

Glutathione depletion causes a JNK and p38^{MAPK}-mediated increase in expression of cystathionine-γ-lyase and upregulation of the transsulfuration pathway in C6 glioma cells

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Abbreviations: CTH, cystathionine-γ-lyase; DA-64, N-[carboxymethylamino] 4,4'-bis [dimethylamino]-diphenylamine; DEM, diethylmaleate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione (γ-glutamylcysteinylglycine); GSH-MCB, glutathione-monochlorobimane; GST, glutathione-S-transferase; JNK, c-Jun-N-terminal kinase; L-αAA, L-α-amino adipate; L-BOAA, L-β-N-oxalylamino-L-alanine; MAPK, mitogen-activated protein kinase; MCB, monochlorobimane; MKK4, mitogen-activated protein kinase kinase 4; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; NAC, N-acetylcysteine; NDA, naphthalenedicarboxaldehyde; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; PPG, propargylglycine; RIPA, radioimmunoprecipitation assay; SAPK, stress activated protein kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; SDS, sodium dodecyl sulphate; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one; TNF, tumour necrosis factor.

Abstract

Cancer cells have a high demand for cysteine as precursor of the antioxidant, glutathione, that is required to promote cell growth and division. Uptake of cystine by the x_c^- cystine-glutamate exchanger provides the majority of cysteine, but a significant percentage may be derived from methionine, via a transsulfuration pathway. Our aim was to evaluate the relative contribution of the exchanger and the transsulfuration pathway to glutathione synthesis in astrocytoma/glioblastoma cells, using the C6 glioma cell line as a model system.

Blockade of the x_c^- exchanger with the gliotoxins L- α -amino adipate or L- β -N-oxalylamino-L-alanine (400 μ M) caused a loss of cellular cysteine and depletion in glutathione to 51% and 54% of control, respectively, after 24 h. Inhibition of the transsulfuration pathway with propargylglycine (1 mM, 24 h) depleted glutathione to 77% of control. Co-incubation of cells with gliotoxin and propargylglycine reduced glutathione to 39 % of control at 24 h and to 20 % at 48 h. Expression of cystathionine- γ -lyase, the rate-limiting enzyme of the transsulfuration pathway, was significantly increased following incubation of the cells with gliotoxins.

Incubation of C6 cells with diethylmaleate for 3 h led to a significant reduction in glutathione (63%), whereas expression of cystathionine- γ -lyase was increased by 1.5-fold. Re-feeding methionine to diethylmaleate-treated cells incubated in the absence of cystine or methionine resulted in a significant recovery in glutathione that was blocked by propargylglycine. Co-incubation of C6 cells with diethylmaleate and the JNK-inhibitor, SP600125, abolished the increase in expression of cystathionine- γ -lyase that had been observed in the presence of diethylmaleate alone. Similar results were obtained with the p38^{MAPK} inhibitor, SB203580.

It is concluded that glutathione depletion causes a JNK- and p38^{MAPK}-mediated increase in expression of cystathionine- γ -lyase that promotes flux through the transsulfuration pathway to compensate for loss of glutathione in C6 glioma cells.

Running title: transsulfuration and glutathione in C6 cells

Key words: cystine-glutamate exchanger, gliotoxin, astrocyte, transsulfuration, cystathionine- γ -lyase, glutathione

1. Introductory Statement

The amino acid cysteine is the rate-limiting precursor for synthesis of the major cellular antioxidant, glutathione (γ -glutamylcysteinylglycine; GSH). In the brain, *de novo* synthesis of GSH via the γ -glutamyl cycle takes place primarily in astrocytes, which then release GSH for export to neurones (Dringen et al., 1999; Wang and Cynader, 2000). Thus, the capacity of neurones for antioxidant defence requires a continuous supply of GSH from astrocytes, which relies on the availability of cysteine.

A large body of evidence has contributed to the view that in astrocytes, the x_c^- cystine-glutamate exchanger is the principal mechanism of ensuring sufficient cytosolic cysteine to provide for GSH synthesis (Kato et al., 1992; Sagara et al., 1993a; Koyama et al., 2000; McBean, 2002). However, recent demonstrations of an active transsulfuration pathway in astrocytes has raised the possibility that a significant proportion of cysteine required for GSH originates as methionine that is converted to cystathionine, which is then hydrolysed to cysteine and α -ketobutyrate (Vitvitsky et al., 2006; Diwakar and Ravindranath, 2007). The regulatory enzymes of this pathway are two pyridoxal-phosphate dependent enzymes, cystathionine- β -synthase (L-serine hydro-lyase EC 4.2.1.22) and cystathionine- γ -lyase (L-cystathionine cysteine-lyase EC 4.4.1.1). Cystathionine- β -synthase is widely expressed in the brain, where it has been localised to astrocytes (Lee et al., 2009), whereas expression of cystathionine- γ -lyase is much lower and limits the flux through this pathway in astrocytes.

The identification of an active transsulfuration pathway in astrocytes has led to a re-evaluation of the x_c^- cystine-glutamate exchanger as the exclusive provider of cysteine for GSH synthesis. For example, new evidence suggests that up-regulation of the exchanger in astrocytes protects against oxidative stress by driving a highly efficient cystine/cysteine cycle that exports cysteine, thus creating a reducing extracellular environment that effectively bypasses the requirement for GSH release (Banjac et al., 2008). If up-regulation of the x_c^- exchanger channels cystine into the cell for release as cysteine in preference to synthesis of GSH, then an alternative source of cysteine, such as the transsulfuration pathway, may be required to maintain intracellular GSH during oxidative stress.

The aim of this study was to determine the relative contribution of the x_c^- exchanger and the transsulfuration pathway to GSH synthesis using both gliotoxin-mediated inhibition of the exchanger and direct inhibition of GSH with diethylmaleate (DEM). Cancer cells have a high demand for cysteine to provide GSH and drive cell growth and proliferation and we

have selected the C6 glioma cell line as a model system for astrocytoma/glioblastoma cells in which to study synthesis of glutathione.

L- α Aminoadipate (L- α AA), L- β -N-oxalylamino-L-alanine (L-BOAA) and L-homocysteate are substrates and/ or inhibitors of the x_c^- cystine-glutamate exchanger (Kato et al., 1993; Patel et al., 2004; Warren et al., 2004) and belong to a group of glutamate analogues that have been termed 'gliotoxins' (Bridges et al., 1992). We have previously used ^{13}C NMR spectroscopy to show that incubation of C6 glioma cells with a sub-toxic concentration of gliotoxins causes metabolic disruption and a decrease in glutathione production (Brennan et al., 2004). Here we show that incubation of cells with a sub-toxic concentration of gliotoxin leads to an increase in the contribution of the transsulfuration pathway to GSH synthesis. We show, for the first time, that expression of the key regulatory enzyme of this pathway, cystathionine- γ -lyase is enhanced following depletion of GSH by gliotoxins or by DEM. Furthermore, we provide evidence that increased expression of cystathionine- γ -lyase by DEM is dependent on activation of the c-Jun-N-terminal kinase (JNK) and p38^{MAPK} pathways in these cells.

2. Experimental procedures

2.1. Reagents

Polyclonal rabbit antibodies for determination of total and phosphorylated forms of MKK4, JNK1/2, c-Jun AKT, p44/42^{MAPK} and p38^{MAPK} were purchased from Cell Signalling, Hertfordshire, UK. Monoclonal antibodies for cystathionine-γ-lyase were purchased from Antibodies Online, Aachen, Germany and α-tubulin from Abcam, Cambridge, UK. L-αAminoadipate, propargylglycine, lipoic acid and N-acetylcysteine were obtained from Sigma-Aldrich, UK and L-BOAA from Lathyrus Technologies, Hyderabad, India. All other reagents and chemicals were purchased from Sigma-Aldrich or Riedel-de-Haën, Seelze, Germany.

2.2. Cell culture

C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) with 2 mM glutamine and supplemented with 10% foetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin at 37° C in a humidified atmosphere of 5% CO₂ and 95% air. Routine screening confirmed that the cultures were mycoplasma free. All experiments were performed on cells between passage 24 and 38.

2.3. Determination of cell viability

The MTT (3, [4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay was used to assess cell viability. Cells were seeded at approx 1x10⁴ cells/100 µl in a 96-well plate and treated with L-αAA or L-BOAA for 24 h at 1 µM-10 mM, as indicated in the text. Control cells were incubated in DMEM for the same time period. Cells were then incubated for a further 3 h at 37° C with fresh DMEM containing 0.5 mg/ml MTT. The medium was removed and 180 µl dimethylsulphoxide (DMSO; 99.5% v/v) was added to each well. The absorbance was measured at 570 nm with a reference filter at 660 nm. There was no evidence of cell death under the conditions used in these experiments (results not shown).

2.4. Fluorimetric measurement of GSH

C6 glioma cells were seeded at a density of 1 x 10⁶ cells/well in a 12-well plate and allowed to adhere for 24 h at 37°C. Intracellular GSH was determined using the sensitive fluorimetric method of Kamencic et al. (2000). Cells were washed with phosphate buffered saline (PBS) and incubated in fresh media containing glutathione-S-transferase (GST;

1U/ml) and monochlorobimane (MCB; 100 μ M) for 30 min at 37°C. The cells were then washed twice with PBS before addition of 500 μ l lysis buffer (0.05 M sodium pyrophosphate, 0.1% SDS, pH 7.4). A 450 μ l sample of the lysate was transferred to an Eppendorf tube containing 140 μ l perchloric acid (6% v/v) and centrifuged at 12000 x g for 10 min. The remaining 50 μ l of cell lysate was retained for quantification of total protein by the Bradford method. The specific fluorescence of the GSH-MCB adduct was read on a fluorimeter at λ_{ex} 380 nm and λ_{em} 470 nm. Extracellular GSH was determined using DMEM (w/o phenol red) supplemented with 2 mM glutamine, 10% foetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Following treatment with gliotoxin, media was transferred to another 12-well plate and the cells were washed and incubated in fresh DMEM. The GSH content in the media and cells were determined using MCB and GST, as described above. The concentrations of intracellular and extracellular GSH were expressed as nmol GSH/mg protein.

Cell treatments: C6 glioma cells were treated with either L- α AA or L-BOAA at a subtoxic concentration of 400 μ M (Brennan et al. 2003) for 2-48 h, unless otherwise stated. C6 glioma cells were also incubated with L- α AA or L-BOAA in the presence of 400 μ M N-acetylcysteine (NAC) or 30 μ M lipoic acid (both suppliers of cysteine) for 24 h or in the presence of 1 mM propargylglycine (PPG; inhibitor of cystathionine- γ -lyase) for 24 or 48 h. For the 48 h gliotoxin treatment, cells were treated with either L- α AA or L-BOAA for 24 h prior to the addition of 1 mM PPG. In another set of experiments, C6 glioma cells were pretreated for 3 h with 100 μ M DEM before replacement with fresh media containing 300 μ M methionine, 300 μ M methionine and 300 μ M cystine or 300 μ M methionine and 1 mM PPG.

2.5. Measurement of intracellular cysteine

C6 glioma cells were seeded at a density of 3×10^6 cells/ 75 ml flask and were grown until 70-80% confluent. The media was removed and the cells were treated with lipoic acid (30 μ M), NAC (400 μ M), L- α AA or L-BOAA (both 400 μ M) for 24 h at 37° C. The concentration of intracellular cysteine was measured according to the spectrophotometric method of Gaitonde (1967), with minor modifications, that gives comparable results to HPLC (Ohmori et al., 2001). Briefly, cells were washed twice with PBS before the addition of 500 μ l lysis buffer containing 0.1 mM L-dithiothreitol. A 450 μ l sample of the lysate was transferred to an Eppendorf tube containing 140 μ l perchloric acid (6% v/v) and centrifuged

at 12000 x g for 10 min. A 500 µl sample of this supernatant was transferred to another Eppendorf tube containing 500 µl 1 M glacial acetic acid, 200 µl 1 M HCl and 300 µl ninhydrin reagent (0.25 mg ninhydrin dissolved in 6 ml 1 M glacial acetic acid). The remaining 50 µl of cell lysate was retained for quantification of total protein. The pH was adjusted to between 0.3-0.9 with 1 M HCl. This reaction mixture was mixed thoroughly and heated in boiling water (100° C) for 10 min to develop the pink product. The mixture was then rapidly cooled in cold water (4° C). This pink product was stabilized by diluting the reaction mixture to 1 ml with 95% ethanol and the absorbance was read at 560 nm. A reagent blank without cysteine was prepared under the same conditions. The intracellular cysteine concentration was calculated from the standard curve (1 nM-10 µM cysteine) and expressed as nmol cysteine/mg protein.

2.6. Activity of cystathionine-γ-lyase

The activity of cystathionine-γ-lyase activity was determined by a sensitive method developed by Ogasawara et al. (2002) with minor modifications. Briefly, C6 glioma cells were grown on 10 cm² petri dishes. For the preincubation studies, the cells were treated with L-αAA, L-BOAA (both 400 µM) or PPG (1 mM) for 24 h at 37°C. The direct effect of the gliotoxins was assessed by adding L-αAA, L-BOAA or PPG (all 6.5 mM) to the reaction mixture (in mM: Tris-phosphate buffer pH 8.0 containing ethylenediaminetetraacetic acid (EDTA) 2.35, pyridoxal 5'-phosphate 0.035 and β-chloro-L-alanine 13.7). The cells were pelleted by centrifugation, sonicated (three 5 sec bursts, on ice) and centrifuged at 13000 x g for 5 min at 4° C. The liver was quickly removed from a 9 month old mouse, rinsed with ice-cold 0.9% NaCl, placed on ice and then homogenized (1 g / 5 ml) in 20 mM PBS (pH 7.4) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. Homogenates were centrifuged at 12,000 x g for 20 min at 4° C and stored at -80° C until use. This homogenate was diluted as required with 100 mM phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and 1 mM EDTA, before assay. This method utilizes colourimetry for the determination of pyruvate produced from β-chloro-L-alanine, catalysed by cystathionine-γ-lyase. Pyruvate formation was measured in C6 glioma cells (66 µg) and in liver (11 µg) for 25 min. The reaction was terminated by the addition of 4,4-dithiopyridine. The pyruvate was oxidized by pyruvate oxidase in the presence of thiamine pyrophosphate and Mg²⁺ to liberate hydrogen peroxide. A leuco dye, DA-64 (N-[carboxymethylamino] 4,4'-bis [dimethylamino]-diphenylamine), is oxidized by hydrogen peroxide with peroxidase to produce

Bindschelder's Green. The absorbance of green dye (727 nm) was measured using a Hitachi U-2000 spectrophotometer. The sample blank was similarly prepared except that the sample was added to the substrate mixture after the addition of 4,4-dithiopyridine. The cystathionine- γ -lyase specific activity in each sample was expressed as the absorbance at 727 nm (corrected against the blank)/ μ g protein. Cystathionine- γ -lyase activity was detected in C6 glioma cells and found to be 13% of the corresponding activity measured in the mouse liver (data not shown).

2.7. Western blot analysis of cystathionine- γ -lyase expression

C6 glioma cells were seeded at a density of 2×10^6 cells/10 cm² Petri dish. When 70-80% confluent, the media was removed and the cells were treated with L-BOAA or L- α AA (both 400 μ M) for 24 h. In a separate set of experiments, cells were treated with 500 μ M L-homocysteate, 20 μ M H₂O₂ or with both L-homocysteate + H₂O₂ for 24 h. C6 glioma cells were also treated with 100 μ M DEM, in the absence and presence of 10 μ M SP600125 (JNK1/2 inhibitor) and SB203580 (p38^{MAPK} inhibitor). Plates were then rinsed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (in mM: Tris-HCl 100 pH 7.4, NaCl 150, EDTA 1, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and protease inhibitors) for 40 min at 4 °C. Samples were centrifuged at 14,000 x g for 30 min at 4 °C. For the preparation of the tissue samples, liver obtained from a 9 month old mouse and brain from a 17 week old rat was rinsed with ice-cold 0.9% NaCl. The tissue was homogenised in ice-cold radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 16000 x g for 30 min at 4 °C. The supernatant was diluted with RIPA buffer to obtain a protein conc. of 1 μ g/ μ l and further centrifuged at 13,000 x g for 20 min at 4 °C. The supernatants were quickly frozen in liquid nitrogen and stored at -80 °C until use. The protein was determined using the Bradford assay and samples were boiled (2 min for cell lysate and 5 min for tissue homogenate) in equal volumes of 4X Laemmli buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 4% 2-mercaptoethanol). Samples of mouse liver (4 μ g protein/lane), rat brain (10 μ g protein/lane) and C6 glioma cells (12 μ g protein /lane) were then separated on a 10 % SDS PAGE gel and transferred onto a nitrocellulose membrane. Blots were incubated with cystathionine- γ -lyase monoclonal antibody (1:500) and α -tubulin (1:1000) overnight at 4 °C. The membrane was incubated with HRP-conjugated anti-mouse IgG (1:5000) for 1 h and the bands were visualised with enhanced chemiluminescence (Western Lighting ECL plus; PerkinElmer, Ireland). The density of bands detected for the cystathionine- γ -lyase protein in the cell

samples were quantified by densitometry analysis using GeneTools and the levels of expression was normalised to the corresponding α -tubulin detected. Cystathionine- γ -lyase expression in the treated group was represented as % of enzyme expression in the untreated group.

The activation of SAPK/JNK pathway by DEM was determined by detecting the total and phosphorylated forms of MKK4, JNK1/2 and c-Jun. Briefly, C6 glioma cells treated for 3 h with 100 μ M DEM and then lysed in RIPA Buffer containing protease inhibitors, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 1 mM sodium orthovanadate. The blots were probed with rabbit phospho-antibodies (1:1000) specific to MKK4 (Thr261), JNK1/2 (Thr183/Tyr185) and c-Jun (Ser63). The total forms of MKK4, JNK1/2 and c-Jun were determined by stripping the blot and re-probing membrane with respective total antibody (1:1000). Similarly, p38^{MAPK}, p42/p44^{MAPK} and AKT activated forms were also assessed using phospho-specific antibodies for p-AKT (Ser473), p44/42^{MAPK} (Thr202/Tyr204) and p38^{MAPK} (Thr180/Tyr182). The blot was stripped and the total forms of p38^{MAPK}, p42/p44^{MAPK} and AKT were determined.

2.8. Statistical analysis

The results are expressed as mean \pm S.E.M. of at least 4 independent experiments measured in triplicate. The difference between the treated and untreated groups (control) was determined by one-way ANOVA (Newman-Keuls post-hoc test) or two-way ANOVA (Bonferroni post-hoc test) as required and the difference between the means was considered significant at *P*-values of less than 0.05.

3. Results

3.1. Measurement of cysteine content in C6 glioma cells treated with L- α AA and L-BOAA

C6 glioma cells were treated with L- α AA or L-BOAA (both at 400 μ M) for 24 h. The cells were also incubated with either lipoic acid (30 μ M) or NAC (400 μ M) for the same time period. The cysteine content in untreated C6 glioma cells was 6.8 ± 0.5 nmol/mg protein. As positive controls, both lipoic acid and N-acetylcysteine significantly increased intracellular cysteine by 35% and 28%, respectively ($P < 0.05$). In contrast, incubation of the cells with L- α AA or L-BOAA significantly decreased the cysteine content in C6 glioma cells by 66% and 74%, respectively ($P < 0.001$; Fig. 1).

3.2. Depletion in cellular GSH by L- α AA and L-BOAA is increased by inhibition of cystathionine- γ lyase

C6 glioma cells were treated with L- α AA or L-BOAA for 24 h or 48 h, in the absence and presence of 1 mM PPG. For the 48 h gliotoxin treatment, PPG was added for the second 24 h period. Incubation of cells with 1 mM PPG alone caused a significant reduction in GSH to 77 % of control ($P < 0.05$). Incubation of the cells with L- α AA or L-BOAA alone for 24 h caused a significant reduction in GSH to 51% and 54% of control, respectively. Incubation of the cells with either gliotoxin for 48 h resulted in no further decrease in the concentration of GSH in the cells (Fig. 2).

After 24 h co-incubation of the cells with both L- α AA and PPG, the GSH concentration was 44 % of control, whereas on co-incubation with L-BOAA and PPG, GSH fell to 38% of control, which is significantly lower than the content of GSH following 24 h incubation with L-BOAA alone. At 48 h incubation with L-BOAA, the effect of PPG was even more pronounced: cellular GSH was 20% of control, which is significantly lower than the concentration with L-BOAA alone (50% of control; $P < 0.01$). A similar reduction in GSH was observed in cells treated with L- α AA and PPG for 48 h (24% of control; $P < 0.01$; Fig. 2), compared to cells treated with L- α AA alone.

3.3. L- α AA or L-BOAA has no effect on cystathionine- γ lyase activity in C6 glioma cells

Cystathionine- γ lyase activity was measured in C6 glioma cells and mouse liver (results not shown) for 25 min. Neither L- α AA nor L-BOAA had any effect on the activity of the

enzyme (Fig. 3). Similarly, pretreatment of the cells for 24 h with 400 μ M L- α AA or L-BOAA had no effect on enzyme activity (data not shown). 1 mM PPG inhibited cystathionine- γ -lyase activity directly (to 43% of control, $P < 0.01$). 24 h treatment of C6 cells with 1 mM PPG resulted in a significant reduction (to 59% of control) in enzyme activity (data not shown).

3.4. Cystathionine- γ -lyase expression in C6 glioma cells, in the absence and presence of L- α AA and L-BOAA

The expression of cystathionine- γ -lyase was examined in mouse liver and rat brain tissue homogenates and in C6 glioma cell lysate by Western blot analysis, using a monoclonal anti-cystathionine- γ -lyase antibody. A single band of molecular weight of approximately 43 kDa was detected for this protein in all three samples (Fig. 4A). A greater expression of cystathionine- γ -lyase was found in the mouse liver compared to the C6 glioma cells, which is consistent with the difference in cystathionine- γ -lyase activity observed between the samples (data not shown). Cells were also treated with L- α AA or L-BOAA (both 400 μ M) and expression of cystathionine- γ -lyase was determined. There was no change in expression of the enzyme following 24 h incubation with either gliotoxin (Fig. 4B). In contrast, after a 48 h there was a significant increase in cystathionine- γ -lyase expression by 100% and 98%, respectively (Fig. 4C, $P < 0.001$).

3.5. Cystathionine- γ -lyase expression in C6 glioma cells following inhibition of the x_c^- exchanger in the absence and presence of H_2O_2

Expression of cystathionine- γ -lyase was determined in C6 glioma cells following incubation with 500 μ M L-homocysteate, 20 μ M H_2O_2 or with both homocysteate and H_2O_2 for 24 h. There was no change in protein expression following treatment with H_2O_2 . However, cystathionine- γ -lyase expression was significantly increased in cells treated with L-homocysteate (by 40%, $P < 0.01$ compared to untreated group) and with both homocysteate and H_2O_2 (by 62%; $P < 0.001$, compared to untreated group, Fig. 5).

3.6. Diethylmaleate increases expression of cystathionine- γ -lyase after 3 h, leading to increased production of GSH at 24 h

C6 glioma cells were treated for 3 h with 100 μ M DEM, after which time the intracellular concentration of GSH and level of expression of cystathionine- γ -lyase was

determined. Treatment of the cells with DEM reduced the cellular concentration of GSH to 63% of control ($P < 0.001$; Table 1), whereas the level of expression of cystathionine- γ -lyase was increased by almost 1.5-fold (Fig. 6). In a separate set of experiments, cells were pre-incubated with 100 μ M DEM for 3 h, after which the media was replaced with fresh media containing 300 μ M methionine with or without 300 μ M cystine or 1 mM PPG, respectively. Untreated cells were incubated for the same time period. Re-incubation of the cells with methionine and cystine increased cellular GSH to 135% of control ($P < 0.05$; Table 1). Addition of 300 μ M methionine following the treatment of cells with DEM increased the GSH content to 65% of control. The increase in GSH in the presence of methionine was abolished by co-incubation of the cells with PPG (Table 1).

3.7. Incubation of C6 glioma cells with diethylmaleate increases expression of cystathionine- γ -lyase via activation of JNK

Incubation of cells with 100 μ M DEM for 3 h caused an increase in the activation of JNK (Fig. 7A) after 1 h and was blocked by co-incubation of the cells with the JNK inhibitor, SP600125 (10 μ M; results not shown). Co-incubation of C6 cells with DEM and SP600125 for 3 h abolished the increase in expression of cystathionine- γ -lyase that had been observed in the presence of DEM alone (Fig. 7B).

Other components of the pathway, MKK4 and c-Jun, showed similar patterns of phosphorylation following treatment with DEM (Fig. 8).

3.8. Activation of p38^{MAPK} and p42/p44^{MAPK} by diethylmaleate

C6 glioma cells were treated with 100 μ M DEM for 0-180 min and the total and phosphorylated (activated) forms of p38^{MAPK} and p42/p44^{MAPK} were determined. This was to establish whether or not the activation of MAPKs by DEM was restricted to the JNK/SAPK pathway. DEM had no effect on p42/p44^{MAPK} phosphorylation (Fig. 10A). In contrast, exposure of the cells to DEM resulted in a rapid phosphorylation of p38^{MAPK} after 5min which reached maximal levels at 1 h (Fig. 9A). Inhibition of p38^{MAPK} by SB203580 (10 μ M) reduced the DEM-mediated increase in cystathionine- γ -lyase expression by 32 % ($P < 0.01$, Fig. 9B).

3.9. Diethylmaleate has no effect on the AKT pathway in C6 glioma cells

Total and phosphorylated forms of AKT were assessed in C6 glioma cells following incubation of the cells with 100 μ M DEM for 0-180 min. The phosphorylation levels of AKT remained unchanged in the cells treated with DEM compared to the untreated group (Fig.10B).

4. Discussion

Glial cells require cysteine as a precursor for γ -glutamate cysteine ligase, catalyst of the rate-limiting step in the γ -glutamyl cycle leading to GSH formation. In astrocytes and glioblastoma /astrocytoma cells, the majority of cysteine originates from reduction of cystine imported via the cystine-glutamate exchanger (Sagara et al., 1993b). The transsulfuration pathway, which supplies cysteine from methionine via homocysteine, has recently been identified as a minor contributor to GSH in astrocytes and in whole brain preparations under normal conditions (Vitvitsky et al., 2006; Diwakar and Ravindranath, 2007). Following from reports that expression of cystathionine- γ -lyase in brain is much lower than that of cystathionine- β -synthase and effectively limits flux through the pathway (Lee et al., 2009), our first experiments focussed on measurement of the activity and expression of this enzyme after incubation of C6 cells with gliotoxins. Our results extend the work of Vitvitsky et al. (2006) and we show, for the first time, direct measurement of the functional activity and expression of cystathionine- γ -lyase in C6 glioma cells. More significantly, we show that expression of this enzyme is enhanced when a decrease in intracellular cysteine causes depletion of GSH and that the relative contribution of the transsulfuration pathway to GSH is increased.

As expected from previous studies with L- α AA and L-BOAA (Kato et al., 1992; Warren et al., 2004), incubation of C6 glioma cells with either gliotoxin for 24 h or 48 h led to a significant reduction in the intracellular concentration of GSH that was mirrored by a 66% depletion in cellular cysteine. Our experiments (results not shown) confirmed that both gliotoxins caused significant inhibition of the x_c^- exchanger in C6 cells. Determination of cellular viability confirmed that the concentration of each toxin used in these experiments was 10-fold lower than its EC_{50} in C6 glioma cells (Brennan et al., 2003, 2004) and that the threshold for the decrease in GSH occurred between 8-10 h (unpublished observations). We rule out inhibition of the transsulfuration pathway by the gliotoxins in causing the observed decrease in intracellular cysteine, since neither gliotoxin had any direct effect on the activity of cystathionine- γ -lyase in C6 glioma cells. These results validate our use of gliotoxins as a tool for studying the contribution of the transsulfuration pathway to GSH synthesis in response to depletion of intracellular cysteine arising from blockade of the x_c^- exchanger. In the second phase of the study, we addressed the question of whether pre-treatment of the cells with DEM would also lead to increased expression of cystathionine- γ -lyase. DEM

causes direct depletion of reduced GSH (Bizzozero et al., 2006) independently of the concentration of cysteine and enabled us to distinguish between lowered intracellular cysteine or GSH as the trigger to increase expression of cystathionine- γ -lyase. The outcome was the same, regardless of whether the drop in GSH was a result of gliotoxins or DEM and we conclude that transsulfuration provides a compensatory mechanism that is upregulated in response to depletion of GSH or oxidative stress in C6 cells.

As already stated, the level of expression of cystathionine- γ -lyase in C6 glioma cells and astrocytes is low compared to hepatocytes, yet from our results with PPG, the transsulfuration pathway normally accounts for approximately 25% of the total GSH concentration in C6 cells, thus providing a minor, yet significant, quantity of cysteine for GSH synthesis. Importantly, there was a significant increase in expression of the enzyme after 48 h incubation of the cells with either L- α AA or L-BOAA. Accordingly, the contribution of the transsulfuration pathway at this time point rose to 45% of total GSH, as illustrated by the fact that PPG caused a significantly greater reduction in residual GSH at 48 h, compared to 24 h, incubation with either gliotoxin. Similarly, blockade of the x_c^- exchanger with L-homocysteate showed an increase in the expression of cystathionine- γ -lyase after 24 h, which achieved greater significance when the cells were exposed to oxidative stress (in the form of H₂O₂) in addition to inhibition of the exchanger.

Incubation of C6 cells with a low concentration of DEM to deplete GSH, followed by re-feeding methionine did not fully restore GSH to control values, but brought it back to a concentration equal to 50% of the level obtained by re-feeding both methionine and cystine (Table 1). This is another illustration of the capacity for upregulation of the transsulfuration pathway in these cells. Notably, in the presence of both methionine and cystine, the concentration of GSH after 24 h exceeded the resting level by 36%. It is not clear what the reason for this is. One possibility may be increased expression of the x_c^- exchanger following DEM treatment (Bannai, 1984; Ruiz et al., 2003), but, as recently demonstrated by Banjac et al. (2008) and discussed below, enhanced activity of the exchanger does not necessarily lead to elevated GSH. Furthermore, Kranich et al. (1996) performed experiments on re-feeding cystine and glutamate to amino-acid starved astrocytes and observed an overcompensation in GSH that could not be a reaction to DEM, since it was not used in their experiments.

In addressing the question of the mechanism of regulation of cystathionine- γ -lyase as a result of GSH depletion in C6 cells, our evidence points to the direct involvement of the JNK and p38^{MAPK} signalling pathways in this response. Incubation of the cells with DEM

caused a significant increase in phosphorylated JNK 1 and 2, cJun and MKK4 and the JNK inhibitor, SP600125 inhibited the stimulation in cystathionine- γ -lyase expression by DEM. The same result was obtained with the p38^{MAPK} inhibitor, SB203580, on expression of cystathionine- γ -lyase following DEM pre-treatment. P38^{MAPK} and c-Jun-N-terminal kinase (c-Jun) are two isoforms of mitogen activated protein kinases (MAPK) that are activated by dual phosphorylation on both a tyrosine and a threonine and become activated by environmental stresses, such as irradiation, DNA-damaging agents, heat shock and inflammatory cytokines (Kyriakas and Avruch, 1996). JNK phosphorylation takes place in the final step of the stress activated protein kinase (SAPK) pathway that occurs in many cells as a prelude to apoptosis. We propose that a depletion in GSH triggers activation of the both pathways, thus stimulating the transsulfuration pathway and channelling methionine towards production of cysteine. It is significant that glutathione depletion caused by mild oxidative stress in hepatocytes led to a sustained increase in p-c-Jun and p-JNK activity, thus sensitizing the cells to TNF-induced apoptosis (Matsumaru et al., 2003), and that moderate oxidative stress acts as a signal to up-regulate cystathionine- γ -lyase activity in foetal hepatocytes during the foetal-to-neonatal transition (Martín et al., 2007). However, this is the first time that a link between intracellular GSH, JNK and p38^{MAPK} activation and cystathionine- γ -lyase activity has been described, giving a new insight into the responsiveness of glioma cells to mild oxidative stress.

It is likely that depletion of intracellular thiols by DEM in C6 cells increases the generation of reactive oxygen species (Bannai, 1984), which may act as a trigger for initiation of apoptotic cell death pathways. However, it is evident that different cells activate alternative stress-activation pathways in response to oxidative stress. Aoshiba et al. (1999) detected activation of p38^{MAPK}, but not c-Jun in lung fibroblasts, following DEM-induced depletion of GSH. On the other hand, GSH depletion in human cancer cells was linked to death receptor 5 upregulation and apoptosis via the c-Jun pathway (Yue et al., 2006). In murine hepatocytes, DEM-mediated cytosolic GSH depletion led to sustained increase in activity of c-Jun and JNK (Matsumaru et al., 2003). GSH depletion by DEM was associated with inhibition of TNF-induced NF- κ B transactivation of anti-apoptotic genes (including inducible nitric oxide synthase). It was concluded that extramitochondrial GSH depletion alters the thiol redox state, leading to inhibition of NF- κ B transactivation of survival genes and to sustained activation of JNK, both of which contribute to the sensitisation of the cells

to TNF-induced apoptosis. Our future experiments will be directed towards identifying the genetic mechanism for upregulation of cystathionine- γ -lyase.

It has been reported that DEM causes increased expression of the xCT subunit of the x_c^- exchanger in human vascular smooth muscle cells that leads to a restoration of GSH by 24 h (Ruiz et al., 2003). Similarly, oxidative stress causes up-regulation of the cystine-glutamate exchanger in glial cells (Dun et al., 2006; Mysona et al., 2009). Both of these observations might argue against increasing flux through the transsulfuration pathway as a compensatory mechanism for augmenting GSH during oxidative stress. However, new evidence suggests that up-regulation of the exchanger in astrocytes drives a highly-efficient cystine/cysteine redox cycle that, through export of cysteine, creates a reducing extracellular environment that effectively by-passes the need to export GSH (Banjac et al., 2008). If that is the case, then it is likely that intracellular GSH requires an alternative source of cysteine, such as we propose would be provided by the transsulfuration pathway.

Threats to neuronal survival, such as an increase in extracellular glutamate, promote GSH release from astrocytes in a presumed bid to provide protection to neurones (Frade et al., 2008). Our results showing upregulation of the transsulfuration pathway following GSH depletion provide an insight into how, under such circumstances, the supply of cysteine for GSH is maintained. An increase in extracellular glutamate could out-compete cystine as a substrate for the x_c^- exchanger, whereas an increase in extracellular GSH would alter the redox balance in favour of cysteine. It is likely that both of these situations would damage normal uptake of cystine by the exchanger, to the extent that continued synthesis of GSH in astrocytes relies on the methionine cycle to provide cysteine by the transsulfuration pathway, in the face of dwindling x_c^- -mediated import of cystine.

Cancer cells have a high demand for GSH to promote cell growth and division. The requirement for cysteine is furnished via the x_c^- exchanger, which, as recorded in the case of gliomas, releases glutamate that damages surrounding neurones, thus providing space for tumour growth (Sontheimer, 2008). Consequently, blockade of the exchanger is viewed as a potential target for cancer therapy (Lo et al., 2008; Savaskan et al., 2008; Chung and Sontheimer, 2009) and has proven successful in experimental models as a means of limiting glutamate release. However, other recent evidence indicates that methionine uptake in gliomas is high and correlates positively with tumour viability (Kato et al., 2008), thus questioning the potential effectiveness of blockade of the x_c^- exchanger as a means of limiting GSH synthesis and tumour cell survival. If, as indicated by our results, the

transsulfuration pathway would be upregulated in response to therapeutic blockade of the x_c^- exchanger, this casts doubt on the effectiveness of a strategy that is aimed at limiting GSH production by this mechanism. Further work on investigating GSH-induced upregulation of this pathway in glioma cells would be central to understanding how GSH production could be effectively limited.

In summary, this work has provided, for the first time, direct evidence for the upregulation of the key regulatory enzyme of the transsulfuration pathway, cystathionine- γ -lyase, following blockade of the x_c^- exchanger and decreased GSH in C6 glioma cells. Whilst the contribution of this pathway to production of GSH is low under normal conditions, we propose that it functions as a compensatory pathway to ensure continued supply of cysteine for GSH synthesis during oxidative stress or when cystine import may be required to drive a cystine/cysteine redox cycle.

Acknowledgement

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Table 1.

Use of methionine in GSH synthesis

Treatment	GSH (nmol/mg protein)
Untreated (27 h)	41.1 ± 3.8
DEM (3 h)	15.1 ± 2.8
DEM (3 h) + Methionine + Cystine (24 h)	55.8 ± 5.8 *** #
DEM (3 h) + Methionine (24 h)	26.7 ± 3.5**
DEM (3 h) + Methionine + PPG (24 h)	10.0 ± 1.9

C6 glioma cells were treated with 100 μ M DEM for 3 h. The media was then replaced with fresh media containing methionine (300 μ M), methionine + cystine (both 300 μ M) or methionine (300 μ M) + PPG (1 mM) for 24 h (n = 4, ** P <0.01 and *** P <0.001 compared to group treated with DEM alone and # P <0.05 compared to untreated group, one-way ANOVA)

Figure legends

Fig. 1. The effect of NAC, lipoic acid, L- α AA and L-BOAA on the cysteine content in C6 glioma cells. Intracellular cysteine was measured following incubation of cells with lipoic acid (30 μ M) or NAC (400 μ M), L- α AA or L-BOAA (both 400 μ M) for 24 h at 37°C (n = 4, * P < 0.05 and *** P < 0.001 compared to untreated group, one-way ANOVA).

Fig. 2. Influence of PPG on gliotoxin-mediated depletion of GSH. C6 glioma cells were treated with L- α AA or L-BOAA (both 400 μ M) for 24 h and 48 h in the absence and presence of 1 mM PPG. For the 48 h gliotoxin treatment, PPG was added for the remaining 24 h of treatment. Control cells (untreated) were incubated in media alone (n = 4, * P < 0.05 and *** P < 0.001 compared to untreated group, one-way ANOVA; $^{\$}$ P < 0.05 and $^{\$}$ P < 0.01 compared using two-way ANOVA).

Fig. 3. Effect of L- α AA and L-BOAA on activity of cystathionine- γ -lyase (CTH). Activity of the enzyme was determined following incubation of cells for 25 min with L- α AA or L-BOAA (both at 6.5 mM). Positive control group with selective inhibitor, PPG, is also shown. Activity of cystathionine- γ -lyase is expressed as A_{727} nm/ μ g protein, respectively (n = 5, ** P < 0.01 compared to untreated group, one-way ANOVA).

Fig. 4. Expression of cystathionine- γ -lyase in C6 glioma cells in the absence or presence of L-BOAA and L- α AA. A. Western blot showing cystathionine- γ -lyase (CTH) expression in mouse liver (4 μ g; lane 1), rat brain (10 μ g; lane 2) and C6 glioma cells (12 μ g; lanes 3-5), using anti-cystathionine- γ -lyase monoclonal antibody. Expression of cystathionine- γ -lyase in C6 glioma cells after 24 h (B) and 48 h (C) incubation with 400 μ M L-BOAA and L- α AA. For each cell sample, 12 μ g protein was loaded per lane and cystathionine- γ -lyase expression was normalised to the corresponding α -tubulin detected. (i) Representative Western blot of cystathionine- γ -lyase and α -tubulin expression in each sample. (ii) Data are presented as a % of the CTH expression in the untreated group (n = 4, *** P < 0.001 compared to untreated group, one-way ANOVA).

Fig. 5. Expression of cystathionine- γ -lyase in the presence of L-homocysteate, H_2O_2 or H_2O_2 + L-homocysteate. C6 glioma cells were treated for 24 h with 500 μ M L-homocysteate, 20

$\mu\text{M H}_2\text{O}_2$ or in the presence of both and the cystathionine- γ -lyase (CTH) expression in each sample (12 $\mu\text{g/lane}$) was determined and quantified by densitometry. The levels of expression was normalised to the corresponding α -tubulin detected. (i) Representative Western blot of cystathionine- γ -lyase and α -tubulin expression in each sample. (ii) Data are presented as a % of the cystathionine- γ -lyase expression in the untreated group ($n = 4$, $**P<0.01$, $***P<0.001$ compared to untreated group, one-way ANOVA).

Fig. 6. Effect of DEM on cystathionine- γ -lyase expression. C6 glioma cells were treated for 3 h with 100 μM DEM and the level of cystathionine- γ -lyase (CTH) expression was measured by Western blot analysis and normalised to the corresponding α -tubulin detected. (i) Representative western blot of cystathionine- γ -lyase and α -tubulin expression in each sample. (ii) Data is presented as a % of the cystathionine- γ -lyase expression in the untreated group ($n = 6$, $***P<0.001$ compared to untreated group, one-way ANOVA).

Fig. 7. Role of JNK phosphorylation in DEM induced cystathionine- γ -lyase expression. C6 glioma cells were treated for 0-240 min with 100 μM DEM (A) or for 3 h with 100 μM DEM \pm 10 μM SP600125 (B). Following each treatment time, the total and phosphorylated (activated) forms of JNK 2 and JNK 1 were determined by Western blot analysis. (i) Representative blots of total and phosphorylated forms of JNK1/2. (ii) Level of phosphorylation of JNK1/2 in each group was normalised to the relative total JNK 1/2 expressed in the cell and data is represented as % of phospho-JNK1/2 in untreated group ($n = 3$, $*P<0.05$, $**P<0.01$ compared to equivalent untreated group, one-way ANOVA).

Fig. 8. Phosphorylation of MKK4 and c-Jun by DEM. C6 glioma cells were treated for 0-240 min with 100 μM DEM and the levels of total and phosphorylated MKK4 (A) and c-Jun (B) were determined after each time point. (i) Representative Western blot of total and phospho-MMK4 (A) and total and phospho-c-Jun (B). (ii) Data is represented as % of phospho-MMK4 (A) or phospho-c-Jun (B) in untreated group ($n = 3$, $*P<0.05$, $**P<0.01$ compared to equivalent untreated group, one-way ANOVA).

Fig. 9. Involvement of p38 in DEM mediated increase of cystathionine- γ -lyase expression. C6 glioma cells were treated for 0-180 min with 100 μM DEM (A) or for 3 h with DEM \pm 10 μM SB203580 (B) and the levels of total and phospho-p38^{MAPK} were detected. (i) Western

blot representative of levels of total and phospho-p38^{MAPK} detected for each group. (ii) phospho-p38^{MAPK} levels detected were normalised to the relative total p38^{MAPK} and expressed as % of phospho-p38^{MAPK} in untreated group (n = 3, **P*<0.05. ***P*<0.01 compared to untreated group and ^{##}*P*<0.01 compared between treated groups, one-way ANOVA).

Fig. 10. Effect of DEM on phosphorylation of p42/p44^{MAPK} and AKT. C6 glioma cells were treated for 0-180 min with 100 μ M DEM and the levels of total phospho-p42/44^{MAPK} (A) and AKT (B) were determined by Western blot analysis (i) Representative blot of total and phospho-p42/44 and AKT. (ii) Data is expressed as % of phospho-p42/44^{MAPK} (A) or phospho-AKT (B) in untreated group (n = 3, one-way ANOVA).

Figure 1

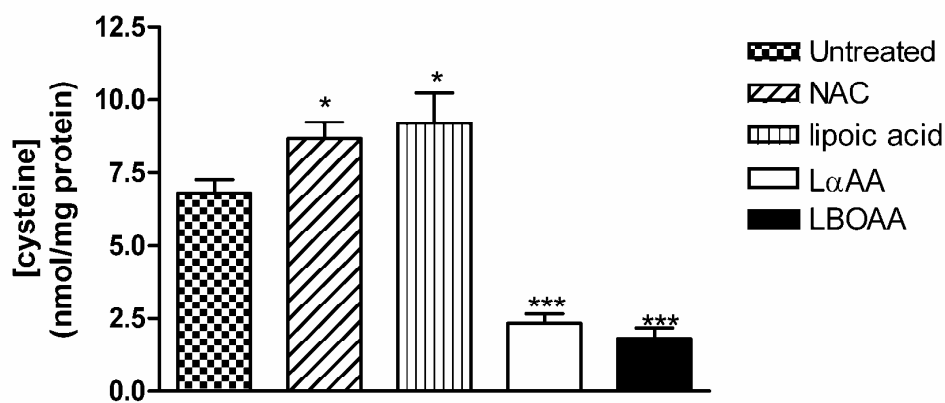


Figure 2

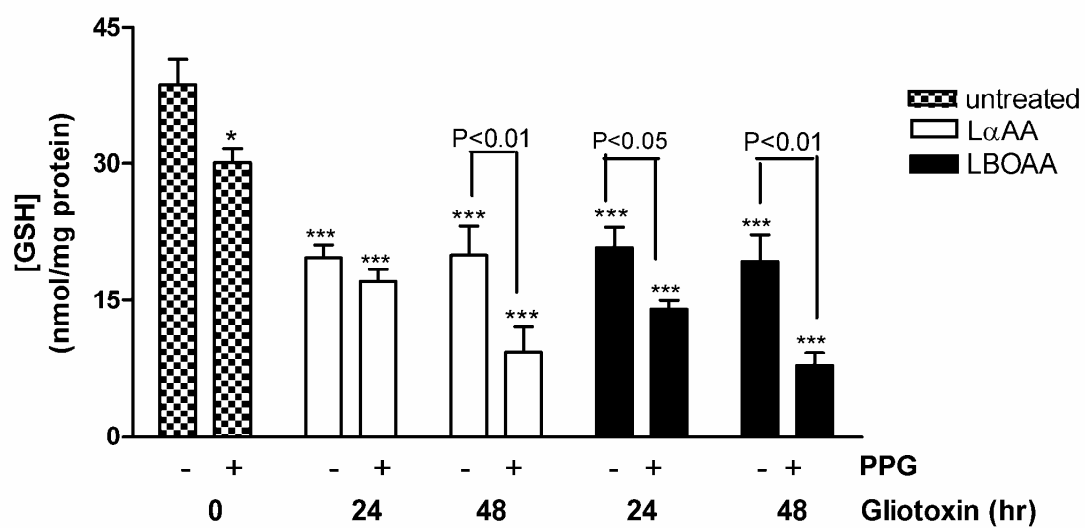


Figure 3

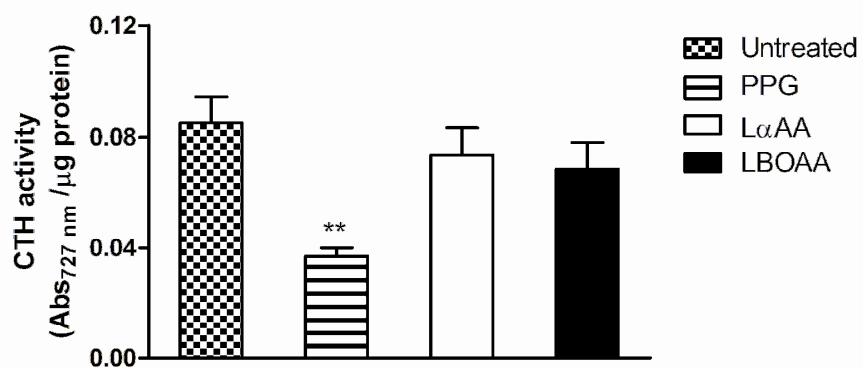
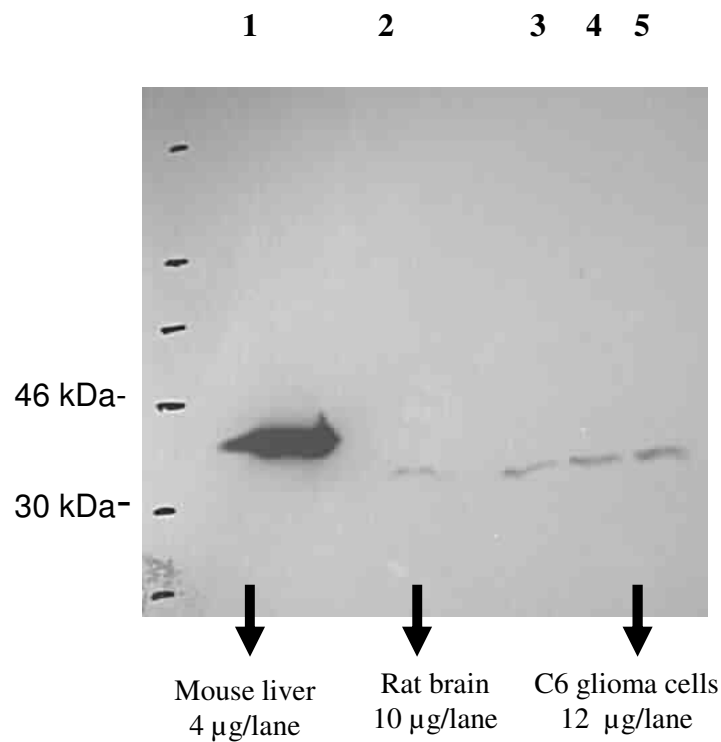
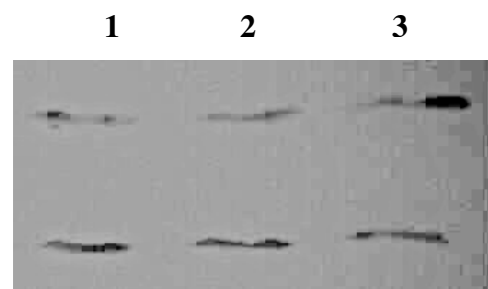


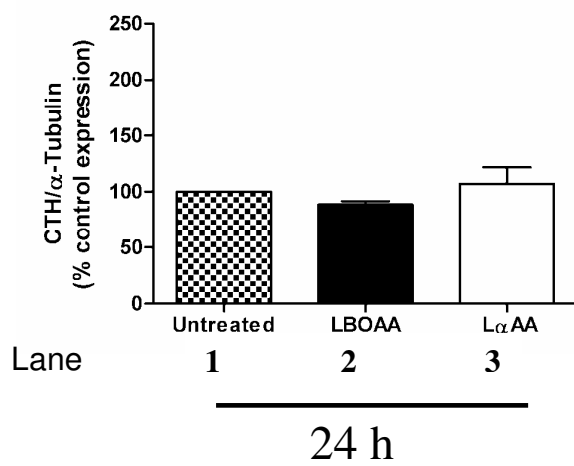
Figure 4
(A)



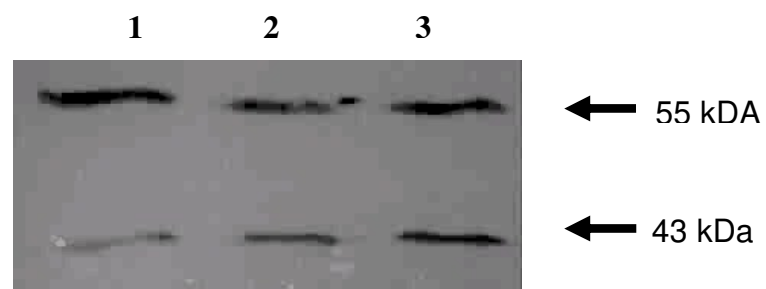
(B) i



ii



(C) i



ii

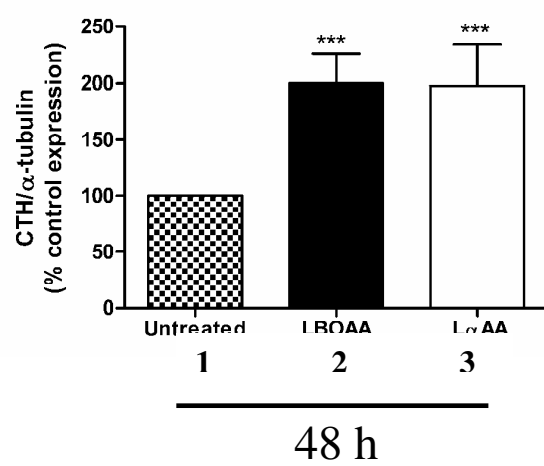
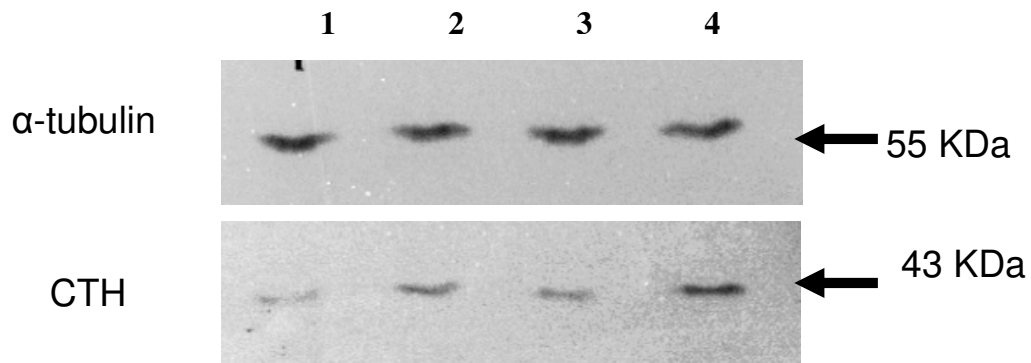


Figure 5

i



ii

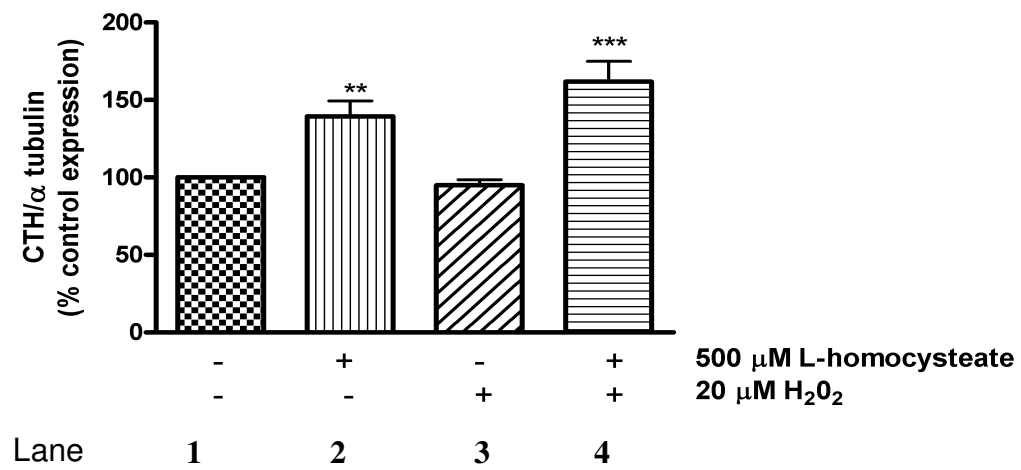


Figure 6

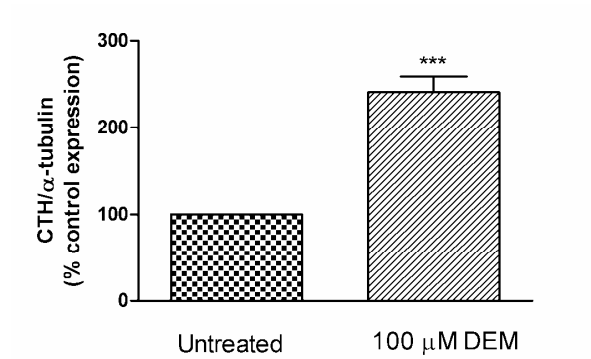
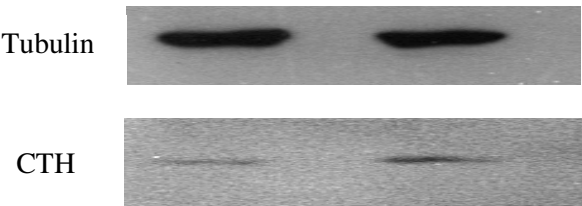
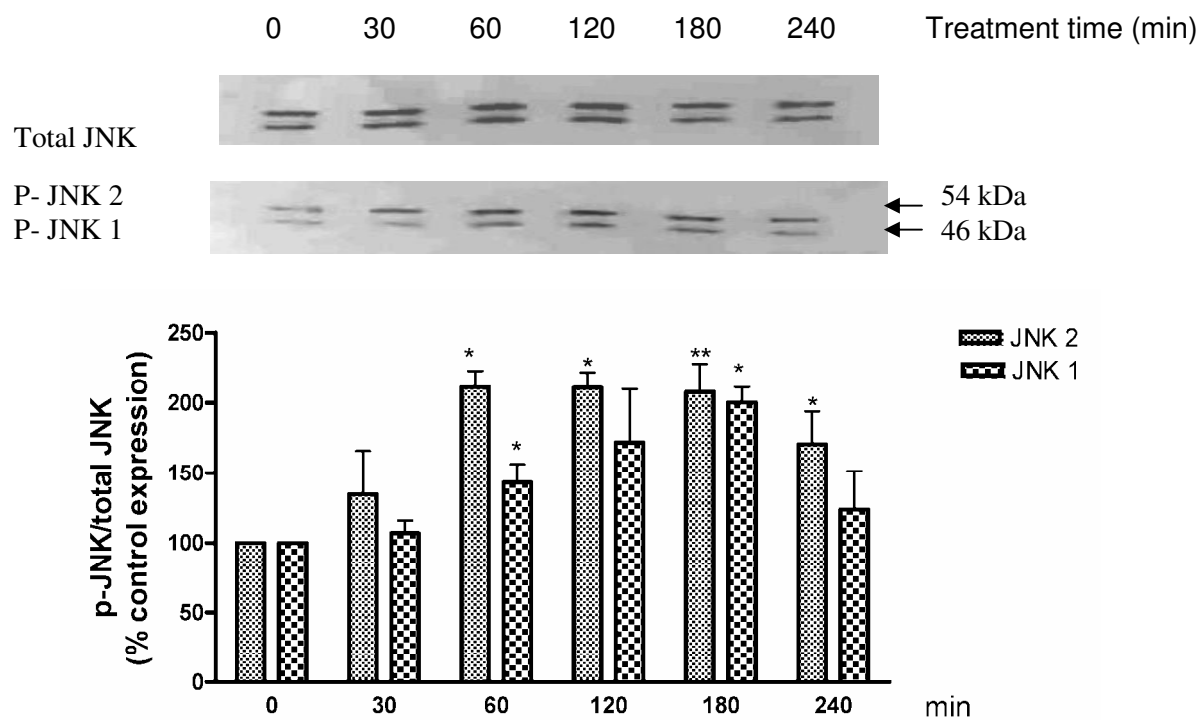


Figure 7
(A)



(B)

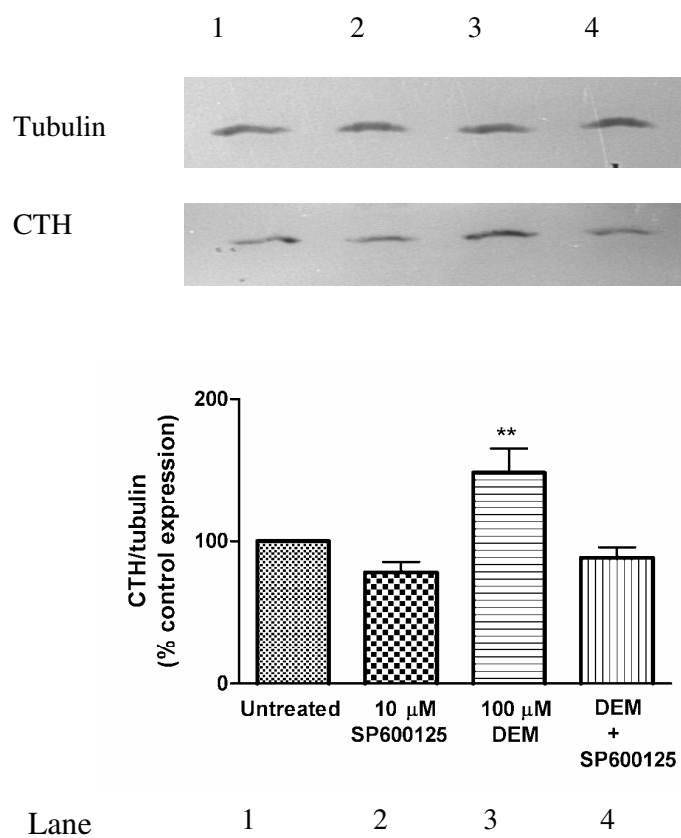
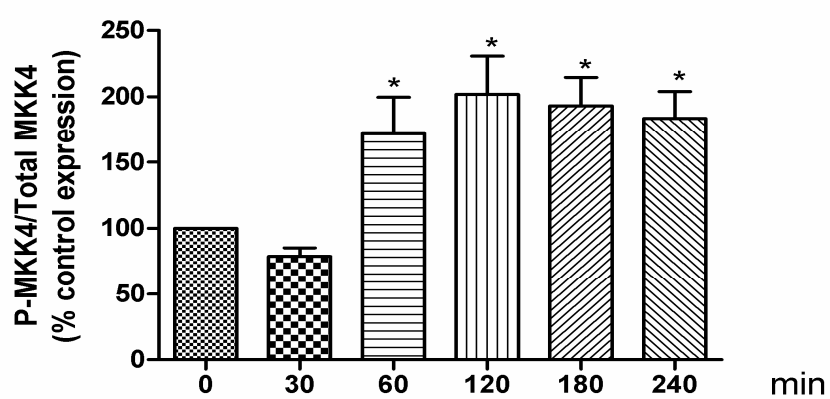
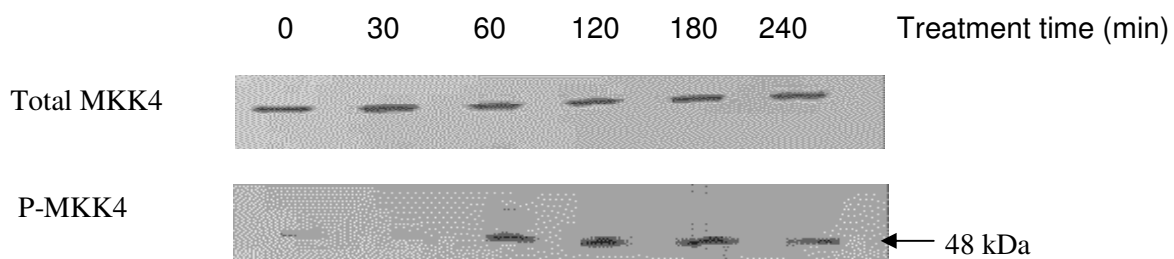


Figure 8
(A)



(B)

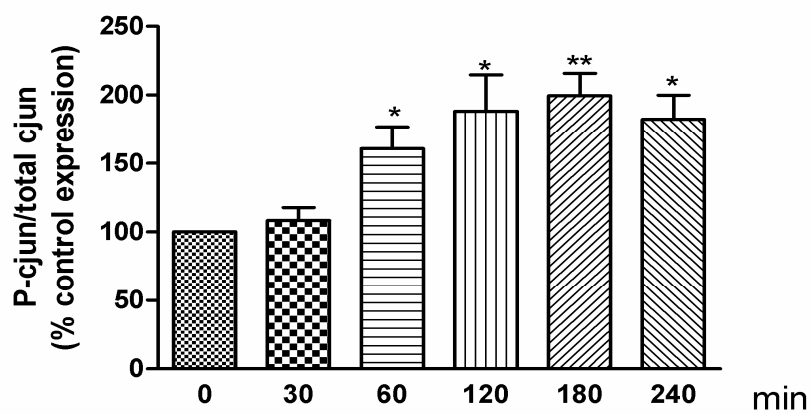
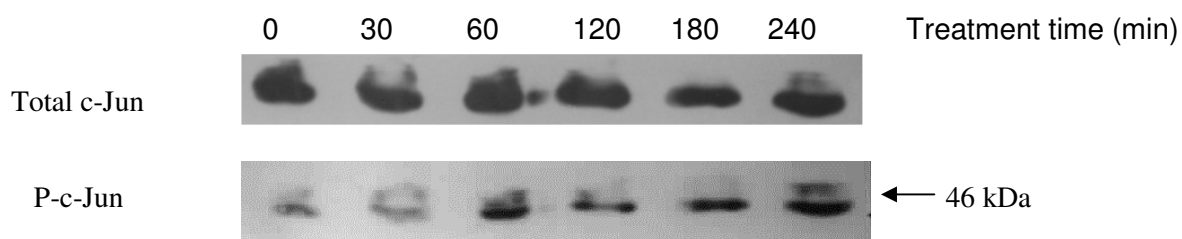
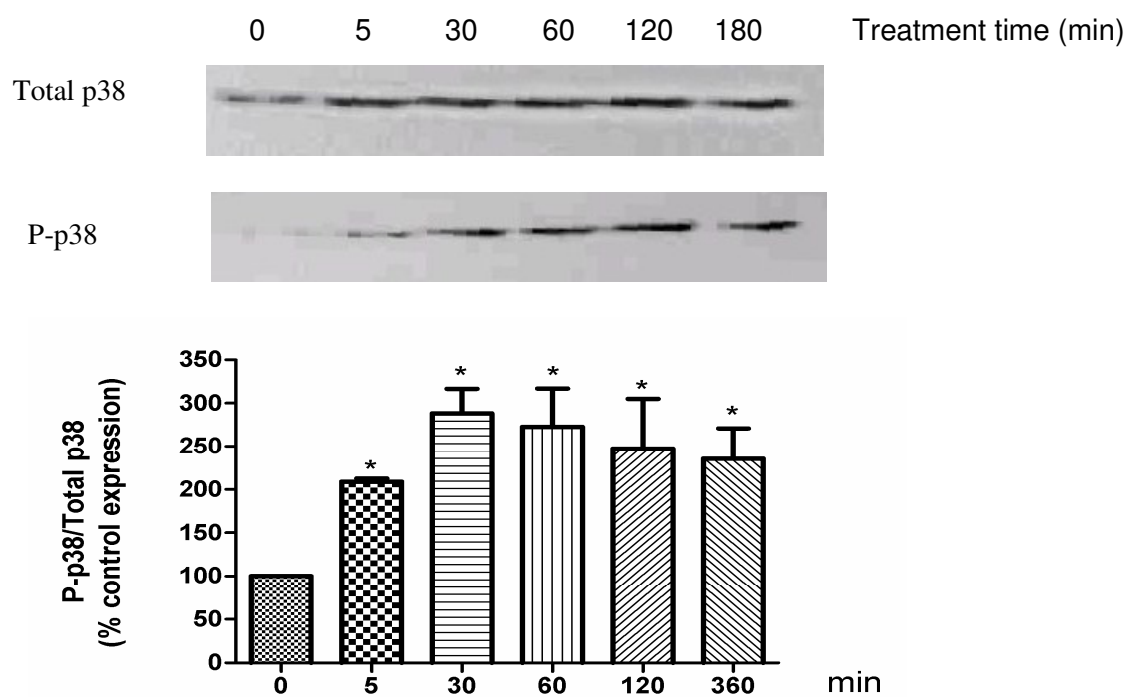


Figure 9
(A)



(B)

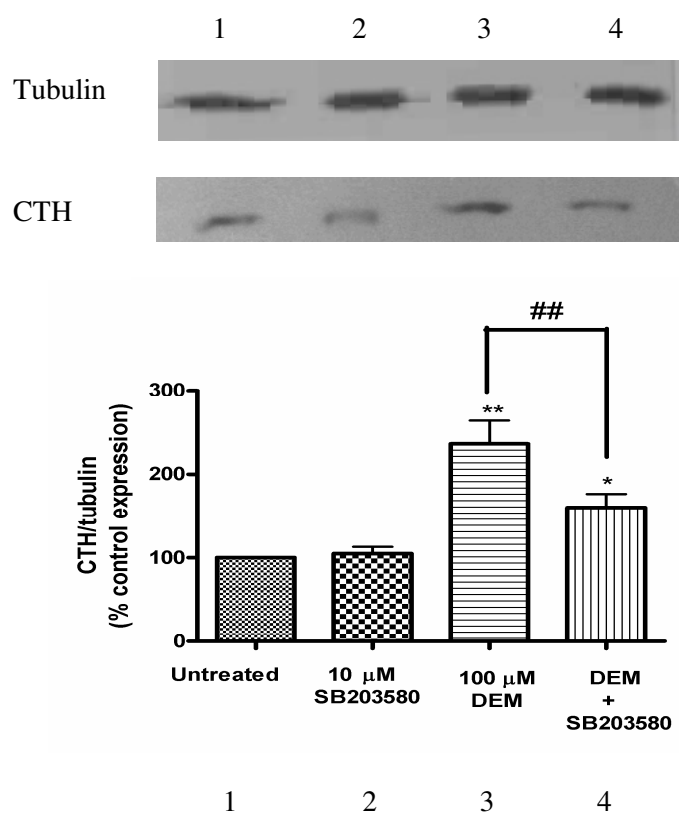
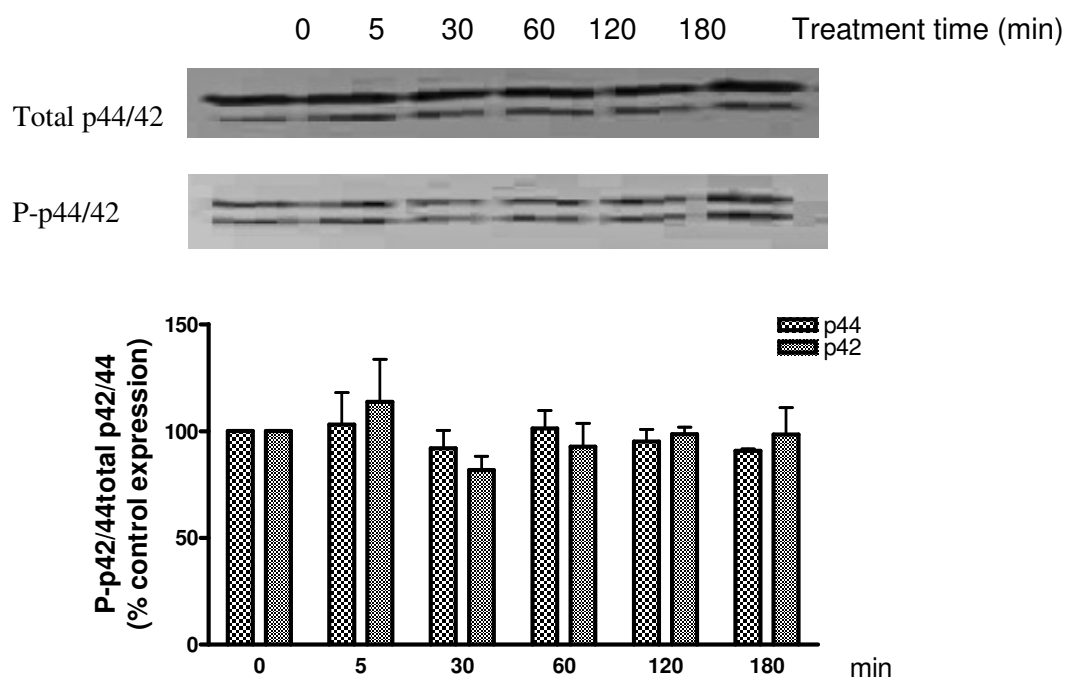


Figure 10
(A)



(B)

