The transsulfuration pathway: a source of cysteine for glutathione in astrocytes

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Key words

Transsulfuration, brain, astrocyte, cysteine, cystathionine-γ-lyase, cystathionine-β-synthase, glutathione

Abbreviations

GSH, L-γ-glutamyl-L-cysteinyl-glycine (glutathione); GSSG, oxidised form of glutathione; DEM, diethylmaleate; IL-6, interleukin-6; JNK, c-Jun-*N*-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factorκB; SAPK, stress activated protein kinase; TNFα, tumour necrosis factorα; xCT, subunit of the x_c^- cystine-glutamate exchanger

Summary

Astrocytes cells require cysteine as a substrate for glutamate cysteine ligase (γ-glutamylcysteine synthase; EC 6.3.2.2) catalyst of the rate-limiting step of the γ-glutamylcycle leading to formation of glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH). In both astrocytes and glioblastoma/astrocytoma cells, the majority of cysteine originates from reduction of cystine imported by the cystine-glutamate exchanger. However, the transsulfuration pathway, which supplies cysteine from the indispensable amino acid, methionine, has recently been identified as a significant contributor to GSH synthesis in astrocytes. The purpose of this review is to evaluate the importance of the transsulfuration pathway in these cells, particularly in the context of a reserve pathway that channels methionine towards cysteine when the demand for glutathione is high, or under conditions in which the supply of cystine by the cystine-glutamate exchanger may be compromised.

An overview of sulfur amino acid metabolism and the transsulfuration pathway

Sulfur amino acid metabolism is critically important in mammalian cells and is linked to provision of methyl groups for a large number of biochemical processes. Dietary methionine is activated by conversion to S-adenosylmethionine in an ATP-dependent reaction catalysed by methionine adenosyltransferase that, through action of methyltransferase, yields S-adenosylhomocysteine, followed by homocysteine (Fig.1). Homocysteine can be either re-methylated back to methionine using a methyl group provided by methyl tetrahydrofolate, or irreversibly converted to cysteine via transsulfuration. In hepatic cells in particular, dietary cysteine acts in a methionine-sparing capacity and promotes re-methylation of homocysteine. Should the supply of cysteine be insufficient, homocysteine is channelled into the transsulfuration pathway by conjugation with serine to provide cystathionine, which is the immediate precursor of cysteine (Fig. 1). Cysteine is the source of both taurine and hydrogen sulfide (H_2S) besides being the essential amino acid constituent in the functional (CXXC) motif of the major cellular antioxidant families, which include GSH, glutaredoxins, thioredoxins and peroxiredoxins (Benight et al., 2009). It has been estimated that, in mammalian liver, approximately 50% of cysteine in GSH is derived from methionine via transsulfuration (Banerjee and Zou, 2005). The focus of this article is to review the current understanding of the transsulfuration pathway and its regulatory enzymes, and to assess the importance of this pathway in antioxidant defence in the brain and in astrocytes, in particular.

Regulatory enzymes of the transsulfuration pathway

The regulatory enzymes of the transsulfuration pathway are the pyridoxal-phosphate dependent enzymes, cystathionine-β-synthase (L-serine hydro-lyase (adding homocysteine); EC 4.2.1.22) and cystathionine-γ-lyase (L-cystathionine cysteine-lyase; EC 4.4.1.1). Cystathionine-β-synthase is a heme-containing enzyme that is subject to regulatory control, as it catalyses the first committed step of the transsulfuration pathway (Aitken and Kirsch, 2005; Banerjee and Zou, 2005). Regulation of metabolic flux through the competing transmethylation and transsulfuration pathways occurs at several levels. S-adenosylmethionine is an allosteric activator of cystathionine-β-synthase that effectively channels excess sulfur towards metabolism when methionine levels are high. The presence of a heme cofactor suggests redox-sensitive regulation of enzyme activity and this has been verified by the observation that reduction of cystathionine-β-synthase to the ferrous state is accompanied by a two-fold loss in activity that can be reversed on re-oxidation with ferricyanide (Banerjee and Zou, 2005). Genetic defects of cystathionine-β-synthase are the most common hereditary causes of hyperhomocysteinemia in humans.

Cysteine is a competitive inhibitor of cystathionine-γ-lyase, which predicts that, at the physiological cysteine: cystathionine ratio in liver, the enzyme is inhibited under normal conditions (Martín et al., 2007). This would prevent accumulation of intracellular cysteine, which would likely undergo auto-oxidation to cystine and loss of redox balance. Cystathionine-γ-lyase in foetal hepatocytes is upregulated at both the mRNA and protein levels by mild oxidative stress and new-born infants and rat pups have a higher cystathionine-γ-lyase activity than fetuses (but not premature infants (Martín et al., 2007). Severe oxidative stress causes inhibition of the enzyme. Kidney cystathionine-γ-lyase is upregulated at the protein level following ischaemia/reperfusion injury in mice (Tripatara et al., 2009). A decrease in plasma homocysteine and upregulation of the enzyme in rat liver and lung following dietary docosahexaenoic acid administration has been noted (Huang et al., 2010).

A deficiency in the transsulfuration pathway leads to excessive homocysteine production, loss of cellular redox homeostasis and reduced GSH production (Rosado et al., 2007). Resulting changes in DNA methylation, coupled with DNA damage due to loss of antioxidant protection, are viewed as potential causes for tumour growth and expansion (Rosado et al., 2007).

H_2S production by cystathionine-β-synthase and cystathionine-γ-lyase

Much information on the activity and localisation of cystathionine-β-synthase and cystathionine-γ-lyase comes from the fact that both enzymes utilise cysteine or homocysteine to produce the gas H_2S (Fig 1). H_2S is an endogenous anti-inflammatory, antioxidant and neuroprotective agent that may have therapeutic potential in a range of neurodegenerative diseases (Kimura, 2010; Lee et al., 2009). There is therefore considerable interest in its mechanism of biosynthesis and several authors have provided details of this process in both nervous and non-nervous tissues. Cysteine is the preferred substrate for H_2S production by either enzyme and accounts for 70% of the total amount of the gas produced under normal conditions. Cystathionine-β-synthase generates serine as co-product of H_2S formation, whereas cystathionine-γ-lyase catalyses conversion of cysteine to pyruvate, H_2S and NH_3 (Lee et al., 2009). Singh et al. (2009) have established that the preferred reaction mechanism of cystathionine-β-synthase is by beta replacement of cysteine by homocysteine, as opposed to the alternative mechanisms of beta-elimination of cysteine to generate serine or condensation of two mol of cysteine to cystathionine (Singh et al., 2009). In the

reaction catalysed by cystathionine-γ-lyase, alpha, beta elimination of cysteine normally accounts for 70% of total H_2S, but in conditions such as hyperhomocysteinemia, the relative contribution of homocysteine to H_2S production increases to as much as 90% (Chiku et al., 2009). Kinetic data have been used to predict the relative contribution of cystathionine-β-synthase and cystathionine-γ-lyase to H_2S production at physiologically-relevant substrate concentrations. It is estimated that cystathionine-β-synthase would account for between 25% - 70% of the total amount of H_2S produced, depending on the level of activation of the enzyme by S-adenosylmethionine (Singh et al., 2009).

Localisation of cystathionine-β-synthase and cystathionine-γ-lyase

Cystathionine-β-synthase is abundant throughout the brain, and has been recorded as being particularly highly expressed in Purkinje cells and in the hippocampus (Li et al., 2009) and in cerebellar Bergmann glia and astrocytes (Kimura, 2010 and references therein). Cystathionine-γ-lyase is expressed more restrictedly than cystathionine-β-synthase, and until recently was thought to be entirely absent from brain, which gave rise to the view that the transsulfuration pathway was not functional in this tissue (Grange et al., 1992).

Accordingly, studies using selective inhibitors for cystathionine-β-synthase and cystathionine-γ-lyase and gene knockout experiments promote the view that cystathionine-β-synthase is responsible for H_2S production in human brain, whereas cystathionine-γ-lyase is the dominant enzyme in the vasculature (Lee et al., 2009). For example, cystathionine-γ-lyase gene deletion in mice caused reduced H_2S production in serum, heart and aorta, but not brain. Similarly, immunolabelling of cystathionine-γ-lyase was observed in vessel walls in human brain, whereas strong staining for cystathionine-β-synthase was detected in astrocytes in the hippocampus and precentral cortex (Lee et al., 2009). Pong et al. (2007) recorded immunostaining for cystathionine-γ-lyase in mouse liver, but not brain, in confirmation of the fact that enzyme activity in liver is 100 times higher than in brain. However, there are conflicting views on the level of expression and activity of cystathionine-γ-lyase in the brain and not all studies concur with the view that the enzyme is confined to non-nervous tissue. Species differences may be important in this regard. For example, activity of the enzyme in human brain is 100 times that of mouse brain (Pong et al., 2007). In the salamander, expression of cystathionine-γ-lyase is detectable in retinal Müller cells, cerebellum and liver (Pong et al., 2007). Similarly, H_2S production in the porcine retina is blocked by the selective inhibitor of cystathionine-γ-lyase, DL-propargylglycine, signifying an active

contribution of the enzyme in these cells (Opere et al., 2009). Functional activity of cystathionine-γ-lyase in the brain of Swiss albino mice was observed by Karunakaran et al. (2007), which was reduced by 68% 18 h after administration of DL-propargylglycine (Diwakar and Ravindranath, 2007).

Cystathionine-β-synthase and cystathionine-γ-lyase in glial cells

The rate of synthesis of H_2S in human astrocytes isolated from surgically resected temporal lobe tissue was seven-fold higher than in microglial cells and ten-fold higher than in neuronal cell lines and was significantly reduced in all these cell types by the cystathionine-β-synthase inhibitor, hydroxylamine, but not by DL-propargylglycine (Lee et al., 2009). Once again, there is conflicting data on the existence of cystathionine-γ-lyase in glial cells: Lee et al. (2006) identified expression of the gene in mouse microglial cells by reverse transcriptase-polymerase chain reaction (RT-PCR) and a corresponding reduction in endogenous H_2S following inhibition of cystathionine-γ-lyase with DL-propargylglycine and a second inhibitor of the enzyme, beta-cyano-L-alanine. However, primary rat astroglial cultures and glioma cells have demonstrable cystathionine-γ-lyase activity (Kandil et al., 2010; Kranich et al., 1998; Vitvitsky et al., 2006) and cystathionine can be used as an alternative to cysteine as a precursor for GSH synthesis (Kranich et al., 1998).

Whilst investigations into the variable distribution and activity of cystathionine-β-synthase and cystathionine-γ-lyase are informative in regard to regional selectivity in the mechanism of H_2S production, evidence of a functional transsulfuration pathway that is geared towards production of cysteine requires co-localisation and demonstrable activity of both cystathionine-β-synthase and cystathionine-γ-lyase. For this reason and because cystathionine-β-synthase is strongly expressed in brain, support for a transsulfuration pathway in this tissue relies on a demonstration of functionally active cystathionine-γ-lyase. Consequently, the lack of consensus in the literature in regard to the expression of this enzyme in brain has prompted the view that transsulfuration has little relevance in cerebral cysteine metabolism and production of GSH (Grange et al., 1992). However, more recent data supports the view that transsulfuration plays a physiological role in GSH production in the brain and in astrocytes in particular.

GSH synthesis in astrocytes – a role for the transsulfuration pathway?

As with the vast majority of cells, the rate of GSH biosynthesis in astrocytes must be regulated to compensate for loss of the thiol due to export following oxidation to GSSG, use in conjugation

reactions, mercapturic acid production and glutathionylation. In the brain, *de novo* synthesis of GSH in astrocytes occurs in a two-step process that is catalysed by γ-glutamate cysteine ligase (γ-glutamylcysteine synthase; EC 6.3.2.2.) and GSH synthase (EC 6.3.2.3), respectively. Both of these reactions require ATP. The rate of biosynthesis is controlled by cysteine availability, activity of γ-glutamate cysteine ligase, which is feedback inhibited by GSH and the level of expression of the enzyme. Astrocytes export GSH for use by neurones (Dringen et al., 1999). Since the first work by Bannai and co-workers almost 30 years ago (Bannai, 1984), a large body of evidence has contributed to the view that cysteine for GSH is supplied to astrocytes in its oxidised form (cystine) from the extracellular medium by the electro-neutral cystine-glutamate (x_c^-) exchanger, which, on entry to the cystosol, is reduced to cysteine (Kato et al., 1993; McBean, 2002; Sato et al., 2000).

Nonetheless, several lines of evidence have challenged the traditional stance that the cystine-glutamate exchanger is the exclusive mechanism for provision of cysteine for GSH in astrocytes. In 1998, Kranich et al. observed that cystathionine could partially substitute for cysteine as precursor for synthesis of GSH in primary rat astroglial cultures, which could only happen if cystathionine-γ-lyase was active (although, oddly, methionine did not substitute for cysteine as GSH precursor in their experiments). More recently, Diwakar and Ravindranath (2007) showed depletion of GSH (38% at 6 h post administration) in mouse brain following subcutaneous application of DL-propargylglycine that was accompanied by a significant reduction in the activity of brain cystathionine-γ-lyase. Vitvitsky et al. (2006) have provided additional evidence for an active transsulfuration pathway in mouse brain slices, cortical neuronal cultures and primary astrocytes. Astrocytes and neurones displayed depletion of GSH following incubation of the cells with DL-propargylglycine, which achieved a level of 40 % of control after 10 h. Further evidence for the existence of a functional transsulfuration pathway in brain slices, astrocytes and neurones came from the observation that incubation with radiolabelled (^{35}S) methionine resulted in incorporation of the label into GSH. Once again, the only possible route for labelling of the S-atom in GSH from methionine is via transsulfuration to cysteine. Further confirmation of an active transsulfuration pathway in rat glioma cells and primary astrocytes has come from data showing a 23% reduction in GSH after 24 h incubation of the cells with DL-propargylglycine (Kandil et al., 2010). In all reports to date, the contribution of the transsulfuration pathway to GSH in brain slices, astrocytes or microglia is between 23-40%, which is less than the contribution observed in hepatic cells (approximately 50%; Beatty and Reed, 1980; Mosharov et al., 2000). Thus, it can be

concluded that under normal conditions, cysteine derived from the transsulfuration pathway is a minor, but significant, contributor to cellular GSH in brain.

Oxidative stress and the transsulfuration pathway

Oxidative stress is a positive regulator of the transsulfuration pathway. In a physiological context, oxidative stress activates cystathionine-β-synthase, thus promoting conversion of methionine to cysteine and enabling increased synthesis of GSH. This process involves endogenous reactive oxygen species-targeted proteolysis of the enzyme by a mechanism that is incompletely understood. At the transcriptional level, cystathionine-β-synthase is activated by glucocorticoids and inhibited by insulin, whereas its catalytic activity is regulated by binding of nitric oxide or carbon monoxide to the heme pocket of the enzymes (Li et al., 2009). There are conflicting reports on the response of cystathionine-β-synthase to oxidative stress in astrocytes. On the one hand, evidence has been presented that epidermal growth factor, cAMP and tumour necrosis factorα (TNFα), all cause upregulation of the enzyme via enhanced transcription (Kimura, 2010). Conversely, Lee et al. (2009), have shown that inflammatory activation of microglia and astrocytes caused induction of nuclear factor kappaB (NF-κB), release of TNFα, interleukin-6 (IL-6) and nitrite ions that all resulted in down-regulation of cystathionine-β-synthase and inhibited production of H_2S. Interestingly, H_2S production in vascular cells, which use cystathionine-γ-lyase rather than cystathionine-β-synthase, was unaffected by inflammatory agents, signifying that different regulatory pathways control the activity of these enzymes in different cell types.

There is increasing evidence that the transsulfuration pathway in astrocytes may function in a reserve capacity that can be used to supply cysteine when provision of the amino acid via the cystine-glutamate exchanger is limited, or when oxidative stress places an increased demand on GSH synthesis. Experiments have shown that flux through the transsulfuration pathway increases during oxidative stress conditions, as achieved by exposure of astrocytes to *tert*-butylhydroperoxide for 10 h, and a concomitant increase in GSH synthesis [facilitated by rapid upregulation of γ-glutamate cysteine ligase; see (Kresja et al., 2010); (Vitvitsky et al., 2006). Similarly, the expression and functional activity of cystathionine-γ-lyase increases following depletion of GSH by diethylmaleate in rat glioma cells and in primary astrocytes (Kandil et al., 2010 and Fig. 2). The same result was observed on depletion of GSH by blockade of the cystine-glutamate exchanger: in this case, the contribution of the transsulfuration pathway to GSH synthesis rose from 23% to 61 % after 24 h and to 80 % after 48 h (Kandil et al., 2010).

Further investigations have established that depletion of GSH in rat glioma cells by mild oxidative stress results in a c-Jun-*N*-terminal kinase (JNK) and p38-mitogen-activated protein kinase ($p38^{MAPK}$)-mediated increase in expression of cystathionine-γ-lyase and up-regulation of the transsulfuration pathway that promotes GSH synthesis (Kandil et al., 2010). A similar mechanism occurs in primary rat astrocytes (Kandil et al., unpublished observations). $P38^{MAPK}$ and JNK are two isoforms of 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. It is likely that GSH depletion in astrocytes triggers activation of both pathways, thus stimulating the transsulfuration pathway and channelling methionine towards production of cysteine. A similar mechanism may take place in foetal hepatocytes, as moderate oxidative stress acts as a signal to up-regulate cystathionine-γ-lyase activity in these cells during the foetal-to-neonatal transition (Martín et al., 2007). Information on the mechanism of upregulation of cystathionine-γ-lyase by GSH depletion in astrocytes requires further investigation. However, it is worth noting that in murine hepatocytes, DEM-mediated cytosolic GSH depletion was associated with inhibition of TNFα-induced NF-κB transactivation of anti-apoptotic genes (including inducible nitric oxide synthase; Matsumaru et al., 2003). It was concluded that extra-mitochondrial GSH depletion alters the thiol redox state, leading to inhibition of NF-κB transactivation of survival genes and to sustained activation of JNK, both of which contribute to sensitisation of the cells to TNF-induced apoptosis.

Functional importance of the transsulfuration pathway in astrocytes

Notwithstanding the evidence that GSH depletion in astrocytes triggers up-regulation of the transsulfuration pathway in astrocytes that enables an increased supply of cysteine, further experimentation is required to establish fully the relative importance of this pathway, compared to the cystine-glutamate exchanger, to intracellular cysteine during oxidative stress. Certainly, exposure of mouse retinal Müller cells to xanthine:xanthine oxidase for 6 h causes up-regulation of the cystine-glutamate exchanger, accompanied by enhanced sodium-independent uptake of radiolabelled glutamate (a measure of exchanger activity) by up to 89% of control (Dun et al., 2006; Mysona et al., 2009). Similarly, 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). Both of these observations might argue against increasing flux though the

transsulfuration pathway as a compensatory mechanism for augmenting GSH during oxidative stress. However, evidence suggests that up-regulation of the cystine-glutamate 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 could be provided by the transsulfuration pathway.

There may be other situations in which import of cystine via the cystine-glutamate exchanger is compromised and would therefore limit the capacity of the cell to synthesise GSH, if an alternative pathway was unavailable. 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). An excess of 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 possible that both of these situations would damage uptake of cystine by the exchanger, to the extent that astrocytes would require provision of cysteine by the transsulfuration pathway.

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 (Chung and Sontheimer, 2009; Lo et al., 2008; Savaskan et al., 2008) and has proven successful in experimental models as a means of limiting glutamate release. However, other evidence indicates that methionine uptake in gliomas is high and correlates positively with tumour viability (Kato et al., 2008), which may indicate a greater dependence on transsulfuration in these cells than in normal astrocytes. Additional research on the extent of upregulation of the transsulfuration pathway is required before one can be sure of the effectiveness of a strategy that is aimed at limiting GSH production by blockade of the cystine-glutamate exchanger.

Conclusion

In summary, research has shown that the transsulfuration pathway in astrocytes is active and may be particularly important as a compensatory process that will supply cysteine for GSH synthesis in as a response to mild or moderate oxidative stress, or when import of this amino acid by the cystine-glutamate exchanger is limited. Further experimentation is required for a full

understanding of how changes in activity of the regulatory enzymes of this pathway, cystathionine-β-synthase or cystathionine-γ-lyase, operate to maintain a balance between the fluctuating demands for H_2S, cysteine and GSH in normal astrocytes and in astrocytoma/glioblastoma cells.

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Figure legends

Fig 1. Schematic representation of the transsulfuration pathway, showing cysteine as precursor of GSH and H_2S. CBS, cystathionine-β-synthase; CTH, cystathionine-γ-lyase; CGL, glutamate cysteine ligase.

Fig 2. Increase in cystathionine-γ-lyase (CTH) expression in rat primary cortical astrocytes by DEM. Cells were treated for 0-4 h with 100 μM DEM and the level of 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 presented as a percentage of the cystathionine-γ-lyase expressed in the untreated group of n = 3 experiments. **p < 0.01, ***p < 0.001 compared to untreated group, using one-way ANOVA.

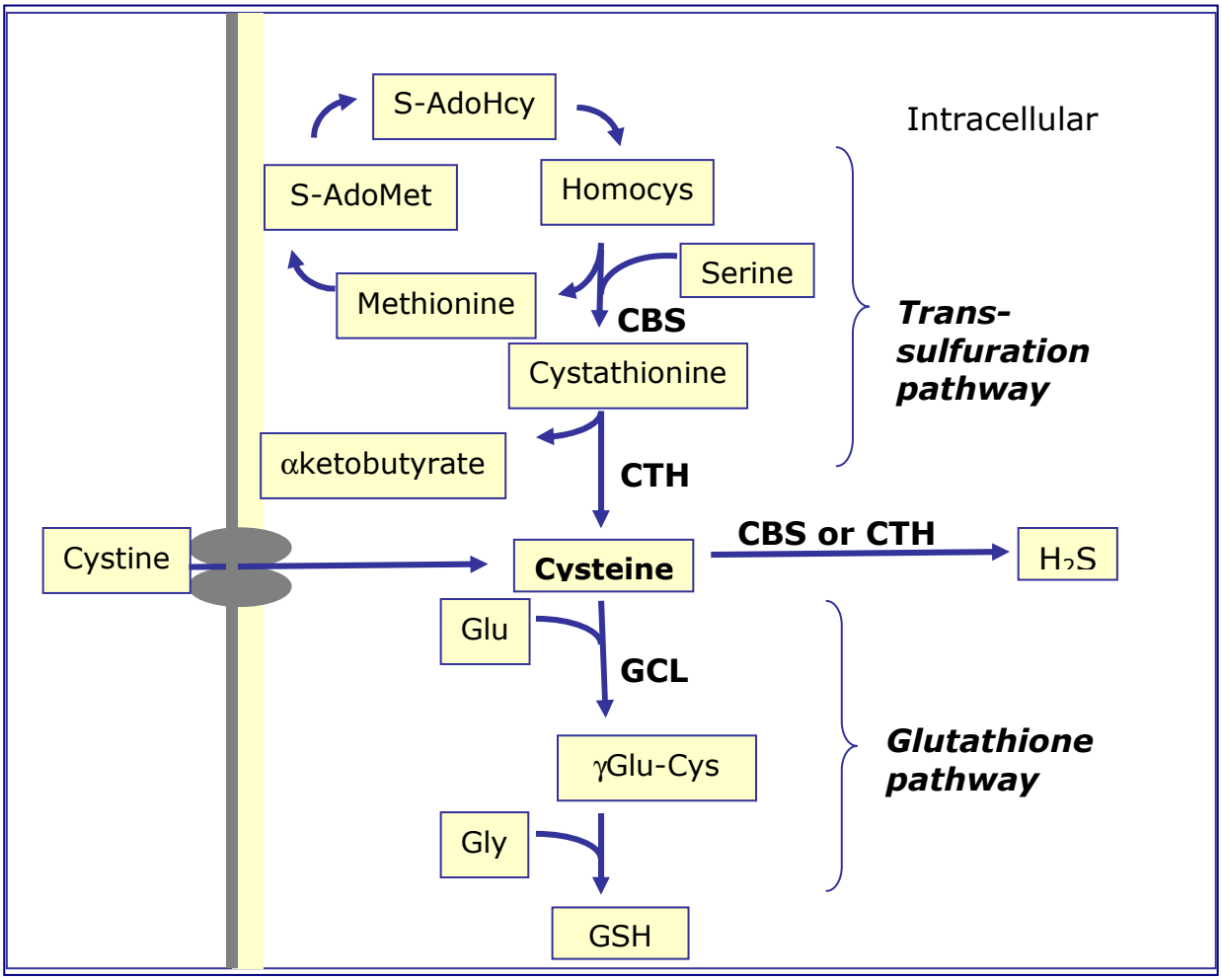
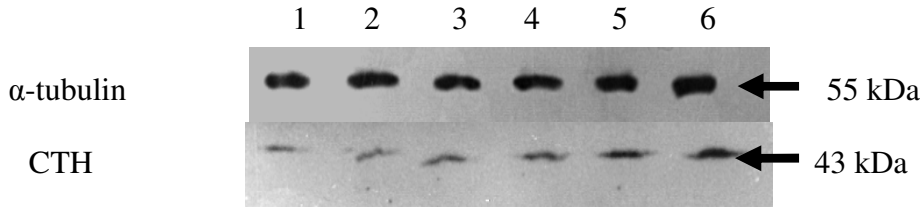
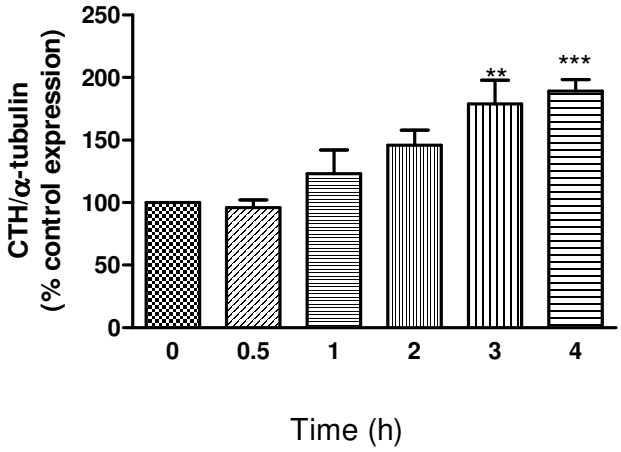


Figure 1 The transsulfuration pathway

Figure 2
(i)


1
2
3
4
5
6
α-tubulin
55 kDa
CTH
43 kDa

(ii)


CTH/α-tubulin
(% control expression)
250
200
150
100
50
0
**
***
0
0.5
1
2
3
4
Time (h)