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Regulation of cysteine dioxygenase and γ-glutamylcysteine synthetase is associated with hepatic cysteine level

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Abstract

Two hepatic enzymes, cysteine dioxygenase (CDO) and γ-glutamylcysteine synthetase (GCS), play important regulatory roles in the response of cysteine metabolism to changes in dietary sulfur amino acid or protein levels. To examine the time-course of changes in CDO and GCS activities, CDO and GCS-catalytic or heavy subunit protein and mRNA levels, and cysteine and glutathione levels, we adapted rats to either a low protein (LP) or high protein (HP) diet, switched them to the opposite diet, and followed these parameters over 6 days. Hepatic CDO activity and amount, but not mRNA level, increased in response to higher protein intake; the $t_{1/2}$ of change for CDO activity or protein level was 22 h for rats switched from a LP to a HP diet and 8 h for rats switched from a HP to a LP diet, suggesting that the HP diet decreased turnover of CDO. Hepatic GCS activity, catalytic subunit amount and mRNA level decreased in response to a higher protein intake. GCS catalytic subunit level changed with a similar $t_{1/2}$ for both groups, but the change in GCS activity in rats switched from a LP diet to a HP diet was faster (~16h) than for rats switched from a HP to a LP diet (~74h). Hepatic cysteine and glutathione levels reached new steady states within 12 h (LP to HP) or 24 h (HP to LP). CDO activity appeared to be regulated at the level of protein, probably by diminished turnover of CDO in response to higher protein intake or cysteine level, whereas GCS activity appeared to be regulated both at the level of mRNA and activity state in response to the change in cysteine or protein availability. These findings support a role of cysteine concentration as a mediator of its own metabolism, favoring catabolism when cysteine is high and glutathione synthesis when cysteine is low.

Keywords: Cysteine; Cysteine dioxygenase; Diet; γ-Glutamylcysteine synthetase; Glutathione; Liver; Protein degradation

1. Introduction

Normal regulation of cysteine metabolism appears to play an important role in health because high levels of cysteine and low levels of sulfate are associated with the occurrence of several chronic neurological and non-neurological diseases associated with aging and with less rapid progression of some disease states [1–5]. Elevated levels of cysteine have been shown to be both cytotoxic and neurotoxic [6–9], and increased levels of plasma total cysteine, as well as of its precursor homocysteine, have been associated with increased risk for pregnancy complications, adverse pregnancy outcomes and cardiovascular disease [10–11].

Two hepatic enzymes involved in cysteine metabolism are clearly regulated in response to sulfur amino acid intake. Hepatic cysteine dioxygenase (CDO; EC 1.13.11.20) increases and hepatic γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2) decreases when animals are fed diets high in protein or sulfur amino acids [12–14]. Marked changes in CDO and GCS levels occur when dietary protein is increased from below-requirement levels to above-requirement levels [15–17]. CDO activity is barely detectable in liver of rats fed low protein diets, but CDO activity increases up to ~ 6 nmol · min^{-1} · mg $protein^{-1}$ in rats fed diets containing high levels of protein or high sulfur amino acids. In contrast, GCS activity is high (~13 nmol · min^{-1} · mg $protein^{-1}$) in liver of rats fed low protein diets and decreases to ~5 nmol · min^{-1} · mg $protein^{-1}$ in liver of rats fed diets containing high levels of protein or sulfur amino acids.

Changes in activities of these enzymes are physiologically important in the regulation of cysteine homeostasis. A large physiological effect of changes in CDO activity on the capacity of an animal to catabolize cysteine to taurine and sulfate/pyruvate has been demonstrated by differences in

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rates of product formation by isolated rat hepatocytes from rats adapted to diets that contained various levels of protein or sulfur amino acid and by different tissue and urinary taurine and sulfate levels in intact rats adapted to these same diets [12,13,15]. Similarly, an effect of hepatic GCS activity on the capacity (at a given cysteine level) of the animal to synthesize glutathione has been demonstrated by differences in the rates of glutathione synthesis by isolated rat hepatocytes from rats adapted to diets that contained various levels of protein or sulfur amino acid [13,18,19].

By catalyzing the first step in the oxidative metabolism of cysteine, CDO clearly plays a central role in cysteine catabolism and, hence, in control of cellular and body cysteine concentrations [15–17]. Cysteine catabolism is also essential for the formation of sufficient sulfate and taurine to meet the body's requirements. Low levels of CDO can result in low rates of inorganic sulfate release from sulfur amino acids and, hence, impaired sulfation reactions [1–3], and low or absent CDO activity is clearly the basis of the requirement of some species for dietary taurine [20].

Synthesis of glutathione from its substituent amino acids by the sequential actions of GCS and glutathione synthetase also plays an important role in cysteine homeostasis: glutathione synthesis removes cysteine from the intracellular cysteine pool, but, at the same time, glutathione serves as a reservoir of available cysteine. Cysteine is generally the rate-limiting substrate for synthesis of glutathione, but extracellular hydrolysis of glutathione by the sequential actions of γ-glutamyl transpeptidase and dipeptidase also is an important source of cysteine in peripheral tissues [21–23].

To further define the mechanisms involved in the response of hepatic CDO and GCS to dietary protein or sulfur amino acids, we determined the time-course of the diet-induced changes in CDO activity and CDO protein and mRNA levels in liver and in GCS activity and GCS-heavy or catalytic subunit (GCS-HS) protein and mRNA levels in both liver and kidney. We also assessed the relation of these changes to changes in tissue cysteine and glutathione levels. The results confirm that hepatic CDO increases and hepatic GCS decreases in response to dietary protein and provide further evidence for the regulation of CDO activity at the level of protein degradation and of GCS-HS at the level of its mRNA and also activity state. Although GCS activity is much higher in kidney than in liver, renal GCS activity and GCS-HS expression were not affected by diet.

2. Experimental procedures

2.1. Animals and dietary treatments

For the animal study, seventy-two male Sprague-Dawley rats (170 to 210 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed individually in polycarbonate cages containing woodchips and paper bedding in a room maintained at 20°C and 60 to 70% humidty with light from 2100 h to 0900 h. Rats had free access to water at all times but had access to food only during the dark cycle. Rats were fed semipurified diets that contained 100 g casein/kg (low protein, LP) or 400 g casein/kg (high protein, HP) and were purchased from Dyets, Inc. (Bethlehem, PA). In addition to casein, the diets also contained (g/kg) 155 dextrinized cornstarch, 100 sucrose, 50 fiber (Solka-Floc), 40 soybean oil, 35 AIN-93M-VX vitamin mix, 10 AIN-93M mineral mix, 2.5 choline bitartrate, 0.008 tert-butylhydroquinone, and either 507.5 (LP) or 207.5 (HP) cornstarch. Food intake was measured and recorded for the entire experimental period.

Rats were randomly divided into two groups; one group was fed the LP diet and the other group was fed the HP diet for 2 weeks. At the end of the light cycle on day 15, the 37 rats fed each diet were blocked by weight into 3 weight groups; one animal from each weight block was randomly assigned to each of 12 diet treatment/time-point groups: initial group (0 time, no additional dietary treatment); 8 groups that were switched to the opposite diet and then killed at 1, 3, 6, 12, 24, 48, 72, or 144 h after the start of the new diet; and 3 groups that remained on the same diet and were killed at 6, 12, or 48 h as additional control groups. Each group contained 3 rats. At the designated time point, rats were weighed, anesthetized with CO_2 and decapitated. Liver and kidneys were removed, rinsed with ice-cold saline, weighed, immediately frozen in liquid nitrogen and stored at –140°C until analysis. The experimental protocol was approved by the Cornell University Institutional Animal Care and Use Committee. Due to time and cost considerations, CDO and GCS-HS protein and mRNA levels were measured on pooled samples for each experimental group; equal amounts of soluble protein or equal amounts of total RNA from each rat were pooled to obtain the pooled samples. Enzyme activity assays were done for liver or kidney from individual rats.

2.2. Analysis of CDO activity, CDO protein and CDO mRNA level

Portions of frozen liver were homogenized in ice-cold 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH. 6.0, or 0.05 M potassium phosphate buffer, pH 6.8, for assay of CDO activity. CDO activity was measured by the method of Bagley et al. [24]. CDO activity was expressed on the basis of total soluble protein; protein was measured by the method of Smith et al. [25].

CDO level was measured by quantitative western blot analysis as described previously [16,17]; a relative standard curve generated on each gel/membrane with previously pooled liver supernatant from rats fed a moderate protein (200 g casein/kg) diet was used to ensure linearity and accurate quantification. Rabbit anti-CDO was raised against recombinantly-expressed rat liver CDO, and the immunoglobulin (IgG) fraction was purified using Affi-Gel (Bio-Rad) and used as the primary antibody. Protein in the

100,000 × *g* supernatant fractions was measured by the method of Smith et al. [25]. Relative levels of CDO were expressed on the basis of total soluble protein in the samples.

CDO mRNA was measured by quantitative northern blot analysis as described previously [16,17] using radiolabeled cDNA probes. Relative standard curves were generated on each gel/membrane using previously pooled total RNA from rats fed a moderate protein diet. Cyclophilin mRNA was measured as an internal standard using a probe generated with Cyclophilin-A-Mouse DECAprobe Template (Ambion, Inc., Austin, TX). The relative levels of CDO and cyclophilin mRNA were expressed on the basis of total RNA in the samples. Total RNA was measured by absorbance at 260 nm.

2.3. Analysis of GCS activity, GCS-HS protein and GCS-HS mRNA level

Portions of frozen tissue were homogenized in 20 mmol/L Tris buffer, pH. 8.2; the 100,000 × *g* supernatant fraction was obtained and used for assay of GCS activity. Assay of GCS activity was carried out as described by Yan and Huxtable [26] except that both γ-glutamylcysteine and glutathione were measured as products because some of the γ-glutamylcysteine was further converted to glutathione using glycine present in the tissue supernatant. Glutathione and γ-glutamylcysteine were quantified by the HPLC method of Fariss and Reed [27] as modified by Stipanuk et al. [28]. Protein was determined by the method of Smith et al. [25].

GCS-HS protein was measured by quantitative western blot analysis as described previously [16,17]; a relative standard curve generated on each gel/membrane with previously pooled liver supernatant from rats fed a moderate protein (200 g casein/kg) diet was used to ensure linearity and accurate quantification. Rabbit anti-GCS heavy, or catalytic, subunit serum [29] was a gift from Dr. Henry Jay Forman (University of Alabama, Birmingham, AL). The specificity of this antibody has been described previously [16]. Protein in the 100,000 × *g* supernatant fractions was measured by the method of Smith et al. [25]. Relative levels of GCS-HS were expressed on the basis of total soluble protein in the samples.

GCS-HS mRNA was measured by quantitative northern blot analysis as described previously [16,17] using radiolabeled cDNA probes. Relative standard curves were generated on each gel/membrane using previously pooled total RNA from rats fed a moderate protein diet. Cyclophilin mRNA was measured as an internal standard. The relative levels of CDO and cyclophilin mRNAs were expressed on the basis of total RNA in the samples. Total RNA was measured by absorbance at 260 nm.

2.4. Cysteine and glutathione levels

Total cysteine and glutathione (thiol plus disulfide forms) in liver and kidney were measured by formation of S-carboxymethyl derivatives followed by chromophore derivatization of primary amines with 1-fluoro-2,4-dinitrobenzene and separation of these derivatives by reversed-phase ion-exchange HPLC as described previously [16,17,27].

2.5. Kinetic and statistical analyses

Degradation constants and half-lives were calculated for the changes in enzyme activity and amount in response to the dietary change by fitting the data to a first-order exponential decay equation (Origin, version 5, Microcal Software, Inc. Northhampton, MA). Student's *t*-test was used to compare results for rats fed the LP vs. the HP diet at a particular time point (Minitab, release 13.1, Minitab, Inc.)

3. Results

3.1. Weight gain, food intake, and tissue weights of rats fed high and low protein diets

Rats had an initial mean weight of 189 g and consumed an average of 22 g (LP) or 21 g (HP) of diet per day during the 2 weeks prior to the diet change (Table 1). During this time, the mean weight gain of rats fed the LP diet was 4.3 g per day, whereas that of rats fed the HP diet was 6.6 g per day, yielding final mean weights of 249 and 271 for rats fed the LP and HP diets, respectively. During the 6 days following the diet switch, the mean daily food intake (i.e., for groups killed at 24, 48, 72 and 144 h after the diet change) was not significantly different ($P \leq 0.05$) for rats fed the two diets. However, rats that were given the HP diet consistently consumed less diet (16 to 18 g per day) than did rats that received the LP diet (20 to 22 g per day), whether the rats had been switched to the opposite diet or maintained on their initial diet (control groups). Over the 6 days after the diet switch, body weight remained slightly higher in rats originally fed the HP diet, but liver and kidney weights and relative liver and kidney weights (g/100 g body weight) were not significantly different ($P \leq 0.05$) between the two groups. Rats consumed diet steadily over the 12 h dark period as judged by measured food intake and the stomach contents of rats killed at 3, 6 and 12 h after introduction of the new diet.

3.2. Cysteine dioxygenase activity, protein and mRNA level in liver of rats switched between high and low protein diets

CDO activity in liver of rats underwent a rapid change in response to the change in protein content of the diet. CDO activity in rats adapted to the low protein diet was 0.19 ± 0.09 nmol · min^{-1} · mg protein^{-1} and increased to 5.0 ± 0.07 nmol · min^{-1} · mg protein^{-1} by 24 h (a 25-fold increase) after rats were switched to the HP diet with an estimated half-life of 21.9 ± 0.5 h (Fig. 1). In contrast, CDO activity in rats

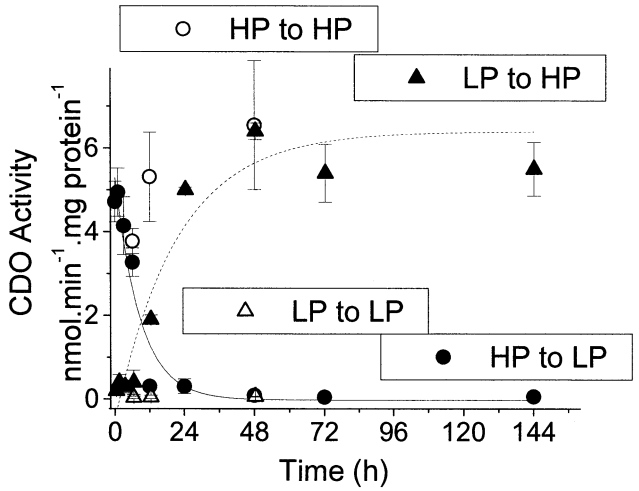

Fig. 1. CDO activity in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●) and in control rats continued on the initial adaptation diet for an additional 6 days (Δ, LP diet; ○ HP diet). Each value is the mean ± SEM for 3 rats.

adapted to the HP diet was 4.6 ± 0.5 nmol · min^{-1} · mg $protein^{-1}$ and decreased to 0.32 ± 0.05 nmol · min^{-1} · mg $protein^{-1}$ by 12 h after rats were switched to the LP diet with an estimated half-life of 8.6 ± 1.8 h. The relative CDO level in rats adapted to the low protein diet was 0.17, and this increased to 4.0 by 24 h (a 23-fold increase) after rats were switched to the HP diet with an estimated half-life of 20.4 h (Fig. 2). In contrast, the relative CDO level in rats adapted to the HP diet was 5.1, and CDO level decreased to 0.37 by 12 h after rats were switched to the LP diet with an estimated half-life of 7.9 h. (Note that CDO protein levels appear to be similar to CDO activity levels only because the immunodetectable CDO values are expressed relative to the amount of CDO in liver of rats fed a moderate protein diet and the CDO activity in liver of rats fed a moderate protein diet happens to be ~1 nmol · min^{-1} · mg $protein^{-1}$.) CDO activity and amount remained constant in control rats that remained on their initial diet for an additional 6, 12 or 48 h. The similar patterns of change in CDO activity and CDO amount are consistent with previous observations indicating that changes in CDO activity reflect changes in CDO concentration [15–17,30].

The apparent half-life of CDO turnover during the transition from one steady state to another was 2.5-times as long in liver of rats switched to the HP diet as in liver of rats switched from the HP to the LP diet, suggesting that degradation of CDO is down-regulated in response to a higher cysteine availability or up-regulated in response to a lack of cysteine. However, estimates of turnover based on changes in enzyme concentration or activity over time are not accurate measures of enzyme synthesis or degradation because they reflect contributions of both. The observed kinetics can be seen as consistent with a relatively slow and constant rate of CDO synthesis and a more rapid rate of CDO degradation in rats fed the LP diet. However, a 2.5-fold change in the rate of CDO degradation alone would not yield a ~20-fold change in the steady-state concentration of CDO. It is possible that CDO degradation rate increased and then decreased over the time-course of the adaptation period or that CDO synthesis was restricted by sulfur amino acid availability in rats fed the LP diet.

The relative abundance of CDO mRNA per unit total RNA was not different in rats fed the LP and HP diets and remained essentially constant between groups of rats switched to the opposite diet for all measurements made at the end of the 12-hr light period (i.e., 24, 48, 72 and 144 h) (Fig. 3A). However, CDO mRNA abundance decreased markedly during the feeding (dark) period to 28 to 35% of the baseline value at 12 h (the end of the dark period) before it returned to baseline by 24 h (the beginning of the dark period, or the end of the fasting/light period). CDO mRNA abundance also decreased in control rats maintained on the same diet (LP or HP) for the entire experimental period; CDO mRNA dropped to 29% of the "time 0" value in the LP control group and to 53% of the "time 0" value in the HP control group (data not shown). Cyclophilin mRNA was measured as a control; levels of cyclophilin mRNA did not vary significantly over the course of the experiment, except for a 20 to 40% dip in cyclophilin mRNA abundance relative to total RNA at 6 to 12 h in rats switched from the HP diet to the LP diet (Fig. 3B). Because CDO mRNA was consistently lower at 12 h, regardless of dietary treatment, it is possible that CDO mRNA was diluted by an increase in total RNA in livers of rats over the course of the 12-hr feeding period. Because we did not make measurements at different time-points within the 24-hr cycle subsequent to day 1, we do not know if this is a diurnal variation or a response to the dietary switch, but the former seems more likely. Nevertheless, it is clear that hepatic CDO mRNA levels did not respond to the dietary treatment over the 6-day time-course of adaptation to the new diet.

3.3. γ-Glutamylcysteine synthetase activity and γ-glutamylcysteine synthetase-heavy subunit protein and mRNA levels in liver of rats switched between high and low protein diets

In contrast to CDO activity, GCS activity in liver of rats underwent a decrease in response to the change in protein content of the diet. GCS activity in rats adapted to the low protein diet was 16 ± 1 nmol · min^{-1} · mg $protein^{-1}$ and decreased to 6 ± 1 nmol · min^{-1} · mg $protein^{-1}$ (37% of initial) by 6 days after rats were switched to the HP diet with an estimated half-life of 16 ± 8 h (Fig. 4). GCS activity in rats adapted to the HP diet was 6 ± 1 nmol · min^{-1} · mg $protein^{-1}$ and increased to 17 ± 2 nmol · min^{-1} · mg $protein^{-1}$ (2.8 times the initial level) by 6 days after rats were switched to the LP diet with an estimated half-life of 74 ± 13 h. The relative GCS-HS level in rats adapted to the low protein diet was 1.4, and this decreased to 0.8 (57% of

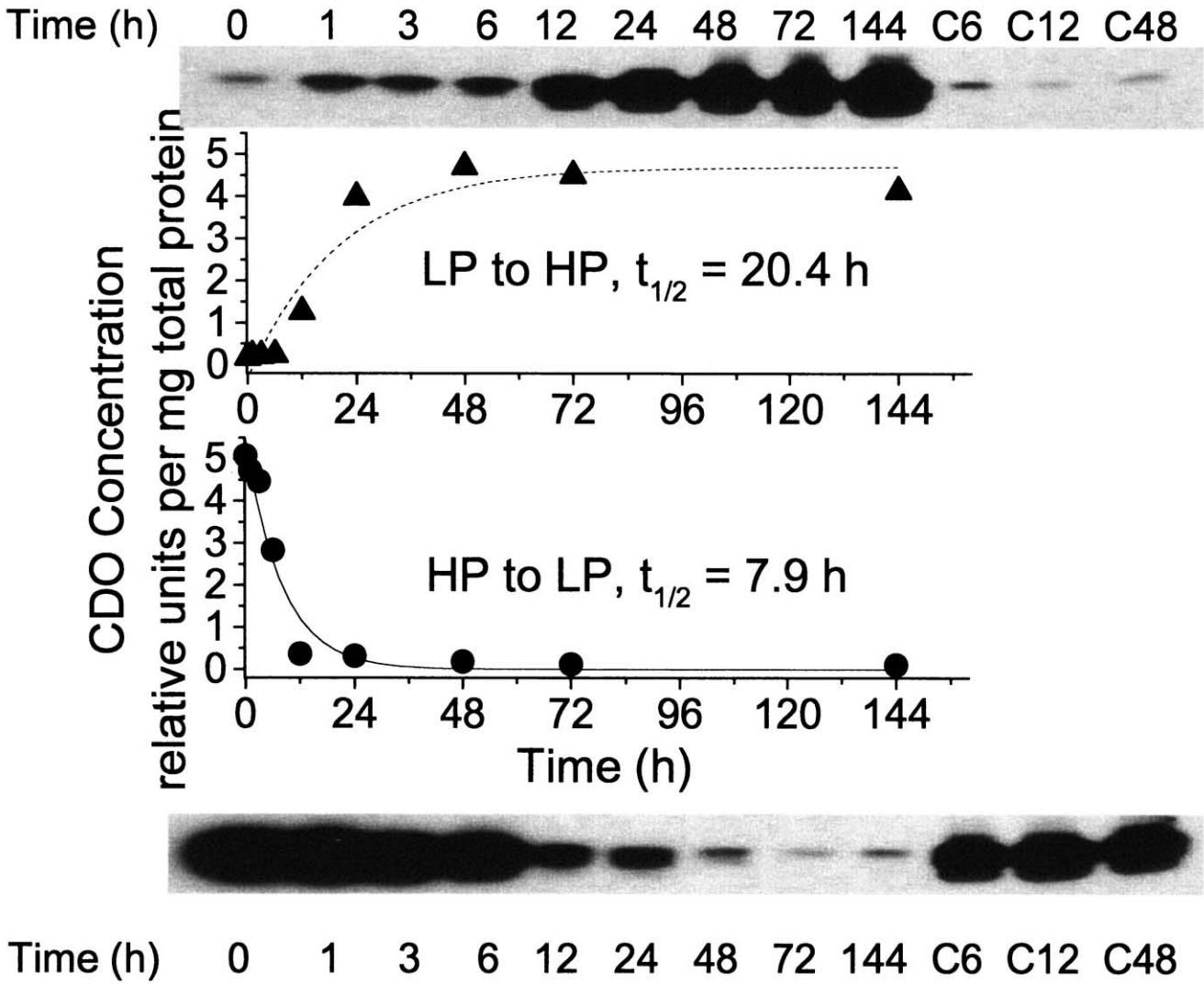

Fig. 2. CDO protein in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●). Each point on the graphs is the average of triplicate determinations on a pooled sample. A representative western blot for rats switched from the LP to HP diet (top) or from the HP to LP diet (bottom) and for control (C) rats maintained on the initial LP (top) or HP (bottom) diet is shown; 70 mg of total soluble protein was loaded in each lane.

initial) by 6 days after rats were switched to the HP diet with an estimated half-life of 70 h (Fig. 5). In contrast, the relative GCS-HS level in rats adapted to the HP diet was 0.9, and GCS-HS amount increased to 1.3 (1.4-times the initial level) by 6 days after rats were switched to the LP diet with an estimated half-life of 62 h. GCS activity and GCS-HS level remained constant in control rats that remained on their initial diet for an additional 6, 12 or 48 h. The changes in GCS activity were greater than those in GCS-HS amount, indicating that only one-third of the change in GCS activity could be attributed to a change in catalytic subunit concentration.

The apparent half-life of GCS-HS turnover during the transition from one steady state to another was similar in liver of rats switched from the LP to the HP diet ($t_{1/2}$ = 70 h) and in liver of rats switched from the HP to the LP diet ($t_{1/2}$ = 62 h), suggesting that the rate of GCS-HS turnover is not regulated in response to cysteine availability. The half-life for the increase in GCS activity in rats switched from the HP to the LP diet was similar (74 ± 13 h), but the decrease in GCS activity in rats switched from the LP diet to the HP diet was notably faster, 16 ± 8 h. This latter observation suggests that the activity state of GCS-HS, as well as GCS-HS concentration, may be down-regulated in liver of rats fed high protein diets, allowing a more rapid decrease than could be accomplished by GCS-HS turnover alone.

The relative abundance of GCS-HS mRNA per unit total RNA was 0.5 in liver of rats adapted or switched to the HP diet and 1.0 in liver of rats adapted or switched to the LP diet. New steady-state GCS-HS mRNA levels were reached by 2 days after the diet switches (Fig. 6). As for CDO mRNA abundance, there were some marked changes in GCS-HS mRNA abundance during the 24-hr light-dark cy-

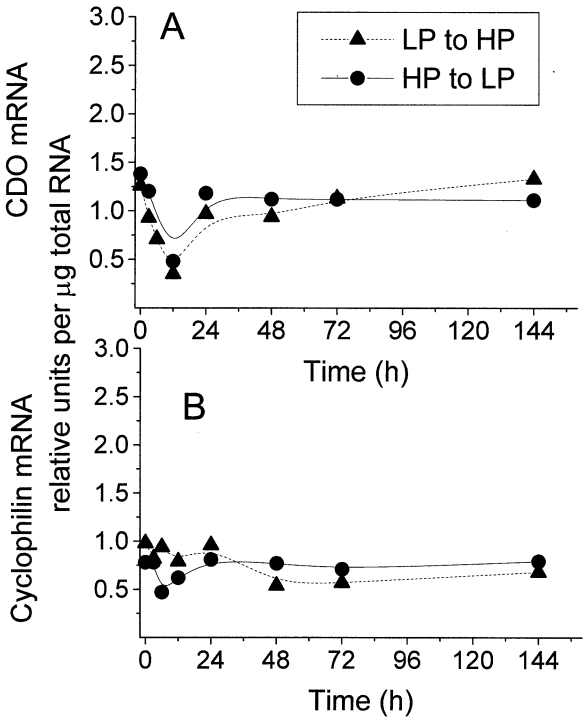

Fig. 3. CDO mRNA (A) or cyclophilin mRNA (B) abundance in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●). Each value is the average of triplicate determinations on a pooled sample.

cle, but these were in the opposite direction with GCS-HS mRNA abundance demonstrating transient increases at 3 h after initiation of the feeding (dark) period. No clear trend in GCS-HS mRNA response was observed in control rats maintained on the same diet throughout the study, probably because a 3-hr time point measurement was not made for control rats.

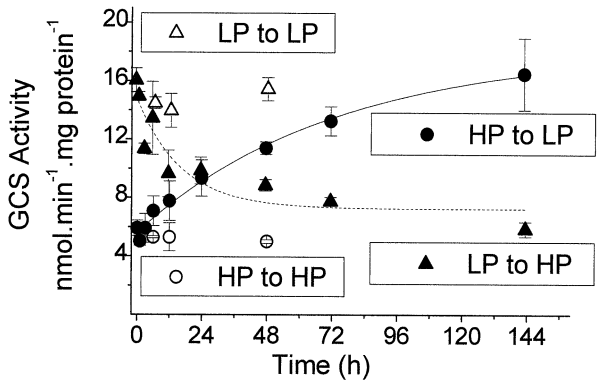

Fig. 4. GCS activity in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●) and in control rats continued on the initial adaptation diet for an additional 6 days (Δ, LP diet; ○ HP diet). Each value is the mean ± SEM for 3 rats.

3.4. Cysteine and glutathione levels in liver of rats switched between high and low protein diets

The total cysteine level in liver of rats adapted over two weeks to the LP diet and HP diet was 39 ± 8 nmol/g and 95 ± 10 nmol/g, respectively. When rats adapted to the LP diet were switched to the HP diet, the liver cysteine level increased markedly and rapidly, reaching a peak of 163 ± 52 nmol/g at the mid-point of the feeding (dark) period and then falling to a fasting plateau value of approximately 87 ± 7 nmol/g by 24 h (Fig. 7A). Cysteine level in livers of control rats that remained on the LP diet did not change significantly during the 12-hr feeding period. When rats adapted to the HP diet were switched to the LP diet, the liver cysteine level decreased gradually over the course of the feeding (dark) period, declining from the initial level of 95 ± 10 nmol/g to a fasting plateau value of approximately 55 nmol/g by 6 h and then falling further after 72 h to reach the final level of 44 ± 1 nmol/g at 144 h. Cysteine level in liver of control rats that remained on the HP diet did not change. Clearly, the observed changes in cysteine level were due to the change in sulfur amino acid or protein content of the diet rather than to diurnal responses to the light-dark/feeding-fasting cycle in general.

Overall, the changes in cysteine level closely paralleled the changes in CDO activity, except the lag in the increase in CDO activity in response to the switch from the LP to the HP diet was accompanied by an overshoot in hepatic cysteine level at 6 to 12 h. Because we did not make measurements at various points during the feeding cycle on subsequent days, we do not know whether this overshoot was a response to dietary intake, to inadequate CDO to maintain hepatic cysteine below 100 nmol/g, or both. The apparent increase in cysteine level at 12 h in the control rats maintained on the HP diet was substantially less than the peak values observed 12 h after the switch from a LP to HP diet, suggesting that both dietary intake and an initial inadequacy of CDO concentration contributed to the overshoot in cysteine concentration.

For GCS measurements, the changes in GCS-HS mRNA most closely followed the changes in hepatic cysteine level. Changes in GCS-HS protein and GCS activity, except for the decrease in GCS activity in rats switched from the LP to the HP diet, occurred more slowly than did the changes in cysteine levels.

Glutathione synthesis in the liver is dependent both on the supply of cysteine as substrate and the activity of GCS. Cysteine level was higher and GCS activity was lower in liver of rats fed the HP diet. Despite the effect of sulfur amino acid intake on GCS activity, the effect of cysteine supply dominated and glutathione levels tended to change in parallel with hepatic cysteine levels. The total glutathione levels in liver of rats adapted to the LP diet and HP diet were 2.8 ± 0.5 μmol/g and 4.9 ± 0.3 μmol/g, respectively. When rats adapted to the LP diet were switched to the HP diet, the liver glutathione level increased markedly and

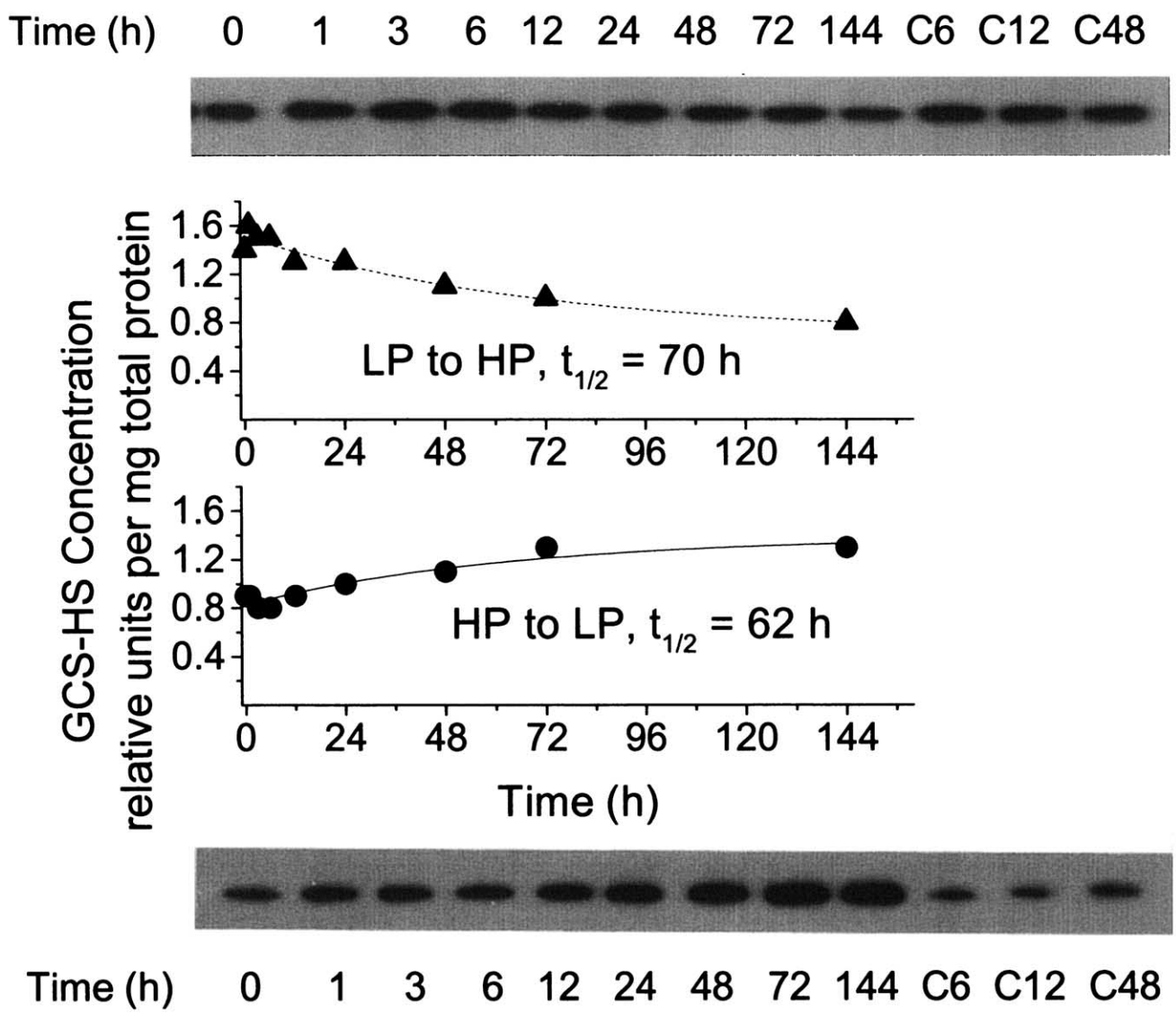

Fig. 5. GCS-HS protein in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●). Each point on the graphs is the average of triplicate determinations on a pooled sample. A representative western blot for rats switched from the LP to HP diet (top) or from the HP to LP diet (bottom) and for control (C) rats maintained on the initial LP (top) or HP (bottom) diet is shown; 70 μg of total soluble protein was loaded in each lane.

rapidly, reaching 10.6 ± 1.7 μmol/g at 6 h and a peak of 11.7 ± 0.3 μmol/g at 12 h (Fig. 7B). Fasting glutathione levels gradually decreased from 7.4 + 0.3 μmol/g at 24 h after the diet switch to 4.3 μmol/g at 144 h after the switch to the HP diet. Changes in glutathione levels in rats switched to the HP diet seemed to lag behind those for cysteine levels (Fig. 4A and B; see 6 and 12 h time points) but to follow the same general pattern. Glutathione levels in control rats that remained on the LP diet were essentially constant and showed no diurnal rhythm or feeding response. When rats adapted to the HP diet were switched to the LP diet, the liver glutathione level decreased gradually over the first 24 h to reach a fasting plateau value of about 2.5 μmol/g. The glutathione level in control rats on the HP diet was increased at 12 h, as was the cysteine level at 6 and 12 h, but had returned to the fasting plateau level by 24 h. The transient increase at 12 h probably reflects the higher hepatic cysteine concentration that provided more substrate for glutathione synthesis.

3.5. Glutamylcysteine synthetase activity, γ-glutamylcysteine synthetase – heavy subunit level and mRNA level, and cysteine and glutathione levels in kidney of rats switched between high and low protein diets

GCS activity in kidney was ~120 ± 3 nmol · min^{-1} · mg $protein^{-1}$, and it was not affected by the dietary protein level nor by the light-dark/feeding-fasting cycle. The relative levels of GCS-HS protein and GCS-HS mRNA were also similar for all treatment groups. However, the levels of GCS activity as well as of GCS-HS protein and GCS-HS mRNA were high in kidney compared to liver: the relative levels of

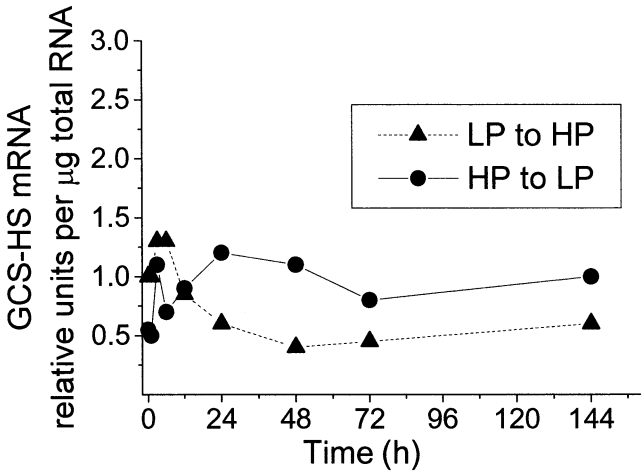

Fig. 6. GCS-HS mRNA abundance in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●). Each value is the average of triplicate determinations on a pooled sample.

GCS-HS protein and GCS-HS mRNA in kidney were 5.9 ± 0.1-times and 32 ± 1-times, respectively, those in liver of rats fed a moderate protein diet. Renal levels of cysteine and glutathione were also similar for all the treatment groups; cysteine averaged 675 ± 37 nmol/g kidney and glutathione averaged 2.5 ± 0.2 μmol/g kidney.

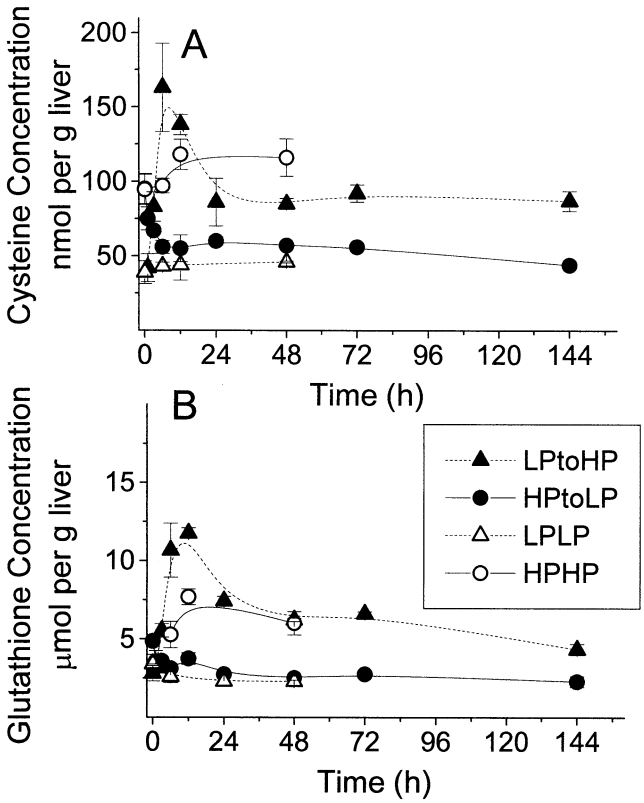

Fig. 7. Cysteine (A) and glutathione (B) levels in liver of rats adapted to a LP diet and switched to a HP diet at 0 time (▲) or adapted to a HP diet and switched to a LP diet at 0 time (●) and in control rats continued on the initial adaptation diet for an additional 6 days (Δ, LP diet; ○ HP diet). Each value is the mean ± SEM for 3 rats.

4. Discussion

4.1. Regulation of cysteine and glutathione levels

Hepatic CDO and GCS activities and cysteine and glutathione levels changed rapidly in response to a switch from a low protein diet to a high protein diet or vice versa, with new steady state levels being approached by or before 6 days. No effect of the diet change was observed on CDO or GCS activities or cysteine or glutathione levels in kidney. The lack of effect in kidney, despite the major role kidney plays in glutathione turnover, is consistent with our previous report of minimal effects of diet on cysteine concentration or levels of cysteine-metabolizing enzymes in the kidney, lung or brain [17].

The liver is able to respond to a large change in dietary protein (or sulfur amino acid) level by rapidly increasing or decreasing CDO activity over a 25-fold range. This increase or decrease in CDO activity was associated with a parallel change in the fasting-state hepatic cysteine level, which varied over a much smaller, 2.4-fold, range. Thus, new steady states were reached in which CDO activity varied markedly but cysteine was held low at levels less than 0.1 μmol/g. Hepatic steady-state glutathione level varied less than 2-fold and tended to lag behind changes in cysteine level, reflecting the role of cysteine as limiting substrate for hepatic glutathione synthesis. This response of liver CDO activity to a change in dietary protein or sulfur amino acid load either removes or conserves cysteine so that a low cysteine level is maintained in liver as well as in circulating fluids and in peripheral tissues.

The liver also responds to a large change in dietary protein or sulfur amino acid level by altering GCS activity over a 2.7-fold range, but in the opposite direction of the change in CDO activity or cysteine and glutathione levels. GCS activity increased in response to a low intake of sulfur amino acids, contributing to maintenance of adequate glutathione synthesis in the face of low cysteine concentrations, but decreased in response to a high intake of sulfur amino acids, favoring cysteine degradation (vs. glutathione synthesis) when cysteine availability was high.

4.2. Regulation of CDO activity

Changes in CDO activity were proportional to changes in CDO amount over the 6-day time course following the dietary switch from a low protein to a high protein diet or vice versa. The changes in response to diet occurred with similar half-lives for CDO activity as for CDO amount, but the apparent half-life was less for rats switched from a low protein to a high protein diet than for rats switched from a high protein to a low protein diet, suggesting that the deg-

radation rate for CDO was slower in rats consuming a high protein diet than in rats consuming a low protein diet. Although time-course studies of enzyme activity or level do not definitively define rate constants for protein synthesis and degradation, the apparent difference in CDO turnover is clearly consistent with regulation of CDO at the level of protein degradation.

The absence of an effect of dietary change on the level of CDO mRNA in liver of rats in this study also indicates that CDO levels are not regulated at the transcriptional level. In addition, the absence of any effect of diet on the polysome profile observed for CDO mRNA [19] and the inability of cycloheximide or emitine, which block protein synthesis, to prevent the accumulation of CDO in response to supplemental sulfur amino acids in primary cultures of rat hepatocytes [31] indicate that CDO is regulated at the level of degradation rather than synthesis.

4.3. Regulation of GCS activity

GCS activity decreased in liver of rats switched from a low protein diet to a high protein diet and increased in liver of rats switched from a high protein diet to a low protein diet. GCS-HS amount and GCS-HS mRNA level followed similar patterns. The apparent half-lives for GCS-HS protein changes were similar for the low to high and high to low protein switches, but the apparent half-life for the decrease in GCS activity with the switch of rats to a high protein diet was shorter (faster k_d) than observed for the increase in GCS activity, indicating that GCS activity decreased more rapidly than did GCS-HS concentration. Clearly, a portion of the change in GCS activity is related to a diet-induced change in GCS-HS mRNA level and GCS-HS protein level. However, the more rapid loss of GCS activity than of GCS-HS itself is consistent with a decrease in apparent activity state of GCS that we have previously reported as a consequence of feeding a high protein diet [16,17].

GCS is known to be transcriptionally regulated, and it is quite possible that protein/sulfur amino acid level brings about a signal for transcriptional regulation of the GCS-HS gene. The particular DNA elements and signal transduction mechanisms for this diet-induced regulation of GCS are not yet known [17,30]. The decrease in GCS-HS activity state in rats fed higher levels of protein has several possible explanations. First, a high protein diet could down-regulate the expression of GCS-LS (light or modifier subunit) to a greater degree than for GCS-HS, resulting in an imbalance of the two subunits. The formation of holoenzyme from the two subunits is associated with more favorable kinetics for γ-glutamylcysteine synthesis. The free GCS-HS has a higher K_m for glutamate and a lower K_i for glutathione and thus would be less active, particularly with the higher glutathione levels present in liver of rats fed the high protein diet [32–34]. A second possibility is simply increased inhibition of GCS-HS by glutathione when the protein content of the diet is increased; this response to tissue glutathione level could occur without any change in GCS-LS amount. Finally, GCS has been reported to undergo reversible phosphorylation with the phosphorylated GCS having less activity than the non-phosphorylated enzyme [35,36]. Phosphorylation of enzymes of amino acid metabolism could be a general mechanism used to increase amino acid removal in liver of rats fed high protein diets as well as in liver during starvation when amino acids from muscle are a major fuel.

4.4. Changes in CDO and GCS-HS mRNA abundance during the feeding (dark) period

Over the first 3 to 12 hr following the diet change (i.e., the dark period, food intake after an overnight fast), CDO mRNA abundance decreased and GCS-HS mRNA abundance increased relative to total RNA. Cyclophilin mRNA tended to decrease in rats switched to the low protein diet, but showed less variation over time. These changes in mRNA abundance were also observed in the control rats who remained on their adaptation diet (no diet switch). Although these changes in relative abundance of specific mRNAs relative to the total hepatic pool of RNA were not dependent upon the diet being fed, they do suggest that time point variation within the lighting/feeding cycle can have a substantial effect on the observed abundance of a specific enzyme mRNA.

4.5. Cysteine as the mediator of changes in CDO and GCS activities

We have previously suggested that the concentration of cysteine, or a closely related molecule, is the intracellular signal for changes in CDO and GCS activities [30]. In this study, we followed the level of both cysteine and glutathione over the 12-hr feeding (dark) period of day 1 and at the end of the fasting (light) period for days 1 through 6 after a switch from a low to a high protein diet or from a high to a low protein diet. When rats adapted to a high protein diet were switched to a low protein diet, the levels of cysteine and glutathione decreased gradually and reached a new plateau level by 12 to 24 h. In contrast, in rats switched from a low protein diet to a high protein diet, cysteine and glutathione levels increased markedly by 3 h and then gradually decreased to reach a new lower plateau level by 24 h. The apparent overshoot in both cysteine and glutathione concentrations may reflect the lag observed for changes in CDO and GCS activities; a few hours were required for CDO to increase to a level that could keep pace with the increased intake of sulfur amino acids. It is also possible, of course, that this overshoot is a response to diet intake alone and would have been observed on subsequent days if observations had been made during the subsequent feeding cycles. Nevertheless, cysteine and glutathione levels had leveled off by the end of the first fasting period, and the somewhat higher fasting levels were maintained in rats

consuming the high protein diet throughout the remainder of the time-course.

The rapid change in cysteine and glutathione levels and the maintenance of different cysteine and glutathione levels in the new steady state is consistent with a role for these thiols in the intracellular signaling that brings about changes in CDO and GCS activities. Inhibition of glutathione synthesis had no effect on the up-regulation of CDO in response to increased cysteine levels [30], but a possible effect of glutathione on the down-regulation of GCS has not been ruled out. This role of thiols on down-regulation of GCS activity may occur via changes in GCS-HS or GCS-LS levels or via feedback inhibition of GCS. The role of cysteine in the up-regulation of CDO appears to be due to a decreased rate of CDO degradation. Further work in our laboratory will seek to define the precise mechanisms involved in the regulation of CDO and GCS in response to changes in cellular cysteine levels.

References

[1] Davies MH, Ngong JM, Pean A, Vickers CR, Waring RH, Elias E. Sulphoxidation and sulphation capacity in patients with primary biliary cirrhosis. J Hepatol 1995;22:551–60.

[2] Bradley H, Waring RH, Emery P, Arthur V. Metabolism of low-dose paracetamol in patients with rheumatoid arthritis. Xenobiotica 1991; 21:689–93.

[3] Bradley H, Gough A, Sokhi RS, Hassell A, Waring R, Emery P. Sulfate metabolism is abnormal in patients with rheumatoid arthritis. Confirmation by in vivo biochemical findings. J Rheumatol 1994;21: 1192–6.

[4] Emery P, Salmon M, Bradley H, Wordsworth P, Tunn E, Bacon PA, Waring R. Genetically determined factors as predictors of radiological change in patients with early symmetrical arthritis. Bmj 1992; 305:1387–9.

[5] Heafield MT, Fearn S, Steventon GB, Waring RH, Williams AC, Sturman SG. Plasma cysteine and sulphate levels in patients with motor neurone, Parkinson's and Alzheimer's disease. Neurosci Lett 1990;110:216–20.

[6] Flynn J, McBean GJ. Kinetic and pharmacological analysis of L-(35S]cystine transport into rat brain synaptosomes. Neurochem Int 2000;36:513–21.

[7] Li H, Dryhurst G. Irreversible inhibition of mitochondrial complex I by 7-(2-aminoethyl)-3, 4-dihydro-5-hydroxy-2H-1, 4-benzothiazine-3-carboxylic acid (DHBT-1): a putative nigral endotoxin of relevance to Parkinson's disease. J Neurochem 1997;69:1530–41.

[8] Montine TJ, Picklo MJ, Amarnath V, Whetsell WO Jr., Graham DG. Neurotoxicity of endogenous cysteinylcatechols. Exp Neurol 1997; 148:26–33.

[9] Reis HJ, Gomez MV, Kalapothakis E, Diniz CR, Cordeiro MN, Prado MA, Romano-Silva MA. Inhibition of glutamate uptake by Tx3-4 is dependent on the redox state of cysteine residues. Neuroreport 2000; 11:2191–4.

[10] El-Khairy L, Ueland PM, Nygard O, Refsum H, Vollset SE. Lifestyle and cardiovascular disease risk factors as determinants of total cysteine in plasma: the Hordaland Homocysteine Study. Am J Clin Nutr 1999;70:1016–24.

[11] El-Khairy L, Vollset SE, Refsum H, Ueland PM. Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine Study. Am J Clin Nutr 2003;77:467–72.

[12] Bagley PJ, Stipanuk MH. The activities of rat hepatic cysteine dioxygenase and cysteinesulfinate decarboxylase are regulated in a reciprocal manner in response to dietary casein level 1, 2, 3. J Nutr 1994;124:2410–21.

[13] Bagley PJ, Stipanuk MH. Rats fed a low protein diet supplemented with sulfur amino acids have increased cysteine dioxygenase activity and increased taurine production in hepatocytes. J Nutr 1995;125: 933–40.

[14] Yokoyama M, Nakazoe JI. Intraperitoneal injection of sulfur amino acids enhance the hepatic cysteine dioxygenase activity in rainbow trout (Oncorhynchus mykiss). Fish Physiology and Biochemistry 1996;15:143–8.

[15] Bella DL, Hahn C, Stipanuk MH. Effects of nonsulfur and sulfur amino acids on the regulation of hepatic enzymes of cysteine metabolism. Am J Physiol 1999;277:E144–53.

[16] Bella DL, Hirschberger LL, Hosokawa Y, Stipanuk MH. Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver in vivo. Am J Physiol 1999;276:E326–35.

[17] Stipanuk MH, Londono M, Lee JI, Hu M, Yu AF. Enzymes and metabolites of cysteine metabolism in nonhepatic tissues of rats show little response to changes in dietary protein or sulfur amino acid levels. J Nutr 2002;132:3369–78.

[18] Bella DL, Kwon YH, Stipanuk MH. Variations in dietary protein but not in dietary fat plus cellulose or carbohydrate levels affect cysteine metabolism in rat isolated hepatocytes. J Nutr 1996;126:2179–87.

[19] Bella DL, Stipanuk MH. Effects of protein, methionine, or chloride on acid-base balance and on cysteine catabolism. Am J Physiol 1995;269:E910–7.

[20] Stipanuk MH, Bagley PJ, Coloso RM, Banks MF. Metabolism of cysteine to taurine by rat hepatocytes. Adv Exp Med Biol 1992;315: 413–21.

[21] Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW. Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci U S A 2000;97:5101–6.

[22] Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chevez-Barrios P, Wang Y, Habib GM, Goodman JC, Huang SL, Lebovitz RM, Matzuk MM. Growth retardation and cysteine deficiency in γ-glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci USA 1996;93:7923–6.

[23] Kumar TR, Wiseman AL, Kala G, Kala SV, Matzuk MM, Lieberman MW. Reproductive defects in γ-glutamyl transpeptidase-deficient mice. Endocrinology 2000;141:4270–7.

[24] Bagley PJ, Hirschberger LL, Stipanuk MH. Evaluation and modification of an assay procedure for cysteine dioxygenase activity: high-performance liquid chromatography method for measurement of cysteine sulfinate and demonstration of physiological relevance of cysteine dioxygenase activity in cysteine catabolism. Anal Biochem 1995;227:40–8.

[25] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150:76–85.

[26] Yan CC, Huxtable RJ. Effects of monocrotaline, a pyrrolizidine alkaloid, on glutathione metabolism in the rat. Biochem Pharmacol 1996;51:375–9.

[27] Fariss MW, Reed DJ. High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. Methods Enzymol 1987;143:101–9.

[28] Stipanuk MH, Coloso RM, Garcia RA, Banks MF. Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. J Nutr 1992;122:420–7.

[29] Shi MM, Iwamoto T, Forman HJ. g-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. Am J Physiol 1994;267:L414–21.

[30] Kwon YH, Stipanuk MH. Cysteine regulates expression of cysteine dioxygenase and γ-glutamylcysteine synthetase in cultured rat hepatocytes. Am J Physiol Endocrinol Metab 2001;280:E804–15.

[31] Stipanuk MH, Londono M, Hirschberger LL. Cysteine-responsive regulation of hepatic cysteine dioxygenase by the ubiquitin-proteasome system. FASEB J 2003;17:B370.
[32] Huang CS, Anderson ME, Meister A. Amino acid sequence and function of the light subunit of rat kidney γ-glutamylcysteine synthetase. J Biol Chem 1993;268:20578–83.
[33] Huang CS, Chang LS, Anderson ME, Meister A. Catalytic and regulatory properties of the heavy subunit of rat kidney γ-glutamylcysteine synthetase. J Biol Chem 1993;268:19675–80.
[34] Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP. Initial characterization of the glutamate-cysteine ligase modifier subunit Gclm(-/-) knockout mouse. Novel model system for a severely compromised oxidative stress response. J Biol Chem 2002;277:49446–52.
[35] Lu SC, Kuhlenkamp J, Garcia-Ruiz C, Kaplowitz N. Hormone-mediated down-regulation of hepatic glutathione synthesis in the rat. J Clin Invest 1991;88:260–9.
[36] Sun WM, Huang ZZ, Lu SC. Regulation of γ-glutamylcysteine synthetase by protein phosphorylation. Biochem J 1996;320:321–8.