Cysteine regulates expression of cysteine dioxygenase and γ-glutamylcysteine synthetase in cultured rat hepatocytes

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Received 5 October 2000; accepted in final form 26 January 2001

Kwon, Young Hye, and Martha H. Stipanuk. Cysteine regulates expression of cysteine dioxygenase and γ-glutamylcysteine synthetase in cultured rat hepatocytes. Am J Physiol Endocrinol Metab 280: E804–E815, 2001.—Rat hepatocytes cultured for 3 days in basal medium expressed low levels of cysteine dioxygenase (CDO) and high levels of γ-glutamylcysteine synthetase (GCS). When the medium was supplemented with 2 mmol/l methionine or cysteine, CDO activity increased by >10-fold and CDO protein increased by 1.5- or 3.2-fold. In contrast, GCS activity decreased to 51 or 29% of basal, GCS heavy subunit (GCS-HS) protein decreased to 89 or 58% of basal, and GCS mRNA decreased to 79 or 37% of basal for methionine or cysteine supplementation, respectively. Supplementation with cysteine consistently yielded responses of greater magnitude than did supplementation with an equimolar amount of methionine. Addition of propargylglycine to inhibit cystathionine γ-lyase activity and, hence, cysteine formation from methionine prevented the effects of methionine, but not those of cysteine, on CDO and GCS expression. Addition of buthionine sulfoximine to inhibit GCS, and thus block glutathione synthesis from cysteine, did not alter the ability of methionine or cysteine to increase CDO. GSH concentration was not correlated with changes in either CDO or GCS-HS expression. The effectiveness of cysteine was equivalent to or greater than that of its precursors (S-adenosylmethionine, cystathionine, homocysteine) or metabolites (taurine, sulfate). Taken together, these results suggest that cysteine itself is an important cellular signal for upregulation of CDO and downregulation of GCS.

OTHER THAN INCORPORATION INTO PROTEIN, the major fates of cysteine in the body are incorporation into the tripeptide glutathione (GSH) or via transsulfuration and catabolism via cysteine-sulfate-dependent pathways (Fig. 1). Because methionine sulfur is almost entirely converted to cysteine sulfur via the transsulfuration pathway before its sulfur is oxidized and excreted, intake of a certain molar amount of methionine results in synthesis of a nearly equimolar amount of cysteine (21, 22). Although substantial amounts of cysteine are incorporated into both protein and GSH, cysteine is ultimately catabolized to taurine or sulfate. We have previously reported results of several rat studies in which both hepatic cysteine dioxygenase (CDO, EC 1.13.11.20) and hepatic γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2) activities responded to changes in the protein or dietary sulfur amino acid levels. These two enzymes responded in opposite directions, with CDO activity increasing and GCS activity decreasing in response to an increase in the protein or sulfur amino acid level (2–5). Increases in CDO activity were associated with increases in the urinary taurine-to-taurine+sulfate ratio (5). Higher levels of CDO activity were also accompanied by greater rates of both taurine and sulfate production as well as by an increase in the proportion of cysteine metabolized to taurine vs. sulfate by isolated hepatocytes incubated with 0.2 mmol/l L-cysteine (2, 3, 5). Likewise, the lower levels of GCS activity in isolated hepatocytes were accompanied by lower rates of GSH synthesis (2, 5).

Increases in CDO activity in response to an increase in the supply of sulfur amino acids could be critical for prevention of potential damage to the cell by rapid removal of excess cysteine. Elevated levels of cysteine have been shown to be both cytotoxic and neurotoxic, and increased levels of homocysteine, a precursor of cysteine in the methionine transsulfuration pathway, have been associated with increased risk for cardiovascular disease and with the occurrence of neural tube defects (22). The lower GCS activity present in animals fed high levels of sulfur amino acids acts to somewhat limit the rate of GSH synthesis and favors the catabolism of cysteine to taurine or sulfate. On the other hand, the higher GCS activities and lower CDO activities that are present in animals fed diets low in sulfur amino acids appear to ensure that cysteine is efficiently used for GSH synthesis, rather than being catabolized to taurine and sulfate, when sulfur amino acid supply is limited.

Studies of the molecular regulation of CDO and GCS activities in rat liver by dietary sulfur amino acid intake indicated that increases in CDO activity were accomplished predominantly by increases in CDO protein concentration, with no effect on CDO mRNA levels.
and a much smaller degree of change in activity state (3, 4). Changes in GCS activity in response to dietary supplementation with methionine were largely accounted for by changes in the concentration of mRNA coding for GCS-heavy (or catalytic) subunit (GCS-HS) (3, 4). The precise mechanisms and signals involved in the sulfur amino acid-induced regulation of CDO and GCS are not known, although the regulation of CDO clearly appears to be posttranscriptional (3, 4).

Recently, we reported the development of a primary hepatocyte model for studies of regulation of CDO and GCS by sulfur amino acids (16). Supplementation of the culture medium with either methionine or cysteine resulted in higher CDO activity and lower GCS activity in cultured rat hepatocytes. These studies demonstrated that hepatocytes in culture could be used as a model for further studies of the regulation of hepatic CDO and GCS at the cellular level.

Thus rat hepatocytes in primary culture were used for studies designed to further elucidate the particular sulfur amino acid or metabolite that was effective at the cellular level in inducing regulatory changes in hepatic CDO and GCS activities and to determine whether the pattern of changes in enzyme activity,
enzyme protein concentration, and enzyme mRNA concentrations in cultured hepatocytes were similar to those observed in liver of intact animals. To determine the particular sulfur amino or metabolite that acts as a signal for regulatory changes in CDO and GCS at the cellular level, we cultured hepatocytes with methionine, cysteine, or one of their metabolites. We also used DL-propargylglycine (PPG), an inhibitor of cystathionine γ-lyase (EC 4.4.1.1), to block transsulfuration of methionine to cysteine (17) and DL-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of GCS, to block GSH synthesis (15) to further assess the roles of cysteine and GSH, respectively, as mediators of the effect of sulfur amino acid availability on CDO and GCS activities.

MATERIALS AND METHODS

Materials. Williams’ medium E (WE medium), murine natural epidermal growth factor (EGF), bovine insulin, and antibiotic-antimycotic mixture that contained the sodium salt of penicillin G, streptomyacin sulfate, and amphotericin B were purchased from GIBCO-BRL (Grand Island, NY). Calf skin collagen type I, collagenase, dexamethasone, Na2SeO3, bathocuprine disulfonate (BCS), BSO, PPG, l-cysteine, l-methionine, S-adenosyl-l-methionine, l-cystathionine, l-homocysteine, and taurine were all purchased from Sigma (St. Louis, MO). Tissue culture dishes (60 × 15 mm) were purchased from Becton-Dickinson (Franklin Lakes, NJ). [35S]Cysteine (2,000 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). All other reagents were of analytical grade and were obtained from commercial sources.

Antibodies. The purified IgG fraction from rabbit anti-CDO serum was a gift from Dr. Yu Hosokawa (National Institute of Health and Nutrition, Tokyo, Japan). Rabbit antibody was raised to CDO purified from rat liver (13). Rabbit anti-GCS-HS serum was a gift from Dr. Henry Jay Forman (University of Alabama at Birmingham, Birmingham, AL). The preparations of these antibodies against rat liver CDO (13) and a peptide sequence of rat kidney GCS-HS (18) have been reported. Specificity of these antibodies has been described previously (4).

Primary cultures of rat hepatocytes. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in stainless steel mesh cages in a room maintained at 20°C and 60–70% humidity with light from 2000 to 0800. Rats were given ad libitum access to water and a nonpurified diet (Prolab RMH 1000, Agway, Syracuse, NY). The care and use of animals was approved by the Cornell University Institutional Animal Care and Use Committee. Rats weighed ~250–300 g when they were used to obtain hepatocytes.

Hepatocytes were isolated aseptically by collagenase perfusion as described by Berry et al. (6). The initial viability of isolated hepatocytes was more than 85% as determined by 0.2% (wt/vol) Trypan blue exclusion. The freshly isolated hepatocytes were resuspended in WE medium to give a cell number of 1.5 × 10⁷ cells per ml; the suspended cells then were diluted with basal WE medium to yield a final cell concentration of 7.5 × 10⁶ cells per ml. The basal WE medium provided 0.49 mmol/l total l-cysteine, 0.08 mmol/l l-methionine, and a negligible amount of GSH (0.16 mmol/l) and was prepared to contain 1 μg/ml insulin, 50 ng/ml EGF, 50 nmol/l dexamethasone, 3 mmol/l Na2SeO3, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Culture dishes were coated with collagen as described previously (16). Five milliliters of the diluted cell suspension (0.18 ml/cm²) were plated on each 60-mm-diameter collagen-coated dish. Cells were allowed to attach in basal medium over a 4-h period. At 4 h, the basal medium was replaced with fresh basal medium or with medium that was supplemented with 2 mmol/l methionine, 2 mmol/l cysteine, or 2 mmol/l of a sulfur amino acid metabolite as indicated in RESULTS. Incubations with thiols (i.e., cysteine or homocysteine) also contained 0.05 mmol/l of BCS, which was added to prevent formation of disulfides (9). The experimental medium was replaced every 24 h (i.e., at 28 and 52 h). To examine the effect of inhibitors, either 1 mmol/l PPG or 100 μmol/l BSO were added to the designated culture medium when medium was changed at 28 and 52 h. Cells were harvested after 72 h of treatment (i.e., total of 76 h in culture).

At the end of the culture period, monolayer cultures were washed three times with 2.5 ml of ice-cold PBS. For measurement of enzyme activities and Western blot analysis, washed cells were collected by scraping. Harvested cells were suspended to 50 mmol/l MES, pH 6.0, to give a final cell concentration of ~6.5 × 10⁶ cells per ml, and the suspension was sonicated for three 15-s periods using a High Intensity Ultrasound Processor (Sonics and Materials, Danbury, CT) to disrupt the cells. A portion of each cell homogenate was centrifuged at 20,000 g for 30 min at 4°C to obtain the supernatant fraction. Protein concentrations in the cell homogenates and supernatant fractions were measured by the method of Lowry et al. (13). Incubations with thiols (i.e., cysteine or homocysteine) also contained 0.05 mmol/l of BCS, which was added to prevent formation of disulfides (9). The experimental medium was replaced every 24 h (i.e., at 28 and 52 h). To examine the effect of inhibitors, either 1 mmol/l PPG or 100 μmol/l BSO were added to the designated culture medium when medium was changed at 28 and 52 h. Cells were harvested after 72 h of treatment (i.e., total of 76 h in culture).

Measurement of enzyme activities. By use of the cell homogenate, which contained ~8–10 mg protein/ml, CDO activity was measured according to the method of Bagley and Stipanuk (1) except that the volume of the reaction mixture was reduced to 0.2 ml and the amount of [35S]cysteine was increased to 10–15 μCi/0.2 ml of reaction mixture. For assay of GCS activity, the cell homogenate was further diluted with 0.5 or 1 volume of N-(2-hydroxyethyl)piperazone-N’-3-panesulfonic acid (EPPS) buffer, pH 8.5, to give a final concentration of 50 mmol/l EPPS buffer. GCS activity was assayed as described previously (4), except that the volume of the reaction mixture was reduced to 0.5 ml and EPPS buffer was used (instead of HEPES) to maintain the incubation mixture at pH 8.1. Under these assay conditions, the GSH present in the cell homogenate had no effect on measured GCS activity (Y. H. Kwon and M. H. Stipanuk, unpublished results).

Western blot analysis. Separation of proteins was carried out by one-dimensional SDS-PAGE (14). Aliquots of supernatants that contained ~20–150 μg of protein were mixed with equal volumes of SDS-reducing buffer and then were loaded onto polyacrylamide gels (10 and 15% wt/vol polyacrylamide for GCS-HS and CDO, respectively, with 4% stacking gels). Medium-range molecular weight markers (Promega, Madison, WI) and Rainbow molecular weight markers (Sigma) were used for estimation of protein molecular weights.

Protein blotting was performed using the procedure for tank transfer described by Hoefer Scientific (San Francisco, CA) to an Immobilon-P polyvinylidene difluoride transfer membrane (Millipore, Medford, MA). Membranes were incubated with the IgG fraction of rabbit polyclonal anti-rat CDO...
for 3 h or with rabbit polyclonal anti-rat GCS-HS for 2 h at room temperature. Immunoreactive protein was detected by chemiluminescence with the use of goat anti-rabbit IgG (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP) and the Supersignal CL-HRP substrate system (Pierce) with exposure to Kodak X-OMAT SRP film. Bands were scanned with a Hewlett Packard Scanjet 3C (Hewlett-Packard, Camas, WA), and two-dimensional quantitative densitometric analysis of the scanned bands was done using the Molecular Analyst program (Bio-Rad Laboratories, Hercules, CA). Relative protein amounts were quantitated using standard curves (pixel density/mm² vs. μg total protein loaded) run on each gel; standard curves were generated by loading incremental amounts (20–50 μg for CDO, 15–45 μg for GCS-HS) of total supernatant protein from hepatocytes cultured in methionine-supplemented medium on each gel. The relative amount of protein was then divided by the actual amount of total protein loaded for each sample.

**Extracting RNA and mRNA analysis.** Total RNA was isolated from cultured hepatocytes using a TOTALLY RNA kit (Ambion) based on the method of Chomczynski and Sacchi (8). Northern blot analysis was done as described by Brown (7) with electrophoresis on a 1% (wt/vol) agarose-formaldehyde gel and blotting onto a Magna Graph nylon membrane (Micron Separations, Westboro, MA). Membranes were prehybridized using herring sperm DNA and then hybridized with one of the 32P-labeled cDNA probes (2 × 10⁶ cpm/ml) for rat CDO, rat GCS-HS, or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Probes for CDO and GCS-HS mRNAs were prepared and labeled as described previously (4). GAPDH mRNA was synthesized using GAPDH mouse DECAprobe template (Ambion). After hybridization, membranes were washed twice in 2× saline-sodium phosphate-EDTA buffer (SSPE) with 0.2% (wt/vol) SDS for 30 min at room temperature and twice in 0.1× SSPE at 60°C for 15 min and then autoradiographed using Kodak X-OMAT film. Probes were stripped from the membrane by boiling the membrane in 0.1× SSPE with 1% (wt/vol) SDS between hybridization with the three probes.

For quantification of relative levels of mRNA, aliquots of total RNA (5–15 μg/dot) were applied to a Magna Graph nylon membrane using a microsample filtration manifold (Minifold, Schleicher and Schuell, Keene, NH). The membranes were hybridized with the [32P]cDNA for CDO, GCS-HS, or GAPDH mRNA. Results were quantified using a Bio-Rad GS-363 Phosphorescence Imaging System (Bio-Rad, Melville, NY) and Molecular Analyst program (Bio-Rad). A standard curve was generated on each membrane by loading incremental amounts (1.25–15 μg) of pooled total RNA from hepatocytes cultured in methionine-supplemented medium. Relative amounts of mRNA for each enzyme mRNA and for GAPDH were calculated using a standard curve of pixel density per square millimeter vs. the amount of total RNA loaded. Relative enzyme mRNA amount was calculated from the standard curve and was corrected for loading by dividing the relative enzyme mRNA amount by the relative GAPDH mRNA amount.

**Statistics.** Data were analyzed either by the paired t-test or by analysis of variance (Minitab 10.5, Minitab, State College, PA) and Tukey’s ω-procedure (20). Differences were accepted at P ≤ 0.05. Data for CDO activity and relative amounts of CDO protein were transformed to log₁₀ before statistical analysis. Correlation coefficients were calculated using Microsoft Excel 5.0 (Microsoft, Cambridge, MA).

## RESULTS

**Effects of methionine, cysteine and their metabolites on the activities of CDO and GCS.** As shown in Table 1, addition of 2 mmol/l methionine, homocysteine, or cysteine to the basal medium was effective in both increasing CDO activity and decreasing GCS activity. Neither S-adenosylmethionine nor cystathionine was effective in significantly increasing CDO activity, although both compounds can be converted to homocysteine and then cysteine. S-Adenosylmethionine and cystathionine did significantly decrease GCS activity, but the decrease was less than that obtained with cysteine, homocysteine, or methionine. Taurine and sulfate, which are catabolites of cysteine, were not effective as regulators of either CDO or GCS activity. The changes in CDO and GCS activities across all treatments were reciprocal, with the correlation coefficient for CDO activity vs. GCS activity being r = −0.87.

**Effects of PPG on the ability of methionine and cysteine to affect expression of CDO and GCS.** As shown in Fig. 2, the addition of PPG significantly decreased the GSH concentration in cells cultured in either basal or methionine-supplemented medium but not in cells cultured in cysteine-supplemented medium. Despite a higher initial GSH concentration in methionine-supplemented cells, the GSH content of cells cultured in either basal or methionine-supplemented medium reached a similar low level of ~10–15 μmol/mg protein (~1.5–2.5 μmol/g wet weight of hepatocytes) after 2 days of treatment with PPG. The marked decrease in the GSH content of cells cultured in basal medium, which provided much more cysteine than methionine [0.49 mmol/l cyst(e)ine and 0.1 mmol/l methionine], to a medium supplemented with excess methionine (+2.0 mmol/l) demonstrates the quantitatively important contribution of methionine as a precursor of cysteine and their metabolites to medium on the activities of CDO and GCS in cultured rat hepatocytes

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Enzyme Activities, %basal</th>
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<tbody>
<tr>
<td></td>
<td>CDO</td>
</tr>
<tr>
<td>Basal + 2 mmol/l methionine</td>
<td>299 ± 39*</td>
</tr>
<tr>
<td>Basal + 2 mmol/l S-adenosyl-methionine</td>
<td>150 ± 12</td>
</tr>
<tr>
<td>Basal + 2 mmol/l homocysteine</td>
<td>227 ± 23*</td>
</tr>
<tr>
<td>Basal + 2 mmol/l cystathionine</td>
<td>86 ± 21</td>
</tr>
<tr>
<td>Basal + 2 mmol/l cysteine</td>
<td>303 ± 38*</td>
</tr>
<tr>
<td>Basal + 2 mmol/l taurine</td>
<td>86 ± 22</td>
</tr>
<tr>
<td>Basal + 2 mmol/l sulfate</td>
<td>76 ± 14</td>
</tr>
</tbody>
</table>

Values are means of percent basal activity (means ± SE for 3 hepatocyte preparations). Activities of cysteine dioxygenase (CDO) and γ-glutamylcysteine synthetase (GCS) were measured after hepatocytes had been cultured in basal Williams’ medium E or basal medium supplemented with the compound indicated for 72 h. Activities were expressed per milligram of total protein. The basal mean activity of CDO was 0.08 mmol·min⁻¹·mg protein⁻¹, and the basal mean activity of GCS was 0.71 nmol·min⁻¹·mg protein⁻¹. *Significantly different for each enzyme activity (P ≤ 0.05) from the value for cells from the same rat liver cultured in basal medium by paired t-test.
tein for GSH synthesis. The level of GSH in cells cultured in cysteine-supplemented medium was not significantly decreased by PPG, as would be expected when excess preformed cysteine is available.

As shown in Fig. 3A, CDO activity was 14 times higher in cells cultured in medium supplemented with methionine and 23 times higher in cells cultured in medium supplemented with cysteine than in cells cultured in basal medium. The addition of PPG to methionine-supplemented medium significantly reduced the activity of CDO to the basal level. In contrast, the activity of CDO in cells in cysteine-supplemented medium remained high, showing no apparent effect of PPG. No effect of PPG on CDO activity was observed in hepatocytes cultured in basal medium in which CDO activity was already very low.

GCS activity is cells cultured in medium supplemented with methionine was 54% of the basal level, and that in cells cultured in medium supplemented with cysteine was 26% of the basal level (Fig. 3B). Addition of PPG to the methionine-supplemented medium significantly reduced the activity of CDO to the basal level. In contrast, the activity of CDO in cells in cysteine-supplemented medium remained high, showing no apparent effect of PPG. No effect of PPG on CDO activity was observed in hepatocytes cultured in basal medium in which CDO activity was already very low.

The relative amounts of CDO and GCS-HS protein, which were quantified by Western blot analysis of samples from individual experiments, are shown in Table 2. The band detected by antibody for CDO corresponded to a molecular mass of ~23.5 kDa. This band corresponds to the lower of the two apparent forms of CDO (23.5 and 25.5 kDa) observed by Bella et al. (4, 5) for rat liver CDO. No other CDO antibody-reactive bands were observed. The level of CDO protein in cells cultured in basal medium was too low to be quantified in some experiments. Nevertheless, this basal level clearly was significantly increased in hepatocytes cultured in medium supplemented with methionine or cysteine to levels that were at least 10 or 15 times basal, respectively. Addition of PPG to the methionine-supplemented medium prevented the increase in CDO protein level, but addition of PPG did not affect the level of CDO protein in cells cultured in cysteine-supplemented medium. These effects on CDO protein are consistent with observed changes in the activity of CDO in response to sulfur amino acids and PPG.

On Western analysis, the band detected by antibody for GCS-HS had a molecular mass of ~74 kDa, which corresponds to the reported molecular mass of rat kidney GCS-HS (18) and to that reported by Bella et al. (4) for rat liver GCS-HS. No other GCS-HS antibody-reactive bands were observed. Addition of methionine or cysteine to the medium resulted in levels of GCS-HS protein that were 10 or 37% less, respectively, than those in cells cultured in basal medium; the difference was significant between cells cultured in basal vs. cysteine-supplemented, but not methionine-supplemented, medium (Table 2). The more marked effect of

Fig. 3. Effect of sulfur amino acids and/or PPG on the activities of CDO (A) and GCS (B) in cultured hepatocytes. Rat hepatocytes were cultured in basal medium or medium supplemented with 2 mmol/l L-methionine (+ Met) or 2 mmol/l L-cysteine (+ Cys) for 72 h. The effect of PPG was assessed by addition of 1 mmol/l PPG to the culture medium for the last 48 h. Results are expressed as means ± SE for experiments with hepatocytes isolated from 3 different rats. Values not followed by a common superscript letter are significantly different (P < 0.05) by ANOVA and Tukey’s ω-procedure.
cysteine than of methionine in decreasing GCS-HS protein level is consistent with the greater effect of cysteine than of methionine in increasing CDO protein. PPG treatment did not have a significant effect on GCS-HS concentration in cells cultured in methionine-supplemented medium, which is not unexpected because methionine supplementation alone did not cause a significant decrease in the GCS-HS protein level in this study. As observed for GCS activity, the treatment of cells cultured in cysteine-supplemented medium with PPG did not significantly affect the concentration of GCS-HS, which remained low.

The relative amounts of CDO and GCS-HS mRNAs, which were determined on individual samples by quantitative dot blot analysis with correction for relative level of GAPDH mRNA in the sample, are reported in Fig. 4. Northern blots for CDO and GCS-HS mRNAs, which were run on pooled samples for each treatment group, are shown below the bar graphs. The cDNA probe for CDO mRNA hybridized with a single band of ~1.7 kb, which is consistent with the reported size of rat liver CDO mRNA (13). The GCS-HS probe hybridized with a single band ~3.7 kb, which is consistent with the reported size of rat kidney GCS-HS mRNA (27).

The CDO mRNA level was significantly higher in cells cultured with either supplemental methionine (1.5-fold higher) or cysteine (4-fold higher) than in cells cultured in basal medium (Fig. 4A). Supplementation with cysteine resulted in significantly higher levels of CDO mRNA than did supplementation with methionine. Treatment of cells with PPG did not significantly reduce the concentration of CDO mRNA in cells cultured in either basal or cysteine- or methionine-supplemented medium.

The concentration of GCS-HS mRNA in cells cultured in methionine- or cysteine-supplemented medium was 88 or 41%, respectively, of that in cells cultured in basal medium (Fig. 4B). Supplemental methionine did not have a statistically significant effect on the GCS-HS mRNA level, whereas supplemental cysteine resulted in a significantly lower GCS-HS mRNA concentration. Addition of PPG to the medium tended to increase the GCS-HS mRNA concentration in cells cultured in either basal or sulfur amino acid-supplemented medium, but the effect of PPG was significant only for methionine-supplemented cells.

Effects of buthionine sulfoximine on the ability of methionine and cysteine to affect expression of CDO and GCS. Although cellular GSH concentrations were not closely correlated with CDO or GCS activity in the studies with PPG, the possible role of GSH concentration in regulation of CDO activity was studied further using buthionine sulfoximine (BSO) to block GSH synthesis. As shown in Fig. 5, the GSH concentration tended to be higher in hepatocytes cultured in medium supplemented with sulfur amino acids and without BSO. Addition of BSO decreased the concentration of GSH by >90% in cells cultured in either basal or sulfur amino acid-supplemented medium. GSH concentration was low and similar in all cells cultured with BSO (~2–4 nmol/mg protein, or ~0.3–0.7 μmol/g wet wt of hepatocytes), regardless of whether or not the medium was supplemented with sulfur amino acid.

Although the cellular GSH level was significantly depleted by addition of BSO to the medium, the activity of CDO was not affected (Fig. 6A). The lack of effect on CDO activity clearly indicates that the response of CDO activity to sulfur amino acids is not mediated by the changes in GSH concentration. The correlation coefficient for CDO activity vs. GSH concentration was 0.19.

The addition of BSO to basal medium decreased GCS activity by ~75%, whereas its addition resulted in an apparent 50% decrease in GCS activity in cells cultured in sulfur amino acid-supplemented media (Fig. 6B). The decrease was statistically significant only for cells cultured in basal medium, which had the highest level of GCS activity in the absence of BSO. Nevertheless, GCS activity was low and similar in cells grown in media with BSO, regardless of whether or not the medium was supplemented with methionine or cysteine. Because BSO was added to inhibit GCS activity and thus to deplete the cellular GSH concentration, low GCS activity was expected.
Results for quantification of CDO and GCS-HS protein by Western blot analysis of samples from individual experiments are shown in Table 3. Treatment with BSO did not significantly affect the level of CDO protein in cells cultured in basal or sulfur amino acid-supplemented medium, which is consistent with the observed lack of response of CDO activity to BSO. As seen in the other studies, supplementation of the culture medium with either methionine or cysteine significantly increased CDO mRNA to 2.4 or 3.3 times the basal level, respectively (Fig. 7A). The 48-h exposure to BSO tended to decrease CDO mRNA, but these apparent decreases were not significant for cells cultured in any of the media.

As shown in Fig. 7B, GCS-HS mRNA was significantly lower in hepatocytes cultured in medium with supplemental methionine or cysteine than in cells cultured in basal medium. The exposure of cultured hepatocytes to BSO significantly increased GCS-HS mRNA concentration in cells cultured in both basal and sulfur amino acid-supplemented media, and this increase in GCS-HS mRNA abundance presumably was in response to the GSH depletion produced by BSO.

**DISCUSSION**

Reciprocal regulation of CDO and GCS. In all three of the studies reported in this paper, CDO activity was greater and GCS activity was less in hepatocytes cultured in medium to which either methionine or cysteine had been added. CDO activity increased up to 24 times the basal level with cysteine supplementation of the medium and up to 15 times the basal level with methionine supplementation of the medium. In contrast, GCS activity in hepatocytes cultured with methi-
onine decreased to as little as 33% of the level found in cells cultured in basal medium, and GCS activity in cells cultured with cysteine decreased to as little as 24% of the basal level. Thus the capacity for GSH synthesis decreased and the capacity for cysteine catabolism increased in response to an increased availability of sulfur amino acid.

Although we used 2 mmol/l of supplemental amino acid or metabolite in this study, we would expect to see significant effects of lower concentrations of cysteine or methionine on CDO and GCS activities. In our initial studies with hepatocytes in culture, we noted a dose-response relationship for both CDO and GCS activities between 0.1 and 0.5 mmol/l cysteine or methionine and a plateau in responses over the range of 0.5–5 mmol/l (16). Changes in sulfur amino acid concentration in the medium were not monitored over the 24-h period after each replacement of medium with fresh medium, but a copper chelator was included in thiol-supplemented medium to minimize thiol oxidation.

This pattern of response of CDO and GCS activities in hepatocytes to sulfur amino acids in the culture medium is consistent with the pattern reported previously for these hepatic enzymes in rats fed diets that contained methionine, cysteine, or protein in excess of the requirement level (3, 4). For example, supplementation of a basal diet that contained 100 g of casein/kg with 10 g of L-methionine/kg resulted in an increase of CDO activity to 35 times the basal level and in a decrease of GCS activity to 47% of the basal level (4). However, although the pattern of response was very similar, it should be noted that the CDO activity observed in cultured hepatocytes was markedly lower than the values reported for liver from intact rats or for freshly isolated hepatocytes (3, 4, 5). CDO activity appears to be lost with dedifferentiation of hepatocytes in culture and is also low in liver cell lines (16). A second notable difference between studies with intact rats and these experiments with cultured rat hepatocytes is that addition of cysteine to the culture medium consistently yielded a greater increase in CDO activity and a smaller decrease in GCS activity than did an equimolar amount of methionine, whereas methionine was more effective than cysteine when the sulfur amino acids were added to a low protein diet (2, 3). Differences in the rates of intestinal absorption or the rates of hepatic uptake of methionine and cyst(e)ine in intact rats, differences in the rate or efficiency of con-

Table 3. Relative levels of CDO and GCS-HS proteins in hepatocytes cultured with sulfur amino acids and/or BSO

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Relative Amount of Protein</th>
<th>Relative Amount of Protein</th>
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<tbody>
<tr>
<td>CDO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>(&lt;2.4, &lt;2, &lt;2.4)$^\text{§}$</td>
<td>(&lt;4, &lt;4, 11.3)$^\text{§}$</td>
</tr>
<tr>
<td>Basal + 2 mmol/l methionine</td>
<td>27.0 ± 2.4$^\text{†}$</td>
<td>17.0 ± 0.9$^\text{†}$</td>
</tr>
<tr>
<td>Basal + 2 mmol/l cysteine</td>
<td>34.2 ± 1.8$^\text{a}$</td>
<td>35.2 ± 6.6$^\text{a}$</td>
</tr>
<tr>
<td>Basal + 2 mmol/l methionine</td>
<td>35.3 ± 0.9$^\text{a}$</td>
<td>39.8 ± 1.5$^\text{a}$</td>
</tr>
<tr>
<td>Basal + 2 mmol/l cysteine</td>
<td>30.8 ± 4.1$^\text{†}$</td>
<td>35.9 ± 5.6$^\text{a}$</td>
</tr>
<tr>
<td>GCS-HS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>19.0 ± 1.6$^\text{†}$</td>
<td>27.7 ± 0.7$^\text{†}$</td>
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</tbody>
</table>

Values are means ± SE for 3 cell preparations. BSO, dL-buthionine-[S,R]-sulfoximine. Supernatants (20,000 $\text{g}$) of cultured hepatocytes (20–175 $\mu$g total protein/lane for CDO, 20–25 $\mu$g total protein/lane for GCS-HS) were analyzed by Western analysis and quantified as described in MATERIALS AND METHODS. Relative amounts of CDO or GCS-HS protein were divided by the amount of total protein loaded per lane. The relative amount of CDO protein in some samples was below the range of the standard curve, even when the maximal amount of total protein was loaded. These values are indicated as less than the lowest value on the standard curve. For the same protein (CDO or GCS-HS), values not followed by a common symbol are significantly different ($P \leq 0.05$) by ANOVA and Tukey’s $\text{ω}$-procedure.
version of methionine to cysteine in intact rats vs. cultured hepatocytes, or differences in the rates of removal of methionine and cysteine by various pathways could explain this apparent difference in response of liver cells to methionine vs. cysteine.

Changes in enzyme mRNA and protein concentrations in response to sulfur amino acid supplementation. The relative changes in CDO and GCS activities, protein concentrations, and mRNA concentrations are summarized in Table 4. Increases in CDO activity in response to sulfur amino acids were associated with similar degrees of changes in CDO protein concentration and with much smaller changes in CDO mRNA abundance ($r = 0.97$ for CDO activity vs. CDO protein and $r = 0.89$ for CDO protein vs. CDO mRNA). In previous studies with rats fed diets supplemented with methionine or cysteine or with high levels of protein, increases in both CDO activity and CDO protein, but no changes in CDO mRNA abundance, were observed. In cultured hepatocytes the increases in CDO activity could be explained by an increase in the mRNA level (2.5–5.0 times basal) and increases in CDO protein or activity that were 4–6 times as much as those predicted from the increases in CDO mRNA. The meaning of the differences in CDO mRNA levels is uncertain, as the amount of CDO mRNA found in freshly isolated hepatocytes decreases markedly with maintenance of these cells in primary culture over 24 h and remains low through 76 h in culture (Y. H. Kwon, L. L. Hirschberger, and M. H. Stipanuk, unpublished observations). Although cultured hepatocytes do not exactly model hepatocytes in situ, the major regulatory change, which was an increase in CDO activity associated with a similar increase in CDO protein, was consistent between the animal and cell culture studies. Thus these studies in cell culture also indicate that regulation of CDO activity in response to sulfur amino acids is accomplished predominantly via changes in CDO concentration. The possible role of changes in CDO mRNA abundance and the significance of the two isoforms of CDO need further study, as does the mechanism by which the concentration of CDO is increased.

Decreases in GCS activity in response to supplementation of the culture medium with sulfur amino acids were associated with similar trends for GCS-HS mRNA abundance and GCS-HS protein concentration ($r = 0.96$ for GCS activity vs. GCS-HS protein and $r = 0.95$ for GCS-HS protein vs. GCS-HS mRNA). How-

Fig. 7. Effect of sulfur amino acids and/or BSO on the relative levels of CDO (A) and GCS-HS mRNAs (B) in cultured hepatocytes. Rat hepatocytes were cultured in basal medium or medium supplemented with 2 mmol/l L-methionine (+ Met) or 2 mmol/l L-cysteine (+ Cys) for 72 h. The effect of BSO was assessed by addition of 100 μmol/l BSO to the culture medium for the last 48 h. The amounts of CDO and GCS-HS mRNAs were adjusted for the amount of GAPDH mRNA in each aliquot of RNA loaded onto the gel. Results are expressed as means ± SE for experiments with hepatocytes isolated from 3 different rats. Values not followed by a common superscript letter are significantly different ($P < 0.05$) by ANOVA and Tukey’s $\alpha$-procedure. Northern blot analysis of CDO and GCS-HS mRNAs, which was done on pooled samples from each treatment group ($n = 3$; samples pooled on the basis of equal amounts of total RNA), is shown below the bar graphs.

Table 4. Relative effects of methionine and cysteine on CDO and GCS activities, concentrations, and mRNA levels in cultured rat hepatocytes

<table>
<thead>
<tr>
<th>Medium</th>
<th>CDO Activity</th>
<th>CDO Protein</th>
<th>CDO mRNA</th>
<th>GCS Activity</th>
<th>GCS-HS Protein</th>
<th>GCS-HS mRNA</th>
<th>GSH Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Basal + 2 mmol/l methionine</td>
<td>12.5</td>
<td>&gt;11</td>
<td>2.5</td>
<td>0.51</td>
<td>0.89</td>
<td>0.79</td>
<td>1.69</td>
</tr>
<tr>
<td>Basal + 2 mmol/l cysteine</td>
<td>18</td>
<td>&gt;15</td>
<td>4.2</td>
<td>0.29</td>
<td>0.58</td>
<td>0.37</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Values are means ± SE of combined $n = 6$ hepatocyte preparations (i.e., cells from 6 rats). All values have been divided by that of the basal group to express values for sulfur amino acid-supplemented hepatocytes relative to basal values of 1.0. Values are based on the mean values of cells cultured with the indicated medium in the studies with PPG (−PPG groups only) and in studies with BSO (−BSO groups only).
ever, the decreases in GCS-HS protein concentration tended to be less than the decreases in GCS activity or GCS-HS mRNA concentration, suggesting that regulation of GCS involves multiple mechanisms. Both the pattern and the magnitude of the changes in GCS activity, GCS-HS protein concentration, and GCS-HS mRNA in response to sulfur amino acid supplementation were similar to those observed in liver of rats fed diets supplemented with sulfur amino acid plus a mixture of nonsulfur amino acids or diets high in casein (3, 4). However, when diets were supplemented with sulfur amino acid alone, the magnitudes of the decreases in GCS-HS protein concentration and GCS activity were similar, suggesting that the decrease in activity state in vivo depends on the presence of nonsulfur amino acids as well as sulfur amino acids (3, 4). Thus again, although the overall changes in response to sulfur amino acids appear to be similar in liver in vivo and in hepatocytes in culture, they are not identical, and this indicates the importance of also doing studies in vivo.

Expression of both subunits of GCS is known to be transcriptionally regulated, and GCS activity also can be modulated by posttranslational mechanisms (24, 25). The initiating signal for GCS induction in response to an increase in cysteine is not clear. It seems likely that cysteine has a regulatory effect on GCS-HS expression that is independent of changes in GSH concentration, because cellular GSH concentrations were not closely associated with GCS-HS mRNA levels in these studies with cultured hepatocytes. Additionally, the apparent decrease in activity state of GCS-HS (GCS activity/GCS-HS protein concentration) observed in cells cultured in medium supplemented with sulfur amino acids does not appear to be explained by feedback inhibition of GCS by GSH. The assay conditions used to measure GCS activity result in GSH concentrations that are too low to have a measurable effect on activity, and cellular GSH concentrations were not closely correlated with GCS activity state. Note, for example, that cells cultured in basal medium had markedly different ratios of GCS activity to GCS-HS protein ratios, as well as different GCS-HS mRNA levels, than did cells cultured in cysteine-supplemented media, despite similar GSH concentrations.

In these studies with cultured rat hepatocytes, we observed induction of CDO at the level of both mRNA and protein, indicating that regulation of CDO in response to cysteine may be complex. In studies with intact rats, no changes in CDO mRNA levels were observed, indicating that regulation of CDO expression in vivo is clearly posttranscriptional (3, 4). Yamaguchi et al. (26) showed that injection of cysteine into rats increased the activity of hepatic CDO without a lag phase, suggesting the rapid activation of a previously existing endogenous CDO by cysteine or the stabilization of CDO by its substrate. The half-life of CDO is on the order of 2 to 3 h and has been reported to be increased to 6 h in the presence of excess cysteine, favoring the stabilization hypothesis (11, 12). However, the induction of CDO activity by cysteine was partially inhibited by cycloheximide (26), suggesting an involvement of protein synthesis as well. Although CDO protein concentration and activity were closely correlated in these studies with cultured hepatocytes, it should be noted that preliminary results from our laboratory indicate that the activity of the CDO isoform with the lower apparent molecular weight, which is the dominant isoform in cultured hepatocytes, is much less than that of the other isoform, which is most abundant in liver in vivo (L. L. Hirschberger and M. H. Stipanuk, unpublished observations). This observation raises the hypothesis that some type of posttranslational modification may be involved in regulation of CDO activity. Clearly the details of the molecular mechanisms involved in upregulation of CDO remain to be elucidated.

**Cellular mediator of response to sulfur amino acids.** When methionine and cysteine and their metabolites were tested for effectiveness in upregulating CDO and downregulating GCS, methionine, homocysteine (an intermediate in the transsulfuration pathway), and cysteine were found to be effective in regulating the activities of both enzymes. S-Adenosylmethionine and cystathionine, intermediates in the transsulfuration pathway, were effective in downregulating GCS activity but not in upregulating CDO. The lesser effect of S-adenosylmethionine and cystathionine may be related to lower rates of cellular uptake of these compounds compared with methionine, cysteine, and homocysteine (21). Taurine and sulfate, which are metabolites of cysteine, had no effect on either GCS or CDO activity. Because cysteine was as effective as any of its precursors, it seems very likely that either cysteine or a closely related compound must play an essential role in the hepatic response to sulfur amino acids.

A key role of cysteine is also supported by the observation that, in these studies with cultured hepatocytes, the magnitudes of the effects of sulfur amino acid supplementation in increasing CDO activity, CDO concentration, and CDO mRNA concentration or in decreasing GCS activity, GCS-HS concentration, and GCS-HS mRNA concentration were consistently greater when cysteine was added than when methionine was added.

To clarify the role of cysteine, we used PPG to block the transsulfuration of methionine to cysteine at the level of cystathionine. Inhibition of cystathionine γ-lyase (cystathionase) blocks the last step of the methionine transsulfuration pathway in which cystathionine is cleaved to release cysteine, α-ketobutyrate, and ammonia. By blocking transsulfuration at the level of cystathionine, addition of PPG should limit cysteine formation from methionine sulfur and serine and also result in higher concentrations of cystathionine and perhaps other transsulfuration intermediates. The marked decrease in GSH concentration that resulted from treatment of cells cultured in either basal or methionine-supplemented medium with PPG indicated the effectiveness of PPG in inhibiting transsulfuration; GSH synthesis depends upon cysteine availability as well as on GCS activity. Addition of PPG
to culture medium supplemented with methionine blocked the increase in CDO activity and the decrease in GCS activity, but PPG had no effect on CDO or GCS activity in hepatocytes cultured in medium supplemented with cysteine. Thus methionine was ineffective in regulating these two enzymes when its conversion to cysteine was blocked, clearly indicating that cysteine, rather than methionine or an intermediate in the transsulfuration pathway, is essential for bringing about sulfur amino acid-induced changes in CDO and GCS activities in hepatocytes.

Because GSH concentration is closely associated with cysteine availability (supplied as cysteine or formed from methionine via transsulfuration), additional studies were done to test the possibility that GSH, rather than cysteine, may be the mediator of the effect of sulfur amino acids on CDO and GCS activities. The effectiveness of BSO in inhibiting GCS, the enzyme that catalyzes the first step in GSH synthesis, is clear from the low GCS activities (but not GCS-HS protein concentrations) and low GSH concentrations in hepatocytes cultured with BSO. The effects of BSO on GCS activity and GSH concentration were observed regardless of the sulfur amino acid level in the medium.

The consistent lack of response of CDO activity, CDO protein, and CDO mRNA to BSO despite the marked decreases in the level of GSH that were induced by BSO indicate that CDO is not regulated by the cellular content of GSH. GSH concentrations were not correlated with CDO activity in either the study with PPG or the study with BSO, also indicating that GSH is not the mediator of the effect of cysteine on CDO activity.

Although GCS activity was low in cells cultured with BSO due to its inhibition of GCS, cells cultured with BSO had higher concentrations of GCS-HS mRNA and GCS-HS protein. This suggests that the low GSH concentration, or oxidative stress related to it, caused upregulation of GSH-HS expression. On the other hand, variations in GSH concentration in cells cultured with or without PPG and with or without sulfur amino acid were not correlated with GCS activity, suggesting that a signal other than cellular GSH concentration and/or oxidative stress is also involved in regulation of GCS activity. It is possible that cysteine itself, in addition to GSH, is involved in the downregulation of GCS in response to sulfur amino acid supplementation.

These studies provide strong evidence that cysteine itself, rather than a precursor or metabolite of cysteine, acts as an initial signal for regulation of CDO and GCS activities in hepatocytes. Clearly, further studies of the mechanisms by which cysteine downregulates GCS activity and upregulates CDO activity need to be conducted, and cultured rat hepatocytes appear to be a suitable model system for use in some of those studies.

We gratefully acknowledge the assistance of Larry L. Hirschberger. We also thank Dr. Jay Forman for the anti-GCS-HS serum, Dr. Yu Hosokawa for the anti-CDO IgG, and Drs. Yu Hosokawa and Nobuyo Tsunoyama for the EcoR I-cut cDNA for CDO.

This research was supported in part by United States Department of Agriculture/Cooperative State Research, Education, and Extension Service Grants 94–34324–0987 and 99–34324–8120.

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