Expression of γ-Glutamylcysteine Synthetase in the Liver of Copper-Deficient Rats (43928)

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Abstract. **Copper deficiency in rats increases hepatic glutathione concentration. The present study was undertaken to determine the biochemical and molecular basis for the glutathione elevation. Weanling Sprague-Dawley rats were fed a purified diet deficient in copper (0.4 μg/g diet) or one containing adequate copper (5.7 μg/g diet) for 4 weeks. Hepatic glutathione concentration, the activity of the rate-limiting enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase (γ-GCS), and the relative amount of mRNA for the enzyme were determined. Hepatic glutathione concentration in copper-deficient rats was significantly elevated (6.6 vs 5.6 μmol/g). The activity of hepatic γ-GCS was 1.6 times higher in the copper-deficient than in the copper-adequate rats (58.0 vs 35.9 nmol NADH/min • mg protein). The steady-state amount of mRNA for γ-GCS was increased 5-fold in the copper-deficient rat liver. The findings demonstrate that the elevated hepatic glutathione concentration in copper-deficient rats results from upregulation of γ-GCS activity. This study provides further understanding of changes in hepatic glutathione metabolism induced by copper deficiency.**

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Copper deficiency in rats increases hepatic glutathione concentration (1). The reason for the glutathione elevation has not been delineated. Several studies (2–5) have shown that copper deficiency reduces activities of both copper-dependent and non-copper-containing antioxidant enzymes, such as copper- and zinc-dependent superoxide dismutase (Cu,Zn-SOD) and glutathione peroxidase (GSHpx). The compromised defense system has been associated with copper deficiency–induced oxidative stress (4–6). It was thus hypothesized that the elevation of hepatic glutathione concentration is a compensatory response to the deficit in Cu,Zn-SOD and GSHpx (7), because glutathione is an important antioxidant that participates in both enzymatic and nonenzymatic protective responses. To pursue this hypothesis, the biochemical and molecular basis for elevation of glutathione concentration needs to be elucidated.

Glutathione is synthesized intracellularly from its constituent amino acids via two sequential enzymatic reactions. The rate-limiting reaction is catalyzed by γ-glutamylcysteine synthetase (γ-GCS). In the present study, we determined the enzymatic activity of γ-GCS and the relative amount of mRNA for this enzyme to examine whether upregulation of γ-GCS activity is involved in the increased hepatic glutathione concentration of copper-deficient rats.

Materials and Methods

Diets and Animals. *Diets.* A copper-adequate diet was formulated according to Reeves *et al.* (AIN-93G diet) (8), except that no antioxidant (tert-butylhydroquinone) was added. The primary ingredients were cornstarch (53%), casein (20%), sucrose (10%), and soybean oil (7%). Vitamins and minerals provided by the diet included the addition of Cu to a final concentration as indicated below. A copper-

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deficient diet was similarly formulated except for the replacement of copper by the corresponding weight of cornstarch. Diet analysis for copper (see below) yielded values of 5.7 mg Cu/kg diet for the copper-adequate diet and 0.4 mg Cu/kg diet for the copper-deficient diet.

Animals. Male, weanling Sprague-Dawley rats (46–57 g; Sasco, Lincoln, NE) were housed in quarters maintained at 22°–24°C with a 12:12-hr light:dark cycle. They were divided into two weight-matched groups having average weights of 52 g each. One group was given free access to the copper-adequate diet; the second group was given free access to the copper-deficient diet. Rats also had free access to deionized water. These experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (9).

Tissue Sample Preparation. After 4 weeks on their respective diets and an overnight fast, each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body wt; Vet Labs, Lenexa, KS). Blood was withdrawn from the inferior vena cava for erythrocyte counting and plasma assays. The liver was removed, flushed with cold 0.9% NaCl via its major vessels, and divided for subsequent assays. Tissue samples for mineral assays were stored at −20°C and samples for enzyme and mRNA assays were placed in liquid nitrogen, then stored at −80°C for less than 72 hr prior to analyses.

Blood Analysis. Hematocrit and hemoglobin content were determined on a Coulter Counter (Model S Plus 4; Hialeah, FL) as described previously (10). A Cobas Fara automated analyzer (Roche Diagnostic Systems, Nutley, NJ) was used to determine serum ceruloplasmin (11).

Analysis of Minerals. Trace element contents of the liver were determined by inductively coupled argon plasma emission spectroscopy (Model 1140; Jarrell-Ash, Waltham, MA) after lyophilization and digestion of the liver with nitric acid and hydrogen peroxide (12). Assay of dietary copper content was performed by a dry ashing procedure (13), dissolution in aqua regia and measurement by atomic absorption spectrophotometry (Model 503; Perkin Elmer, Norwalk, CT). Mineral contents of National Institute of Standards and Technology (NIST) reference samples (#1577a, bovine liver for organs; #1572, citrus leaves for diets) were within the specified ranges by NIST, thus validating our assay procedure.

Glutathione Assay. Hepatic tissue was homogenized in 5% (w/v) 5-sulfosalicylic acid (1:10) at 4°C. The homogenate was centrifuged at 10,000*g* for 15 min and the supernatant was assayed for GSH by the DTNB-glutathione reductase recycling assay (14). The 1-ml reaction mixture contained 190 μl stock buffer (143 m*M* sodium phosphate and 6.3 m*M* Na_4-EDTA), pH 7.5, 700 μl 0.248 mg NADPH/ml in stock buffer, 100 μl 6 m*M* DTNB, and 10 μl sample. The assay was initiated by addition of 10 μl 266 U glutathione reductase/ml. The standard assay was done under the same conditions including the same concentration of 5-sulfosalicylic acid. The amount of GSH was determined from the standard curve, in which the equivalents present (1, 2, 3, and 4 nmol) is plotted against the rate of change of absorbance at 412 nm, and was expressed as micromoles of GSH per gram of tissue.

Determination of γ-Glutamylcysteine Synthetase Activity. Tissue samples from liver were homogenized in 0.2 *M* Tris-HCl buffer (1:5), pH 8.0, using a variable speed tissue tearer (Biospec Products, Inc.) at about 20,000 rpm for 30 sec on ice. The homogenates were centrifuged at 10,000*g* for 50 min at 4°C. The enzyme activity was determined by the assay described by Seelig and Meister (15). The 1-ml reaction mixture contained 0.1 *M* Tris-HCl buffer, 150 m*M* KCl, 5 m*M* Na_2ATP, 2 m*M* phosphoenolpyruvate, 10 m*M* 1-glutamate, 10 m*M* L-α-aminobutyrate, 20 m*M* $MgCl_2$, 2 m*M* Na_2-EDTA, 0.2 m*M* NADH, 17 μg of pyruvate kinase, and 17 μg of lactate dehydrogenase. The reaction was initiated by addition of 50 μl tissue homogenate to the reaction mixture and the decrease in absorbance at 340 nm was followed. A sample blank contained all reagents except the tissue sample for which 50 μl of buffer was substituted. Another control assay was also done by omission of L-α-aminobutyrate in the presence of tissue sample. Specific enzyme activity was expressed as nanomoles of NADH oxidized to NAD per minute per milligram of cytosolic protein. Protein was determined by the assay described by Smith *et al.* (16).

Analysis of mRNA for γ-Glutamylcysteine Synthetase. Total liver RNA was isolated from quick frozen livers by using the RNAzol B method (Cinna/Biotecx, Friendswood, TX) and quantified spectrophotometrically. Total RNA, 15 μg, was then subjected to a 1% denaturating agarose gel and transferred to a GeneScreen Plus membrane (DuPont). The equal amount of RNA loaded on the gel was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA. Hybridization and wash procedure were conducted by the method described by Church and Gilbert (17). The probe corresponding to the 764–base pair PstI fragment of human γ-GCS complementary DNA was obtained from Dr. Timothy Mulcahy at the University of Wisconsin Comprehensive Cancer Center (18). The probe was labeled with ^{32}P dCTP using the random-prime method of Feinberg and Vogelstein (19). After autoradiography, the membrane was stripped and rehybridized with human β-actin cDNA to further ensure the integrity of the RNA sample and the equality of the amounts of RNA loaded onto all lanes. Autoradiographic images were scanned and an-

alyzed by using the MCID system from Imaging Research Inc. (Ontario, Canada). Densitometric values were then determined from digitized images of autoradiograms. Values were corrected for background and expressed as a percentage of the average signal of six individual controls.

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA). The data (all tables and figures) were presented as the value of mean ± SD for each determination from 6 replicate animals for each treatment. Differences between treatments were considered significant at $P < 0.01$.

Results and Discussion

In Table I characteristics of rats fed a copper-deficient diet are compared with those of rats fed a copper-adequate diet. Depressed liver copper concentration, reduced activities of the copper-dependent ceruloplasmin, and decreased hematocrit and hemoglobin concentrations were observed in the rats fed the copper-deficient diet. Under the same experimental condition, depressed plasma copper concentration and reduced Cu,Zn-SOD activity in the liver also have been observed (20). All of these changes have been reported previously and are typically indicative of severe copper deficiency (2).

Glutathione concentration in the liver was measured by using the DTNB-glutathione reductase recycling assay. As shown in Table II, hepatic glutathione concentration in the copper-deficient rats was significantly elevated. Although this assay only measures the total glutathione content (reduced and oxidized forms), previous studies (1) have shown that copper deficiency increases the amount of reduced but not oxidized glutathione in the liver.

The rate-limiting step in *de novo* glutathione synthesis is catalyzed by γ-GCS. Therefore, in an attempt to determine the biochemical basis responsible for the glutathione elevation, we examined the γ-GCS activity. The results presented in Table II show that copper deficiency indeed elevated the γ-GCS activity in the liver. To determine whether the increased γ-GCS activity resulted from enhanced gene expression, we measured the relative amount of mRNA for γ-GCS.

Table I. Characteristics of Rats Fed Copper-Deficient Diet and Those of Rats Fed Copper-Adequate Diet

	CuD	CuA
Liver Cu (nmol/g dry wt)	32 ± 7[a]	177 ± 13
Hematocrit	0.34 ± 0.04[a]	0.40 ± 0.02
Hemoglobin (g/l)	111 ± 12[a]	134 ± 7
Ceruloplasmin (mg/l)	29 ± 2[a]	373 ± 37

Note. Values are means ± SD ($n = 6$).
[a] Significantly different from those of CuA ($P < 0.01$).

Table II. Glutathione Levels and γ-GCS Activities in the Liver of Copper-Deficient and -Adequate Rats

	CuD	CuA
Glutathione (μmol/g · wt)	6.6 ± 0.6[a]	5.6 ± 0.6
γ-GCS (nmol NADH/min · mg protein)	58.0 ± 9.5[a]	35.9 ± 7.6
γ-GCS mRNA (% autoradio-image intensity)	500 ± 60[a]	100 ± 25

Note. Values are mean ± SD ($n = 6$).
[a] Significantly different from those of CuA ($P < 0.01$).

Total RNA was isolated from the liver and subjected to Northern analysis utilizing a ^{32}P-labeled probe corresponding to a 764–base pair PstI fragment within the coding region of hlGCS-50, a full-length γ-GCS clone isolated from a human liver complementary DNA library (18). As shown in Figure 1, the probe hybridized to a 2.4-kb transcript, which was elevated about five times (Table II) in the liver of copper-deficient rats. The elevated γ-GCS activity and the amount of mRNA thus correspond to the increased glutathione concentration.

The γ-GCS activity feedback regulated by the amount of cellular glutathione (21). This feedback mechanism controls the upper limit of cellular glutathione concentration. Although high concentration of glutathione inhibit the γ-GCS activity, glutamate competes with glutathione for binding at the regulatory site (21). It has been shown that elevation of glutamate uptake in dog erythrocytes is associated with the increased amount of cellular glutathione (22). However, the increased hepatic glutathione concentration in copper-deficient rats cannot be explained by either the glutathione- or glutamate-regulated synthesis. The present study shows that the γ-GCS activity itself is increased by copper deficiency, suggesting that the in-

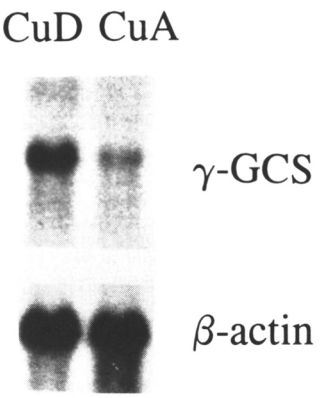

Figure 1. Northern blot analysis of γ-GCS expression in the liver of copper-deficient (CuD) and -adequate (CuA) rats. The experimental procedure is described in Materials and Methods. Autoradiographic image analysis results are shown in Table II. The autoradiography is a representative of six animals for each treatment.

creased glutathione synthesis is unlikely to result from the regulation of substrates or products.

Copper deficiency causes a wide diversity of metabolic and pathologic changes (2). Although the biochemical and molecular basis for these changes has not been extensively investigated, several studies suggest that oxidative stress is involved in the pathophysiology of copper deficiency (23–25). Because glutathione is an important antioxidant, which participates in cellular protection against damage induced by oxygen-derived free radicals, the increased glutathione synthesis in the liver of copper-deficient rats may reflect a general adaptation to oxidative stress. Mechanisms for such an adaptation have been shown to include transcriptional and translational control of gene expression (26). In the present study, the relative amount of mRNA for γ-GCS was elevated in the liver of copper-deficient rats, which correlated with the increased γ-GCS activity and the corresponding elevation of glutathione concentration. The results thus demonstrate that the elevation of hepatic glutathione concentration most likely results from the enhanced gene expression of γ-GCS.

It was noted that the level of elevated γ-GCS activity (1.6-fold) was not parallel with the increased amount (5-fold) of mRNA for this enzyme. Previous studies by Lu *et al.* (27) have shown that hepatic GSH synthesis is downregulated by two distinct hormone-mediated signal transduction pathways. Therefore, the γ-GCS activity may be regulated by phosphorylation. It is, however, unknown whether Cu deficiency affects post-translational regulation of this enzyme activity. Further studies need to be done with this regard.

The present study therefore provides further insight into the mechanism(s) responsible for the changes in glutathione metabolism caused by copper deficiency. Additional studies are required to define whether the elevated gene expression of γ-GCS results from (i) copper deficiency *per se;* (ii) a compensatory response to the deficit in SOD and GSHpx; and/or (iii) an adaptation to the oxidative stress induced by copper deficiency. The information generated from the present study will certainly facilitate such investigations.

In summary, the present study examined the activity of γ-GCS and the relative amount of mRNA for the enzyme in an attempt to elucidate the biochemical and molecular basis for the elevated hepatic glutathione concentration in copper deficiency. The data obtained demonstrate that the elevation of hepatic glutathione concentration results from upregulation of γ-GCS activity. This study provides additional information about the changes in glutathione metabolism induced by copper deficiency. This will facilitate further studies of the mechanism(s) responsible for the biochemical alteration.

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