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Inhibition of cystathionine-γ-lyase leads to loss of glutathione and aggravation of mitochondrial dysfunction mediated by excitatory amino acid in the CNS

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Abstract

Oxidative stress has been implicated in the pathogenesis and progression of neurodegenerative disorders and antioxidants potentially have a major role in neuroprotection. Optimum levels of glutathione (γ-glutamylcysteinyl glycine), an endogenous thiol antioxidant are required for the maintenance of the redox status of cells. Cystathionine γ-lyase is the rate-limiting enzyme for the synthesis of cysteine from methionine and availability of cysteine is a critical factor in glutathione synthesis. In the present study, we have examined the role of cystathionine γ-lyase in maintaining the redox homeostasis in brain, particularly with reference to mitochondrial function since the complex I of the electron transport chain is sensitive to redox perturbation. Inhibition of cystathionine γ-lyase by L-propargylglycine caused loss of glutathione and decrease in complex I activity in the brain although the enzyme activity in mouse brain was 1% of the corresponding hepatic activity. We then examined the effect of this inhibition on the neurotoxicity mediated by the excitatory amino acid, L-β-oxalyl amino-L-alanine, which is the causative factor of a type of motor neuron disease, neurolathyrism. L-β-Oxalyl amino-L-alanine toxicity was exacerbated by L-propargylglycine measured as loss of complex I activity indicating the importance of cystathionine γ-lyase in maintaining glutathione levels and in turn the mitochondrial function during excitotoxicity. Oxidative stress generated by L-β-oxalyl amino-L-alanine itself inhibited cystathionine γ-lyase, which could be prevented by prior treatment with thiol antioxidant. Thus, cystathionine γ-lyase itself is susceptible to inactivation by oxidative stress and this can potentially exacerbate oxidant-induced damage. Cystathionine γ-lyase is present in neuronal cells in human brain and its activity is several-fold higher compared to mouse brain. It could potentially play an important role in maintaining glutathione and protein thiol homeostasis in brain and hence afford neuroprotection.
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Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH), a non-protein thiol is present in millimolar amounts in mammalian cells. GSH is less prone to oxidation than cysteine and is ideal for maintaining intracellular redox potential. It exists predominantly in the reduced (thiol, GSH) form and the oxidized (disulfide, GSSG) form accounts for less than 1% of the total glutathione. In the cell, almost 90–95% of glutathione is in the cytosol, about 5% in the mitochondria and a small percentage in the endoplasmic reticulum and in the nucleus (Meister, 1991). Important functions of GSH include its antioxidant activity (Lu, 1999), specifically its role in maintaining protein thiol homeostasis, reaction partner for the detoxification of xenobiotics (Rahman et al., 1999), cofactor in isomerization reactions (Dringen et al., 2000) and as storage and transport form of cysteine (Lu, 1999). In brain, GSH is an important antioxidant since brain is highly sensitive to perturbation in the equilibrium between antioxidant system and reactive oxygen species (Clarke and Sokolof, 1999). Oxidative stress has been implicated in the pathogenesis and progression of several neurodegenerative diseases (Bains and Shaw, 1997) and maintenance of redox status as well as the antioxidant capability of CNS during oxidative stress is vital for neuroprotection.

GSH is synthesized from cysteine, glutamate and glycine by the consecutive actions of two cytosolic ATP-dependent

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enzymes, γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (Meister, 1995). The first step in GSH biosynthesis is catalyzed by GCS, the rate-limiting enzyme, while GSH synthetase has apparently no regulatory role (Lu, 1999). Physiologically, GCS is regulated either by competitive, nonallosteric inhibition by GSH (Richman and Meister, 1975) or by the availability of its precursor amino acids. Availability of cysteine is therefore critical for GSH synthesis.

Cysteine is synthesized through the transulfuration pathway wherein dietary methionine is converted to cysteine. ATP-dependent activation of methionine leads to the production of *S*-adenosylmethionine (catalyzed by methionine adenosyltransferase), and subsequent demethylation and removal of the adenosyl moiety yields homocysteine. Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine β-synthase (using pyridoxal 5′ phosphate as cofactor). The last enzyme of the transsulfuration pathway is cystathionine γ-lyase (CGL, γ-cystathionase, EC 4.4.1.1), which is also a pyridoxal 5′ phosphate dependent enzyme. It catalyzes the conversion of L-cystathionine into L-cysteine, α-ketobutyrate and ammonia and is the rate-limiting enzyme for the synthesis of cysteine from methionine. Although, cysteine levels in cells may also be enhanced by transport of cystine through specialized transporter systems (Shanker and Aschner, 2001), the importance of the transulfuration pathway in generating cysteine for GSH synthesis is well recognized in liver because any perturbation of this pathway leads to decreased levels of cellular GSH (Meister, 1995). While the enzyme is well characterized in liver, we have very little information available on the role of this pathway in the brain (Heinonen, 1973).

We have examined the constitutive expression of cystathionine γ-lyase in brain and studied its role in brain during excitotoxicity using a model excitatory amino acid, L-BOAA (L-β-oxalyl amino-L-alanine) present in the chickling pea from plant *Lathyrus sativus* (Rao et al., 1964). Consumption of the chickling peas for prolonged periods leads to a neurodegenerative disorder called as *neurolathyrism* in humans (Selye, 1957) which is manifested by damage of upper motor neurons, degeneration of anterior horn cells and loss of axons in pyramidal tracts of lumbar spinal cord (Streifler et al., 1977; Cohn and Streifler, 1981). L-BOAA exerts neurotoxicity through the AMPA subclass of glutamate receptors (Pearson and Nunn, 1981; Ross et al., 1989) and causes inhibition of mitochondrial complex I (NADH ubiquinone oxidoreductase) in motor cortex and lumbosacral cord (Sriram et al., 1998) of mice.

1. Experimental procedures

1.1. Materials

L-BOAA was obtained from Research Biochemicals (Natick, MA). TRI reagent was purchased from Molecular Research (Cincinnati, OH). Ubiquinone 1 was obtained as a gift from Eisai Pharmaceutical Company (Tokyo, Japan). Northern blot analysis was performed using digoxigenin-labeling kit from Roche Biochemicals (Mannheim, Germany). All other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, MO) or Qualigens (Mumbai, India). The plasmid containing cDNA for cystathionine γ-lyase was provided by Dr. Michael Lieberman, Baylor College of Medicine, Houston, USA.

Human brain tissues were obtained from post-mortem samples of male traffic accident victims with no known neurological disorders from Human Brain Tissue Repository for neurobiological studies, Department of Neuropathology, NIMHANS. The average interval between death and autopsy was 8 ± 17 h. The average age of the victims was 28.33 ± 13.35. Human brain obtained was washed with sterile ice-cold normal saline (sodium chloride, 0.9%, w/v). The meninges and blood vessels were removed from the brain. The brain regions were dissected out using standard anatomical landmarks. The brain regions were stored at −70 °C until use.

1.2. Animals

Male Swiss albino mice (3 months old) were obtained from the Animal Research Facility. Animals had free access to pelleted diet (Lipton Calcutta, India) and water *ad libitum*. All animal experiments were carried out according to National Institutes of Health guidelines for care and use of laboratory animals. All efforts were made to minimize animal suffering, to reduce number of animals used and to employ alternatives to in vivo techniques, if available. L-Propargyl glycine, (cystathionine γ-lyase inhibitor; 100 mg/kg body weight) was administered subcutaneously before L-BOAA and animals were sacrificed at different time periods after L-BOAA. Control animals received equal volume of vehicle.

1.3. Processing of tissue

Tissues were processed for measurement of activities of complex I, cystathionine-γ-lyase and estimation of total GSH (GSH + GSSG) levels. Tissue was homogenized in 0.25 M sucrose and homogenate was centrifuged at 24,000 × *g* for 30 min to obtain cytosol and used for estimation of cystathionine-γ-lyase activity. The homogenate was centrifuged at 1000 × *g* for 10 min to obtain post-nuclear supernatant. The post-nuclear supernatant was centrifuged again at 14,000 × *g* for 30 min to obtain the mitochondrial pellet. Mitochondrial pellet was suspended in sucrose (0.25 M), and freeze-thawed for assay of complex I. For assay of GSH, frozen tissue was homogenized in 9 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. An aliquot of homogenate was added to an equal volume of 5-sulfosalicylic acid (10%, w/v), mixed and centrifuged at 10,000 × *g* for 10 min and supernatant was used for estimation of total GSH. Protein concentration was estimated by a dye-binding method (Bradford, 1976).

1.4. Estimation of total glutathione

Total glutathione (GSH + GSSG) was estimated by the enzymatic recycling method (Tietze, 1969). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 0.8 mM dithiobisnitrobenzoic acid and 0.5 U/ml glutathione reductase in a total volume of 0.44 ml. The acid-soluble tissue supernatant (0.01 ml) prepared as described above was added. The reaction was initiated by adding 0.05 ml of 1.2 mM NADPH. The increase in absorbance was measured at 412 nm over 3 min. A standard curve was generated using known amount of GSSG.

1.5. Assay of NADH:ubiquinone oxido-reductase (complex I) activity

Complex I activity was assayed in mitochondrial preparations as rotenone-sensitive NADH–ubiquinone oxido-reductase (Sriram et al., 1998; Kenchappa et al., 2002). The assay was performed in 35 mM potassium phosphate buffer (pH 7.4) containing sodium cyanide (2.65 mM), magnesium chloride (5 mM), EDTA (1 mM), bovine serum albumin (1 mg/ml) and antimycin (2 μg/ml). Brain mitochondria (70–80 μg) and ubiquinone 1 (0.05 mM) were added to the assay buffer to a final volume of 0.48 ml. After pre-incubation of reaction mixture at room temperature for 2 min, reaction was initiated by addition 0.02 ml of 5 mM NADH solution. Test and blank reactions were run separately. The rate of decrease of absorbance at 340 nm was monitored over 3 min. Assay

was also carried out in presence of rotenone to determine rotenone-sensitive enzyme activity and less than 2% of the total activity was rotenone insensitive. The enzyme activity is expressed as nmoles of NADH oxidized/min/mg protein.

1.6. Assay of cystathionine-γ-lyase

Cystathionine-γ-lyase activity was measured in cytosol prepared from mouse brain or liver according to Heinonen (1973). The reaction mixture (total volume 0.5 ml) having cytosolic protein from liver (400 μg) or brain (2 mg), 100 μl Tris–HCl buffer (5 mM) and 10 μl of pyridoxal 5′-phosphate (1 mM) was considered as blank. The test sample contained all the above and 10 μl of L-cystathionine (2 mM), which is the substrate. The samples were incubated for 2 h at 37 °C and the reaction was terminated by placing the tubes in ice-water bath at 4 °C. DTT (5 mM, 10 μl) was added to each tube to reduce all cysteine. Ninhydrin reagent was added to all the tubes and heated in boiling water bath for 10 min. Tubes were cooled under tap water, the absorbance of the pink coloured complex was measured at 560 nm by spectrophotometric method (Gaitonde, 1967) using cysteine as standard.

1.7. Northern blotting

Whole brain and liver in case of mice, was homogenized in TRI reagent and total RNA was extracted. mRNA was extracted from total RNA using mRNA isolation kit purchased from Qiagen according to manufacturers protocol. Amount of mRNA was quantitated and separated on agarose gel electrophoretically with total RNA. Then RNA was transferred to positively charged nylon membrane by capillary transfer UV cross-linked and hybridized with digoxigenin labeled cRNA, which was prepared using cDNA. The blot was washed, incubated with anti-digoxigenin antibody conjugated with alkaline phosphate and visualized using a chromogenic substrate for alkaline phosphate.

1.8. In situ hybridisation

The paraffin sections from mouse and human brain regions were deparaffinized by heating followed by treatment with xylene and chloroform. Then sections were hydrated to facilitate action of proteinase K then again dehydrated and hybridized with digoxigenin labelled cRNA synthesized by cDNA. After hybridization the sections was washed and incubated with blocking reagent. Antibody to digoxigenin conjugated to peroxidase was added after which sections were treated with biotinylated tyramide followed by streptavidin–fluorescein. The sections were observed under fluorescent microscope.

2. Results

2.1. Cystathionine γ-lyase activity in mouse brain and liver

Cystathionine γ-lyase activity was measured in cytosol prepared from whole brain and liver of mice. The activity was measured as rate of formation of cysteine using cytosolic preparation from mouse brain and liver. The rate of formation of cysteine increased with increasing protein concentration up to 1.6 mg protein/ml of reaction mixture in brain. In liver, where the activity was 100 fold higher than brain, the activity was linear up to 0.4 mg protein/ml of reaction mixture (Fig. 1a). Cystathionine γ-lyase activity in mouse brain was only 1% of the corresponding activity in liver.

2.2. Constitutive expression and localization of cystathionine γ-lyase in mouse liver and brain

The constitutive expression of cystathionine γ-lyase mRNA in mouse liver and brain was examined by northern blot analysis. The (poly A^+) RNA from mouse liver and brain were electrophoresed under denaturing conditions and transferred to nylon membrane. The blot was hybridized using antisense riboprobe synthesized using the cDNA of mouse liver cystathionine γ-lyase. A transcript of molecular mass of approximately 1.7 kb was observed in mouse liver and brain indicating the constitutive expression of cystathionine γ-lyase (Fig. 1b). Fluorescence *in situ* hybridisation (FISH) studies demonstrated the presence of cystathionine γ-lyase mRNA predominantly in neuronal cells in mouse brain regions. The neuronal cells in cerebral cortex showed intense cytosolic staining, indicating the presence of cystathionine γ-lyase mRNA (Fig. 1c). The laminar architecture of different cortical layers was clearly visible. In cerebellum, the Purkinje cells showed intense fluorescence, while the granule cell layer was relatively less intensely stained (Fig. 1c). Intense fluorescence was seen in hippocampus, in the pyramidal neurons of CA2 and CA3, and in the granule cell layer of dentate gyrus (Fig. 1c). In midbrain, the reticular neurons were selectively labelled, indicating predominant presence of cystathionine γ-lyase mRNA in these cell populations (Fig. 1c).

2.3. Effect of inhibition of cystathionine γ-lyase on GSH levels and mitochondrial complex I

Administration of L-propargylglycine (PPG) to rat increases the serum cystathionine content by inhibiting cystathionine γ-lyase (Awata et al., 1984), while under normal condition cystathionine is not found in the serum. We therefore, examined the effect of PPG on brain cystathionine γ-lyase activity and the consequences of cystathionine γ-lyase inhibition following neurotoxic insult. A single dose of PPG (100 mg/kg body weight) was administered to mice and cystathionine γ-lyase activity was estimated in brain after varying time periods. The inhibition of cystathionine γ-lyase activity by PPG in mice brain leads to loss of GSH. Maximum GSH loss was seen at 6 h (38%) and partial recovery was observed at 18 h (28% loss). Decrease in GSH due to administration of PPG also leads to inhibition complex I at 6 (30% inhibition) and 18 h (37% inhibition; Fig. 2).

2.4. Effect of cystathionine γ-lyase inhibition on mitochondrial complex I loss mediated by L-BOAA

Earlier studies in our laboratory have shown that in male mice a single dose of L-BOAA causes loss of GSH and mitochondrial complex I in lumbosacral cord. This inhibition can be reversed by DTT, *in vitro*, and by pretreatment of α-lipoic acid *in vivo*, indicating that the inhibition is caused by oxidation of critical thiol groups in complex I subunits (Sriram et al., 1998; Kenchappa et al., 2002). We therefore, examined the effect of cystathionine γ-lyase inhibition on L-BOAA mediated complex I dysfunction, since inhibition of cystathionine γ-lyase per se leads to loss of GSH and complex I. Administration of L-BOAA or PPG alone caused similar effects in terms of loss of GSH in lumbosacral cord. However, administration of PPG followed L-BOAA induced greater GSH loss (48% loss, Fig. 3a). In a similar manner, complex I loss was

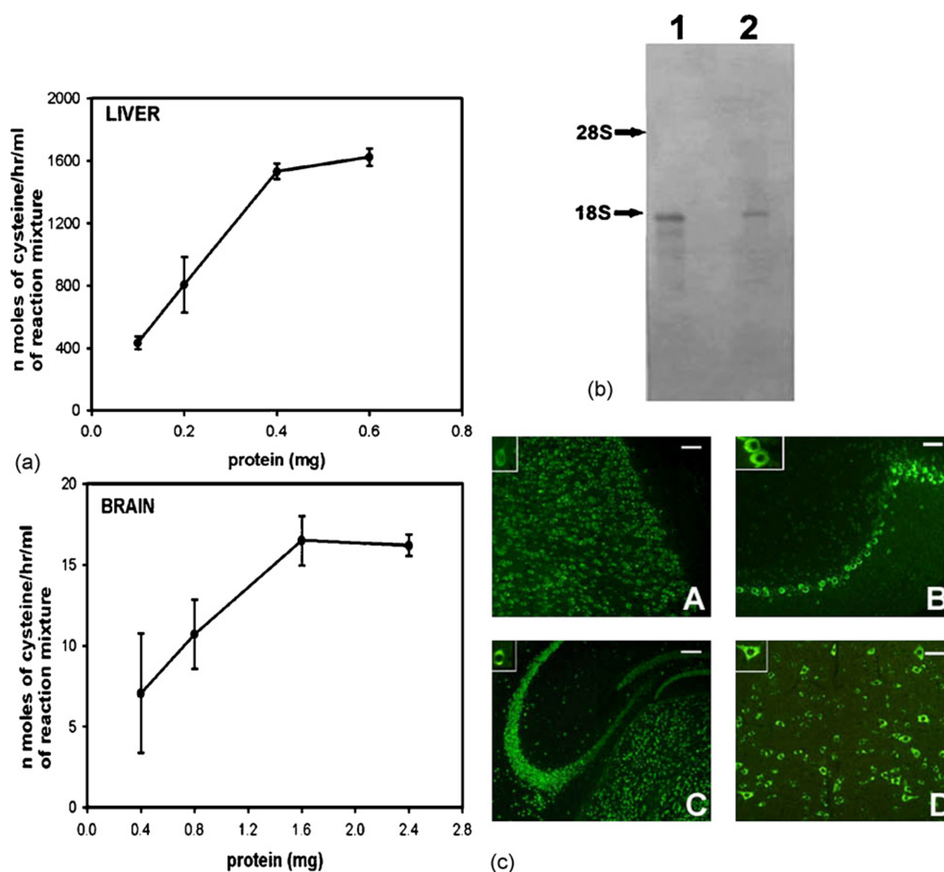

Fig. 1. Constitutive expression of cystathionine γ-lyase in mouse brain. (a) Effect of varying concentrations of protein on cystathionine γ-lyase activity in cytosol prepared from mouse brain and liver. The activity is expressed as nmoles of cysteine formed/h/mg protein. Values are mean ± S.D. (n = 6 animals). (b) The mRNA and total RNA from mouse liver and brain were electrophoresed under denaturing conditions. After transfer to nylon membrane, the blot was hybridized with riboprobe prepared using cDNA from mouse liver cystathionine γ-lyase. Lanes 1 and 2 were loaded with 5 μg of (poly A+) RNA from liver and brain, respectively. (c) Presence of cystathionine γ-lyase in the neurons of cerebral cortex (A; bar = 50 μm). Inset: magnified view of a cortical neuron. Fluorescent labeling of the Purkinje cells in the mouse cerebellum is depicted (B; bar = 50 μm). Inset: magnified view of the Purkinje cell in cerebellum. Fluorescence labeling was seen in granule cell layer of dentate gyrus. The CA2, and CA3 subfields of the hippocampus were intensely fluorescent (C; bar = 200 μm). Inset: magnified view of the pyramidal neuron in hippocampus. (D) Intense fluorescence was seen in reticular neurons in the midbrain which express cystathionine γ-lyase mRNA (bar = 50 μm). Inset: magnified view of a reticular neuron of midbrain.

more severe when PPG and L-BOAA were given together rather than when given alone (Fig. 3a).

2.5. DTT reverses complex I inhibition caused due to pretreatment of L-propargylglycine followed by L-BOAA

Earlier studies from our laboratory had shown that complex I inhibition caused by L-BOAA can be reversed by treating isolated mitochondria with the disulfide reductant, dithiothreitol (DTT; Sriram et al., 1998). We therefore, examined if similar reversal could take place in PPG treated mice. Mice were pretreated with PPG (100 mg/kg body weight, s.c.) for 6 h followed by L-BOAA (10 mg/kg body weight, s.c.) for 1 h and mitochondria was isolated from lumbosacral cord. The inhibition of complex I caused by PPG alone or PPG and L-BOAA was reversed by DTT (Fig. 3b) indicating that the inhibition is caused by oxidation of critical thiols of complex I subunits. These results reiterated that maintenance of thiol status is important for complex I activity and its recovery after neurotoxic insult.

2.6. Prolonged exposure to L-BOAA inhibits cystathionine γ-lyase activity in mouse brain and spinal cord

A single dose of L-BOAA (10 mg/kg body weight, s.c.) was administered to mice and they were sacrificed at 1 and 30 h. Later, cystathionine γ-lyase activity was measured in cytosol prepared from brain and spinal cord. L-BOAA inhibited the activity of cystathionine γ-lyase in brain (35%) and spinal cord (40%) at 30 h, while it had no effect after 1 h exposure (Fig. 4a). These results suggested that cystathionine γ-lyase is not affected at early intervals after L-BOAA, but exposure for longer periods inhibits cystathionine γ-lyase activity.

2.7. Cystathionine γ-lyase inhibition caused by L-BOAA was not reversed by DTT

A single dose of L-BOAA (10 mg/kg body weight) was administered to mice and sacrificed after 30 h. Cytosol prepared from whole brain and spinal cord was incubated with and without DTT for 1 h at 37 °C. The resin was removed by brief

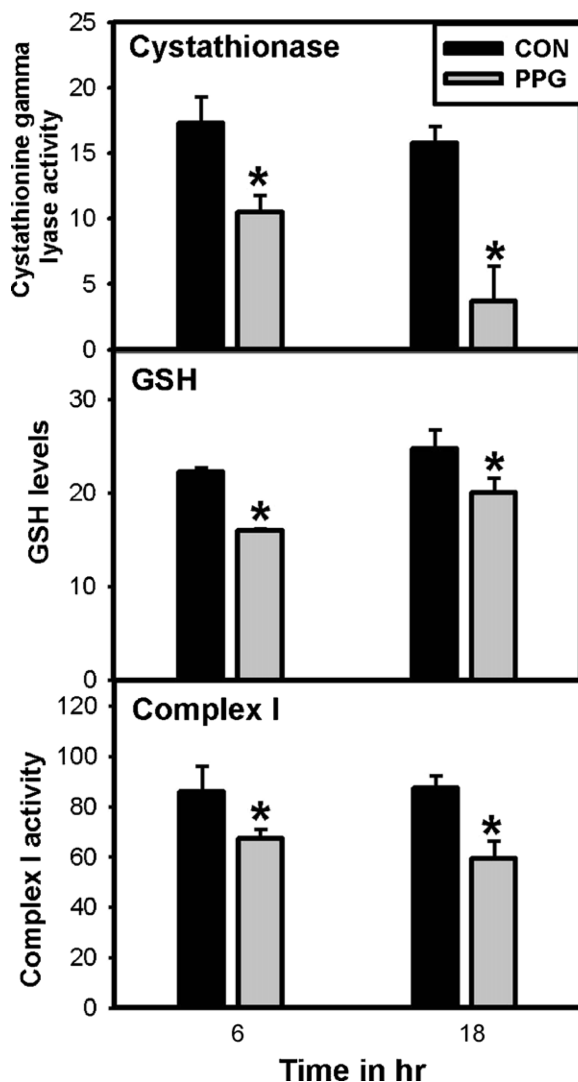

Fig. 2. Effect of L-propargylglycine (PPG) on the activity of cystathionase γ-lyase, complex I and GSH levels in mice brain. PPG (100 mg/kg body weight, s.c) was administered to mice and animals were killed at 6 and 18 h after dosage (gray bar). Control animals received vehicle alone (black bar). Complex I and cystathionine γ-lyase activities and GSH levels were estimated in whole brain of mice. The activity of complex I is expressed as nmoles of NADH/min/mg protein. cystathionine γ-lyase activity is expressed as nmoles of cysteine/h/mg protein and GSH levels are expressed as nmoles of GSH/mg protein. Values are mean ± S.E.M. ($n = 6$ animals). Asterisks indicate values significantly different from controls ($P < 0.002$).

centrifugation and cystathionine γ-lyase activity was measured in the cytosol. DTT did not reverse the inhibition of cystathionine γ-lyase caused by L-BOAA (Fig. 4b).

2.8. Pretreatment of α-lipoic acid protects against inhibition of cystathionine γ-lyase and complex I activity by L-BOAA

Mice were pretreated 1 h before with α-lipoic acid (20 mg/kg body weight, s.c.) followed by L-BOAA and sacrificed after 30 h and complex I activity was measured in motor cortex and lumbosacral cord. L-BOAA causes loss of complex I activity in motor cortex and lumbosacral cord. However, pretreatment with α-lipoic acid prevented loss of complex I activity following L-BOAA treatment for 30 h (data not shown). These results suggest that maintenance of thiol homeostasis is important to ensure mitochondrial function. Cystathionine γ-lyase activity was also measured in brain and spinal cord following treatment of animals with α-lipoic acid and L-BOAA. Prolonged exposure to L-BOAA caused loss of cystathionine γ-lyase activity in brain and spinal cord. However, when mice were pretreated with α-lipoic acid the loss of cystathionine γ-lyase was prevented (Fig. 4c).

2.9. Constitutive expression and localization of cystathionine γ-lyase in human brain

Cystathionine γ-lyase activity was measured in cytosol prepared from human brain regions obtained at autopsy from traffic accident victims. No significant difference was observed in cystathionine γ-lyase activity amongst brain regions such as cortex, cerebellum, striatum, thalamus and pons except in hippocampus where the activity was significantly lower (36%; Fig. 5a). Cystathionine γ-lyase activity in human brain was 100-fold higher than that observed in mouse brain (Fig. 5).

The (poly A^+) RNA isolated from human brain was electrophoresed under denaturing conditions and transferred to a nylon membrane. The blot was hybridised using the antisense riboprobe synthesised using cDNA of cystathionine γ-lyase from mouse liver, which has 90% homology with human liver cDNA. The northern blot analysis revealed the presence of cystathionine γ-lyase transcript with molecular weight of approximately 1.7 kb (Fig. 5b).

In order to localize cystathionine γ-lyase mRNA in human brain regions, fluorescence *in situ* experiment was performed using paraffin-embedded human brain section. Fluorescence *in situ* hybridisation (FISH) studies demonstrated the presence of cystathionine γ-lyase mRNA predominantly in neuronal cells in human brain regions (Fig. 5c). Cystathionine γ-lyase mRNA was seen in the neurons in cerebral cortex. In the cerebellum, Purkinje cells showed intense fluorescence, while the granule cell layer was less intensely stained. Intense fluorescence was observed in the granule cell layer in the dentate gyrus and CA3 pyramidal neurons in the hippocampus. The reticular neurons in the midbrain were also stained indicating the presence of cystathionine γ-lyase mRNA in these cell populations.

3. Discussion

Cystathionine γ-lyase activity in mouse brain was very low and represented 1% of the hepatic activity. However, significant amount of cystathionine γ-lyase activity was detectable in human brain. In fact, the activity was 100 times more than the corresponding activity in mouse brain. Such differences have not been noted in the activity of γ-glutamyl cysteinyl synthase, the rate-limiting enzyme in GSH synthesis or in GSH levels per se between human and mouse brain (Mirecki et al., 2004; Schulz et al., 2000). It is presumable that cystathionine γ-lyase plays an important role in human brain. The enzyme activity was similar in most regions of the brain, except in hippocampus

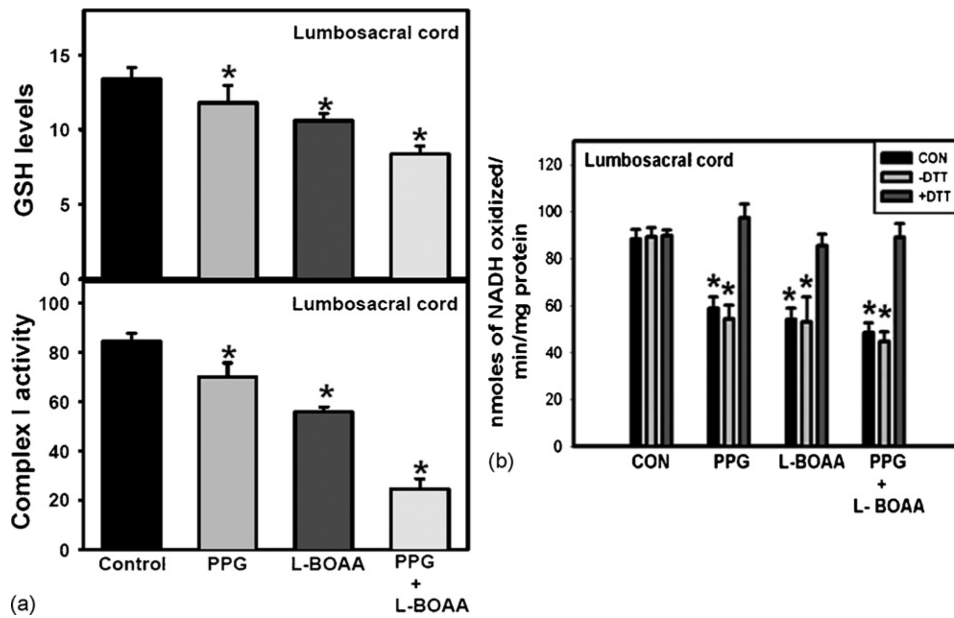

Fig. 3. Effect of L-propargylglycine pretreatment on L-BOAA toxicity and its reversal by thiol reductant. (a) Mice were pretreated for 6 h with L-propargylglycine (100 mg/kg body weight, s.c.) prior to administration of L-BOAA (10 mg/kg body weight, s.c.) and sacrificed 1 h after L-BOAA dose. Control animals received vehicle alone. GSH and complex I activity were estimated in lumbosacral cord. The values are expressed as nmoles of GSH/mg protein and nmoles of NADH oxidized/min/mg protein, respectively. Values are mean ± S.E.M. ($n = 6$ animals). Asterisks indicate values significantly different from controls ($P < 0.002$). (b) Mitochondria were isolated from lumbosacral cord of mice pretreated with 6 h of L-propargylglycine (100 mg/kg body weight, s.c.) followed L-BOAA (10 mg/kg body weight, s.c.) for 1 h. Mitochondrial samples were incubated with (+DTT) and without (−DTT) for 30 min at 37 °C. The loss of complex I activity was reversed by dithiothreitol indicating that oxidation of sulphydryl was responsible for the inhibition. Complex I activity is expressed as nmoles of NADH/min/mg protein. Values are depicted as mean ± S.D. ($n = 4$ animals). Asterisks indicate values significantly different from controls ($P < 0.002$).

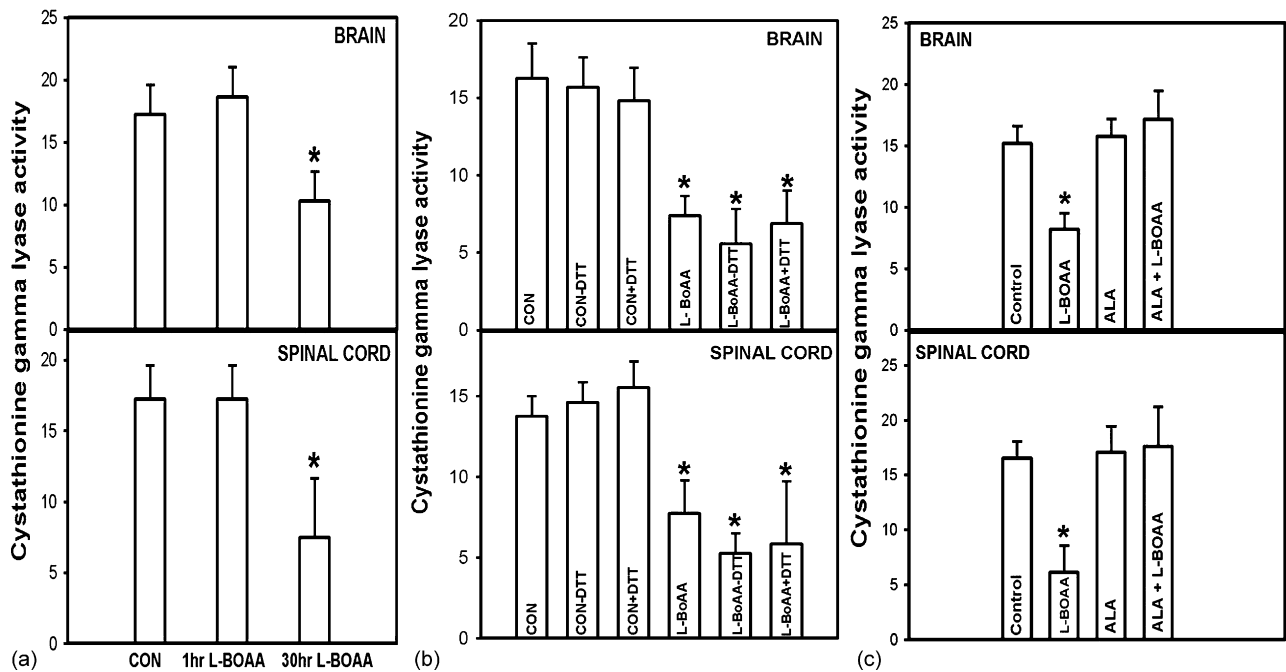

Fig. 4. Effect of L-BOAA on cystathionine γ-lyase activity in mouse brain and spinal cord. (a) Animals were dosed with L-BOAA (10 mg/kg body weight, s.c.) and sacrificed at 1 and 30 h later. Cystathionine γ-lyase activity was measured in brain and spinal cord. Values are mean ± S.D. ($n = 6$ animals) and asterisks indicate values significantly different from vehicle treated controls ($P < 0.002$). (b) Cytosol isolated from brain and spinal cord of mice treated with vehicle or L-BOAA (10 mg/kg body weight, s.c.) for 30 h were incubated with (+DTT) and without (−DTT) for 30 min at 37 °C. Cystathionine γ-lyase activity was measured. Data are depicted as mean ± S.D. ($n = 4$ animals). Asterisks indicate values significantly different from controls ($P < 0.002$). (c) Animals were pretreated with α-lipoic acid (20 mg/kg body weight, s.c.) 1 h prior to administration of L-BOAA (10 mg/kg body weight, s.c.) and sacrificed 30 h after the L-BOAA dose. Activity of cystathionine γ-lyase was measured in the brain and spinal cord. Values are mean ± S.D. ($n = 6$ animals) and asterisks indicate values significantly different from vehicle treated controls ($P < 0.002$).

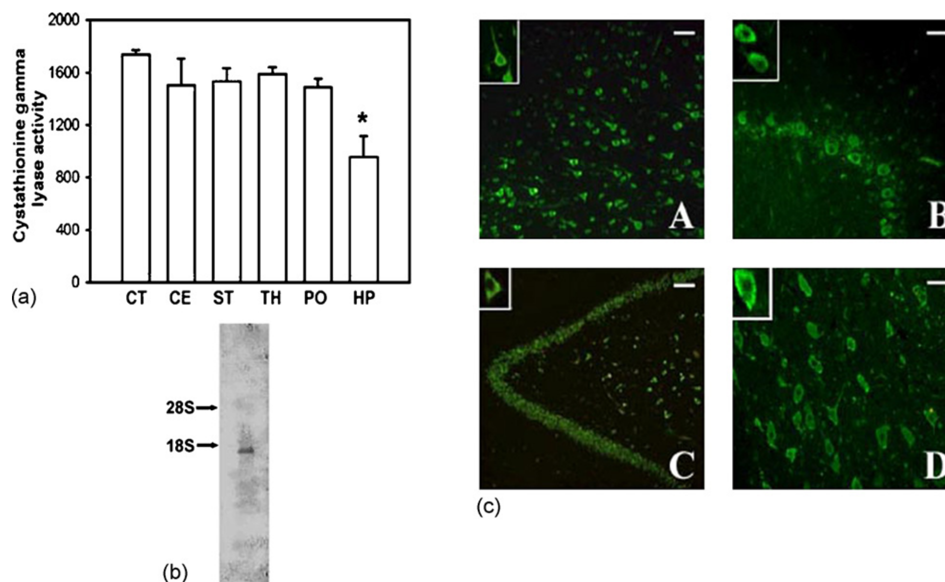

Fig. 5. Cystathionine γ-lyase activity and expression in human brain regions. (a) Activity of cystathionine γ-lyase was estimated in cytosol prepared from human brain regions and is expressed as nmoles of cysteine formed/h/mg protein. The brain regions are represented as: CT, cortex; CE, cerebellum; ST, striatum; TH, thalamus; PO, pons; HP, hippocampus. Data are mean ± S.E.M. values ($n = 6$ human brain samples). Asterisks indicate values significantly different from cortex ($P < 0.05$). (b) Human brain mRNA (5 μg) and total RNA was electrophoresed under denaturing conditions. After transfer to nylon membrane, the blot was hybridized with ribo-probe prepared using cDNA from mouse liver cystathionine γ-lyase. The cystathionine γ-lyase mRNA was seen as a band at approximately 1.7 kb in human brain cortex. (c) Intense fluorescence was seen in the neuronal cell layers of the human brain cortex (A; bar = 200 μm). Inset: magnified view of a cortical neuron. Fluorescent labeling of the Purkinje cells and the granule cell layer was seen in the human cerebellum (B; bar = 100 μm). Inset: magnified view of Purkinje cells of cerebellum. The presence of cystathionine γ-lyase mRNA was seen in the pyramidal neurons of CA3 and and granule cell layer of dentate gyrus (C; bar = 100 μm). Inset: magnified view of a pyramidal neuron of hippocampus. Reticular neurons in the midbrain express cystathionine γ-lyase mRNA (D; bar = 100 μm). Inset: magnified view of a reticular neuron in midbrain.

where it was significantly lower as compared to cortex (Fig. 5). This is the first report of the presence of cystathionine γ-lyase activity in human brain. It is known that the activity in human liver is about 28 mU/mg protein and in AIDS patients the activity was substantially reduced, which has been causally related to low levels of plasma glutathione in AIDS patients (Martin et al., 2001). The impairment in the enzyme activity results in the accumulation of cystathionine leading to cystathionuria, which causes mental retardation in human brain (Vina et al., 1995).

The expression of cystathionine γ-lyase in all regions of mouse and human brain as observed by *in situ* hybridization showed predominant localization in neuronal population. PPG (a specific inhibitor) was able to inhibit cystathionine γ-lyase, which resulted in loss of GSH indicating that availability of adequate amount of cysteine is important for maintenance of GSH level in the CNS. The loss of GSH in CNS caused by PPG administration could be exploited to generate in vivo models of GSH depletion since L-buthionine sulfoxamine (an inhibitor of γ-glutamyl cysteine synthase), which is often used to deplete GSH does not cross the blood brain barrier.

Inhibition of cystathionine γ-lyase results in loss of GSH which precedes complex I inhibition. It is debatable if the decrease in complex I activity is caused due to GSH loss, per se, or if it is an indirect effect of reactive oxygen species, such as superoxide and hydrogen peroxide that are generated as a consequence of GSH depletion. This potentially causes oxidative modification of proteins, such as carbonylation and nitrosolylation, which are not reversible by disulphide reductants. The complex I dysfunction seen in the present study is predominantly due to reversible disulphide formation since the activity can be recovered, in vitro, by reducing agents such as dithiothreitol. Our earlier studies have shown that loss of GSH in brain results in extensive glutathionylation of proteins (Ravindranath and Reed, 1990) and glutathionylation of critical thiol groups in the 52 and 75 kDa subunits of complex I results in loss of complex I activity (Taylor et al., 2003). This probably underlies the complex I dysfunction seen after PPG exposure. Previous studies have shown that exposure of isolated mitochondria to acrolein results in dose dependent increase in reactive oxygen species (ROS) and decrease in GSH levels (Luo and Shi, 2005), which is accompanied by mitochondrial permeability transition. Further, mitochondrial dysfunction caused by increased ROS leads to necrosis and such mechanisms may play a role in neurodegenerative disorders (Luo et al., 2005).

A study in frog (*Rana temporaria*) brain in the course of the fall migration to wintering sites and the period of hibernation, has shown that there is diminished level of GSH in the brain, which correlates with the changes observed in the activity of cystathionine γ-lyase and other enzymes for cysteine delivery. In addition, diminished mitochondria-related oxidative metabolism was also observed (Wrobel, 2001). Thus, cystathionine γ-lyase is important for GSH synthesis which in turn regulates thiol status of enzymes like complex I, which are important for mitochondrial function.

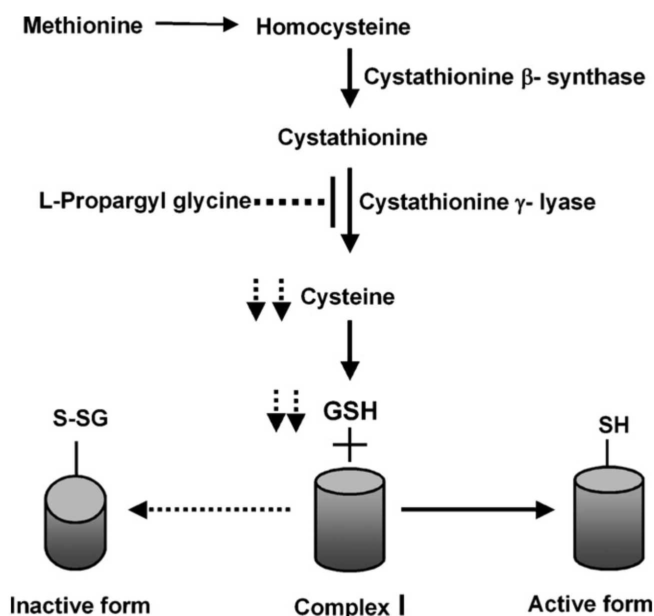

Fig. 6. Influence of cystathionine γ-lyase activity on glutathione levels and complex I activity. Transsulfuration pathway is the main source for cysteine derived from dietary methionine. ATP-dependent activation of methionine leads to the production of *S*-adenosylmethionine (catalyzed by methionine adenosyltransferase), and subsequent demethylation and removal of the adenosyl moiety yields homocysteine. Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine β-synthase (using pyridoxal 5′ phosphate as cofactor). Cystathionine γ-lyase (a pyridoxal 5′ phosphate dependent enzyme) is the regulatory enzyme in the synthesis of cysteine. It catalyzes the conversion of L-cystathionine into L-cysteine, α-ketobutyrate and ammonia. Inhibition of cystathionine γ-lyase by L-propargyl glycine causes loss of glutathione due to decrease in cysteine availability. Decreased glutathione level results in glutathionylation of proteins leading to formation of protein glutathione mixed disulfides. This results in inhibition of enzymes, which have thiol groups in their active site, such as complex I.

The inhibitory effect of cystathionine γ-lyase by PPG exacerbates the excitotoxicity caused by L-BOAA. The additive effect resulted in greater GSH loss and complex I inhibition. The inhibition of mitochondrial complex I by PPG and L-BOAA in lumbosacral cord can be reversed by thiol reductant DTT (dithothreitol) showing that the loss of complex I activity was due to thiol oxidation. Although cystathionine γ-lyase activity is low in brain, its inhibition leads to thiol oxidation and complex I inhibition indicating a causal link between cysteine synthesis, GSH availability and mitochondrial function.

Increased production of ROS by mitochondria is a major contributor to oxidative damage seen in many neurodegenerative disorders. Complex I is the largest component of respiratory chain and a major source of reactive oxygen species. There is selective loss of complex I function in certain neurodegenerative disorders, such as, Parkinson's disease (Schulz et al., 2000). Earlier studies from our lab have demonstrated that alterations in glutathione pool inhibits complex I function (Balijepalli et al., 1999), which can be reversed *in vitro* by the disulfide reductant, dithothreitol and *in vivo* by pretreatment with α-lipoic acid, indicating that inhibition occurs through oxidation of critical thiol groups in complex I subunits (Sriram et al., 1998; Kenchappa et al., 2002).

The excitatory amino acid L-BOAA, per se, inhibited cystathionine γ-lyase activity 30 h after the initial exposure (Fig. 4a), indicating that the enzyme itself is susceptible to inactivation by the oxidative stress generated by the excitatory amino acid. We then ascertained if the inhibition was due to reversible oxidation of cysteine residues in the enzyme, which can then be reversed by DTT, *in vitro*. Inhibition of cystathionine γ-lyase caused by L-BOAA was not reversible by DTT (Fig. 4b) indicating that the inhibition was not due to reversible oxidation of cysteine residues to disulphides. Prior treatment with α-lipoic acid, a thiol antioxidant prevented oxidative modification of cystathionine γ-lyase caused by L-BOAA (Fig. 4c). These studies suggest that maintenance of protein thiol homeostasis is not only important for mitochondrial function but also for enzymes involved in GSH synthesis. The inhibition of cystathionine γ-lyase by L-BOAA may explain the sustained loss of GSH seen in the lumbar spinal cord following L-BOAA (Sriram et al., 1998).

Cystathionine γ-lyase mRNA was localized in brain and found to be present predominantly in neuronal cells in mouse brain indicating the potential role of the enzyme in synthesis of cysteine in neuronal cells. The other source of cysteine, which is the sodium dependent transporter(s) for cysteine, has been reported in cultured neurons and glia (Bannai, 1986). However, it remains to be elucidated whether such transporter exists in brain and if so, their role in transport of cysteine in neuronal cells needs to be defined.

In conclusion, cystathionine γ-lyase is important for maintaining GSH levels in brain, which in turn is critical for mitochondrial function. Dysfunction of cystathionine γ-lyase results in mitochondrial dysfunction due to oxidative modification of complex I subunits (Fig. 6). Importantly, cystathionine γ-lyase can be inactivated by oxidative stress, which can have far-reaching implication in terms of aggravating oxidative damage and limiting the recovery.

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