Livers from copper-deficient rats have lower glutathione peroxidase activity and mRNA levels but normal liver selenium levels

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Activity of the selenoprotein glutathione peroxidase (GPX) is lower in liver and plasma of copper (Cu)-deficient rats and mice. The cause is not known, but altered selenium (Se) levels may be a factor. The purpose of the current experiments was to determine the temporal relationship in liver GPX activity during Cu deficiency in growing male and female Sprague-Dawley rats, and to measure liver GPX mRNA and Se levels. Rats were fed a Cu-deficient (0.5–0.6 mg Cu/kg), Se-adequate (0.16 mg Se/kg) diet. Control rats received Cu in the drinking water (20 μg/mL). In experiment one, rat dams were divided into the two dietary treatments at parturition. Offspring were weaned to the treatment of their dams. In experiment two, treatment began with weanling rats. Specific cuproenzyme activities were markedly reduced in unsupplemented male and female rats in both experiments. In the postweanling model, growth was not impaired and mild anemia was evident only in Cu-deficient males. Livers of 60-day-old Cu-deficient rats from both experiments had 10-fold lower Cu levels, significantly reduced GPX activity, but unchanged Se levels. The steady-state liver GPX mRNA level was lower in Cu-deficient male rats in both experiments and in Cu-deficient female rats in experiment one, and highly correlated with enzyme activity. The corresponding levels of total mRNA were not influenced by Cu status nor sex. Female rats have higher liver Se, GPX activity, and GPX mRNA than male rats. The explanation for lower liver GPX activity in Cu-deficient rats appears unrelated to total liver Se or modulation of enzyme activity.

Keywords: copper deficiency; glutathione peroxidase; rat liver mRNA; selenium; liver enzymes

Introduction

Biochemical functions of essential metals are expressed through specific proteins, many of which catalyze important cellular reactions. For Cu such specific cuproenzymes include ceruloplasmin (EC 1.16.3.1), copper, zinc-superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1), and cytochrome *c* oxidase (CCO, EC 1.9.3.1). These enzymes respond to changes in dietary Cu such that when Cu is limiting, activities are reduced. There are many other enzymes, which are not Cu containing, for which activities also change following dietary Cu deficiency.[1] One such enzyme is the selenoprotein glutathione peroxidase (GPX, EC 1.11.1.9).[2]

GPX activity was shown recently to be lower in liver and plasma of Cu-deficient rats and mice but normal in six other organs studied.[3] The cause of the lower GPX activity and the organ specificity of this change is not known, however selenium pool size may be a factor. When rats are fed a diet with limited selenium, there is a rapid coordinated loss of liver GPX activity, protein, and mRNA levels.[4,5] White et al.[6] reported that rats fed a Cu-deficient diet (0.6 mg Cu/kg) supplemented with 3 mg Se/kg had lower liver GPX activity and total Se levels than rats fed a diet supplemented with 5 mg Cu/kg. Furthermore, compared with controls, Cu-deficient rats had lower liver

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retention of an intraperitoneal dose of Se, ^{75}Se-selenite, 3 days after injection.[2] Thus, a reasonable hypothesis that could explain why both liver and plasma GPX activities are lower is that selenium is limiting in Cu deficiency.

The purpose of the experiments described was to determine the temporal relationship between liver GPX activity and changes in Cu-dependent enzymes during Cu deficiency in growing male and female Sprague-Dawley rats. A second purpose was to determine the effect of Cu deficiency on liver GPX mRNA and Se levels.

Methods and materials

Animals and diets

Pregnant Sprague-Dawley rats were purchased commercially (Harlan Sprague-Dawley, Indianapolis, IN, USA) and 2 days following parturition litter size was adjusted to eight pups. In experiment one, dietary treatments were employed during lactation and postweanling. Treatments began the day pups were born, and at 20 days weaned pups were transferred to stainless-steel cages and housed individually on the same treatment as their dams for an additional 40 days. In experiment two, dietary treatments were only employed postweaning to induce a milder Cu deficiency. Weanling 20 day-old-male and female offspring from three litters of Cu-adequate control dams were randomly assigned into two dietary groups and were fed for 40 days.

Rats were maintained at 24° C with 55% relative humidity on a 12-hr light cycle (07:00–19:00hr) in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. All protocols were formally approved by the University of Minnesota Animal Care Committee.

Rat dams and offspring were maintained on one of two dietary treatments, Cu-deficient or Cu-adequate, that consisted of feeding a Cu-deficient purified diet (Teklad Laboratories, Madison, WI, USA) and either low Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was formulated according to the American Institute of Nutrition AIN-76A diet and contained the following major components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10; DL-methionine, 3; choline bitartrate, 2; and ethoxyquin, 0.01.[7,8] Cupric carbonate was omitted from the AIN-76 mineral mix. The salt mix provided sodium selenite at 0.1 mg Se/kg diet. The purified diet used was analyzed for Cu, Fe, Se, and Zn content. Offspring and dams on the Cu-deficient treatment drank deionized water containing 0.2 ng Cu/L by analysis, whereas Cu-adequate treatment groups drank water that contained 20 mg Cu/L by adding Cu to the drinking water as $CuSO_4$. Diet and drinking water were available ad libitum.

Sample collection

In experiment one, rats, male and female (N = 3 or 4), were randomly sampled from three pooled litters of each dietary treatment at age 20, 40, and 60 days. In experiment two, rats were sampled only at day 60. Blood samples were drawn into heparinized microhematocrit tubes from trunk blood following decapitation after light ether anesthesia. A small alliquot was also removed for hemoglobin analysis. Plasma was obtained by centrifugation. Livers were removed, rinsed with deionized water, weighed, and processed for enzymatic and metal analysis. Portions of livers from 60-day-old rats were quick frozen in liquid nitrogen and stored at −70° C until RNA was extracted. For enzymatic analysis, aliquots of liver were homogenized for 30 sec in 39 volumes of 0.05 mol/L potassium phosphate (pH 7.0) with a Tissumizer and microprobe (SDT-080EN, Tekmar Co., Cincinnati, OH, USA).

Chemical analyses

Portions of liver and 1-g samples of diets were wet-digested with a 4 mL of concentrated HNO_3 (AR select grade, Mallinckrodt, St. Louis, MO, USA), and the residue was brought to 4.0 mL with 0.1 mol/L HNO_3. Samples were then analyzed for metals by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT, USA). Analyses were checked with a certified standard, U.S. National Bureau of Standards 1577 bovine liver (Gaithersburg, MD, USA). Total selenium in diet and liver was determined by neutron activation analysis and verified by certified standards.[9] Hemoglobin was determined spectrophotometrically as metcyanhemoglobin.[10] Total protein content was determined by analysis of the liver homogenates using a modified Lowry method with bovine albumin as a reference.[11]

Enzymatic analyses

Details of the enzyme assays are described elsewhere.[3] Ceruloplasmin activity was determined by measuring the ability of plasma to oxidize *o*-dianisidine using a modification of the method of Lehman et al.[12] Cu,Zn-SOD was measured by following inhibition of pyrogallol autooxidation at 320 nm as described previously.[10] Homogenates were treated with 0.4 volumes of chloroform:ethanol (15:25) to inactivate manganese SOD. Cytochrome *c* oxidase activity was determined (experiment one) on fresh homogenates, as loss of activity upon storage was noted previously. Initial velocity was measured at 25° C and the rate of ferricytochrome c formation (μmol/min) was determined using a molar extinction coefficient of 19,600 for reduced-oxidized cytochrome c. GPX was quantified by a coupled enzyme procedure at 37° C, monitoring loss of NADPH at 340 nm. The reaction mixture contained 0.1 mol/L HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (pH 7.5), 0.1 mmol/L diethylenetriaminepentaacetic acid (DTPA), 1 mmol/L GSH, 0.11 mmol/L NADPH, 2 μg of yeast glutathione (GSH) reductase (Sigma Chemical Co., St. Louis, MO, USA), and sample. The cuvette was preincubated for 3 min and the volume brought to 0.5 mL by adding the reaction substrate 10 μL of *t*-butylhydroperoxide (12.5 mmol/L in 50% ethanol). Use of only 0.25 mmol/L *t*-butylhydroperoxide as substrate ensured that only GPX was detected because, at this concentration, the "GSH peroxidase" activity of the glutathione transferases is minimal. Greater than 98% of the GPX enzyme activity in the assay described was inhibited by 0.4 mmol/L mercaptosuccinic acid, an inhibitor of GSH peroxidase.[13] A unit of GPX activity was defined as that amount of enzyme catalyzing oxidation of 1 μmol/min of GSH. The activity of glutathione transferases (EC 2.5.1.18, GSH-T) was determined by following a protocol established elsewhere.[14] In this assay the conjugation of GSH with the hydrophobic substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was followed spectrophotometrically at 340 nm and 25° C. Kinetic enzyme assays were run in duplicate with a temperature-controlled spectrophotometer (Beckman DU-8, Beckman Instruments, Fullerton, CA, USA). Activities, with the exception of ceruloplasmin, were all expressed as units/mg protein.

Northern blot analysis

Total liver RNA was isolated from quick-frozen livers of 60-day-old rats and from two additional 4.5-month-old rats that were fed either a selenium-adequate or selenium-deficient diet based on Torula yeast as described elsewhere.[15] RNA was isolated from 1 g portions of liver by centrifugation (20 hr) on 5.7 mol/L CsCl after homogenization in the presence of guanidine isothiocyanate and sarkosyl as described previously.[4] Total RNA, 30 μg, was then subjected to denaturating agarose gel electrophoresis and the positions of 18S and 28S ribosomal RNA were noted after staining with acridine orange. RNA was capillary transferred to modified nylon 66 membranes (Biotrans nylon membrane, ICN, Irvine, CA, USA) and hybridized with a ^{32}P-labeled 0.7 kb probe for murine GPX (Oligolabelling kit, Pharmacia LKB, Piscataway, NJ, USA and [α-^{32}P]dCTP DuPont NEN, Wilmington, DE, USA). After autoradiography, the membrane was stripped and rehybridized with a 0.4 kb probe for chicken β-actin to determine actin mRNA levels in rat liver.[4]

Liver RNA samples were also slot-blotted (PR600, Hoeffer Scientific Instruments, San Francisco, CA, USA) to modified nylon 66 membranes (Nytran, Schleicher & Schuell, Keene, NH, USA). RNA was vacuum dried and resuspended by adding 39 μL of a solution containing 50% deionized formamide, 6.5% formaldehyde, and 1X SSC (0.1 mol/L NaCl, 0.015 mol/L sodium citrate, pH 7.0). Samples were heated at 68° C for 15 min, rapidly chilled, and diluted with 80 μL of 1X SSC prior to transfer to the slot-blot apparatus. After initial application to the membrane, slots were washed twice with 0.5 mL portions of 1X SSC. Baked membranes were subjected to hybridization to the 0.7 kb murine GPX probe as described for northern analysis.

Autoradiograms were scanned with a transmission densitometer at 540nm (EC 910, EC Apparatus, St. Petersburg, FL, USA) interfaced with a Macintosh SE/30 computer and the peak areas were quantified using manual integration (Hoeffer GS370). Replicate scans were averaged to determine mean areas.

mRNA analysis

The relative level of total mRNA in each sample was determined by slot-blot hybridization using a ^{35}S-labeled synthetic poly dT probe as described by Hollander and Fornace.[16] The product incorporated 83% of the [α-^{35}S]dTTP (DuPont NEN) used to construct the synthetic poly dT using a poly A template and reverse transcriptase. RNA, 200 and 400 ng samples estimated carefully by absorbance at 260nm, was slot blotted to modified nylon 66 membranes (Biotrans, ICN Pharmaceuticals, Irvine, CA, USA) in 0.05 mol/L sodium phosphate (pH 6.85) and immobilized by heating at 80° C for 2 hr. RNA was hybridized using the described protocol with addition of 0.25 μg/mL of unlabeled poly dT (Sigma Chemical Co.). Hybridization of the slot-blot membrane was carried out in a sealed bag with 5 mL of hybridization solution containing 5×10^6 dpm of ^{35}S. After drying the washed membranes, individual slots were cut, transferred to scintillation vials, and counted (1900 TR, Packard Instrument Co., Downers Grove, IL, USA) with 3 mL of scintillant (Packard Ultima Gold). Binding of the ^{35}S-labeled poly dT probe was linear ($r^2 = 0.994$) between 50–1000 ng of total RNA.

Statistical analysis

Data were analyzed by utilization of factorial analysis of variance (ANOVA), $\alpha = 0.05$ and $\alpha = 0.01$, using a Macintosh computer and statistical software (Statview 512+, Brain Power, Calabasas, CA, USA). Some data were analyzed by regression analysis.

Results

Diets used in the two experiments contained the following mean levels (mg/kg) of trace elements: Cu (0.5, 0.6); Fe (44, 46); Se (0.16, 0.14); and Zn (40, 49). These diets are therefore characterized as low in Cu but adequate in Se.

Experiment one sampled young rats that were derived from dams maintained on either Cu-deficient or Cu-adequate treatment throughout lactation, at 20, 40, and 60 d of age. The dams were killed at weaning and Cu status was evaluated. The dams of both treatment groups were equivalent in size and hemoglobin level. The Cu-deficient dams had markedly lower plasma ceruloplasmin activity (1.3 ± 0.4 units/L), lower liver Cu levels (1.70 ± 0.11 μg Cu/g), and higher liver iron levels (204 ± 27.4 μg Fe/g) than Cu-adequate dams (152 ± 16 units/L, 4.49 ± 0.19 μg Cu/g, and 102 ± 20.3 μg Fe/g, respectively) ($P < 0.05$). Thus, the dietary treatments produced dams with the distinct characteristics of Cu deficiency.

Offspring were weaned to the treatment of their dams and three per group were sampled to establish weanling values. Others were continued on treatment for up to 40 additional days and animals were sampled at 40 days and 60 days. Data were analyzed by factorial ANOVA considering diet, sex, and age as three interacting variables *(Table 1)*. All rats grew during the experimental period and, not surprising, there were sex differences in growth *(Figure 1)*. At the end of the experiment, Cu-deficient males weighed less than Cu-adequate males. Liver Cu levels were influenced by both age and diet but not sex *(Figure 1)*. Plasma ceruloplasmin activity and blood hemoglobin concentration were influenced in a similar manner.

Four liver enzymes were assayed *(Figure 2)*. The two Cu-dependent enzymes, Cu,Zn-SOD and CCO, fell in activity in Cu-deficient rats as they aged suggesting that a more severe Cu deficiency developed with time. There were no sex differences observed for either Cu,Zn-SOD, or CCO activity *(Table 1)*. Liver GPX activity, in contrast, was influenced by age, sex, and diet *(Figure 2, Table 1)*. By age 40 days the sex difference was apparent; however, a significant effect of diet was not observed until age 60 days *(Figure 2, Table 2)*. The activity of liver GSH-T was not altered by dietary Cu deficiency but did increase with age in both male and female rats *(Figure 2)*. Livers from the two older rats fed the torula yeast-based diet were assayed for liver enzymes for comparative purposes. GPX activity in the liver from the Se-adequate rat was 3.05 units/mg compared to 0.01 units/mg for the Se-deficient rat. There were no differences in Cu,Zn-SOD activity between the two rat liver samples (data not shown).

Se analysis of livers from the 60-day-old rats in experiment one clearly indicated that Cu deficiency

Table 1 Factorial ANOVA for Cu-deficient and Cu-adequate, male and female, 20-, 40-, and 60-day-old Sprague Dawley Rats

Characteristic	Main Effects			Interactions			
	Age	Sex	Diet	A × S	A × D	S × D	A × S × D
Body weight	S	S	S	S	S	NS	S
Hemoglobin	S	NS	S	NS	S	NS	NS
Ceruloplasmin	S	NS	S	NS	S	NS	NS
Liver Cu	S	NS	S	NS	S	NS	NS
Cu, Zn-SOD	S	NS	S	NS	S	NS	NS
CCO	S	NS	S	NS	S	NS	NS
GPX	S	S	S	S	S	NS	NS
GSH-T	S	NS	NS	NS	NS	NS	NS
Liver protein	NS	NS	NS	NS	NS	NS	NS

Factorial ANOVA (2 × 2 × 3) was performed on characteristics measured in developing rats. Experiment one, $n = 3$ or 4 per group (See *Figures 1 and 2* for data). A significant (S) result was accepted for $P < 0.01$. NS, not significant.

did not affect liver Se for either males or females *(Table 2)*. Females did have 70% higher liver Se levels than males. The same liver samples were analyzed for total Cu and confirmed that Cu deficiency was evident for both males and females, as liver Cu was markedly lower in those rats not supplemented with $CuSO_4$ *(Table 2)*. Iron was elevated only in livers of Cu-deficient males.

The postweanling Cu-deficient model, experiment two, confirmed the biochemical observations noted in experiment one when the data were analyzed by factorial (2 × 2) ANOVA. In Cu-deficient male rats, liver Cu and GPX activity were reduced to the same extent *(Table 3)*. The corresponding reductions in female Cu-deficient rats were attenuated somewhat, especially GPX activity, when comparing experiment one *(Table 2)* to experiment two *(Table 3)*. Nevertheless, this second study showed that livers with marked reduction in Cu levels have no changes in total Se levels *(Table 3)*. In experiment two, the average liver Se, on a wet-weight basis, for Cu-adequate males was 0.61 μg/g and for females 1.1 μg/g. Also in experiment two, livers from both male and female Cu-deficient rats have higher Fe levels than Cu-adequate rats, confirming another biochemical feature of Cu deficiency *(Table 3)*.

In experiment two, the mean hemoglobin levels of Cu-deficient female rats (12.9 g/100 mL) were not different from mean levels in Cu-adequate females (13.3 g/100mL). Levels in Cu-deficient males (9.0 g/100 mL) were lower than Cu-adequate males (13.1 g/100 mL) but not as low as the 60-day-old Cu-deficient males in experiment one *(Figure 1)*. Growth was not altered by Cu deficiency in either males or females in experiment two (data not shown) in contrast to experiment one *(Figure 1)*. Thus, the Cu-deficient rats in

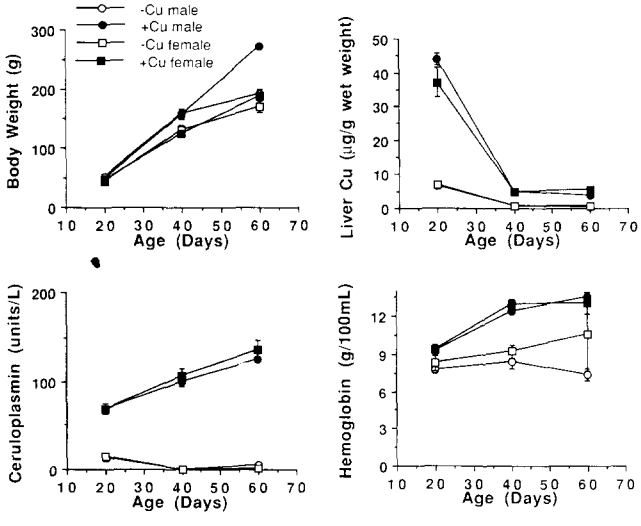

Figure 1 Body weight, liver copper, plasma ceruloplasmin activity, and blood hemoglobin levels of copper-adequate (+Cu) and copper-deficient (−Cu) rats nursed by dams on their respective treatments from birth and weaned at 20 days. Values are means ± SEM, data analysis is presented elsewhere *(Table 1)*.

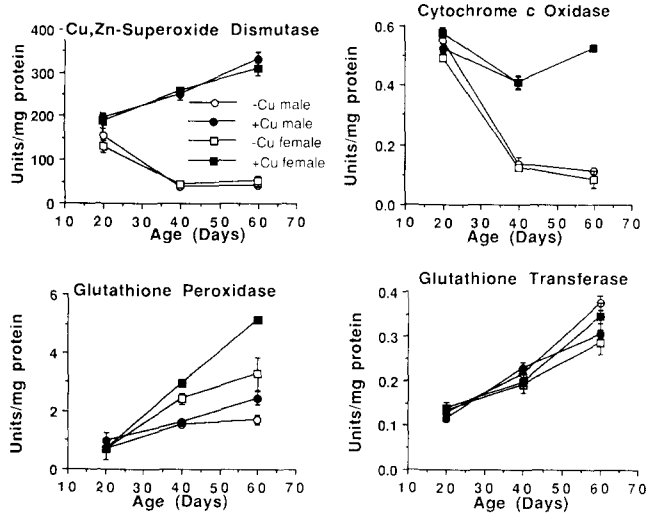

Figure 2 Liver enzyme activities of copper-adequate (+Cu) and copper-deficient (−Cu) rats nursed by dams on their respective treatments from birth and weaned at 20 days. Values are means ± SEM, data analysis is presented elsewhere *(Table 1)*.

Table 2 Experiment one, GPX activity, copper, iron, and selenium levels in livers of 60-day-old copper-deficient and copper-adequate rats[a]

Group	Cu (μg/g)	Fe (μg/g)	Se (μg/g)[b]	GPX (units/mg)
Males				
+Cu	3.99 ± 0.17	59.0 ± 2.8	2.18 ± 0.11	2.45 ± 0.23
−Cu	0.44 ± 0.04	111 ± 4.5	2.29 ± 0.14	1.70 ± 0.15
Females				
+Cu	5.76 ± 0.13	148 ± 4.9	3.65 ± 0.15	5.14 ± 0.04
−Cu[c]	0.55 ± 0.22	143 ± 19	3.36 ± 0.32	3.27 ± 0.57
		ANOVA		
Diet	S	NS	NS	S
Sex	S	S	S	S
Diet × Sex	S	S	NS	NS

[a]Values are means ± SEM ($N = 3$). Data were analyzed by Factorial ANOVA (2 × 2), S, (significant) $P < 0.01$. NS, not significant.
[b]Dry weight.
[c]$N = 2$.

Table 3 Experiment two, GPX activity, copper, iron, and selenium levels in livers of 60-day-old copper-deficient and copper-adequate rats[a]

Group	Cu (μg/g)	Fe (μg/g)	Se (μg/g)[b]	GPX (units/mg)
Males				
+Cu	3.57 ± 0.09	75.4 ± 5.9	1.93 ± 0.06	2.42 ± 0.18[c]
−Cu	0.42 ± 0.04	127 ± 19	2.00 ± 0.09	1.64 ± 0.04[c]
Females				
+Cu	4.18 ± 0.10	163 ± 14	3.49 ± 0.06	5.45 ± 0.21
−Cu	0.66 ± 0.06	254 ± 29	3.52 ± 0.08	4.86 ± 0.09
		ANOVA		
Diet	S	S	NS	S
Sex	S	S	S	S
Diet × Sex	NS	NS	NS	NS

[a]Values are means ± SEM ($N = 3$ males, $N = 4$ females). Data were analyzed by factorial ANOVA (2 × 2). S, (significant); $P < 0.01$. NS, not significant.
[b]Dry weight.
[c]$N = 2$.

experiment two, especially females, did not have signs of severe Cu deficiency such as growth impairment and anemia. In agreement with experiment one, Cu-deficient male and female rats in experiment two had significantly ($P < 0.01$) reduced plasma ceruloplasmin activity (0.2 and 0.2 units/L, respectively, versus 138 and 160 for Cu-adequate rats), had significantly reduced liver Cu,Zn SOD activity (35.8 and 60.2 units/mg protein, respectively, versus 271 and 288 for Cu-adequate rats), but unchanged GSH-T activity.

Northern blot hybridization analysis was performed on representative samples of the livers from 60 day rats in experiment one *(Figure 3)*. A single 13S RNA species was detected with the murine GPX probe. Total liver RNA from Se-adequate and Se-deficient rats, analyzed for comparison purposes, indicated that this procedure readily detected a 95% drop in GPX mRNA due to Se deficiency. Blots of individual total RNA samples showed that the liver GPX mRNA levels were lower in Cu-deficient compared to Cu-adequate male and female rats *(Figure 3)*. It is also clear that Cu-adequate female rats have higher steady-state levels of GPX mRNA than Cu-adequate male rats. The observed reduction in GPX mRNA in Cu-deficient rats was not due to loading less RNA on the gel nor to a general reduction in liver mRNA species, as indicated by rehybridization of the blots with a 0.4 kb β-actin probe. Actin mRNA levels were not lower in samples from Cu-deficient rats and perhaps were slightly higher *(Figure 3)*. Reanalysis of the same samples by slot-blot hybridization to the murine GPX probe using equivalent amounts of total mRNA, determined by poly dT hybridization, yielded an equivalent profile (*Figure 4*, experiment one).

The density of the spots on the autoradiograms from Northern hybridization (experiment one) and slot-blot hybridization (experiments one and two, total $n = 25$) were quantified by scanning densitometry. The GPX:actin density ratios were higher in Cu-adequate males (14.3 and 8.81) than in Cu-deficient males (6.25 and 5.29). The GPX:actin density ratios of Cu-adequate females were also higher (21.8 and 23.1) than

Cu-deficient females (8.72 and 14.3) *(Figure 3)*. Results from the slot-blot hybridization indicated good separation and resolution of blotted samples *(Figure 4)*. Peak areas were integrated and mean densities were calculated for both experiments one and two *(Figure 5)*. Pooled data analyzed by factorial ANOVA indicated that there was a highly significant effect of sex ($P < 0.001$) and a modest effect of diet ($P < 0.05$) on the density of liver GPX mRNA. There was no significant overall effect between groups for the total RNA yield per gram of liver (data not shown) nor for the relative level of total liver mRNA *(Figure 5)*.

Within each treatment group there was variation in the level of GPX activity and mRNA *(Figure 5, Tables 2 and 3)*. However, there was high correlation between the GPX mRNA levels determined by densitometry and the GPX enzymatic activities for all liver samples examined regardless of sex or diet *(Figure 6)*. For the 13 liver samples in experiment one, including the Se-deficient and Se-adequate rats $r = 0.96$, and for the 12 rat liver samples in experiment two $r = 0.94$.

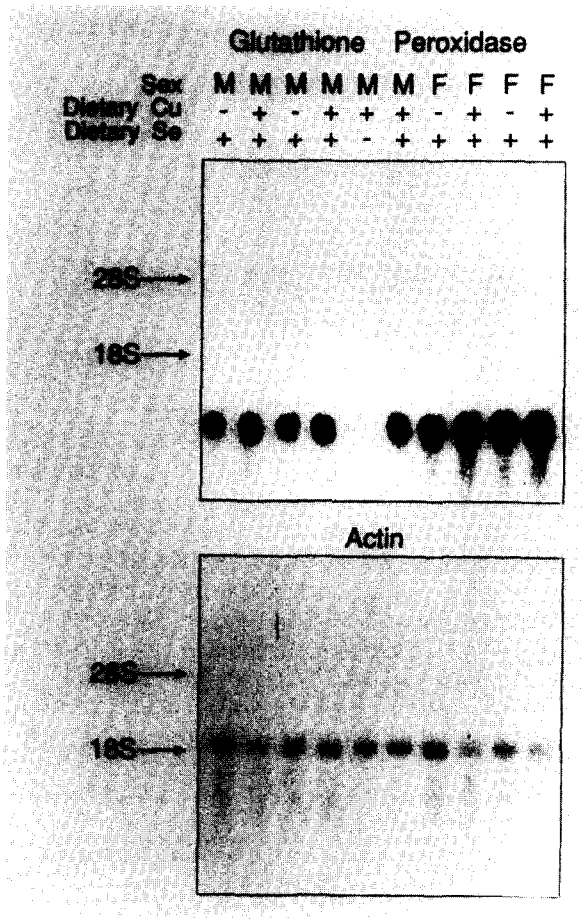

Figure 3 Northern blot hybridization analysis of rat liver total RNA (30μg/lane) subjected to denaturing electrophoresis and binding to ^{32}P-labeled DNA probes specific for murine GPX and chicken β-actin. Arrows indicate migration position of 18S and 28S ribosomal RNA.

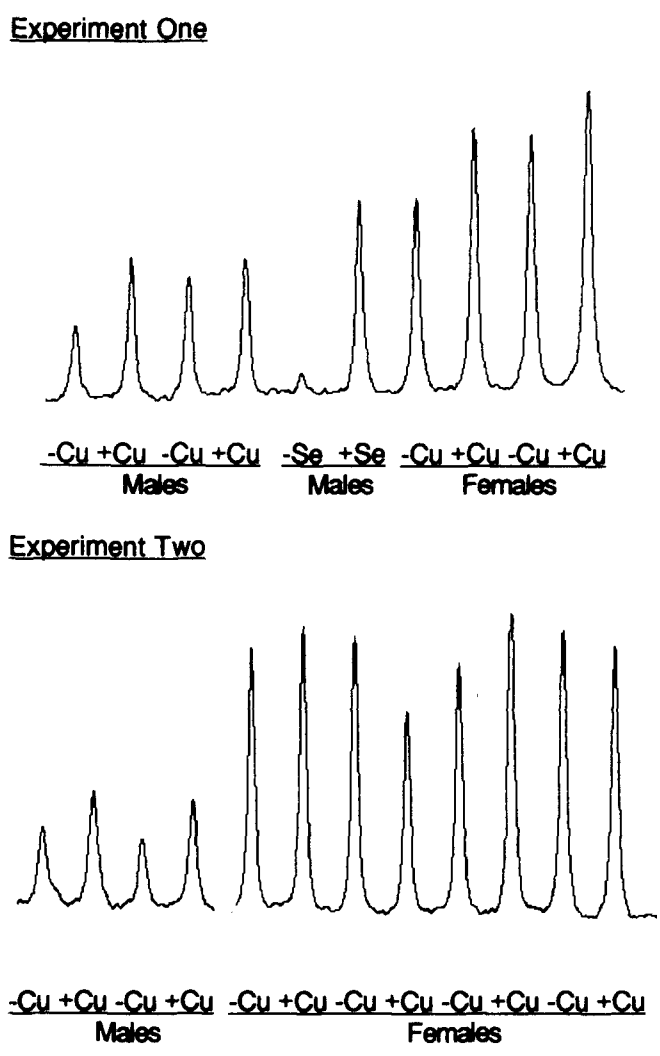

Figure 4 Densitometric scan of autoradiograms of slot-blot hybridization analysis of rat liver total RNA probed with ^{32}P-labeled murine GPX. Each slot was loaded with the same amount of mRNA, determined by poly dT hybridization. The average load was 6.5 μg of total RNA.

Discussion

Both models of Cu deficiency, the lactation plus postweanling model (experiment one) and the less severe postweanling model (experiment two), were characterized by significant reduction in the activities of cuproenzymes plasma ceruloplasmin and liver Cu,Zn-SOD, confirming that the dietary protocol was successful. In experiment one, Cu deficiency was severe enough to impair growth and result in anemia. In experiment two, the more moderate Cu deficiency resulted in rats that grew normally and only males had slight anemia. However, in both experiments significant decreases in liver GPX activity were observed 40 days after weaning.

This change in GPX activity in livers of Cu-deficient rats appeared later than the reduction in the cuproenzymes ceruloplasmin; Cu,Zn-SOD; and cytochrome c

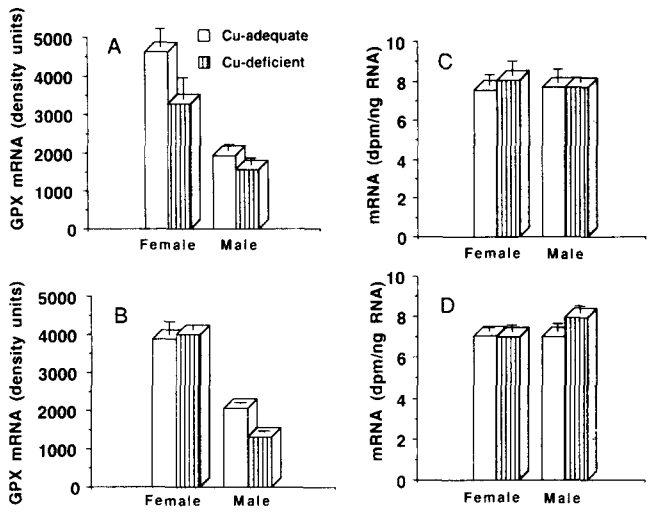

Figure 5 Mean ± SEM integrated densitometric areas from slot-blot analyses for liver GPX mRNA levels, panels A and B, and mean ± SEM liver total mRNA (^{35}S-labeled poly dT binding), panels C and D.

oxidase. Liver GPX activity was reduced significantly in both male and female Cu-deficient rats. This observation for male rats has been reported previously and has been reviewed elsewhere.[3] However, this is the first report that shows that female rat liver GPX activity is also lower in Cu-deficient animals. The mean decrease in liver GPX activity in Cu-deficient male rats in experiment one was 31% and in experiment two was 32%; in Cu-deficient female rats in experiment one the decrease was 36%. In experiment two, the reduction in liver GPX activity in Cu-deficient female rats (10%, $P < 0.05$) occurred in rats that were not anemic and grew normally, indicating that changes in GPX activity can occur in Cu-deficient animals that are not moribund. Cu deficiency does not lower all liver enzyme activities, as GSH-T activity was not influenced by diet. This is consistent with earlier work on male rats.[2,3,17]

These experiments show that the significant decreases in liver GPX activity in Cu-deficient rats are not due to changes in the liver Se content. In both the severe Cu deficiency (experiment one) and moderate Cu deficiency (experiment two), liver Se concentrations were unaffected even though liver Cu levels were markedly lower. This indicates that the modulation of GPX activity in Cu deficiency is not due to simple secondary Se deficiency and rejects that hypothesis.

Both experiments also show that female rats have higher liver Se levels than male rats. Liver Se levels measured in these studies are comparable to those reported in separate studies on male and female rats fed diets adequate in Se.[18,19] The calculated ratio of GPX activity to Se level *(Tables 2 and 3)* is higher in females, 1.4 and 1.6, respectively, for experiments one and two, than in males (1.1 and 1.2). This suggests that factors other than liver Se concentration influence the steady-state level of liver GPX in rats.

Results of both experiments indicated that dietary Cu deficiency was associated with a reduction in liver GPX mRNA. The mean decrease in Cu-deficient males in experiment one was 20% and in experiment two was 35%; the mean decrease in Cu-deficient females in experiment one was 29% and in experiment two there was no significant drop. Data indicated that Cu deficiency had no influence on the yield of total liver RNA nor total liver mRNA. Control blotting with a β-actin probe further shows that this effect of Cu deficiency is not a general effect. It is intriguing that lower GPX activity levels in Cu deficiency are associated with lower GPX mRNA steady state levels. Recently, for example, Mercer et al.[20] reported that livers of mice with low Cu levels have normal steady state mRNA levels for the cuproprotein ceruloplasmin. Thus, the apparent reduction in GPX mRNA level may be a specific effect of Cu deficiency.

Data in this study also show that female rats have higher steady-state levels of liver GPX mRNA. These liver GPX mRNA levels parallel the higher liver GPX activity in female rats. It has been known for some time that female rats have higher liver GPX activity than male rats.[21]

Our results do confirm, however, that liver GPX activity is correlated with liver GPX mRNA.[5] Animals with low GPX activity had low GPX mRNA levels, and this was true for males and females regardless of diet. This is an important observation because it indicates that the decreases in liver GPX activity can be explained by the reduction in GPX mRNA level. This, along with data (not shown) on mixing experiments, strongly suggests that the measured decreases in GPX

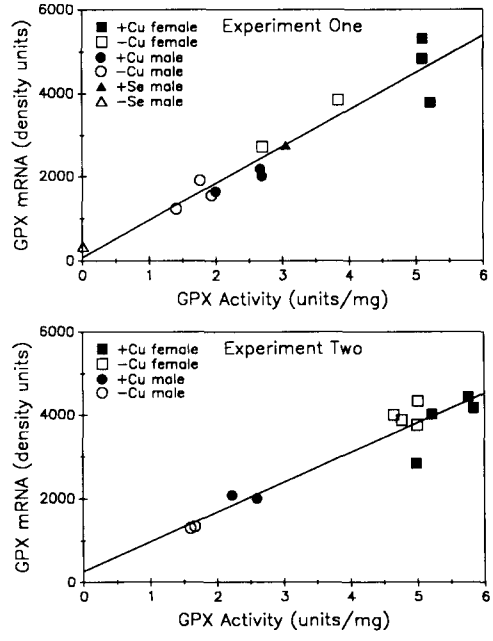

Figure 6 Regression analysis of the average integrated densities for liver GPX mRNA was highly correlated with liver GPX enzyme activity ($P < 0.01$).

activity in livers of Cu-deficient animals are not due to inhibitor modulations of the enzyme.

The mechanism causing lower GPX mRNA levels remains obscure. It is clearly preliminary to suggest that the expression of GPX mRNA is directly under the influence of cellular Cu concentration. One possible mechanism might involve the secondary effect of Cu status on the metabolite hydrogen peroxide, which in turn might effect GPX induction.[22] Another hypothetical mechanism might involve an enzymatic role for Cu in Se metabolism. Clearly, additional work is necessary to elucidate the role of dietary Cu on regulation of GPX.

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