Research article

# Redox regulation of homocysteine-dependent glutathione synthesis

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In certain tissues, glutathione biosynthesis is connected to methionine metabolism via the trans-sulfuration pathway. The latter condenses homocysteine and serine to cystathionine in a reaction catalyzed by cystathionine β-synthase followed by cleavage of cystathionine to cysteine and α-ketoglutarate by γ-cystathionase. Cysteine is the limiting amino acid in glutathione biosynthesis, and studies in our laboratory have shown that approximately 50% of the cysteine in glutathione is derived from homocysteine in human liver cells. In this study, we have examined the effect of pro- and antioxidants on the flux of homocysteine through the trans-sulfuration pathway in the human hepatoma cell line, HepG2. Our studies reveal that pyrrolidine dithiocarbamate and butylated hydroxyanisole enhance the flux of homocysteine through the trans-sulfuration pathway as has been observed previously with the pro-oxidants, $H_2O_2$ and tertiary butyl hydroperoxide. In contrast, antioxidants such as catalase, superoxide dismutase and a water-soluble derivative of vitamin E elicit the opposite effect and result in diminished flux of homocysteine through the trans-sulfuration pathway. These studies provide the first evidence for the reciprocal sensitivity of the trans-sulfuration pathway to pro- and antioxidants, and demonstrate that the upstream half of the glutathione biosynthetic pathway (*i.e.* leading to cysteine biosynthesis) is redox sensitive as is the regulation of the well-studied enzymes in the downstream half (leading from cysteine to glutathione), namely, γ-glutamyl-cysteine ligase and glutathione synthetase.

## INTRODUCTION

In aerobic organisms, a delicate balance exists between oxygen-dependent metabolism and the generation of reactive oxygen species as side products. Glutathione, a thiol-containing tripeptide plays a key role in intracellular defense, and decreased levels of this antioxidant are correlated with the increased frequency of reactive oxygen species-mediated mitochondrial damage[1–3] and apoptosis.[4] The intracellular glutathione pools are large and vary from 1–10 mM depending on the cell type.[5–7] The final two steps in the biosynthesis of glutathione are catalyzed by γ-glutamyl-cysteine ligase and glutathione synthetase, enzymes that are believed to be present ubiquitously (Fig. 1). However, in tissues that have the largest concentrations of glutathione, and, therefore, the highest demand for cysteine (the limiting amino acid), the trans-sulfuration pathway is present (Fig. 1). The latter presents an avenue for the conversion of the essential amino acid, methionine, to cysteine. Thus, the trans-sulfuration pathway catalyzes the sequential conversion of homocysteine, a key junction metabolite in the methionine cycle, to cystathionine and then cysteine.

Studies on the effect of pro-oxidants on glutathione biosynthesis have focused overwhelmingly on the last two enzymes in the biosynthetic pathway.[8–13] A number of conditions and agents elicit the transcriptional activation of γ-glutamyl-cysteine ligase and some also activate glutathione

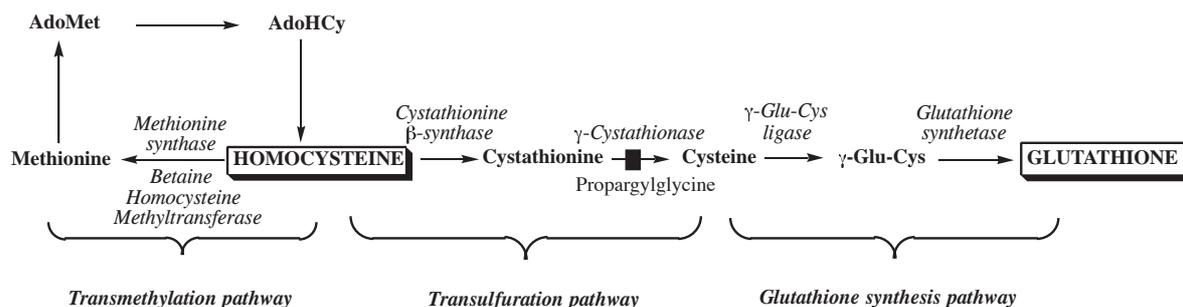*Abbreviations*: ARE, antioxidant response element; NF-κB, nuclear factor kappa B; AP, activator protein; t-BuOOH, tertiary butyl hydroperoxide; PDTC, pyrrolidine dithiocarbamate; BHA, butylated hydroxyanisole; MEM, Eagle's minimal essential medium; PBS, phosphate buffered saline; FBS, fetal bovine serum; SOD, superoxide dismutase

**Fig. 1**. Metabolic pathway for glutathione biosynthesis in liver cells showing the upstream trans-sulfuration and the downstream glutathione synthesis reactions. AdoMet and AdoHcy denote S-adenosylmethionine and S-adenosylhomocysteine respectively.

synthetase. Three *cis*-acting elements that may be important in mediating this response have been identified in the gene encoding the heavy subunit of γ-glutamyl-cysteine ligase, which harbors the catalytic activity.[14–16] These are the antioxidant response element (ARE-4), nuclear factor kappa B (NF-κB) and activator protein (AP-1). The promoter for the light subunit gene of γ-glutamyl-cysteine ligase also contains ARE and AP-1 elements.

In contrast, the responsiveness of the trans-sulfuration pathway enzymes to changes in redox status brought on by pro- or antioxidants has received scant attention. This stems largely from the lack of recognition of the importance of this pathway in the regulation of redox homeostasis. We have recently demonstrated that the trans-sulfuration pathway plays a quantitatively major role in the maintenance of the intracellular glutathione pool in transformed human liver cells in culture.[17] The two homocysteine junction enzymes, methionine synthase and cystathionine β-synthase, display reciprocal sensitivity to ambient redox tone under *in vitro* conditions.[18,19] These observations led us to hypothesize that redox changes may exert an additional layer of regulation at this metabolic junction. The rationale for such a regulation, if it existed, could be that increased flux of homocysteine through the trans-sulfuration pathway under oxidizing conditions would lead to enhanced synthesis of glutathione and thus serve as an autocorrective response to an oxidative insult. Under the opposite set of conditions, homocysteine would be re-directed preferentially to the transmethylation pathway leading to its recycling.

In a recent study using the human hepatoma cell line, HepG2, we demonstrated that both $H_2O_2$ and tertiary butyl hydroperoxide (t-BuOOH) elicit a dose-dependent increase in cystathionine production.[17] This was accompanied by a corresponding increase in incorporation of radioactivity from [$^{35}$S]-methionine into the glutathione pool. In this study, we have compared the effect of pro-oxidants ($H_2O_2$ and t-BuOOH) and substances that are considered to be antioxidants (pyrrolidine dithiocarbamate [PDTC], butylated hydroxyanisole [BHA], vitamin E, catalase, ascorbate and superoxide dismutase [SOD]) on

the flux of homocysteine through the trans-sulfuration pathway and on glutathione levels in HepG2 cells. We found that catalase, a water-soluble form of vitamin E, and SOD elicit a small, but significant, reduction in the intracellular glutathione concentration. Furthermore, the decrease in the glutathione pool size is paralleled by a decrease in the flux of homocysteine through the trans-sulfuration pathway. In contrast, PDTC and BHA increase flux of homocysteine through the trans-sulfuration pathway. These studies provide the first experimental evidence for reciprocal sensitivity of cystathionine β-synthase activity to pro- and antioxidants in a model cell culture system.

MATERIALS AND METHODS

*Reagents and cell lines*

Eagle's minimum essential medium, DL-propargyl-glycine, $H_2O_2$, t-BuOOH, PDTC, BHA, vitamin E (± α-tocopherol), superoxide dismutase, catalase, ascorbate, thymol and trypan blue were purchased from Sigma. The water-soluble analog of vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich. The antibiotic/antimycotic solution was from Gibco BRL. Fetal bovine serum (FBS) was from HyClone Company. Transformed human hepatocytes (HepG2) were from ATCC. [$^{35}$S]-Methionine (>1000 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

*Cell culture*

Cells were grown until they reached 70–80% confluency in 60 x 15 mm Falcon 3004 tissue culture dishes in MEM with 10% FBS, 2 mM L-glutamine, 2.2 g/l sodium bicarbonate and 10 ml/l antibiotic/antimycotic solution. Each dish contained 4 ml of medium, which was changed every 4 days and 2 h before the start of the

experiment. Experiments were initiated by adding the desired reagents to the dishes. [$^{35}$S]-Methionine solution was diluted in phosphate buffered saline (PBS) to give a specific activity of 1.25 µCi/µl and 1 µl of this solution was added per ml of cell culture medium to each dish. At the desired time intervals, cells were harvested and the protein concentration in samples was determined as described earlier.[17] In experiments in which 1 mM H$_2$O$_2$ was used, cell viability was measured using the trypan blue exclusion method. Briefly, H$_2$O$_2$ was added to a concentration of 1 mM to HepG2 cells at 70–80% confluency and viability was determined after 2, 4, 10, and 20 h. The medium (4 ml) was removed and 0.5 ml of 0.4% trypan blue solution in 0.5 ml PBS was added. After 2 min, the cells were washed with 2 × 1 ml PBS, covered with 2 ml PBS and the fraction of (dead) blue cells in a view area was determined. The average value from four view areas was calculated.

*Metabolite analysis*

Concentrations of glutathione and cystathionine in cell extracts were estimated using an HPLC method as described previously.[17] The concentration of individual metabolites was determined by comparing integrated peak areas with previously generated calibration curves for each compound. To represent the thiol concentrations in units of µmol/l of cells, the amount of protein in HepG2 cells was taken to be 53 g protein/l of cells as estimated previously.[17] Incorporation of [$^{35}$S]-methionine into the glutathione pool was evaluated by measuring radioactivity in the relevant chromatographic fraction. Statistical significance was analyzed using the ANOVA test.

RESULTS AND DISCUSSION

*Kinetics of glutathione homeostasis following H$_2$O$_2$ exposure*

We have previously shown that relatively low concentrations of peroxides such as H$_2$O$_2$ and t-BuOOH result in enhanced flux of homocysteine through the trans-sulfuration pathway.[17] However, at the oxidant concentrations used in the earlier study (10–200 µM), there was no detectable change in the glutathione pool immediately following exposure to the oxidants. In order to study the kinetics of recovery of the intracellular glutathione pool following oxidative stress, we examined the time-dependent changes in the concentration of glutathione and glutathione disulfide following exposure to 1 mM H$_2$O$_2$ (Fig. 2). Under these conditions, a decrease in the intracellular glutathione concentration concomitant with an increase in glutathione disulfide is observed. The ratio of glutathione
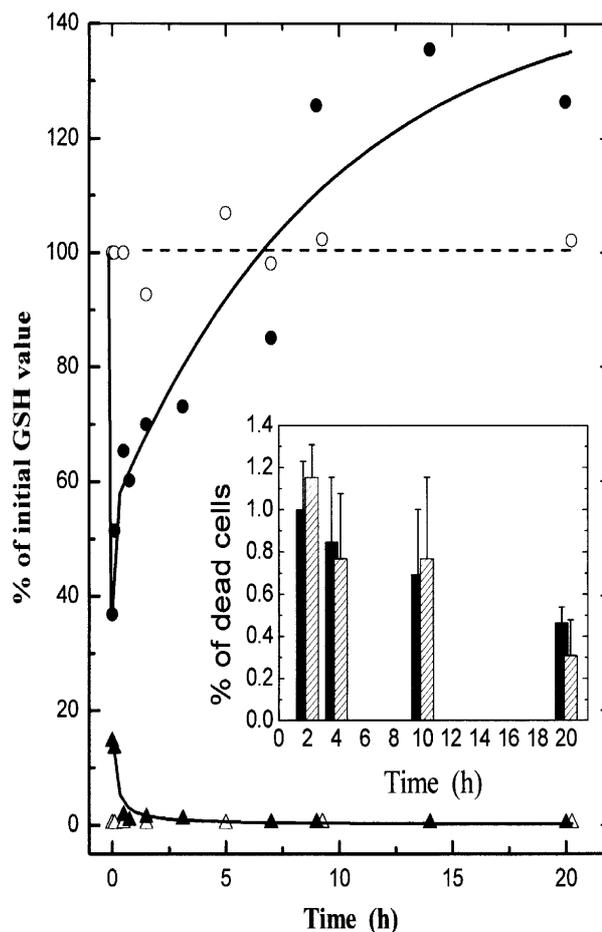


**Fig. 2.** Kinetics of recovery of the intracellular glutathione pool following oxidative challenge. The levels of glutathione and glutathione disulfide in untreated cells is represented by the open circles. In cells treated with a single bolus of H$_2$O$_2$ (1 mM), the glutathione (filled circles) level rapidly declines before recovering, while the glutathione disulfide pool responds reciprocally (filled triangles). The culture dish contained ~4 x 10$^6$ cells in 4 ml medium which corresponds to a ratio of ~1 µmol H$_2$O$_2$/10$^6$ cells. Inset: comparison of viability of cells treated with a single bolus of 1 mM H$_2$O$_2$ (hatched bars) *versus* untreated controls (solid bars) at fixed times following addition of peroxide or an equal volume of PBS to the culture medium.

to glutathione disulfide, which is known to be a marker for the redox status of the cells, changes from an initial value of 400 ± 80 to 2–5 during the first few minutes following H$_2$O$_2$ treatment. This is followed by a recovery phase during which the glutathione level, as well as the glutathione-to-glutathione disulfide ratio, are restored over a period of 6–8 h. Glutathione disulfide displays a reciprocal response and following an initial increase, diminishes to control levels within 2 h. The increase in glutathione concentration during the recovery phase is reflected in the enhanced incorporation of [$^{35}$S]-methionine into this pool (not shown), demonstrating the contribution of the

trans-sulfuration pathway in glutathione-linked redox homeostasis. The glutathione levels finally overshoot the pool size in untreated cells. A similar observation has been reported in L2 cells (derived from type II pneumocytes of adult rat lungs) treated with 4-hydroxy-2-nonenal, a major lipid peroxidation metabolite.[20]

The kinetics of glutathione recovery as well as the correspondence between our data set and that reported for L2 cells,[20] indicate that HepG2 cells treated with a single bolus of 1 mM $H_2O_2$ remained viable during the time course of the experiment. This was confirmed by monitoring the viability of HepG2 cells treated with a single bolus of 1 mM $H_2O_2$ using the trypan blue exclusion assay. As seen in Figure 2 (inset) there is no difference in the number of viable cells in cells treated with 1 mM $H_2O_2$ *versus* untreated controls, and ≤ 1% of the cells were stained blue during the time course of the experiment.

*Effect of catalase, superoxide dismutase, ascorbate and vitamin E, on intracellular glutathione concentration*

Addition of superoxide dismutase, a water-soluble form of vitamin E, and catalase resulted in a decrease in the intracellular glutathione pool, which was reflected in the diminished incorporation of $^{35}S$ from methionine into glutathione (Table 1). In contrast, neither ascorbate nor water-insoluble vitamin E had a statistically significant effect. The limited solubility of vitamin E likely resulted in its low availability in these experiments. Simultaneous addition of catalase, water-soluble vitamin E, and superoxide dismutase did not have an additive effect. Since the effect of catalase was more pronounced than that of superoxide dismutase and water-soluble vitamin E, the cellular response to catalase was further characterized. The dose dependence of the catalase response is presented in Table 2. Addition of 100 or 1000 U/ml of catalase to the medium resulted in a 17% decrease in the intracellular glutathione concentration whereas 10 U/ml did not have a statistically significant effect. The decrease in glutathione concentration was paralleled by a decrease in incorporation of radioactivity. Since thymol is added as a preservative in the commercially available catalase that was initially employed in this study (since this preparation has a high specific activity (48,000 U/mg protein)), an additional control was performed with thymol alone, which was found to have no effect on intracellular glutathione concentration and on $^{35}S$ incorporation from methionine to glutathione (Table 2). We had initially employed catalase containing thymol. We have also repeated these experiments with thymol-free catalase (specific activity, 15,500 U/mg protein). The intracellular glutathione concentration and radioactivity incorporation were found to be 82 ± 3% and 87 ± 6% (*n* = 6), respectively, in the presence of 100 U/ml catalase relative to untreated controls.

In principle, the effect of catalase on the reduced glutathione pool size could be due to its effects on the upstream trans-sulfuration pathway, the downstream glutathione synthesis pathway, or both. To determine whether or not catalase affects the trans-sulfuration pathway, we examined the flux of homocysteine through cystathionine β-synthase in cells treated with propargylglycine. The latter is a suicide inhibitor of γ-cystathionase,[21,22] the second enzyme in the trans-sulfuration pathway, and its administration leads to a time-dependent increase in the intracellular cystathionine

**Table 1**. Effect of various pro- and antioxidants on intracellular glutathione concentration and on incorporation of radioactivity from [$^{35}S$]-methionine

| Additions | Relative intracellular glutathione concentration | | | Relative incorporation of [$^{35}S$] into glutathione | | |
|---|---|---|---|---|---|---|
| | Value | *n* | *P* value | Value | *n* | *P* value |
| Control | 100 ± 1 | 37 | | 100 ± 2 | 36 | |
| Catalase | 83 ± 2 | 23 | ≤ 0.001 | 86 ± 3 | 23 | ≤ 0.001 |
| SOD | 92 ± 3 | 9 | ≤ 0.02 | 92 ± 4 | 9 | ≤ 0.1 |
| Ascorbate | 101 ± 5 | 4 | NS | 100 ± 2 | 3 | NS |
| Vitamin E | 95 ± 4 | 8 | NS | 106 ± 7 | 8 | NS |
| Vitamin E water-soluble | 90 ± 5 | 6 | ≤ 0.05 | 81 ± 6 | 6 | ≤ 0.01 |
| Catalase + SOD + water-soluble vitamin E | 87 ± 3 | 8 | ≤ 0.001 | 77 ± 3 | 8 | ≤ 0.001 |
| t-BuOOH | 110 ± 3 | 6 | ≤ 0.01 | 131 ± 4 | 6 | ≤ 0.001 |
| PDTC | 147 ± 9 | 7 | ≤ 0.001 | 191 ± 18 | 6 | ≤ 0.001 |
| PDTC + t-BuOOH | 160 ± 6 | 6 | ≤ 0.001 | 257 ± 45 | 5 | ≤ 0.01 |
| BHA | 111 ± 6 | 6 | ≤ 0.1 | 153 ± 10 | 6 | ≤ 0.001 |

The concentrations of the various additives employed were catalase (100 U/ml of medium), SOD (100 U/ml of medium), ascorbate (150 μM), vitamin E (200 μM), t-BuOOH (200 μM), PDTC (60 μM), BHA (200 μM), for 10 h following which the concentration of glutathione and the label incorporation into glutathione were determined.

*n*, number of independent determinations; NS, not significant.
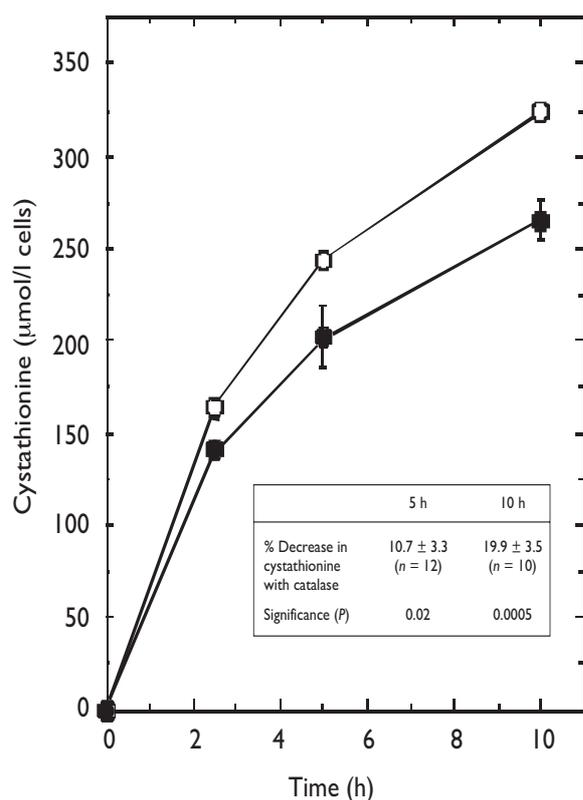
**Fig. 3**. Effect of extracellular catalase treatment on flux of homocysteine through the trans-sulfuration pathway. Cells were treated with 2.5 mM proparglycine only (open circles) or propargylglycine and catalase (100 U/ml; filled circles). The concentration of cystathionine was measured at the indicated times, and a representative data set is presented. The statistical analysis of the data is presented in the inset where *n* is the number of independent determinations.

pool which is too small to be readily detected under normal conditions.[17] Addition of 100 U/ml catalase to cells treated with 2.5 mM propargylglycine resulted in a decrease in cystathionine accumulation and corresponded to a ~20% decrease after 10 h (Fig. 3) which is virtually identical to the decrease in glutathione concentration observed at the same time (Table 2). These results demonstrate, for the first time,

the sensitivity of the upstream trans-sulfuration pathway to antioxidants, and its effect on the pool size of the downstream product, glutathione.

### Effect of PDTC and BHA on intracellular glutathione concentration

PDTC is a relatively stable dithiocarbamate, a family of compounds that can chelate both free and protein-bound metals and is widely used in medicine.[23] The antioxidant behavior of dithiocarbamates results from their radical scavenging properties and cellular responses including inhibition of lipid peroxidation,[24,25] and inhibition of oxidative activation of the transcription factor, NF-κB.[26,27]

However, addition of 60 μM PDTC resulted in an increase in intracellular glutathione after 10 h that is similar to, but more pronounced than, the response observed with the oxidant, t-BuOOH (Table 1). Simultaneous addition of tBuOOH and PDTC had an additive effect resulting in a ~1.6-fold increase in glutathione concentration. The increase in incorporation of [$^{35}$S]-methionine into glutathione was marginally greater than the fold-increase in glutathione concentration (*e.g.* ~1.9-fold *versus* 1.5-fold with PDTC; Table 1) suggesting enhanced use of the trans-sulfuration pathway-derived cysteine in glutathione biosynthesis under these conditions.

BHA elicited a similar response under these conditions. Thus, addition of 200 μM BHA resulted in 1.1-fold increase in intracellular glutathione after 10 h *versus* untreated controls (Table 1), and a 1.5-fold increase in incorporation of $^{35}$S from the methionine pool. These results suggest that the effects of PDTC and BHA derive either from oxidation reactions or from other presently unknown effects, rather than an antioxidant effect on the upstream trans-sulfuration pathway. While the antioxidant behavior of both these compounds are well known,[24,25] they have also been reported to have pro-oxidant effects. Specifically, PDTC has been shown to elicit time- and dose-dependent increases in the

**Table 2**. Effect of varying concentrations of extracellular catalase on intracellular glutathione concentration and on incorporation of radioactivity from [$^{35}$S]-methionine

| Additions | Relative intracellular glutathione concentration | | | Relative incorporation of [$^{35}$S] into glutathione | | |
|---|---|---|---|---|---|---|
| | Value | *n* | *P* value | Value | *n* | *P* value |
| Control | 100 ± 1 | 19 | | 100 ± 3 | 19 | |
| Catalase (10 U/ml) | 95 ± 3 | 9 | NS | 100 ± 10 | 9 | NS |
| Catalase (100 U/ml) | 83 ± 2 | 23 | ≤ 0.001 | 86 ± 3 | 23 | ≤ 0.001 |
| Catalase (1000 U/ml) | 82 ± 3 | 9 | ≤ 0.001 | 86 ± 8 | 9 | ≤ 0.1 |
| Thymol (150 nM) | 99 ± 4 | 8 | NS | 105 ± 10 | 8 | NS |

*n*, number of independent determinations; NS, not significant.

mRNAs encoding the catalytic and regulatory subunits of γ-glutamyl-cysteine ligase in HepG2 cells.[28] γ-Glutamyl-cysteine ligase is believed to be the rate-limiting enzyme in glutathione biosynthesis. BHA enhances the activity of glutathione reductase[29] and functions as a transcriptional activator of γ-glutamyl-cysteine ligase.[30] A 1.5–2-fold increase in glutathione levels has been reported in mice 12 days after feeding with BHA.[30] The observed elevation in glutathione levels in response to PDTC and BHA in our studies is thus consistent with previous reports, while the increased incorporation of radioactivity from the methionine to the glutathione pool additionally suggests enhanced flux through the trans-sulfuration pathway under these conditions. These results confirm and extend our previous observations with peroxide-based pro-oxidants, which demonstrated that the glutathione pool size is modulated by the sensitivity of the trans-sulfuration pathway to oxidative stress.

Recently, using a reporter construct containing one of the two functional promoters for cystathionine β-synthase, decreased promoter activity was reported in HepG2 cells treated with ≥ 50 μM $H_2O_2$.[31] Although these results are at odds with a published report of enhanced flux of homocysteine through cystathionine β-synthase in HepG2 cells treated with $H_2O_2$ and the absence of changes in cystathionine β-synthase protein levels under control of the **endogenous** promoters,[17] this discrepancy was not discussed. In this study, we have confirmed and extended the observation that oxidative stress exerts a reciprocal effect on homocysteine metabolism and leads to enhanced flux through the trans-sulfuration pathway. The metabolic rationale for the observed increase in flux through the trans-sulfuration pathway is that it would help restore the intracellular glutathione pool under oxidative stress conditions. The opposite effect, *i.e.* diminished cystathionine β-synthase activity due to transcriptional repression, would not appear to confer a metabolic advantage and is not supported by our observations on the levels of the endogenous protein.

## CONCLUSIONS

Changes in glutathione levels are linked to a number of disease pathologies and the regulation of the two penultimate enzymes in the biosynthetic pathway, γ-glutamyl-cysteine ligase and glutathione synthetase, has been extensively studied. This study was designed to evaluate the role of redox regulation on the trans-sulfuration pathway and, therefore, its effect on glutathione homeostasis. Our results demonstrate that flux through the trans-sulfuration pathway is reciprocally responsive to the presence of antioxidants and pro-oxidants and modulates glutathione synthesis in transformed human liver cells. Oxidants elicit an increase in metabolic flux from

methionine to glutathione and an increase in intracellular glutathione level while antioxidants such as catalase, SOD, and water-soluble vitamin E produce the opposite effect. Of the antioxidants tested in this study, extracellular catalase had the most significant effect and resulted in a ~20% decrease in intracellular glutathione level and on flux through the trans-sulfuration pathway. Interestingly, a combination of antioxidants did not have an additive effect on glutathione metabolism. The effects of PDTC and BHA on the trans-sulfuration pathway in HepG2 cells could be derived either from oxidation reactions or from other, presently unknown, effects.

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