The Quantitatively Important Relationship between Homocysteine Metabolism and Glutathione Synthesis by the Transsulfuration Pathway and Its Regulation by Redox Changes†

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ABSTRACT: Homocysteine is a key junction metabolite in methionine metabolism. It suffers two major metabolic fates: transmethylation catalyzed by methionine synthase or betaine homocysteine methyl transferase and transsulfuration catalyzed by cystathionine β-synthase leading to cystathionine. The latter is subsequently converted to cysteine, a precursor of glutathione. Studies with purified mammalian methionine synthase and cystathionine β-synthase have revealed the oxidative sensitivity of both junction enzymes, suggesting the hypothesis that redox regulation of this pathway may be physiologically significant. This hypothesis has been tested in a human hepatoma cell line in culture in which the flux of homocysteine through transsulfuration under normoxic and oxidative conditions has been examined. Addition of 100 μM H_2O_2 or tertiary butyl hydroperoxide increased cystathionine production 1.6- and 2.1-fold from 82 $\pm$ 7 μmol h^{-1} (L of cells)$^{-1}$ to 136 $\pm$ 15 and 172 $\pm$ 23 μmol h^{-1} (L of cells)$^{-1}$, respectively. The increase in homocysteine flux through the transsulfuration pathway exhibited a linear dose dependence on the concentrations of both oxidants (50−200 μM H_2O_2 and 10−200 μM tertiary butyl hydroperoxide). Furthermore, our results reveal that approximately half of the intracellular glutathione pool in human liver cells is derived from homocysteine via the transsulfuration pathway. The redox sensitivity of the transsulfuration pathway can be rationalized as an autocorrective response that leads to an increased level of glutathione synthesis in cells challenged by oxidative stress. In summary, this study demonstrates the importance of the homocysteine-dependent transsulfuration pathway in the maintenance of the intracellular glutathione pool, and the regulation of this pathway under oxidative stress conditions. Aberrations in this pathway could compromise the redox buffering capacity of cells, which may in turn be related to the pathophysiology of the different homocysteine-related diseases.

Homocysteine is a sulfur-containing amino acid that is derived from the hydrolysis of *S*-adenosylhomocysteine (AdoHCy),[1] the spent form of the major cellular methyl donor, AdoMet (Figure 1). Elevated levels of homocysteine are correlated with several different pathologies, including cardiovascular diseases (reviewed in ref *1*), neural tube defects (*2*, *3*), and Alzheimer's disease (*4*). It is estimated that up to 40% of the population at risk for cardiovascular diseases has elevated levels of homocysteine. Despite these significant statistics, the mechanism by which cells regulate homocysteine metabolism and the etiology of homocysteine-related diseases remain poorly understood. Homocysteine occupies a unique cofactor-studded metabolic junction position and performs key physiological functions. It is metabolized via either transsulfuration or transmethylation reactions. Its conversion to methionine catalyzed by the B_{12}-dependent zinc protein, methionine synthase, serves to release CH_3-H_4folate, the circulating form of the vitamin, as H_4folate. The latter is then available to support a number of folate-dependent one-carbon transfer reactions. Transmethylation catalyzed by the zinc-containing betaine homocysteine methyltransferase represents an intermediate step in the catabolism of choline, but exhibits restricted tissue distribution (*5*). Transsulfuration catalyzed by the heme and B_6-dependent cystathionine β-synthase leads to cystathionine and subsequently to cysteine and glutathione biosyntheses in addition to providing a catabolic route leading to sulfate. The transsulfuration reaction thus provides a direct link between homocysteine and glutathione, the major redox buffer in mammalian cells. It is therefore not surprising that a number of enzymes at this metabolic nexus display sensitivity to redox changes.

The two isoforms of methionine adenosyl transferase (MATI and -III) found in hepatic cells are responsive to oxidative stress (*6*, *7*). Recent studies on purified mammalian methionine synthase and cystathionine β-synthase have revealed reciprocal sensitivity of the two major homocysteine utilizing enzymes to oxidative conditions (*8*, *9*). Thus, the activity of methionine synthase is reduced under oxidizing

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[1] Abbreviations: AdoHCy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; CH_3-H_4folate, 5-methyltetrahydrofolate; H_4folate, tetrahydrofolate; FBS, fetal bovine serum; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; tBuOOH, tertiary butyl hydroperoxide; H_2O_2, hydrogen peroxide; DTT, dithiothreitol.

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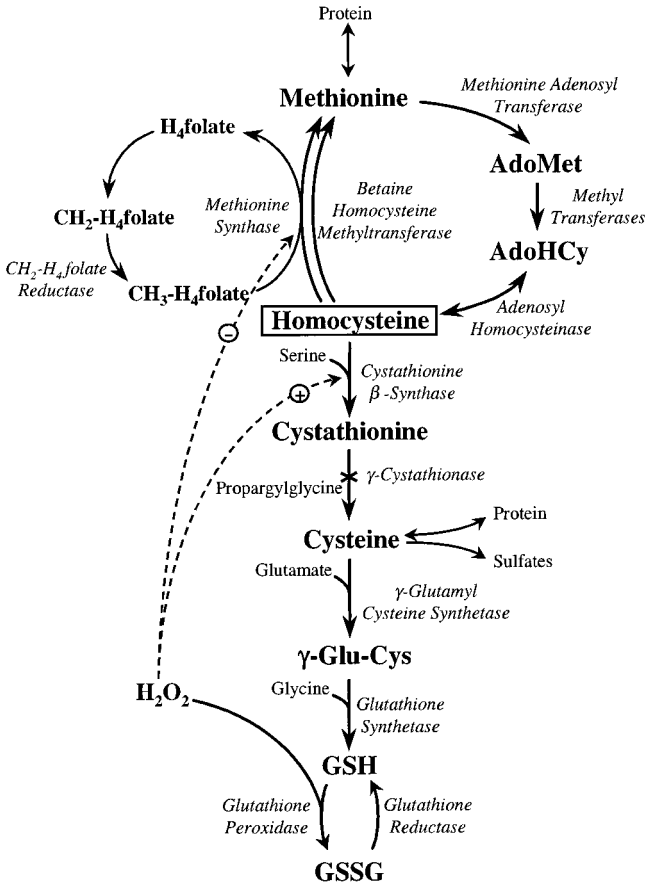

FIGURE 1: Intracellular metabolism of homocysteine. The postulated role of peroxides at this junction is indicated by the dashed lines. GSH and GSSG are glutathione and glutathione disulfide, respectively.

conditions most likely due to the lability of the reactive cofactor intermediate, cob(I)alamin (*8*), whereas the activity of the heme-containing cystathionine β-synthase is enhanced under the same conditions (*9*).

These in vitro studies led us to posit that redox changes may serve a regulatory role under physiological conditions whereby oxidative stress would increase the flux of homocysteine through the transsulfuration pathway. The immediate consequence of this would be an increase in cystathionine levels that could lead to an increase in the concentrations of the downstream metabolites, cysteine and glutathione. Such a regulatory switch could be rationalized as representing a self-correcting response to depleted glutathione levels in cells faced with an oxidative challenge. To test this hypothesis, we have employed a human hepatoma cell line, HepG2, to examine whether the sizes of metabolite pools in this pathway change when cells are exposed to normoxic versus oxidative stress conditions. Our results furnish a quantitative estimate of homocysteine flux through the transsulfuration pathway in human liver cells in culture, and provide evidence for the sensitivity of this pathway to oxidative stress that leads to enhanced cystathionine and glutathione synthesis. Furthermore, they reveal the key role played by homocysteine in the maintenance of the cellular glutathione pool.

MATERIALS AND METHODS

Materials. Minimal essential medium Eagle, H_2O_2, tBuOOH, D,L-propargylglycine, γ-glutamylcysteine, *N*-acetyl-L-cysteine, Hepes, metaphosphoric acid, iodoacetic acid, 2,3-dinitrofluorobenzene, phenylmethanesulfonyl fluoride, Nonidet P-40, disodium EDTA, DTT, leupeptin, pepstatin, and aprotinin were purchased from Sigma. [^{35}S]Methionine (151 Ci/mol) and [U-^{14}C]serine (1000 Ci/mmol) were from Amersham Bioscience. Gentamycin and trypsin were from Gibco BRL. FBS was from HyClone Co. HepG2 cells were from ATCC.

Cell Culturing. Cells were grown in 60 mm × 15 mm tissue culture dishes in minimal essential medium with 10% FBS, 2 mM L-glutamine, 2.2 g/L sodium bicarbonate, and 50 mg/mL gentamycin until they were 60−80% confluent. The culture medium was changed every 4 days and, for the last time, 3 h before the start of the experiment. Cells were preincubated for 1 h with 2.5 mM propargylglycine, when needed. Then, 25−200 μM H_2O_2, 25−200 μM tBuOOH, 1 mM γ-glutamylcysteine, or 1 mM *N*-acetyl-L-cysteine was added. In experiments in which incorporation of radioactive serine into cystathionine was monitored, [U-^{14}C]serine was diluted with cold serine to give a 0.5 M solution with a specific activity of 27 mCi/mol. Radioactivity (110 nCi) was added to the culture medium (4 mL) at the same time as tBuOOH. The final concentration of serine in the culture medium was 1 mM. For experiments in which the incorporation of radioactive methionine into glutathione was monitored, [^{35}S]methionine was diluted with PBS to give a 1.25 μM solution with a specific activity of 125 Ci/mmol. Radioactivity (5 μCi) was added to the medium (4 mL) 30 min prior to tBuOOH addition. The final concentration of methionine in the medium was 100 μM. Since the volume of the additions did not exceed 5% of that of the culture medium, the change in concentrations of medium components was negligible in all cases.

Sample Collection. Culture dishes were placed on ice in a dark room, and the medium was removed by aspiration prior to harvesting. The cells were washed twice with ice-cold PBS and collected by scraping. The cells were divided into two portions, and the first (50 μL) was centrifuged (10000*g* for 2 min). The supernatant (40 μL) was discarded, and 90 μL of lysing buffer [20 mM Hepes (pH 7.4) containing 25 mM potassium chloride, 1 mM phenylmethanesulfonyl fluoride, and 0.5% Nonidet P-40] was added. The suspension was mixed well and placed on ice for 10 min. Following centrifugation (10000*g* for 5 min), the protein concentration in the supernatant was determined using the Bradford reagent (Bio-Rad) with BSA as the standard and represents the concentration of total intracellular protein.

The second aliquot of cells was deproteinized by mixing with an equal amount of fixing solution (16.8 g/L metaphosphoric acid, 5 M sodium chloride, and 5 mM disodium EDTA). After incubation at room temperature for 10 min, the mixture was centrifuged (10000*g* for 3 min), and the supernatant was used immediately for thiol determination.

Measurement of Intracellular Thiol Concentrations. The concentration of thiols in deproteinized cell extracts was measured as described previously (*10*). Briefly, 50 μL of the deproteinized cell extract was mixed with 2 μL of a saturated potassium carbonate solution and 4 μmol of iodoacetic acid. After 1 h in the dark at room temperature, 57 μL of an alcoholic solution of 2,3-dinitrofluorobenzene (1.5% v/v in absolute ethanol) was added and the reaction was allowed to proceed for 4 h in the dark at room

temperature. *N*-Dinitrophenyl derivatives of cystathionine, cysteine, glutathione, and glutathione disulfide were separated by HPLC on a Phenomenex Bondclone NH_2 column (300 mm × 3.9 mm, 10 μm) at a flow rate of 1 mL/min. Solvent A was a 4:1 methanol/water mixture, and solvent B was prepared as follows: 272 g of sodium acetate trihydrate, 122 mL of water, and 378 mL of glacial acetic acid were mixed, and 100 mL of the resulting solution was added to 400 mL of solvent A. The elution conditions were as follows: from 0 to 10 min, isocratic 30% solvent B; from 10 to 30 min, linear gradient from 30 to 100% solvent B. Prior to each injection, the column was equilibrated with 30% solvent B for 10 min. Elution of peaks was monitored by their absorbance at 355 nm. The concentration of individual thiols was determined by comparing integrated peak areas with previously generated calibration curves for each compound. To measure the level of incorporation of the radioactive label into the metabolites of interest, the fractions containing cystathionine and glutathione were collected, and the radioactivity was measured by scintillation counting and normalized for the protein concentration in the sample.

To represent the thiol concentrations in units of micromoles per liter of cells, the amount of protein in 1 L of HepG2 cells was estimated. The cell suspension was divided into two fractions. The cells in one fraction were lysed by repeated freeze−thaw cycles, and the protein concentration was determined. The second fraction of cells was placed in a glass capillary fused at one end and centrifuged at 2500*g* for 10 min. The ratio of the cell pellet volume to the total cell suspension volume was estimated by determining the relative heights of the pellet versus that of the total sample. From this analysis, HepG2 cells were estimated to have 53 g of protein per liter of cells.

Analysis of Cystathionine β-Synthase and Methionine Synthase Levels by Western Analysis. For Western analysis, cell cultures were treated with 200 μM tBuOOH for 6 h, after which the medium was removed by aspiration and the cells were washed with 2 × 3 mL of ice-cold PBS. Lysis buffer (containing 0.15 M NaCl, 30 mM Tris, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 10 μg/mL leupeptin, 2 μg/mL pepstatin, 1.9 μg/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride) was added to cover the cells which were incubated on ice for 30 min. Cell membranes were separated by centrifugation at 14000*g* for 10 min at 4 °C, and the supernatant was used for Western analysis. Cell extracts (80 μg) from control and tBuOOH-treated samples were loaded in each lane and separated on a 7.5% (for methionine synthase) or 10% (for cystathionine β-synthase) polyacrylamide gel under denaturing conditions. The proteins were transferred to a PVDF membrane by electroblotting and detected by Western analysis. Polyclonal antibodies against cystathionine β-synthase and methionine synthase, available in our laboratory, were employed for detection of these proteins. The secondary antibody, anti-rabbit IgG/alkaline phosphatase conjugate (Sigma), was diluted 1:6250, and chemiluminescent detection was performed using the Immun-Star substrate (Bio-Rad).

RESULTS AND DISCUSSION

Studies on the isolated mammalian methionine synthase and cystathionine β-synthase have revealed the redox sensitivity of both enzymes (*8*, *9*). These observations led us to hypothesize that changes in the ambient redox potential could result in changes in the flux of homocysteine between the two competing pathways (*9*). Thus, under oxidizing conditions, increased cystathionine β-synthase activity would be predicted, leading to elevated cystathionine levels, which could in turn lead to elevated glutathione levels. The outcome of such a regulatory switch would be a compensatory increase in the glutathione pool diminished in response to an oxidative insult. In this study, we have tested this hypothesis by measuring changes in the concentration of cystathionine and glutathione in hepatoma cells exposed to oxidative stress conditions.

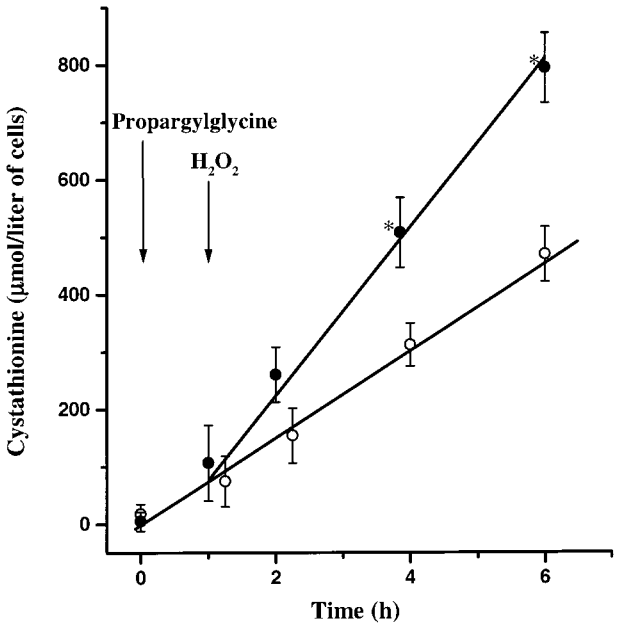

FIGURE 2: Kinetics of intracellular cystathionine accumulation in hepatocytes exposed to 2.5 mM propargylglycine with (●) and without (○) 100 μM H_2O_2. Arrows indicate the times of propargylglycine and H_2O_2 addition to the medium. The observed rates of cystathionine synthesis were 83 ± 7 and 136 ± 15 μmol h^{-1} (L of cells)$^{-1}$ in the absence and presence of H_2O_2, respectively. The results represent the means ± the standard deviation of five experiments. Asterisks indicate that the means are significantly different from control values at $p = 0.005$, as determined by the ANOVA test ($n = 4$).

The Level of Cystathionine Synthesis Is Increased under Oxidative Stress Conditions. Since the intracellular cystathionine concentration is low, it is difficult to directly monitor changes in the cystathionine pool without inhibiting the downstream pathway enzyme, γ-cystathionase, with propargylglycine (Figure 1). The latter is a suicide inhibitor (*11*) that is highly specific for γ-cystathionase at the concentrations employed (*12*). Under these conditions, further metabolism of cystathionine is averted, permitting evaluation of (i) the relative flux of homocysteine through cystathionine β-synthase in the presence and absence of peroxide and (ii) the quantitative repercussions of thwarting cystathionine entry to the cysteine and thereby glutathione pools.

Addition of propargylglycine to the culture medium results in a linear time-dependent increase in the concentration of cystathionine (Figure 2, lower trace), permitting estimation of the steady-state flux of homocysteine through cystathionine β-synthase. Under these conditions, the rate of cystathionine production is 82 ± 7 μmol h^{-1} (L of cells)$^{-1}$.

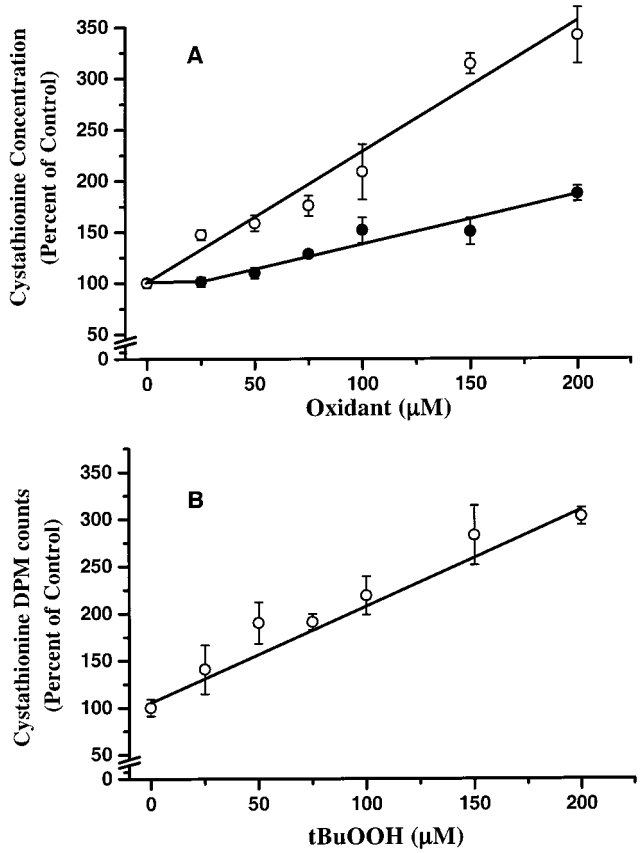

FIGURE 3: Effect of varying concentrations of H_2O_2 (●) and tBuOOH (○) on hepatic cystathionine levels measured by (A) integration of peak areas in chromatographic traces and (B) by radioactivity eluting in the cystathionine fraction (see Materials and Methods). Confluent HepG2 cells were incubated for 6 h in a medium containing 2.5 mM propargylglycine, 1 mM [U-^{14}C]serine, and the indicated concentrations of peroxides. Results are the means ± the standard deviation of seven experiments.

H_2O_2 is a well-studied oxidant, whose addition to the cell culture medium induces oxidative stress response genes (*13*). In culture, response to oxidative stress has been observed within 30−60 min in cells exposed to 100−200 μM peroxide (*13*, *14*). To test the effect of oxidative stress on cystathionine formation, cells treated with propargylglycine were exposed to 100 μM H_2O_2. At this H_2O_2 concentration, the flux of homocysteine through the transsulfuration pathway increased 1.6-fold compared with that of the untreated control to 136 ± 15 μmol h^{-1} (L of cells)$^{-1}$ (Figure 2, upper trace). This is very similar to the 1.7-fold increase in cystathionine β-synthase activity observed under in vitro conditions (*9*). The dose dependence of cystathionine concentration on H_2O_2 is shown in Figure 3A (lower trace). A linear increase in the rate of cystathionine formation was observed at concentrations of >50 μM H_2O_2.

The effect of another oxidant, tBuOOH, on homocysteine flux through the transsulfuration pathway was also examined. tBuOOH had a similar albeit slightly larger effect on the size of the cystathionine pool compared to H_2O_2 (Figure 3A). Addition of 100 μM tBuOOH increased the flux of homocysteine through the transsulfuration pathway 2.1-fold to 172 ± 23 μmol h^{-1} (L of cells)$^{-1}$. Unlike H_2O_2, the increased production of cystathionine was linearly dependent on tBuOOH at all concentrations that were examined. The throughput of homocysteine through the transsulfuration

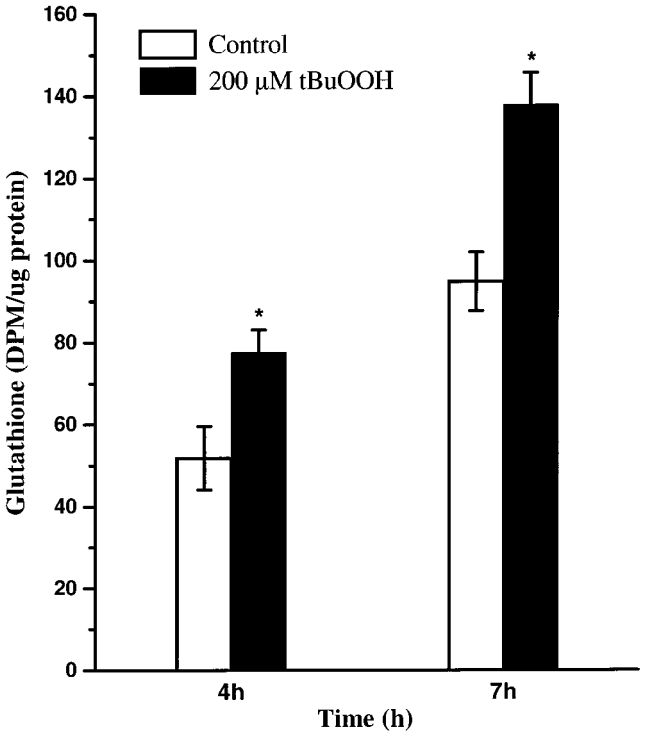

FIGURE 4: Incorporation of [^{35}S]methionine into the glutathione pool in hepatocytes exposed to oxidative challenge. HepG2 cells were incubated in the absence (white bars) and presence (black bars) of 200 μM tBuOOH and harvested at different time points. A 1.5- and 1.45-fold increase in the amount of radioactivity was observed after 4 and 7 h, respectively, in the tBuOOH-treated samples vs controls. The results represent the means ± the standard deviation of four experiments. Asterisks indicate that the means are significantly different from control values at the $p = 0.01$ level, as determined by the ANOVA test ($n = 5$).

reaction was also monitored by measuring the level of incorporation of [^{14}C]serine into cystathionine in the presence of propargylglycine. Serine is the second substrate for cystathionine β-synthase, and the increase in the level of radioactive serine incorporation into cystathionine (Figure 3B) paralleled the increase in the cystathionine pool estimated by integration of the peak area in a chromatographic trace (Figure 3A).

Glutathione Synthesis Is Increased in Response to Oxidative Stress Conditions. While the studies described above indicate that oxidative conditions enhance transsulfuration, they suffer from having been conducted in the presence of the inhibitor, propargylglycine, which could in principle have affected the metabolic response that was being studied. To address this potential limitation, the effect of tBuOOH was examined in the absence of propargylglycine by measuring the level of incorporation of [^{35}S]methionine into the glutathione pool. [^{35}S]Methionine enters glutathione synthesis only via homocysteine and the transsulfuration reaction (Figure 1). This is confirmed by the lack of incorporation of radiolabel from [^{35}S]methionine into glutathione in the presence of propargylglycine (data not shown). As reported previously (*15*), ~90% of the radiolabel from [^{35}S]methionine is associated with proteins at any given time, with the remainder being present in cytosolic metabolites. An ~1.5-fold increase in the level of incorporation of radioactivity into the glutathione pool was observed in cells exposed to 200 μM tBuOOH compared to untreated controls (Figure

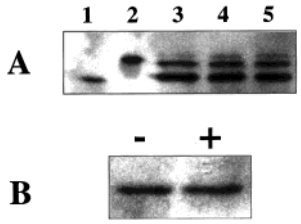

FIGURE 5: Detection of cystathionine β-synthase and methionine synthase levels in HepG2 cells exposed to tBuOOH and in untreated cells. Cell extracts were prepared as described in Materials and Methods. To ensure equal loading in the lanes, the level of methylmalonyl-CoA mutase was detected by Western analysis (not shown). Representative blots are shown here; the Western analysis was performed on eight independent samples and controls for cystathionine β-synthase and methionine synthase, respectively. (A) Analysis of cystathionine β-synthase. Lanes 1 and 2 contained purified recombinant truncated and full-length forms of cystathionine β-synthase; lane 3 contained the control (untreated cells), and lanes 4 and 5 contained extracts from cells treated with 100 and 200 μM tBuOOH, respectively. The full-length recombinant enzyme has an additional 11 amino acids at the N-terminus, and is slightly larger than the wild-type enzyme (*9*). (B) Detection of methionine synthase levels in extracts from untreated (−) and tBuOOH (200 μM)-treated cells (+).

4). This is lower than the ~3-fold increase in cystathionine concentration that is elicited by 200 μM tBuOOH in the presence of propargylglycine (Figure 3). This could be explained by the fact that incorporation of homocysteine into the glutathione pool requires three additional steps beyond formation of cystathionine, and the increase in concentration of the downstream metabolite, glutathione, lags behind the increase observed for cystathionine. Alternatively, the net outcome of the increased flux through the transsulfuration pathway under the observed conditions may simply be an ~1.5-fold increase in the glutathione pool.[2]

The observed increase in the level of synthesis of glutathione from methionine under oxidative stress conditions in the absence of propargylglycine validates the results obtained in the presence of the inhibitor that reveal an increased level of synthesis of cystathionine under the same conditions. Together, these data provide strong support for the hypothesis that redox regulation is important in modulating the flux of homocysteine through the transsulfuration pathway.

Levels of Cystathionine β-Synthase and Methionine Synthase Do Not Change in Response to tBuOOH Treatment. Increased homocysteine flux through the transsulfuration pathway could, in principle, result from an increase in the levels of methionine adenosyl transferase and/or cystathionine β-synthase or a decrease in methionine synthase activity. To determine whether the oxidizing conditions used in our studies affected the expression level of the junction enzymes, methionine synthase and cystathionine β-synthase, Western analysis of cell extracts was performed. As shown in Figure 5, there is no detectable difference in the expression levels of methionine synthase or cystathionine β-synthase in the presence of tBuOOH as compared to those of the untreated controls.

Western analysis using polyclonal antibodies to cystathionine β-synthase routinely revealed the presence of two bands that correspond to the full-length and truncated forms of the enzyme which exist in the tetrameric and dimeric states, respectively (*16*). A hypersensitive site results in the facile cleavage of the full-length enzyme by proteases in vitro (*17*), and during the lengthy purification of the wild-type enzyme (*18*), but has not been observed during purification of the recombinant human enzyme expressed in *Escherichia coli* (*9*).

The results from the Western analysis indicate that redox regulation of the transsulfuration pathway is not exerted at the protein expression level of the junction enzymes, cystathionine β-synthase and methionine synthase. The increased flux through the transsulfuration pathway could result instead from changes in enzyme activity. Since the equilibrium midpoint potential of the heme bound to cystathionine β-synthase has not been determined, it is not known whether the enzyme exists in the ferric or ferrous form at the ambient redox potential inside the cell. However, characterization of the enzyme under in vitro conditions indicates that a 1.7-fold increase in the level of cystathionine formation is correlated with oxidation of ferrous to ferric heme in cystathionine β-synthase (*9*). This is lower than the 3-fold increase that is observed with 200 μM tBuOOH in this study and indicates that the observed response of the transsulfuration reaction to oxidative stress cannot be accounted for simply by potential oxidation state changes of the heme leading to activation of cystathionine β-synthase. The individual or combined changes in the activities of the other redox-sensitive enzymes in this pathway such as a decrease in the level of methionine synthase or an increase in the level of methionine adenosyl transferase would result in an increase in homocysteine concentration (Figure 1). This in turn would increase the extent of conversion of homocysteine to cystathionine since cystathionine β-synthase has a high K_m for homocysteine [5 mM (*9*)] and will be sensitive to changes in the concentration of this substrate. The rate-limiting enzyme in glutathione biosynthesis is γ-glutamylcysteine synthetase, which is upregulated at a transcriptional level under oxidative stress conditions (*19*). Increased flux through the transsulfuration pathway under these conditions would result in an increased availability of cysteine, the limiting substrate for this enzyme.

Homocysteine Plays a Major Role in the Maintenance of the Intracellular Glutathione Pool. In liver cells, there are two sources of intracellular cysteine that can support glutathione synthesis. The first is through import of the amino acid present extracellularly, and the second is via the homocysteine-dependent transsulfuration pathway. To examine the relative importance of the transsulfuration pathway for the maintenance of the intracellular glutathione pool, the concentrations of cysteine and glutathione were determined in the absence and presence of propargylglyine (Table 1). Inhibition of γ-cystathionase leads to a decrease in the intracellular cysteine concentration. After incubation with propargylglycine for 25 h, the cysteine concentration was 65 ± 13 μmol (L of cells)$^{-1}$ as compared to 160 ± 20 μmol (L of cells)$^{-1}$ in untreated controls. Diminution of the cysteine pool was paralleled by depletion of the glutathione pool whose concentration decreased from 6.2 to 2.6 mM (Figure 6). The glutathione to glutathione disulfide ratio,

[2] The total concentration of glutathione increased 117% ($n = 11$) after 7 h in cells treated with 200 μM tBuOOH as compared to that in untreated controls ($n = 15$). The means are significantly different at the $p = 0.001$ level, as determined by the one-way ANOVA test.

Table 1: Concentration of Metabolites in HepG2 Cells with and without Inhibition of γ-Cystathionase

metabolite	concentration	
	untreated cells	propargylglycine-treated cells[a]
cysteine	160 ± 20 μM	65 ± 13 μM
glutathione	6.2 ± 0.4 mM	2.6 ± 0.3 mM
glutathione disulfide	123 ± 11 μM	76 ± 11 μM
glutathione to glutathione disulfide ratio	51	35

[a] The concentration of metabolites reported here was measured 25 h after addition of 2.5 mM propargylglycine.

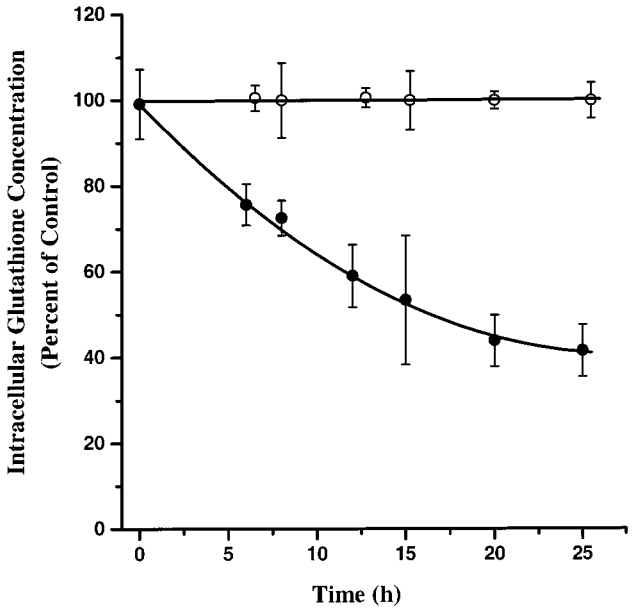

FIGURE 6: Effect of γ-cystathionase inhibition by propargylglycine on intracellular glutathione concentrations. Confluent HepG2 cells were exposed to medium lacking (○) or containing (●) 2.5 mM propargylglycine. The values represent the means ± the standard deviation of four independent experiments.

which is an indicator of the redox status of cells, was decreased 1.5-fold in the presence of propargylglycine as compared to control cells after 25 h (Table 1).

In principle, the observed decrease in the level of glutathione synthesis could result from inhibition by propargylglycine of γ-glutamylcysteine synthetase or glutathione synthetase (Figure 1). Blockage at γ-cystathionase but not at the other two enzymes could be bypassed by provision of glutathionine precursors such as *N*-acetylcysteine or γ-glutamylcysteine. Both compounds lead to restoration of glutathione levels to control values in the presence of propargylglycine (data not shown). These data rule out the possibility that the observed depletion of glutathione was due to a blockage in any of the enzymes downstream of γ-cystathionase in the pathway. Instead, these data indicate that homocysteine metabolism plays a major and quantitatively significant role in the maintenance of the intracellular glutathione pool in human liver cells. Approximately half of the glutathione is derived via homocysteine metabolism, whereas the remainder is presumably derived from cysteine released during protein turnover or imported from the extracellular medium. The concentration of cysteine in the medium was 100 μM and approximates the level found in plasma.

The importance of the transsulfuration pathway to glutathione biosynthesis was first described 20 years ago by Reed and co-workers (*20*). They observed that treatment of rat hepatocytes with 1 mM propargylglycine for 2 h resulted in 35% depletion of the glutathione pool compared to untreated controls. In addition, they reported that either homocysteine or methionine could support the synthesis of glutathione when added as the sole sulfur source to hepatocytes depleted of glutathione with diethyl maleate. Our results with human liver cells in culture are very similar with respect to the proportion of the glutathione pool that is derived from homocysteine.

Relevance to the Pathophysiology of Transsulfuration Defects. These data have potentially important implications to pathologies in which the transsulfuration pathway is disturbed. Functional deficiency of cystathionine β-synthase is accompanied by pleiotropic clinical and pathological abnormalities (*21*). One of the clinical manifestations is dislocation of the optic lens. It has been hypothesized that disruption of protein disulfides by elevated concentrations of homocysteine may be involved in the etiology of lens dislocation (*22*). Our results imply that impaired cystathionine β-synthase may affect this process both by causing homocysteine levels to rise, which leads to protein damage, and by causing glutathione levels to fall, compromising glutathione-dependent repair of mixed disulfides (*23*). Characterization of the transsulfuration pathway in the lens and surrounding tissues would therefore be of interest.

Whereas impaired cystathionine β-synthase leads to elevated homocysteine levels and is expected to perturb glutathione synthesis, γ-cystathionase deficiency is predicted to affect only glutathione synthesis. In contrast to cystathionine β-synthase defects, γ-cystathionase deficiency is likely to be a benign disorder (*21*). The transsulfuration pathway does not exist in all cell types (*5*, *24*). Our results predict that impaired γ-cystathionase would affect the glutathione pool in tissues where the transsulfuration pathway operates. The susceptibility of these tissues (viz., liver and kidney) to oxidative stress or to xenobiotics that are detoxified via conjugation to glutathione may be enhanced by cystathionine β-synthase or γ-cystathionase deficiency.

In summary, our studies provide evidence for an additional and previously unknown level of regulation of the transsulfuration pathway involved in homocysteine metabolism and establish the importance of this reaction sequelae to the maintenance of the glutathione pool in human liver cells. Our studies predict that glutathione metabolism would be perturbed by aberrations in the transsulfuration pathway which may lead to compromised resilience to oxidative and xenobiotic challenges. The 2−3-fold enhancement of homocysteine flux through transsulfuration in response to oxidative stress, albeit modest, could be physiolgically significant. A housekeeping enzyme such as cystathionine β-synthase is unlikely to undergo many-fold changes in activity to effect changes in metabolite fluxes. A recent global analysis of the gene expression profile of aging revealed that the magnitude of change for most genes involved in energy metabolism and biosynthesis is ~2-fold, thus demonstrating that subtle changes in enzyme activity may have profound consequences physiologically (*25*).

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