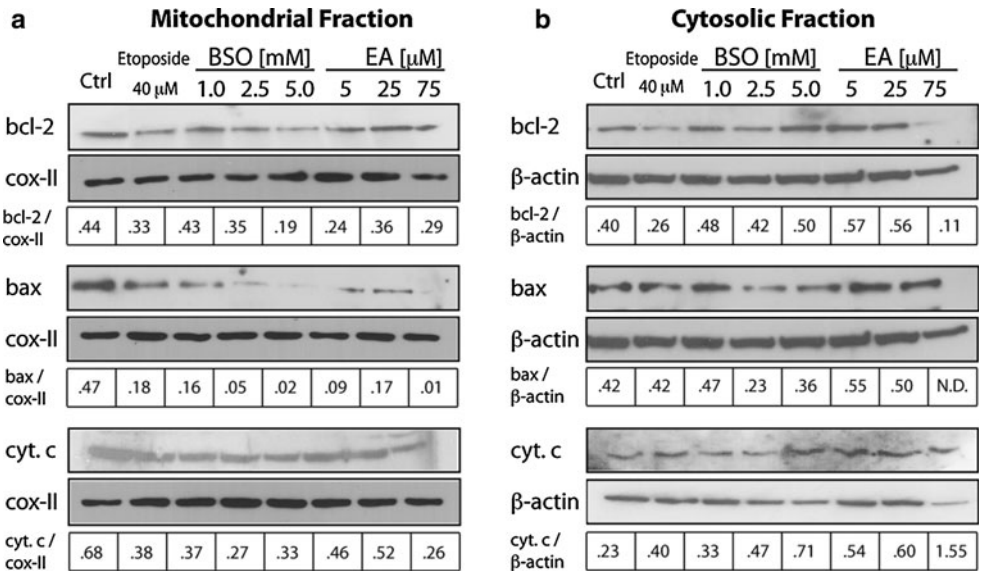


Fig. 4 Immunoblotting of apoptotic indicators bcl-2, bax and cytochrome c in SK-N-SH subcellular fractions Photomicrographs of data obtained by immunoblotting of bcl-2, bax, and cytochrome c from the mitochondrial (**a**) and cytosolic (**b**) fractions of SK-N-SH cells treated with varying concentrations of BSO and EA for 24 h. Etoposide was used as a positive control, β-actin and cytochrome c oxidase subunit II (Cox-II) were utilized, respectively, as internal loading control for cytosolic fraction and mitochondrial fraction. Ratio of band intensities was calculated using ImageJ software

SK-N-SH cells. Subunit II of Cytochrome c oxidase (Cox-II) was used as a mitochondrial marker. In the mitochondrial fraction (Fig. 4a), bcl-2 expression was reduced by BSO treatment as well as EA treatment, bax expression was significantly reduced by either BSO or EA treatments and cytochrome c expression showed a dose-related

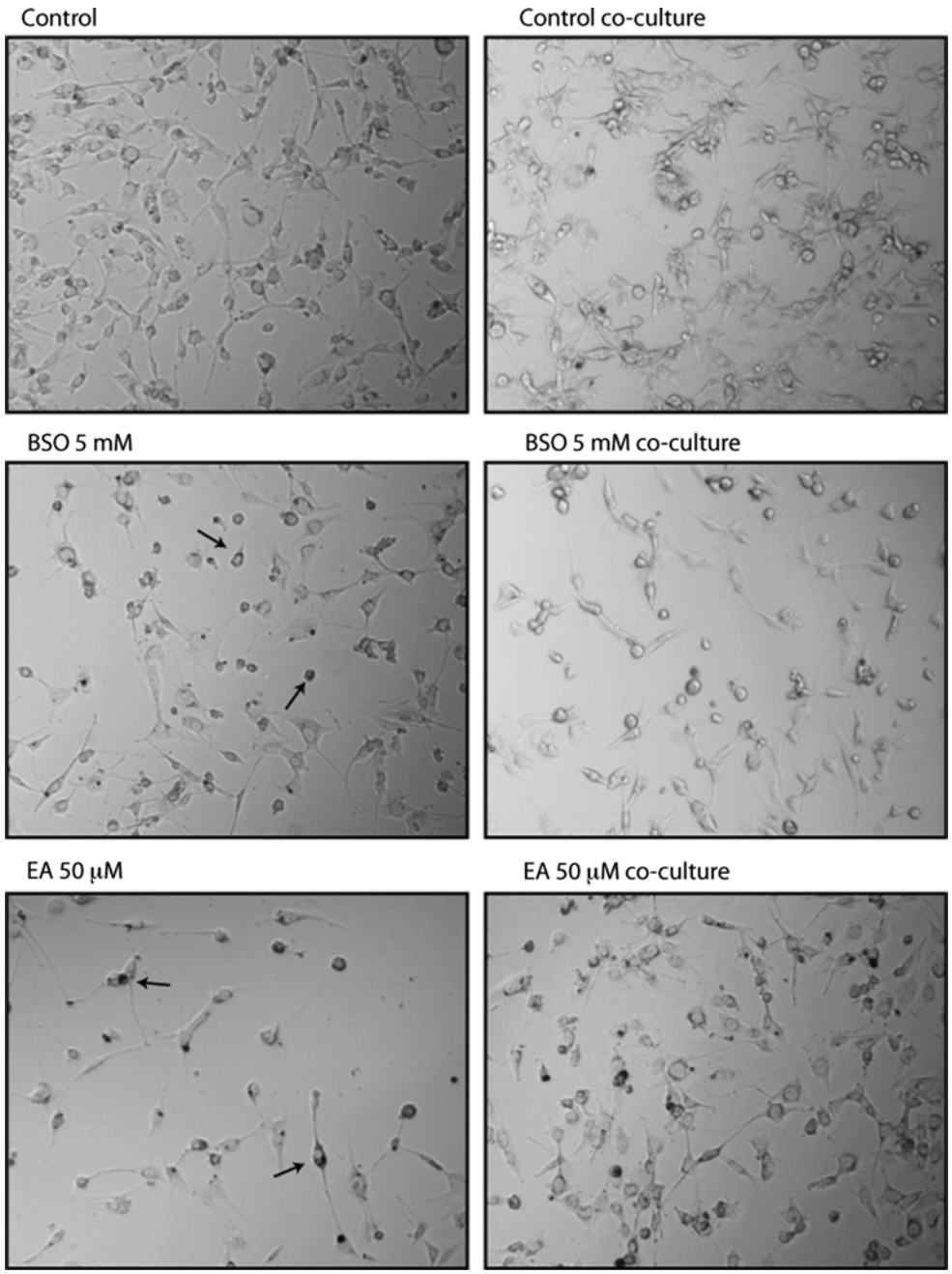


Fig. 5 Protection of SK-N-SH cells by U-87 cells against GSH depletion Photomicrograph of brightfield images of SK-N-SH cells either cultured alone or in co-culture with U-87 cells after 24 h treatment with 5 mM BSO or 50 μM EA. Representative photomicrographs at magnification of ×100. Treatment with BSO or EA decreased the number of viable cells as well as altered their morphology as evident from dark aggregates (*arrows*). Co-culture with U-87 cells protected SK-N-SH cells against EA treatment as evident by their increased number

decrease upon BSO or EA treatment. In the cytosolic fraction (Fig. 4b), bcl-2 expression was significantly decreased by treatment of cells with etoposide (40 μM) and EA (75 μM), bax expression was significantly reduced by treatment of cells with EA (75 μM) or with BSO (2.5, 5 mM). Cytochrome c expression in mitochondria was unaltered whereas that in the cytosolic fraction showed an increase (Fig. 4a, b). GSH depletion-induced effect on bcl-2 and cytochrome c indicated mediation of apoptotic process in death of SK-N-SH cells. Thus, our data suggest that cytoplasmic or mtGSH depletion can lead to mitochondria-mediated apoptosis.

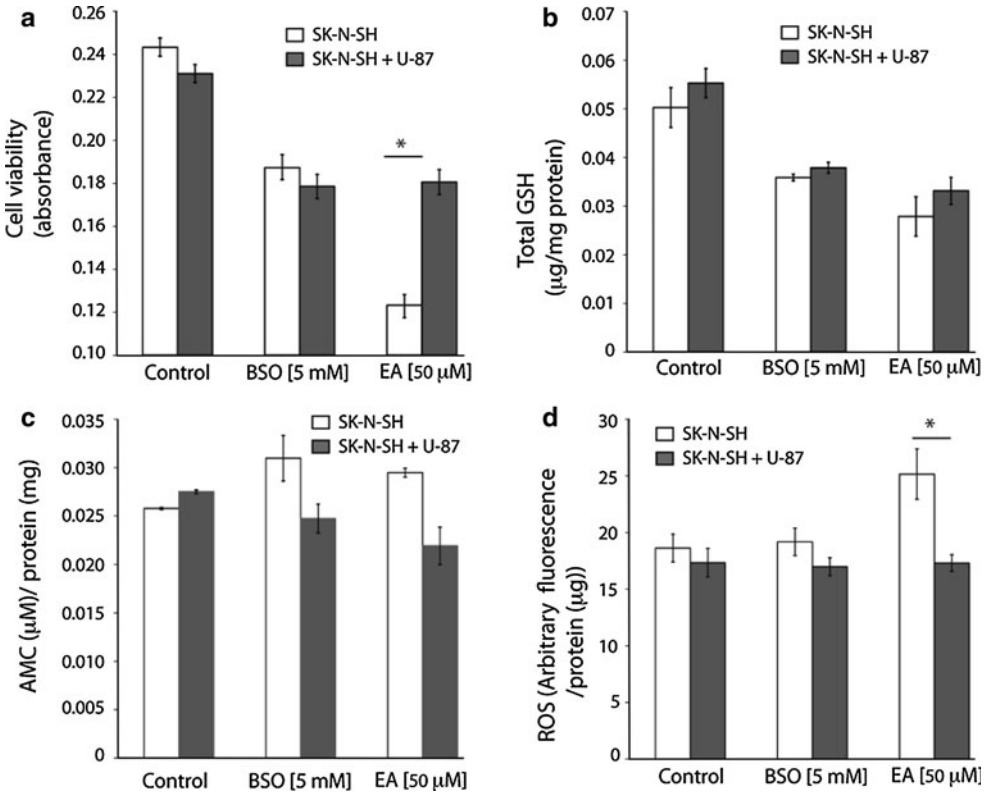


Fig. 6 U-87 cells in co-culture protected SK-N-SH cells against glutathione depletion-induced by EA **a** Cell viability assay, **b** GSH content estimation, **c** caspase-3 activity determination, and **d** ROS measurement of SK-N-SH cells treated with BSO (5 mM) or EA (50 μM) and cultured either alone (*open bars*) or in co-culture with U-87 cells (*closed bars*). All values are mean ± SEM of at least three different experiments analyzed by one-way ANOVA followed by Dunnett's post hoc test for SK-N-SH cells alone or in co-culture with U-87 cells

Co-culture of U-87 cells protects SK-N-SH cells from GSH depletion-induced cell death

Co-culture of SK-N-SH cells with U-87 cells was tested as a neuroprotection strategy. We utilized a lower concentration of EA (50 μM) for neuroprotection experiments since the EA 75 μM treatment toxicity was high and cells were likely in the committed stage for cell death based on our earlier data. Photomicrographs were taken after treatment with BSO (5 mM) or EA (50 μM) for 24 h. Treatment of BSO or EA showed a decrease in number of cells and also dark intracellular aggregates in cells were visible (Fig. 5, arrows). Interestingly, co-culture with U-87 cells protected EA-treated SK-N-SH cells based on their higher cell number and 'normal' morphology whereas the same co-culture rendered only slight protection against BSO treatment (Fig. 5). Restoration of cell viability of SK-N-SH cells grown in co-culture confirmed that U-87 cells could protect SK-N-SH cells against EA treatment (Fig. 6a). Co-culturing of SK-N-SH cells with U-87 cells also significantly decreased ROS generation induced by EA (Fig. 6d): this strategy also led, respectively, to a trend of increased GSH levels and reduced caspase-3 levels (Fig. 6b, c). Thus, U-87 cells in co-culture can protect SK-N-SH cells against GSH depletion induced by EA treatment and the mechanism of this neuroprotection, at least in part, could involve neutralization of ROS.

Bcl-2 overexpression or ebselen treatment do not confer protection to SK-N-SH cells against GSH depletion

We investigated anti-apoptotic bcl-2 overexpression and treatment with ebselen, a scavenger of peroxides, for their neuroprotection potential. Comparisons of bcl-2 expression in stably transfected SK-N-SH cells (with bcl-2 or without bcl-2 (empty vector) and wild type SK-N-SH cells showed increased expression in bcl-2-overexpressing SK-N-SH cells when compared to that in wild type SK-N-SH cells (Fig. 7a). Bcl-2 overexpression did not render neuroprotection against GSH depletion by BSO/EA as assessed by cellular viability (Fig. 7b). We measured GSH content in bcl-2 overexpressing cells and observed that there was no significant change in GSH content (Fig. 7c). We also measured caspase-3 expression (complete and cleaved form) and observed that bcl-2-overexpressing SK-N-SH cells showed significant decrease in total and active form of caspase-3 compared to control SK-N-SH cells (Fig. 7d). Thus, our data demonstrates that protection against GSH depletion needs to be offered at the earlier steps of apoptosis induction. Ebselen treatment at 1 and 3 μM with or without either BSO (5 mM) or EA (50 μM) and cellular viability determination at the end of 24 h showed no neuroprotection by ebselen against GSH depletion (Supplemental Fig. 2).

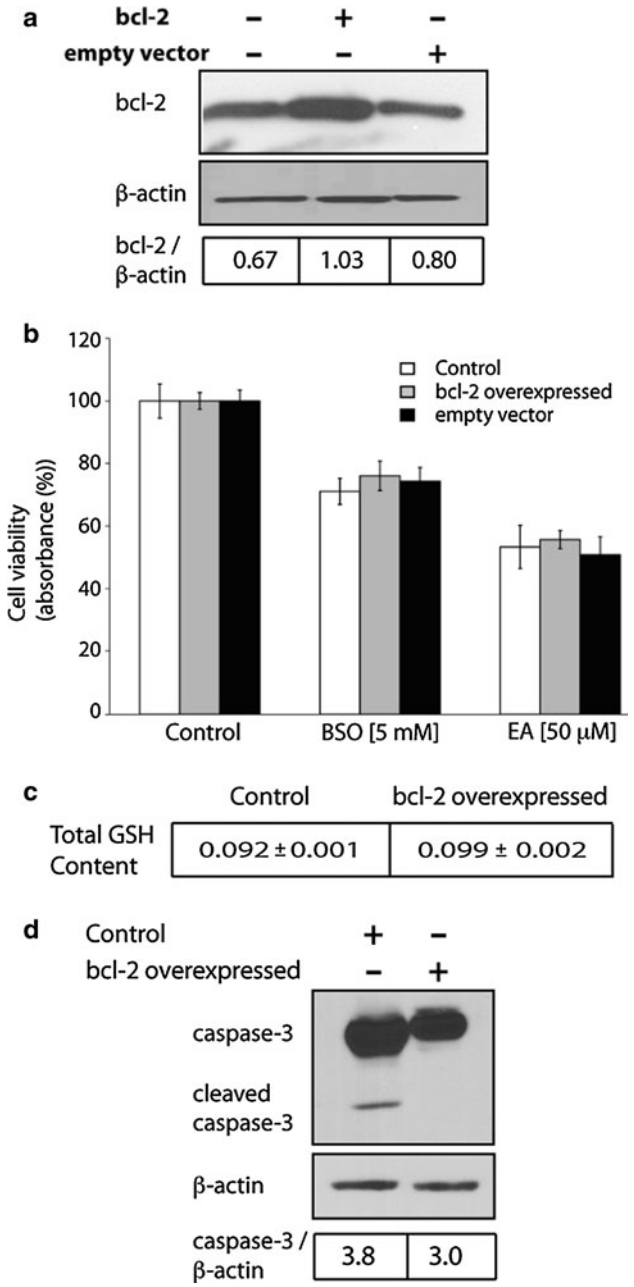


Fig. 7 Neuroprotective potential of bcl-2 overexpression against glutathione depletion by BSO and EA. **a** Immunoblotting with monoclonal bcl-2 antibody was used to confirm bcl-2 overexpression in SK-N-SH cells. Antibody against β-actin was utilized as internal control, **b** SK-N-SH cells (wild type), bcl-2 overexpressed SK-N-SH cells, and empty vector expressed SK-N-SH cells were subjected to treatment with BSO or EA for 24 h after which their cellular viability was measured. **c** Total GSH content was measured in SK-N-SH cells (control) and bcl-2 overexpressing SK-N-SH cells. **d** Expression of the total and cleaved caspase-3 was obtained in SK-N-SH cells (control) and bcl-2 overexpressing SK-N-SH cells. All values are mean ± SEM of at least three different experiments analyzed by one-way ANOVA followed by Dunnett's post hoc test or Student's *t* test

Discussion

Our study underlines the importance of mitochondrial GSH (mtGSH) in oxidative stress- and apoptosis-mediated death of neuron-like SK-N-SH cells and shows that U-87 (glioblastoma) cells in co-culture can protect from GSH-depletion induced neuronal death. We utilized ethacrynic acid (EA) to deplete mtGSH and L-buthionine-(S, R)-sulfoximine (BSO) that depletes only cytosolic GSH pool (Fig. 2, [7]). The comparison of effects of these two inhibitors on death of SK-N-SH cells allowed us to assess the link between mtGSH, oxidative stress and mitochondria-mediated apoptosis. Treatment with BSO or EA decreased the viability of SK-N-SH cells in a dose- and time-dependant manner (Fig. 1). EA treatment decreased mitochondrial and cytosolic GSH but BSO treatment only decreased cytosolic GSH (Fig. 2). Unlike BSO, EA treatment increased cellular ROS levels, decreased complex-I activity and resulted in a large number of annexin V/PI positive cells (Figs. 2, 3). These data, combined with their dissimilar impact on expression of bcl-2/bax and cytochrome c, demonstrated the regulatory role of mtGSH and its depletion in cell death.

A key finding from our study that U-87 glioblastoma cells protect neuron-like SK-N-SH cells against GSH depletion induced by EA (Figs. 5, 6) and our results suggest that decreasing ROS generation (Fig. 6d) and restoring mitochondrial function could potentially be the key mechanisms underlying the neuroprotection confered by U87 cells. Increasing evidence suggests that astrocytes and neurons interact extensively in neural function as seen in GABA-glutamate cycling, ammonia detoxification, supply of energy substrates and trophic factors, synaptic modulation and K^+ redistribution [27]. Emerging evidence further suggests that astrocytes may protect neurons against oxidative stress insults and the probable underlying mechanism includes glutathione cycling between neurons and glial cells. Astrocytes may also provide neurons with glutathione intermediates [15, 28]. Similarly, primary culture of astrocytes protected neurons against ischemic conditions through delivering GSH to neurons [29].

Generation of ROS by mt-electron transport chain (ETC), impairment of mitochondrial structures including mtDNA and decrease in the function of ETC, especially complex-I, is implicated in neurodegenerative disorders such as PD and AD [30]. We employed rotenone to inhibit complex-I of ETC. Similar to rotenone treatment, EA reduced the activity of complex-I and increased ROS levels whereas BSO treatment increased complex-I activity and did not increase ROS. This finding, combined with dissimilar impact of EA and BSO

treatments on mtGSH, suggests a possible correlation between the status of mtGSH and ETC activity. Next, we measured expression of bcl-2, bax and cytochrome c to assess the effect of GSH depletion on mitochondria-mediated apoptosis; a process that often involves mitochondrial outer membrane permeabilization (MOMP) [31]. Bcl-2 family proteins that include anti-apoptotic bcl-2, bcl-X_L and pro-apoptotic bax, bad proteins regulate MOMP [32, 33]. Once MOMP occurs, cytochrome c is released from mitochondria into cytosol where it associates with APAF-1 and procaspase-9 to form an apoptosome which executes apoptosis by activating a caspase cascade [34]. In addition, BSO and EA both reduced the expression of bcl-2 and cytochrome c in mitochondria and increased the level of bcl-2 and cytochrome c in cytosolic fractions. These data confirms the presence of apoptotic process. For bax expression, the results are difficult to interpret; a decrease in cytosol was observed only for BSO 2.5 mM and EA 75 μM. We did not observe increase in the mitochondrial expression of bax as expected from the literature [32, 33]. However, some other studies have also observed no changes in bax expression despite presence of apoptotic process [35]. Taken together, our results appear to point to a potential connection between mtGSH depletion and its regulation of mt-mediated apoptosis.

We examined the effects of two other protective strategies on survival of SK-N-SH cells. We chose glutathione peroxidase mimetic ebselen as it neutralizes peroxide species with the help of reduced GSH [19]. In addition, we overexpressed anti-apoptotic protein bcl-2 to protect SK-N-SH cells from GSH depletion. That these two strategies did not provide adequate protection for SK-N-SH cells against GSH depletion suggests that a preventive approach rather than restorative approach is needed to confer full neuroprotection.

Glutathione depletion-induced oxidative stress can cause mitochondrial dysfunction and abnormal mitochondrial morphology [36–39]. In addition, we have also shown that depletion of mitochondrial DNA results in decrease in GSH and alterations of antioxidant enzymes [40]. Moreover, GSH depletion and resultant oxidative stress and apoptosis has been studied [41–43]. Wullner et al. [44], however, indicated that EA-induced GSH depletion resulted in necrotic changes and cell death in cerebellar granule neurons. Our study indicates that mtGSH depletion leads to mitochondrial dysfunction and mt-mediated apoptosis.

We have previously shown that glutathione depletion by BSO increases manganese neurotoxicity in SK-N-SH and U-87 cells and demonstrated that SK-N-SH and U-87 cells are good cell models in vitro for mechanistic studies implicating GSH depletion [45]. Our novel study demonstrates occurrence of mt-mediated apoptosis in GSH depletion and also underlines the importance of mtGSH pool in this process. We have demonstrated that glioblastoma cells could protect neuroblastoma cells against GSH depletion. Consequently, neuroprotective factors secreted by astrocytes could be tested in primary cultures as potential candidates for rescuing neurons undergoing oxidative stress and degeneration. As such, our results may have implications in the betterment of pharmacotherapy for neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease in which GSH depletion is observed.

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