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Expression of glutathione peroxidase and catalase in copper-deficient rat liver and heart

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Oxidative stress resulting from dietary copper deficiency in rats differentially regulates the expression of copper/zinc- and manganese-superoxide dismutases in the liver and heart. It was hypothesized that the expression of catalase and glutathione peroxidase, the non-copper–containing antioxidant enzymes, in the rat heart and liver may also be regulated by dietary copper deprivation. The expression of the activities, protein concentrations, and mRNA levels for glutathione peroxidase and catalase were studied in the liver and heart of rats made copper-deficient for 4 weeks. The activities and mRNA levels for liver selenium (Se)-dependent glutathione peroxidase and catalase as well as the immunoreactive protein concentration for the catalase were reduced to a similar extent in copper-deficient animals, which suggests transcriptional regulation. The mRNA, activity, and protein concentration for the myocardial catalase were increased in copper-deficient rats. However, the increase was larger for the mRNA than for the activity and protein concentration; this indicates that its regulation probably involves both transcriptional and post-translational mechanisms. Se-dependent glutathione peroxidase was not changed in the heart of copper-deficient rats. These results demonstrate that dietary copper deficiency in rats differentially alters the expression of Se-dependent glutathione peroxidase and catalase in the liver and heart. (J. Nutr. Biochem. 6:256–262, 1995.)

Keywords: glutathione peroxidase; catalase; copper deficiency; free radicals; liver; heart

Introduction

Reactive oxygen metabolites (superoxide anion, O_2^-; hydrogen peroxide, H_2O_2; and hydroxyl radical, ·OH) have been implicated in a number of diseases such as cardiovascular or neurodegenerative diseases as well as in ischemic/reperfusion injury in many organs.[1–4] The reactive oxygen intermediates are generated metabolically.[1–3] Aerobic cells also produce many antioxidants and several antioxidant enzymes to scavenge the reactive oxygen metabolites. Dismutation of O_2^- by copper/zinc- and manganese-containing superoxide dismutase (Cu/Zn- and Mn-SOD) and the degradation of H_2O_2 by glutathione peroxidase (GPX) and catalase limit the cytotoxic effects of these reactive oxygen metabolites.[1–3]

Copper is a cofactor for a number of important metalloenzymes, including Cu/Zn-SOD. Animals fed diet deficient in copper have decreased activity of Cu/Zn-SOD and other copper-containing enzymes in many organs[5] and develop cardiovascular abnormalities resembling ischemic heart disease.[6] Because cardiovascular damage induced by copper deficiency can be reduced by antioxidants,[7–9] it has been suggested that these defects may be the result of reduced Cu/Zn-SOD activity and increased injury caused by reactive oxygen radicals.[6–9] Recently, we reported that Cu/Zn- and Mn-SOD are differentially regulated in copper-deficient rat organs.[10] Does copper deficiency also affect the regulation of GPX (glutathione peroxidase) or catalase, the noncopper containing antioxidant enzymes? The effects of copper deficiency on the activities of GPX and catalase were examined in several earlier studies.[11–19] For example, GPX activity in liver was reduced in copper-deficient rats.[12–19] One study also reported that dietary copper defi-

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Received April 28, 1994; accepted November 10, 1994.

Nutritional Biochemistry 6:256–262, 1995

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00014-Q

ciency decreased the level of rat liver GPX mRNA.[17] Liver catalase was not changed[18] or reduced[11,15] by copper deprivation. In the heart, GPX activity was not significantly altered,[16,18] whereas catalase activity was moderately reduced in copper-deficient animals.[18] Despite these studies, the mechanisms for altered glutathione peroxidase and catalase activities in different organs of copper-deficient rats remain to be investigated. The present study was undertaken to examine the effects of dietary copper deficiency on the activities, mRNA abundance of GPX, and catalase in rat heart and liver. In addition, the immunoprotein content for catalase was also measured. Our data show that dietary copper deprivation differentially affects the expression of GPX and catalase in both organs.

Materials and methods

Materials

Hydrogen peroxide, cumene hydroperoxide, nicotinamide adenine dinucleotide phosphate reduced (NADPH), glutathione reduced (GSH), yeast glutathione reductase, potassium cyanide, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO USA). Sheep anti-bovine catalase IgG fraction and alkaline phosphatase-conjugated donkey anti-sheep secondary antibody was obtained from The Binding Site Inc. (San Diego, CA USA). Aprotinin was ordered from Boehringer Mannheim Corp. (Indianapolis, IN USA). 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP), p-nitroblue tetrazolium chloride (NBT), prestained standard protein markers, and reagents for electrophoresis were supplied by Bio-Rad Laboratories (Richmond, CA USA). Pentobarbital sodium was purchased from Butler Co. (Columbus, OH USA).

Poly $(A)^+$ RNA was purchased from Sigma Chemical Co. [$\alpha-{}^{32}$P]dATP was obtained from DuPont-New England Nuclear Corp. (Boston, MA USA). Oligo dT primer, oligo (dT) cellulose, and random primer labeling kit for cDNA probes were supplied by GIBCO BRL (Grand Island, NY USA). Proteinase K was ordered from AMRESCO (Solon, OH USA). RNAsin (RNAse inhibitor) and AMV reverse transcriptase were acquired from Promega (Madison, WI USA). Nitrocellulose and Nytran membranes were ordered from Schleicher & Schuell (Keene, NH USA). Denhardt's solution was obtained from Research Genetics (Huntsville, AL USA). All other reagents for mRNA isolation and hybridization were of the highest grade available. cDNA probes for human catalase and rat GPX were kindly provided by Dr. Ye-Shih Ho of Wayne State University, Detroit, MI.

Animals and diets

Male weanling Sprague–Dawley rats (Sasco, Omaha, NE USA), assigned to groups of 10 by mean weight (50 g), were fed ad libitum a purified diet for 32 to 33 days based on sucrose (62%), egg white (20%), corn oil (10%), and Jones Foster salts mix without copper or zinc. The diet contains adequate amounts of all nutrients known to be essential for rats but is deficient in copper and zinc.[10,20,21] Animals were maintained similarly to an earlier description with cages of stainless steel, silicone stoppers in water bottles, etc.[10,21] All animals received a drinking solution containing 10 μg of Zn/mL (as acetate). Rats that were to be made copper-deficient received no copper in water, but the solution given to the control rats was supplemented with 3 μg of Cu/mL (as sulfate).[10] After anemia and hypercholesterolemia were detected in blood collected from the tail vein, and necroscopy of 3 rats revealed cardiovascular pathology, the remaining rats were flown from Grand Forks, ND, to Toledo, placed into similar cages, and fed the same diets and drinking solutions as before. Rats were injected i.p. with 0.7 mL of heparin 30 min prior to being anesthetized with pentobarbital (100 mg/kg body weight, i.p.). Hearts and livers (after perfusion with normal saline) were removed, wrapped with aluminum foil, frozen in dry ice, and kept at −70°C for enzyme activity, Western and Northern blot analyses.

Cholesterol determination

Plasma cholesterol was measured according to Allain et al.[22]

Enzyme assays

For the measurement of the GPX activity, tissue samples were homogenized in 10 vol of 0.25 M sucrose and 0.5 mM EDTA solution. The homogenates were centrifuged at 15,000g for 30 min at 4°C. GPX activity in supernatant was determined by the method of Lawrence and Burk which used either H_2O_2 or cumene hydroperoxide as substrate.[23] The activity measured using 1.5 mM cumene hydroperoxide as substrate corresponds to the total GPX (i.e., selenium- and non-selenium–dependent) activity, and that measured using 0.25 mM hydrogen peroxide as substrate corresponds to Se-dependent GPX (Se-GPX) activity. Aliquots (0.1 mL) of enzyme sample were mixed with 0.8 mL of assay mixture at 25°C; then, 0.1 mL of 15 mM cumene hydroperoxide or 2.5 mM hydrogen peroxide was added to initiate the reaction. The final reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3 (to inhibit catalase), 0.2 mM NADPH, 1 U/mL of glutathione reductase, 1 mM GSH, and 1.5 mM cumene hydroperoxide or 0.25 mM hydrogen peroxide. Absorbance at 340 nm was recorded for 3 min. One unit of GPX equals 1 μmol glutathione oxidized per minute.

For the measurement of catalase activity, the enzyme samples were prepared according to the method of Cohen et al.[24] Ethanol (1.0%, vol/vol) and Triton X-100 (1.0%, vol/vol) were added to homogenates to stabilize and to release, respectively, the membrane-bound catalase. Catalase activity was determined by following the disappearance of H_2O_2.[25] Absorbance at 240 nm was recorded for 1 min at 25°C. One unit of catalase is defined as half of the peroxide oxygen liberated from the H_2O_2 solution (10 mM) in 100 sec at 25°C.[25]

Northern blot analysis

Poly $(A)^+$ RNA was isolated as previously described by using a combination of proteinase K digestion, oligo (dT) cellulose selection, and spin column separation.[26] The relative amount of poly $(A)^+$ RNA in each sample was determined by slot-blot hybridization by using ^{35}S-labeled synthetic poly(dT) probe as described by Hollander and Fornace.[27] Binding of the ^{35}S-labeled poly(dT) probe was linear between 25 and 300 ng of poly $(A)^+$ RNA. This linear range was used for adjusting the amount of mRNA loaded in the Northern gel electrophoresis.

Two and 6 μg of mRNA from liver and heart samples, respectively, were subjected to denaturing formaldehyde agarose gel electrophoresis, transferred to a Nytran membrane, UV immobilized (80,000 μJ/cm, Model CL-1000, UVP, San Gabriel, CA USA), and hybridized to ^{32}P-labeled probes. Briefly, Northern blots were prehybridized in the prehybridization solution (5× Denhardt's solution, 50% formamide, 0.2 mg/mL of denatured salmon sperm DNA, 1 mM EDTA at pH 8.0, 2× SSC, and 0.1% SDS) for 4 to 6 hr at 42°C in a shaking water bath. The blots were then hybridized in fresh prehybridization solution containing ^{32}P-labeled rat GPX or human catalase cDNA probe at 42°C for 12 to

16 hr. The blots were washed sequentially with 1× SSC + 0.1% SDS for 2 × 15 min and 0.2× SSC + 0.1% SDS for 2 × 15 min at room temperature. Occasionally one additional washing with 0.1× SSC + 0.1% SDS was carried out at 45°C for 30 min. The efficiency of washing was monitored with a survey meter. Autoradiograms were obtained at −70°C by using Kodak XAR film with an intensifying screen. The intensities of autoradiograms were scanned with a Bio-Rad imaging densitometer (Model GS-670) and peak areas were quantified. The ratio of the intensity of GPX or catalase mRNA to that of the corresponding poly $(A)^+$ RNA provides a relative quantitative measurement of each sample.

Western blot analysis

About 200 mg of liver or one fourth of each heart was homogenized in 2 mL of solution (0.3 M sucrose, 1 mM EDTA, and 200 KIU aprotinin) with Polytron PT 10 (Brinkman, Westbury, NY USA) at a setting of 3 for 2 × 30 sec. Homogenates were filtered through double layers of cheesecloth, SDS was added to the filtrate for a final concentration of 0.1%, and 2 μg of protein from liver and 40 μg of protein from heart were electrophoresed on 10% denaturing polyacrylamide gels.[28] Separated proteins were transferred to nitrocellulose membrane and probed with sheep bovine catalase antisera. Bound antibody was incubated with donkey anti-sheep secondary antibody conjugated to alkaline phosphatase which was detected by NBT-BCIP.[29] The labeled bands were quantitated by scanning with a Bio-Rad imaging densitometer (Model GS-670). Protein concentrations were determined by the BCA method (Pierce Chemical Co., Rockford, IL USA) using bovine serum albumin as standard.

Copper analysis

Liver copper content was analyzed as previously described.[10] Briefly, about 0.5 g of perfused liver was digested with 5 mL of 70% nitric acid at 80°C for 2 hr. Cu was measured by atomic absorption spectrophotometry (Perkin-Elmer, Model 5000, Norwalk, CT USA).

Statistical analysis

Student's unpaired *t*-test was used for comparing the sample means between copper-supplemented (CuS) and copper-deficient (CuD) rats. Significant difference was accepted at $P < 0.05$.

Results

Assessment of copper deficiency

After 32–33 days of dietary copper deprivation, body weight and hematocrit were decreased 23 and 29%, respectively when compared with copper-supplemented animals (*Table 1*). Liver copper content was decreased 75% by copper deficiency. In contrast, copper deficiency caused a small but significant increase in plasma cholesterol level (*Table 1*).

Enzyme activities

Copper deficiency did not affect the total GPX (Se- and non-Se–dependent) activities in liver (CuS, 0.192 ± 0.006; CuD, 0.190 ± 0.004 U/mg of protein, mean ± SEM, n = 16) and in heart (CuS, 0.042 ± 0.002; CuD, 0.044 ± 0.001 U/mg of protein, mean ± SEM, n = 8). *Figure 1* shows that the activity of Se-GPX (assayed with H_2O_2) was significantly decreased (22%) in copper-deficient liver. This implies that the non-Se–GPX activity was increased. The Se-GPX activity in the heart was not changed by dietary copper deprivation. *Figure 1* also demonstrates that while copper deficiency significantly decreased (46%) the activity of catalase in the liver, it significantly elevated (27%) the activity in the heart.

Table 1 Characteristics of copper deficiency

	Copper-supplemented	Copper-deficient
Body weight (g)	235.2 ± 7.7*	181.6 ± 10.0[a]
Liver copper (μg/g)	2.73 ± 0.21	0.67 ± 0.07[a]
Cholesterol (mmol/L)	2.59 ± 0.11	2.92 ± 0.11[b]
Hematocrit (%)	49 ± 0.7	35 ± 3.4[c]

*Values are mean ± SEM from 9 copper-supplemented and 10 copper-deficient rats.
[a] $P < 0.001$.
[b] $P < 0.05$.
[c] $P < 0.004$.

Relative mRNA abundance

Autoradiograms shown at the top of *Figure 2* indicate that cDNA probes for GPX and catalase recognize a 0.9 and 2.4 kb message, respectively, in both organs examined. The relative mRNA contents measured as the ratios of the intensities of mRNA for GPX and catalase to those of poly $(A)^+$ RNA were shown on the bottom of *Figure 2*. Dietary copper deficiency decreased the catalase and GPX mRNA concentrations in liver by 48% and 40%, respectively. In contrast, heart catalase mRNA concentration was significantly increased (62%), whereas GPX mRNA was not altered in copper-deficient rats.

Western blot analysis

To further examine the effect of copper deficiency on the regulation of catalase, the protein contents of catalase were estimated by immunological staining of Western blots with antibodies raised against purified bovine enzyme. Because immunological staining of Western blot with anti-human GPX IgG, the only antibody against GPX available commercially, showed molecular sizes different from those of rat GPX,[30] the measurement of immunoreactive protein for GPX was not done. *Figure 3* indicates that the antibody specifically detects protein with molecular sizes corresponding to catalase in both copper-supplemented and copper-deficient rats. The intensities of stained bands provide a relative quantitative measurement of the amounts of catalase present. The immunoreactive protein level for liver catalase was significantly reduced by 25% whereas that for heart catalase was significantly increased by 27% in copper-deficient animals.

Discussion

In the present study, copper deficiency was directly confirmed by a large reduction of copper content in liver. Autopsy of several rats dead from copper deficiency revealed ascites, hemopericardium, and ventricular aneurysm. These

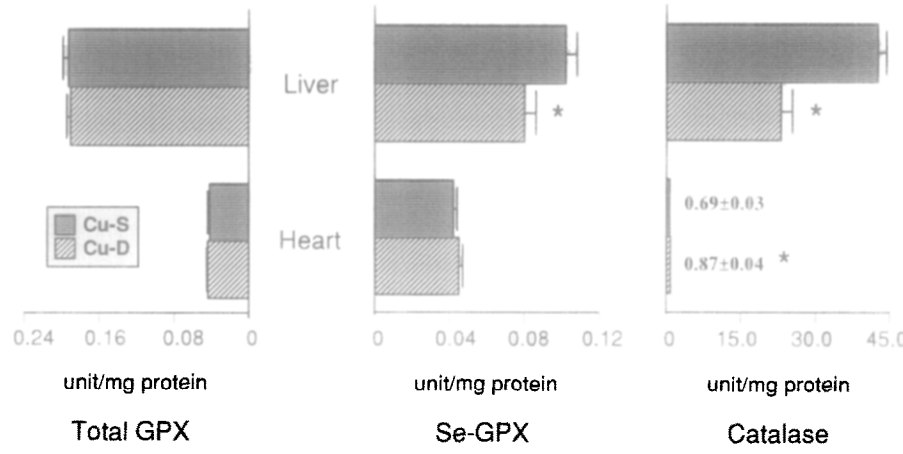

Figure 1 The activities of glutathione peroxidase (GPX) and catalase in the liver and the heart of copper-supplemented (CuS) and copper-deficient (CuD) rats. Total and Se-dependent glutathione peroxidase activities were assayed in 1.5 mM cumene hydroperoxide and 0.25 mM hydrogen peroxide, respectively. Data were expressed as mean + SEM of the activities for total and Se-dependent glutathione peroxidase (n = 16 and 8 for the liver and heart, respectively) and catalase (n = 8 for both the liver and the heart). *$P < 0.05$.

pathological findings are common in copper deficiency.[31,32] These observations infer that these changes were caused by copper deprivation because copper intake is the only difference between these two groups of rats. Furthermore, the reduction of mRNAs cannot be due to the general cytotoxic effects of copper deficiency, which has been found to increase liver β-actin mRNA[17] and Mn-superoxide dismutase mRNA.[10]

The primary findings of the present experiment were that copper deficiency reduced the activities and the mRNA concentrations for catalase and Se-GPX and the immunoreactive protein content for catalase in the liver. In contrast, dietary copper deprivation increased the activity, mRNA, and protein concentrations for catalase, while leaving Se-GPX unaltered, in the heart. These results indicate that catalase and Se-GPX in the heart and liver of copper-deficient animals are differentially regulated.

In the present experiment, moderate but significant decreases in the activity (22%) and mRNA concentration (40%) for liver Se-GPX were found in copper-deficient animals. Similar results have also been reported.[17] Because GPX mRNA was decreased in the absence of any change in

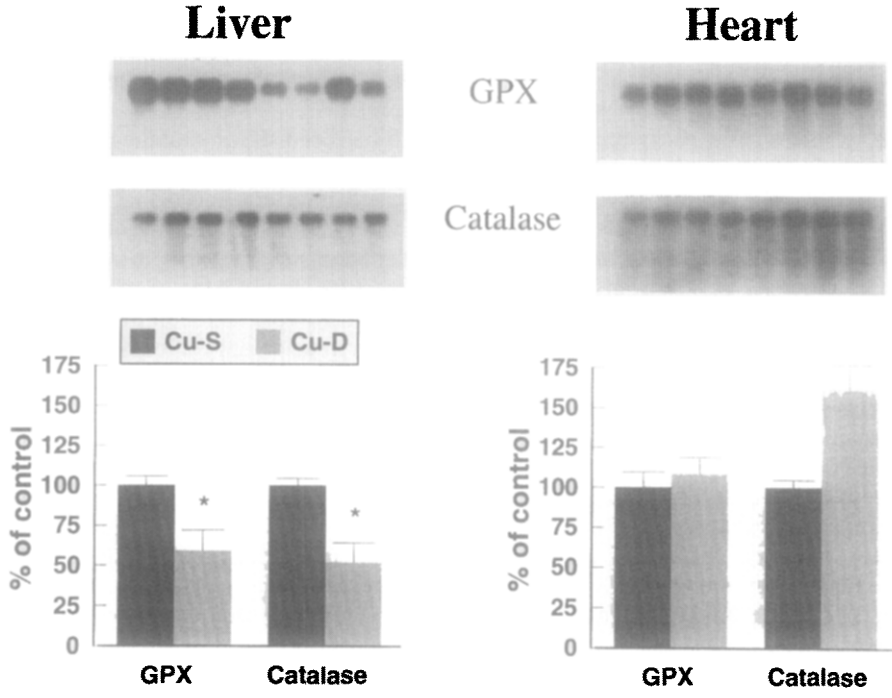

Figure 2 Northern blot analyses of glutathione peroxidase (GPX) and catalase in the liver and the heart of copper-supplemented (CuS) and copper-deficient (CuD) rats. On the top mRNA autoradiograms, the left and the right four samples were from copper-supplemented and copper-deficient rats, respectively. The relative intensities of the autoradiograms were normalized to those of poly $(A)^+$ RNA (data not shown). Data were expressed as percentage + SEM of the means (n = 8) from the corresponding copper-supplemented rats. *$P < 0.05$.

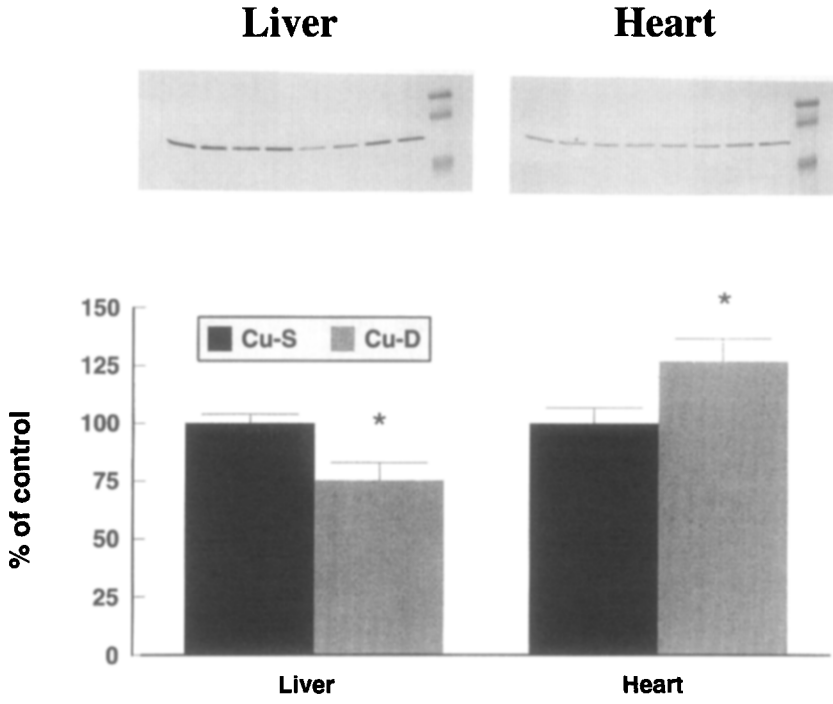

Figure 3 Western blot analysis of catalase in the liver and the heart of copper-supplemented (CuS) and copper-deficient (CuD) rats. The left and right four stained bands of each blot represent enzymes from copper-supplemented and copper-deficient rats, respectively. Molecular weight markers are phosphorylase b (97,400 daltons), bovine serum albumin (66,200 daltons), and ovalbumin (45,000 daltons). Data were expressed as percentage + SEM of the means (n = 8) from the corresponding copper-supplemented rats. *$P < 0.05$.

liver Se content, the lower liver Se-GPX activity found in copper-deficient rats may have resulted from reduced GPX mRNA.[17] In agreement with most other reports,[11,15] liver catalase activity was reduced in copper-deficient rats. In addition, we have found that the concentrations of mRNA and immunoreactive protein for liver catalase were also decreased by copper deprivation. These results suggest that the concentrations of these two antioxidant enzymes from liver of copper-deficient rats are at least partly regulated by its mRNA content. No significant change in heart Se-GPX activity, which is in agreement with most previous studies,[16,18] was observed in copper-deficient animals. In addition, the present results showed that mRNA for myocardial Se-GPX was not changed in copper-deficient rats. In contrast to one report which showed a moderate decline in catalase activity,[18] we found that the activity of heart catalase was increased in copper-deficient animals. The difference may be the result of differences in the diets, strains of animals, or duration of copper deficiency. It is also possible that the very low catalase activity in the heart renders its measurement less reliable. However, in the present study, we also assayed the concentrations of protein and mRNA for heart catalase; they were increased in copper-deficient animals. Analogous to the liver Se-GPX and catalase, these results suggest that the amount of myocardial catalase is partly regulated by its mRNA level in copper-deficient animals. Alterations of mRNA concentrations may be caused by changes in the transcription of the genes, processing of hnRNA (heterogeneous nuclear RNA) transcripts, or mRNA turnover. The mechanisms for the down-regulation of liver catalase and Se-GPX mRNAs or up-regulation of myocardial catalase mRNA are not unknown. Evidence for a primary role of copper as a transcriptional factor in regulating the expression of these enzymes is lacking. One possible mechanism may involve the secondary effect of copper deficiency on the tissue level of H_2O_2, which in turn alters the expression of these enzymes (see below).

It has been shown that the non-Se–GPX activity assayed with cumene hydroperoxide is due to the activity of glutathione-S-transferases (GST).[33,34] Although the significance of the peroxidative activity of GST is not known, several observations suggest that it may play an important role in reducing hydroperoxide. For example, liver contains a relatively high concentration of GST.[34] GST activity was increased in selenium deficiency when the Se-GPX was reduced.[14,34] In the present experiment, the non-Se–GPX activity was enhanced following dietary copper deprivation. However, no change in hepatic non-Se–GPX has also been reported in copper-deficient rats.[19] One study reported that liver GST activity was not altered or was slightly increased in copper-deficient rats.[16] Another study showed that copper deficiency had no effect on liver GST activity.[15]

Copper deficiency reduces liver copper[10,35]: in contrast, it elevates liver iron concentration.[35–38] Excess iron may induce cell injury by serving as a catalyst for lipid peroxidation as well as for the formation of ·OH radicals.[3] Indeed, enhanced lipid peroxide formation was found in copper-deficient rat heart[39] and aorta.[40] Although the existence of free radicals was not checked in the present study, they have

been detected by a spin trapping technique in liver but not heart of copper-deficient rats fed fructose.[41] The severity of copper deficiency can be reduced by deferoxamine, an iron chelator.[42] Furthermore, low dietary iron ameliorated the symptoms of copper-deficient rats fed fructose.[41] These results strongly suggest that iron plays an important role in causing the defects found in copper deficiency. Taken together, these observations and ours suggest that liver iron overload may be an important factor involved in the down-regulation of Se-GPX and catalase in copper-deficient rats. During oxidant stress, as may occur in copper-deficient rat organs, production of O_2^- and H_2O_2 are increased. H_2O_2 can readily cross biological membranes, including nuclear membrane. Iron-induced nuclear DNA damage has been demonstrated in the isolated liver nuclei incubated with iron.[43] Thus, site-specific DNA damage may occur in copper-deficient rat liver nuclei resulting in reduced transcription for catalase, GPX, and Cu/Zn-superoxide dismutase.

Our study and that of others[44] showed that heart contains less that 2% of catalase found in liver, yet it generates a greater amount of H_2O_2/g of tissue than any other organs.[45] On the other hand, Cu/Zn-SOD and Se-GPX activities in the heart are about ¼ and ½, respectively, of that in the liver.[10,44,46] Thus, it has been suggested that SOD and Se-GPX play the major role in the detoxification of reactive oxygen metabolites in heart.[46] In the absence of any change in Se-GPX activity, one could then argue that a small increase in heart catalase activity observed in our experiment is of limited significance. Nonetheless, induction of catalase mRNA similar to that which occurs when cultured tracheoepithelial cells are exposed to H_2O_2[47] and increased mitochondrial numbers or the mitochondrial/myofibrillar ratio[48,49] found in copper-deficient rat heart could contribute to the increase in catalase, which is localized in cytosol and mitochondrial matrix.[50] In contrast to the increase in liver iron, copper deficiency caused a decrease in heart iron in addition to a reduction in copper[35,37,41] which could greatly reduce the production of radicals by copper- or iron-catalyzed Fenton or Haber–Weiss reaction. As previously noted, the liver, but not the heart, of copper-deficient male rats fed fructose showed the presence of free radicals,[41] which indicates less oxidant stress was imposed on heart than liver. However, these observations do not exclude the role of reactive oxygen metabolites in cardiovascular defects found in copper-deficient animals because antioxidants have been shown to inhibit cardiovascular damage associated with Cu deficiency.[7,8]

In summary, dietary copper deprivation in rats down-regulates liver Se-GPX and catalase and up-regulates heart catalase possibly at their mRNA levels. Dietary copper depletion does not alter heart Se-GPX. Excess intracellular iron load and enhanced oxidative stress may reduce the activities, protein concentrations, and mRNA contents for liver Se-GPX and catalase. On the other hand, the increase in heart catalase may result from the cardiac pathological alterations and mRNA induction. These observations indicate that catalase and Se-GPX from different organs display differential susceptibility to oxidative stress. Together with our previous observation,[10] these data suggest that dietary copper deficiency may affect the gene expression of antioxidant enzymes. Future experiments will investigate the nuclear transcriptional efficiency for these enzymes.

Acknowledgment

We greatly appreciate the generosity of Dr. Ye-Shih Ho (Wayne State University, Detroit, MI USA) in providing cDNA probes for rat glutathione peroxidase and human catalase. We thank Eugene Orlowski for technical help. This research was supported in part by NIH grant HL45554 and BRS 2S07RR05700-23.

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