Cu,Zn-Superoxide Dismutase Is Lower and Copper Chaperone CCS Is Higher in Erythrocytes of Copper-Deficient Rats and Mice

ELIZABETH C. WEST AND JOSEPH R. PROHASKA[1]

Department of Biochemistry & Molecular Biology, University of Minnesota, Duluth School of Medicine, Duluth, Minnesota 55812

Discovery of a sensitive blood biochemical marker of copper status would be valuable for assessing marginal copper intakes. Rodent models were used to investigate whether erythrocyte concentrations of copper,zinc–superoxide dismutase (SOD), and the copper metallochaperone for SOD (CCS) were sensitive to dietary copper changes. Several models of copper deficiency were studied in postweanling male Holtzman rats, male Swiss Webster mice offspring, and both rat and mouse dams. Treatment resulted in variable but significantly altered copper status as evaluated by the presence of anemia, and lower liver copper and higher liver iron concentrations in copper-deficient compared with copper-adequate animals. Associated with this copper deficiency were consistent reductions in immunoreactive SOD and robust enhancements in CCS. In most cases, the ratio of CCS:SOD was several-fold higher in red blood cell extracts from copper-deficient compared with copper-adequate rodents. Determination of red cell CCS:SOD may be useful for assessing copper status of humans. Exp Biol Med 229:756–764, 2004.

Key words: copper-deficient; erythrocytes; superoxide dismutase; chaperone CCS

Copper is one of several essential trace metals that are important in supporting biological functions. Early reports of an essential role for copper in supporting normal levels of hemoglobin in blood and to prevent anemia came out in the middle of the 19th century (1). Since then, evidence has accumulated that supports a requirement for dietary copper in humans for optimal function of all biological systems. The current adult recommended dietary allowance (RDA) for copper is 0.9 mg. The amount of dietary copper available for most individuals is near but certainly not in excess of this RDA. In fact, for some age groups the average intake may be below the RDA (2). Currently, it is difficult, if not impossible, to assess marginal copper status in humans. Thus, the need for more sensitive and reproducible markers of copper status is a worthwhile research goal.

Copper is a cofactor for approximately a dozen enzymes (cuproenzymes). These cuproenzymes represent the biological roles for copper and reflect an essential need for dietary copper (3). Ceruloplasmin is a plasma cuproenzyme whose activity responds to changes in dietary copper but may be unsuitable for detecting marginal copper deficiency. Ceruloplasmin is known to rise following inflammation because it is an acute-phase protein (3). Another cuproenzyme is the protein copper,zinc superoxide dismutase (SOD). The activity of SOD is altered by dietary copper deficiency. This was first shown in copper-deficient rat brains 30 yrs ago (4). The original work in rat brain was rapidly supported in red blood cells. Because red blood cells are an easy sampling source, the use of a copper-dependent enzyme in blood is appealing for the assessment of copper status. In fact, SOD activity was shown to be reduced in red blood cells of copper-deficient pigs, rats, chickens, sheep, and humans (5–9). Some have suggested that red cell SOD activity be used as an indicator of human copper nutrition (10, 11).

Many laboratories have contributed to the wealth of knowledge about erythrocyte SOD in copper deficiency. There has been very good agreement that the enzyme activity of erythrocyte SOD was lower following nutritional copper deficiency. However, there has been some disagreement about whether or not the reduction in activity is related to the loss of copper, the cofactor, or whether there is

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[1] To whom correspondence should be addressed at University of Minnesota Duluth, Department of Biochemistry and Molecular Biology, 1035 University Drive, Duluth, MN 55812. E-mail: jprohask@d.umn.edu

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actually a change in the concentration of SOD protein. One group of investigators found that, while enzyme activity was reduced, there was evidence of apo-SOD protein such that the overall SOD protein was not affected by copper deficiency (12). Conversely, another group of investigators using a slightly different method to measure immunoreactive SOD found a significant reduction in both enzyme activity and SOD protein in erythrocytes from copper-deficient rats (13). Our laboratory has been actively involved in evaluating SOD protein concentration in organs of both rats and mice following dietary copper deficiency (14, 15). It was logical, therefore, to extend this work and evaluate red blood cells following dietary copper deficiency in both rats and mice with regard to both SOD activity and protein.

Recently, our laboratory reported that the copper chaperone for SOD (CCS) protein was highly elevated in brain, heart, and liver following dietary copper deficiency in both rats and mice (16). Other investigators reported that CCS was higher in red blood cells of copper-deficient than in copper-adequate rats (17). In that study, the CCS measurements were performed by Western immunoblotting in which hemoglobin was used as a reference for loading gels. We believe that this technique leads to an error because it is well known that erythrocytes from copper-deficient rats are hypochromic. Thus, loading equal amounts of hemoglobin could potentially overload the lanes with CCS. Therefore, other objectives of the current experiments were to extend the observation on CCS from rats to mice, explore the impact of lactation on CCS, and evaluate the issue of normalizing gel loading of extracts from red blood cells of copper-deficient rodents. Furthermore, we evaluated the possibility that the ratio of CCS to SOD might be a useful way to assess copper status.

Materials and Methods

Experimental Animals and Diets. Holtzman rats and ND4 Swiss Webster mice were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN). Animals received one of two dietary treatments: copper-deficient (–Cu) or copper-adequate (+Cu), consisting of a copper-deficient purified diet (Teklad Laboratories, Madison, WI); and either low-copper drinking water or copper-supplemented drinking water, respectively. The purified diet was formulated according to the AIN-76A diet and contained the following major components: sucrose, 500 g/kg; casein, 200 g/kg; cornstarch, 150 g/kg; corn oil, 50 g/kg; cellulose, 50 g/kg; modified AIN-76 mineral mix, 35 g/kg; AIN-76A vitamin mix, 10 g/kg; DL-methionine, 3 g/kg; choline bitartrate, 2 g/kg; and ethoxyquin, 0.01 g/kg. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained 0.4 mg Cu/kg and 48 mg Fe/kg by chemical analysis. Offspring and dams on the copper-deficient treatment drank deionized water, whereas copper-adequate treatment groups drank water that contained 20 mg Cu/L by adding $CuSO_4$ to the drinking water. Animals were given free access to diet and drinking water. All animals were maintained at 24°C with 55% relative humidity on a 12:12-hr light-dark cycle (0700–1900 hrs). All protocols were formally approved by the University of Minnesota Institutional Animal Care and Use Committee.

Established models of perinatal and postnatal copper deficiency were used with slight modifications (14, 18). Pregnant rats were placed on the copper-deficient treatment 7 days after mating. Two days following parturition, litter size for each dam was adjusted to 10 pups. Pups were weaned at P18 and transferred to stainless steel cages. Offspring were sampled at P13 and P24. A total of eight litters (four +Cu and four –Cu) were sampled. Rat dams were killed when pups were P18.

Pregnant mice were placed on treatment 17 days after mating. Male offspring were weaned at age 21 days and placed in stainless steel cages for 1 week. A total of eight litters (four +Cu and four –Cu) were sampled at P28. Mouse dams were killed when pups were P21.

Postnatal copper deficiency was initiated with two groups (n = 5 each) of male P19 Holzman rats that were fed the copper-deficient diet for 30 days and drank either deionized water (–Cu) or copper-supplemented water (+Cu), and maintained in stainless steel cages as described above.

Sample Collection. Trunk blood was collected from rats and mice into heparinized microfuge tubes following decapitation after light ether anesthesia. A small aliquot was removed for hemoglobin analysis. Another 0.2-ml aliquot was added to a preweighed microfuge tube. This sample was centrifuged at 8000 *g* for 7 mins and the plasma was removed. The pellets were gently resuspended in 0.2 ml of 0.9% NaCl and centrifuged at 500 *g* for 5 mins. The supernatant was discarded and the red cell pellet was washed and centrifuged again. The supernatant was discarded and the pellet weighed. The final pellet was osmotically shocked by adding four volumes of lysis buffer, 10 mM Tris pH 7.2, and 1 µl of mammalian protease inhibitor cocktail (P8340; Sigma Chemical Co., St. Louis, MO). The sample was vortexed vigorously and an aliquot was taken for hemoglobin analysis or recentrifuged at 13,000 *g* for 7 mins before assay. We found that the last centrifugation removed a substantial portion of the total hemoglobin in rat lysates but not in mouse lysates. We then assessed hemoglobin content of washed red cells after lysis but prior to centrifugation. Liver was removed, rinsed with deionized water, weighed, and processed for metal analysis. The remainder was quick-frozen in liquid nitrogen.

Chemical Analyses. Portions of liver and 1-g samples of diets were wet-ashed with 4 ml of concentrated HNO_3 (TraceMetal grade; Fisher Scientific, Pittsburgh, PA), and the residue was brought to 4.0 ml with 0.1 M HNO_3. Samples were then analyzed for copper and iron by flame atomic absorption spectroscopy (Model 1100B; Perkin-Elmer, Norwalk, CT). Total protein content of red cell

extracts was determined by using a modified Lowry method with bovine albumin as a reference (19). Hemoglobin was determined spectrophotometrically as metcyanohemoglobin, as described previously (20).

Enzyme Assays. Plasma ceruloplasmin diamine oxidase activity was determined using *o*-dianisidine as a substrate, as described elsewhere (20). Activity of SOD1 was measured spectrophotometrically by monitoring inhibition of pyrogallol autooxidation (0.04 mM) at 320 nm, 25°C, and pH 8.2 as described previously (20). Under these conditions, the change in absorbance is 0.02/min. Red blood cell lysates were also assayed spectrophotometrically for potential enzymes to normalize electrophoresis loading, because hemoglobin was found to be unsuitable. Two glycolyic enzymes were chosen. Lactate dehydrogenase (LDH) was measured by following NADH oxidation at 340 nm using pyruvate as substrate (pH 7.0), as outlined previously (21). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assayed by monitoring NADH formation at 340 nm as glyceraldehyde 3-phosphate was oxidized, as described elsewhere (22).

Western Blot Analyses. SOD-containing protein extracts were size-fractionated on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gels, whereas CCS extracts were fractionated on 12% gels. Proteins were electroblot-transferred to 0.2 μM nitrocellulose membranes (Protran; Schleicher & Schuell, Keene, NH) and processed for immunoblotting as described elsewhere (23). Membranes were stained with Ponceau S (Sigma Chemical) to check for equal protein loading. It was necessary to treat red cell extracts used for SOD with 0.4 volumes of chloroform:ethanol (15:25, v/v) because the SOD and hemoglobin subunit sizes are similar, and immunoblots yielded altered signals for SOD.

Immunoblotting was carried out with rabbit anti-bovine Cu,Zn-SOD (AB 1237; Chemicon International Inc., Temecula, CA) as primary antibody diluted 1:5000 followed by secondary antibody (donkey anti-rabbit IgG conjugated to horseradish peroxidase, Amersham NA 934; Amersham Pharmacia, Piscataway, NJ) diluted 1:50,000. For CCS, primary antibody was a 1:2000 dilution of rabbit antisera developed (Sigma Chemical) against a KLH-peptide, KGRKESAQPPAHL, corresponding to residues 262–274 of human CCS (24). Secondary antibody was diluted 1:10,000. Some CCS membranes were reprobed for actin as described elsewhere (23). Membranes were processed for chemiluminescence detection (SuperSignal; Pierce, Rockford, IL) and the images were directly captured using the FluorChem system (Alpha Innotech, San Leandro, CA). The size of the immunoreactive bands was estimated from regression analysis using standard peptides (Bio-Rad, Hercules, CA).

Statistical Analyses. Means and SEM were calculated. A Student's unpaired, two-tailed *t* test was used when comparing data between the two diet treatments or two genotypes, $\alpha = 0.05$ (Statview 4.5; Abacus Concepts, Inc., Berkeley, CA). Variance equality was tested using the F-test. When inequality was observed, data were log-transformed and the means were retested.

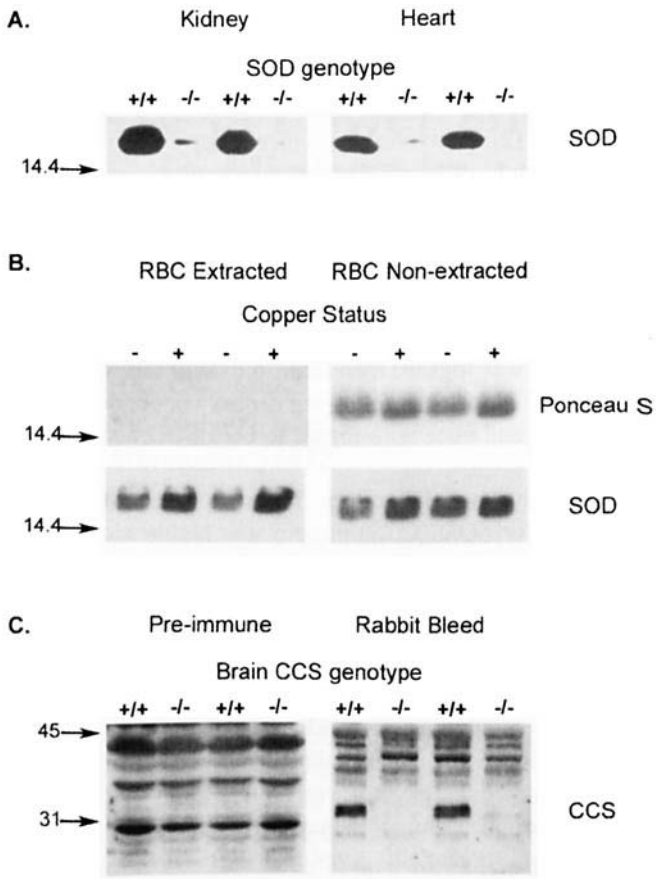

Figure 1. Antibody specificity. (A) Kidney and heart extracts (2 μg protein) from two pairs of wild-type C57BL mice (+/+) and two pairs of sod1-null mice (–/–), a generous gift from Dr. Xin Gen Lei, were subjected to Western immunblot techniques and probed with a commercial antibody (Chemicon AB 1237) rabbit anti-bovine Cu, Zn-SOD. Migration of lysozyme (14.4 kDa) is shown for reference. (B) Erythrocyte lysates (8 μg protein) from Cu-deficient (–) or Cu-adequate (+) rats P24 following perinatal copper deficiency were subjected to Western immunoblot analysis following organic solvent extraction or loaded directly (Right panel). Membranes were stained for total protein with Ponceau S, then blotted for SOD as described in (A). (C) Brain extracts (20 μg protein) from two pairs of wild-type (+/+) or CCS-null mice (–/– Ref. 15) were subjected to Western immunoblot analysis using either preimmune rabbit serum (Left panel) or serum from a rabbit immunized with a CCS peptide antigen as described in "Materials and Methods." Migration positions of carbonic anhydrase (31 kDa) and ovalbumin (45 kDa) are shown for reference.

Results

SOD and CCS Antibody Specificity. Commercial antibody against bovine erythrocyte SOD detected a single immunoreactive band of approximately 16 kDa in heart and kidney extracts from sod1 +/+ (wild-type) mice (Fig. 1A). In contrast, in sod1 –/– mice this band was nearly absent, supporting the veracity of the antiserum. Red cell extracts were separated on 15% SDS-PAGE gels and stained for protein with Ponceau S. A prominent band at 16 kDa was

Table 1. Copper Status of 7-week-old Male Rats Following Dietary Copper Deficiency[a]

Characteristics	Cu-adequate	Cu-deficient
Body weight (g)	282 ± 10.3	278 ± 11.1
Hemoglobin (g/L)	158 ± 2.63	125 ± 16.0
Liver copper (μg/g)	3.65 ± 0.10	0.61 ± 0.05*
Liver iron (μg/g)	70.2 ± 3.35	104 ± 10.52*

[a]Values are mean ± SEM (n = 5) of each group. Rats were maintained on dietary treatment for 30 days. Liver metal levels were determined by flame atomic absorption following wet ashing.
*$P < 0.05$

evident (Fig. 1B). We presume this was hemoglobin because the globin size is about 16 kDa, and hemoglobin is the most abundant red cell protein. Treatment of the red cell lysate with organic solvents removes most proteins but does not remove immunoreactive SOD. In fact, the SOD signal was more robust and reliable following organic solvent treatment (Fig. 1B).

The antiserum harvested from a rabbit immunized against a tridecapeptide near the C-terminus of human CCS was used to evaluate its specificity and suitability for use with rodent samples (Fig. 1C). Compared with preimmune rabbit serum, the immune serum gave a robust signal at approximately 35 kDa, in CCS wild-type (+/+) mice compared with blank lanes in extracts from CCS-null (–/–) mice. Other differences between preimmune and immune sera were not as evident and do not represent CCS-specific peptides.

Copper Status of Rats and Mice Following Dietary Copper Deficiency. Following 30 days of dietary treatment, 7-week-old (P49) male Holtzman rats were evaluated (Table 1). Rats that drank deionized water (–Cu) had 17% of the copper content in their liver compared with rats that drank copper-supplemented water (+Cu). Conversely, the liver iron content of –Cu rats was 148% that of +Cu rats. Both these changes are consistent with copper-deficient characteristics. We failed to detect any significant difference in either body weight or hemoglobin concentration in this cohort of rats. These –Cu rats represent reasonable comparisons to early work by others on red cell SOD (12, 13).

Male mice at P28 derived from –Cu and +Cu dams were compared for their copper status (Table 2). Compared with +Cu mice, –Cu mice were smaller and had hemoglobin levels that were only 58% those of +Cu mice. Liver copper levels in –Cu mice were 48% of +Cu values, which is consistent with severe copper deficiency. Reductions of liver copper in –Cu mice were less severe than reductions in –Cu rats (Table 1). The liver iron concentration in –Cu mice was 177% that of the value in +Cu mice. Together, these characteristics support that mice of two distinct dietary treatment types, –Cu and +Cu, were studied.

Table 2. Copper Status of 4-week-old Male Mice Following Dietary Copper Deficiency[a]

Characteristics	Cu-adequate	Cu-deficient
Body weight (g)	17.5 ± 0.99	13.5 ± 0.48*
Hemoglobin (g/L)	160 ± 2.43	92.5 ± 4.78*
Liver copper (μg/g)	3.89 ± 0.06	1.88 ± 0.07*
Liver iron (μg/g)	60.5 ± 6.88	107 ± 5.42*

[a]Values are mean ± SEM (n = 5) per group. Pups were born to and nursed by Cu-deficient or Cu-adequate dams. Treatment began 4 days prior to parturition. Pups were maintained on the same treatment of their dams for 1 week prior to killing. Metal levels were determined by flame atomic absorption spectroscopy following wet ashing.
*$P < 0.05$

Erythrocyte Enzyme Levels. Plasma was removed from heparinized blood after centrifugation from rats and mice whose characteristics were described. Red cells were washed and then osmotically shocked to prepare solutions with equal weights of cells per volume of lysis solution. Lysed cells were centrifuged to remove red cell ghosts, and the lysate was analyzed for several "soluble" proteins (Table 3). Even after correcting for differences in hematocrit, the concentration of hemoglobin in –Cu mice was lower than that observed in +Cu mice. We found the same result in a confirmatory experiment studying P28 female mice. The lysate hemoglobin for five +Cu mice was 51.2 ± 1.70 mg/ml (mean ± SEM) compared with 43.0 ± 1.19 mg/ml for five –Cu mice ($P < 0.01$). Blood hemoglobin for the same mice was 135 ± 5.27 g/L for +Cu mice compared with 70.9 ± 5.20 g/L for –Cu mice ($P < 0.01$).

We found that hemoglobin was lost in the pellet when we centrifuged lysed red cells from rat blood (data not shown). Thus we have no solid hemoglobin data on lysates

Table 3. Erythrocyte Lysate Hemoglobin and Selected Enzyme Comparisons in Rats and Mice Following Dietary Copper Deficiency[a]

	Rats		Mice	
Characteristic	Cu-adequate	Cu-deficient	Cu-adequate	Cu-deficient
Hemoglobin (mg/ml)	—	—	44.4 ± 1.22	40.8 ± 0.85*
SOD1 (units/ml)	3939 ± 251	1130 ± 77.5*	3136 ± 142	2342 ± 77.4*
LDH (units/ml)	2.27 ± 0.05	2.58 ± 0.06*	2.30 ± 0.13	2.71 ± 0.08*
GAPDH (units/ml)	2.24 ± 0.02	2.24 ± 0.06	2.83 ± 0.19	2.80 ± 0.04

[a]Values are mean ± SEM (n = 5). SOD1, LDH, and GAPDH activities were determined spectrophotometrically. Lysate hemoglobin estimates on rats were not valid.
*$P < 0.05$

for the postnatal rats in this study (Table 3). However, in a recent experiment with P25 female –Cu rats from our perinatal model (a more severe copper-deficient challenge), we confirmed the mouse data. The whole blood hemoglobin in the +Cu rats was 108 ± 3.6 g/L (4) compared with 76.2 ± 4.7 g/L (4) for –Cu rats ($P < 0.01$). The hemoglobin concentration in the unspun lysates was 50.7 ± 0.96 mg/ml for +Cu rats compared with 41.0 ± 3.12 mg/ml for –Cu rats ($P < 0.05$). These data in both rats and mice confirm that using hemoglobin to normalize red cell extracts for electrophoresis loading experiments might result in faulty conclusions.

We confirmed that although hemoglobin was lost in the pellet of lysed rat erythrocytes after centrifugation, SOD activity was the same in the upsun lysate and the supernatant (data not shown). Therefore, we explored the use of other red cell proteins to normalize extracts for loading. Our initial attempt was to use LDH, a glycolytic enzyme, for this purpose. We found, however, that for both rats and mice used in these studies, LDH activity was significantly higher in extracts from –Cu rodents compared with that of +Cu controls (Table 3). We confirmed this observation in other rat and mouse experiments (data not shown). We then measured a second glycolytic enzyme, GAPDH. The GAPDH gene is often used as a loading control for RNA blots. GAPDH activity was not altered by diet history and was found suitable for the purpose of loading equal amounts of red cell proteins for immunoblotting. For both rats and mice the activity of SOD was lower in –Cu extracts compared with +Cu extracts, confirming the copper status inferred by metal analysis (Table 3).

SOD and CCS Content of Red Cells in Male Rats Following Postnatal Copper Deficiency. Red cell extracts from P49 male rats were used to detect SOD and CCS on separate gels. The CCS membrane was stripped and reprobed for actin. This was not possible for the SOD membrane because extracts were treated with organic solvents to remove interfering proteins (Fig. 2A). It is quite apparent that there is a clear difference in both SOD and CCS expression in –Cu rats compared with +Cu rats, whereas actin is unchanged. The mean SOD density of –Cu samples was 41% that of +Cu samples ($P < 0.01$; Fig. 2B). This decrease in immunoreactive SOD was less than the decrease in SOD activity. SOD activity in –Cu rats was 29% that of +Cu rats (Table 3).

One of the five –Cu rats had a weak CCS signal, though actin was normal; but the other four –Cu rats had a robust, enhanced CCS signal compared with the +Cu CCS density (Fig. 2A). After log transformation to establish equal variance, mean CCS density of the –Cu rats tended to be higher than in the +Cu rats ($P < 0.06$). In fact, the mean CCS density from –Cu samples was more than 2.5-fold higher (Fig. 2B).

SOD and CCS Content of Red Cells in Male Mice Following Perinatal Copper Deficiency. Red cells from male mice subjected to a different copper depletion protocol were evaluated for SOD, CCS, and actin in a parallel manner to confirm and extend the rat experiment. The SOD density of the first –Cu sample was as high as the +Cu values, whereas the other four SOD densities were markedly lower (Fig. 3A); however, statistical comparison of the SOD densities yielded a tendency for the –Cu samples to be lower than the +Cu samples ($P < 0.09$). The mean –Cu SOD density was 88% that of the +Cu values (Fig. 3B). The mean SOD activity of –Cu samples was 70% that of +Cu samples (Table 3). Despite the more modest reduction in both SOD activity and density in –Cu mice compared with that of rats, there was a significant correlation between SOD enzyme activity and SOD density in the –Cu mouse samples ($r = 0.93$, $P < 0.01$).

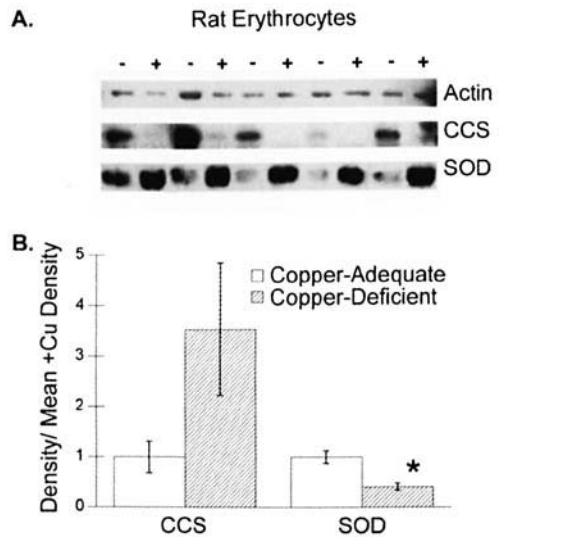

Figure 2. (A) Western immunoblots of erythrocyte extracts from P49 male Holtzman rats. Two separate membranes were prepared, one for actin and CCS and one for SOD. For the actin and CCS blot, each lane was loaded with 0.0044 units of GAPDH activity (approximately 28 μg of protein) and fractionated by SDS-PAGE. For the SOD blot, 0.0006 units (approximately 3.6 μg of protein) were loaded per lane. Five copper-deficient (–) and five copper-adequate (+) rats were analyzed. (B) Mean erythrocyte density of CCS and SOD from the Western blots in (A). Bars represent relative density/mean copper-adequate (+Cu) density ± SEM. Mean +Cu density was set at 1.0. Means from copper-deficient rats for SOD were significantly different than copper-adequate rats, $^*P < 0.01$.

Red cell content of CCS was more than 2-fold higher in –Cu samples compared with +Cu samples ($P < 0.01$; Fig. 3). Actin levels in red cells were not affected by copper status of the mice (Fig. 3A).

SOD and CCS Content of Red Cells in Rat and Mouse Dams Following Copper Deficiency. Comparison of rat dams and mouse dams demonstrated some interesting differences due to dietary copper restriction (Table 4). Copper-deficient rat dams had liver copper levels 33% those of +Cu rat dams and red cell SOD activity was 30% that of +Cu rats. We did not detect a difference in liver iron due to diet despite a mild anemia in –Cu rat dams.

Copper-deficient mouse dams had liver copper levels

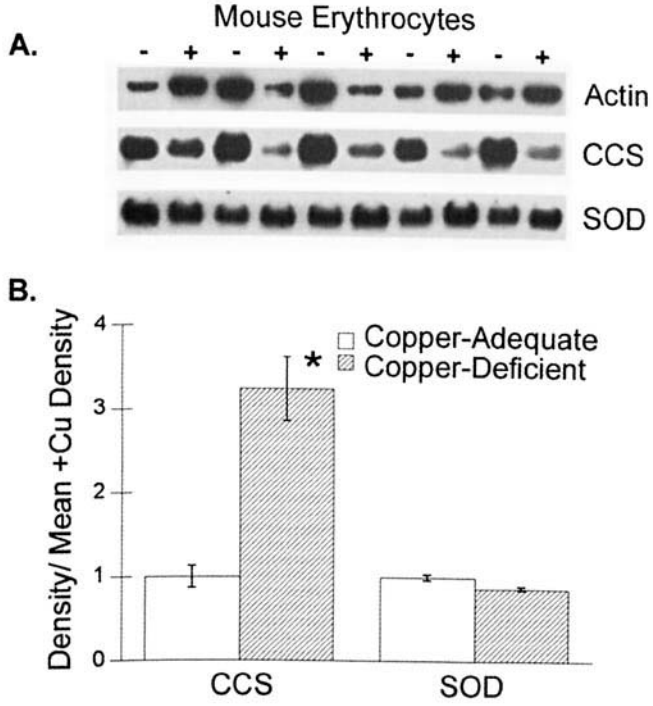

Figure 3. (A) Western immunoblots of erythrocyte extracts from P28 male Swiss-Webster mice. Two separate membranes were prepared, one for actin and CCS and one for SOD. For the actin and CCS blot, each lane was loaded with 0.0056 units of GAPDH activity (approximately 87 μg of protein) and fractionated by SDS-PAGE. For the SOD blot, 0.0007 units (approximately 11.1 μg of protein) were loaded per lane. Five copper-deficient (–) and five copper-adequate (+) mice were analyzed. (B) Mean density of CCS and SOD in erythrocytes from the Western blots in (A). Bars represent relative density/mean copper-adequate (+Cu) density ± SEM. Mean +Cu density was set at 1.0. Means from copper-deficient mice for CCS were significantly different than copper-adequate mice, $^*P < 0.01$.

49% those of +Cu mouse dams and red cell SOD activity was 76% that of +Cu mice. However, –Cu mouse dams had liver iron levels 5.4-fold higher than +Cu mice and more severe anemia than –Cu rat dams. There was a 25% reduction in hemoglobin levels in –Cu mice compared with 16% in –Cu rats (Table 4). For both rat dams and mouse dams, the –Cu group displayed characteristics consistent with copper deficiency.

Immunoblots were evaluated from red blood cell extracts for both rat and mouse dams (Fig. 4). Density of SOD in –Cu rat dams was 39% of that detected in +Cu dams ($P < 0.01$). Consistent with SOD activity measurements, a similar relation in SOD protein was observed in –Cu mouse dams, in which the mean density of –Cu samples was 55% that of +Cu mouse dams ($P < 0.05$; Fig. 4B).

The CCS immunoblots restripped for actin did not give an adequate signal, so a portion of the Ponceau S–stained membrane near the CCS band is shown to illustrate equal protein loading (Fig. 4A). The GAPDH activity of mouse dam erythrocyte lysates was not affected by dietary treatment and averaged 0.45 ± 0.02 units/ml. GAPDH activity was not measured in rat dams, so loading was performed by LDH activity that was not altered by diet in this data set, averaging 1.6 ± 0.1 units/ml. The mean CCS density of –Cu rat dams was 2.3-fold higher than that of +Cu samples ($P < 0.01$; Fig. 4). For –Cu mouse dams there was no statistically significant elevation in CCS compared with +Cu samples because of a weak signal for one –Cu mouse dam (Fig. 4, Lane 7).

Erythrocyte CCS/SOD Content of Rodents Following Copper Deficiency. There was some variability in the intensity of the CCS and SOD signals following immunoblots from –Cu rodents, so that we failed to detect significant statistical differences (Fig.2, CCS; Fig. 3, SOD; and Fig.4, mouse CCS). However, when CCS:SOD ratios were calculated for all rodents in all four experimental groups, it was clear that a strong dietary treatment effect was present ($P < 0.05$; Table 5). For all –Cu rodents compared with controls, there was a 1.5-fold to 9-fold increase in CCS:SOD. The effect was stronger in the two rat models compared with that of mice (Table 5).

Discussion

Antibody specificity is critical for immunoblot quantification and validity. The SOD antibody that was used in these studies reacted only with Cu,Zn-SOD because tissue extracts from sod1 –/– mice did not reveal any detectable binding, whereas wild-type mice gave a strong signal, estimated from size markers to be 16 kDa, the size of an SOD subunit. Furthermore, the immunoreactivity of the SOD antibody was not dependent on copper content, because apo-SOD gave the same immunoreactive signal as holo-SOD (data not shown). Thus, our studies indicate that red blood cell extracts from copper-deficient rats contain lower concentrations of SOD protein than control rats.

Table 4. Copper Status of Rat and Mouse Dams Following Dietary Copper Deficiency[a]

	Rat Dams		Mouse Dams	
Characteristic	Cu-adequate	Cu-deficient	Cu-adequate	Cu-deficient
Body weight (g)	330 ± 9.9	312 ± 9.6	33.0 ± 1.43	32.4 ± 2.57
Hemoglobin (g/L)	158 ± 1.9	132 ± 8.8*	126 ± 9.1	60.6 ± 10.7*
Liver copper (μg/g)	4.40 ± 0.40	1.45 ± 0.20*	4.57 ± 0.19	2.25 ± 0.11*
Liver iron (μg/g)	115 ± 10.4	148 ± 40.8	56.9 ± 8.5	362 ± 52.3*
SOD1 (units/ml)	3724 ± 138	1111 ± 112*	1987 ± 57	1505 ± 86*

[a]Values are mean ± SEM (n = 4). Liver metal levels were determined by flame atomic absorption following wet ashing.
*$P < 0.05$

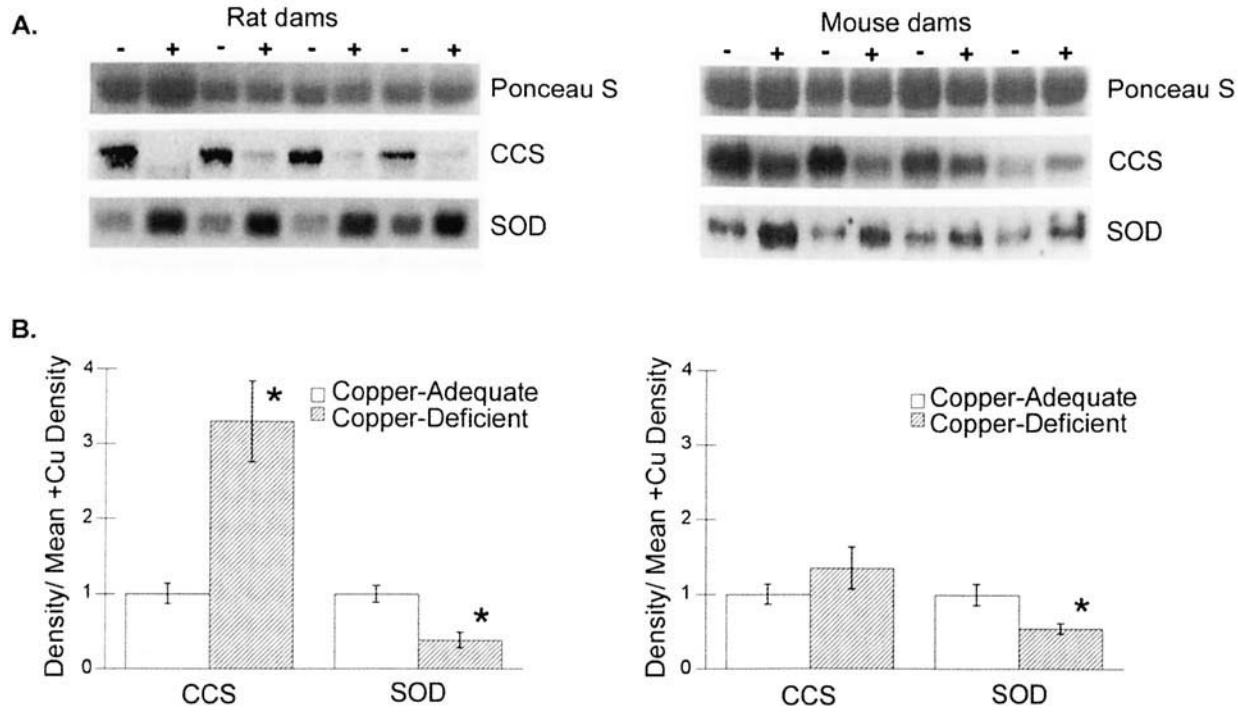

Figure 4. (A) Western immunoblots of erythrocyte extracts from Holtzman rat dams and Swiss-Webster mouse dams. Two separate membranes were prepared for each group, one for CCS and the other for SOD. For the rat CCS blot, each lane was loaded with 0.0031 units of LDH activity and fractionated by SDS-PAGE. For the rat SOD blot, 0.001 unit was loaded per lane. For the mouse CCS blot, each lane was loaded with 0.0009 units of GAPDH activity (approximately 18.4 μg of protein) and fractionated by SDS-PAGE. For the mouse SOD blot, 0.0001 unit (approximately 2.35 μg of protein) was loaded per lane. Four copper-deficient (–) and four copper-adequate (+) animals were analyzed for each group. (B) Mean density of CCS and SOD in erythrocytes from the Western blots in (A). Bars represent relative density/mean copper-adequate (+Cu) density ± SEM. Mean +Cu density was set at 1.0. Means from copper-deficient rats for both CCS and SOD were significantly different than copper-adequate rats, $^*P < 0.01$. Means from copper-deficient mice for SOD were significantly different than copper-adequate mice, $^*P < 0.05$.

These findings confirm the prior observations of others indicating that following postnatal copper deficiency in rats, there is a decrease in the total amount of immunoreactive SOD (13). Our current data disagree with earlier studies by DiSilvestro, who detected no overall decrease in SOD immunoreactive protein following dietary copper deficiency in rats (12). We do agree with DiSilvestro's observation that apo-SOD is present in the red cells of copper-deficient rats. When extracts were loaded with equal amounts of enzyme activity per lane, there was a much stronger immunoreactive signal in samples from copper-deficient rats than from controls (unpublished data). However, overall, our results from rats are consistent with previous work from our laboratory and with that of others who have shown a decrease in immunoreactive SOD1 per total protein in other organ extracts (14, 25, 26).

Our results also extend to another species, the mouse, in which we were able to demonstrate a decrease in SOD immunoreactive protein in red blood cells following copper deficiency. This extends recent previous results in other organs of mice that copper deficiency leads to an overall reduction in SOD1 protein (15). Thus, both rats and mice have a decrease in SOD1 protein that is roughly proportional to the degree of deficiency. In the current studies, the decrease in SOD1 immunoreactive protein was more severe in rats than in mice. Current studies also show that adult females, rats and mice alike, have decreased erythrocyte SOD immunoreactive protein following dietary copper deficiency and the physiological challenge of lactation. Erythrocyte SOD immunoreactive protein is also less in younger male –Cu rats and –Cu mice (data not shown). Overall, the SOD1 enzyme activity and immunoreactive protein seem to be decreased following dietary copper deficiency in most, if not all, organs of copper-deficient rodents.

In our experiments we found that hemoglobin was

Table 5. Ratio of Erythrocyte CCS to Copper,Zinc-SOD Following Dietary Copper Deficiency[a]

Experimental Group	Cu-adequate	Cu-deficient
Postnatal P49 Rats	0.94 ± 0.20	7.90 ± 2.05*
Perinatal P28 Mice	0.99 ± 0.11	3.72 ± 0.47*
Rat Dams	1.04 ± 0.19	10.2 ± 2.69*
Mouse Dams	1.03 ± 0.17	2.48 ± 0.52*

[a]Values (CCS:SOD1) are mean ± SEM (n = 4 or 5) per group. Mean density from immunoblots of Cu-adequate rodents was assigned a value of 1.0 for both CCS and SOD1. Individual densities for all animals were then calculated relative to those values and sample means were then compared statistically.
$^*P < 0.05$

unsuitable as a way to normalize loading for electrophoresis with red blood cells. Hemoglobin interfered with the SOD signal on immunoblots. Also, hemoglobin is lower in concentration per cell following copper deficiency. Recent work with rats has confirmed the decrease in mean corpuscular hemoglobin associated with copper deficiency (27).

Current experiments found that the glycolytic enzyme GAPDH was a suitable protein, unaffected by copper deficiency, for use in normalizing protein loading. Another enzyme, LDH, was not suitable because, in most cases for both rats and mice, LDH activity was higher in extracts from copper-deficient rodents. Further research will be necessary to establish why LDH is higher and whether there are any consequences to the red cell because of this alteration. Recent studies are consistent with an LDH enhancement, as nuclear magnetic resonance studies on erythrocytes of copper-deficient rats showed higher flux through the lactate dehydrogenase locus compared with that of controls (28). The LDH enhancement was detected in both copper-deficient rats and mice.

There are a number of interesting contrasts between rats and mice. In the current studies, for example, –Cu rat dams were mildly anemic, had no statistical elevation of iron in liver, yet had a very severe reduction in erythrocyte SOD activity. In contrast, –Cu mouse dams were severely anemic, had a much higher increase in liver iron, but had a much more modest decrease in liver copper, and a much attenuated decrease in red cell SOD activity. Contrasting effects of copper status on rats and mice has been noted previously for cytochrome oxidase, glutathione peroxidase, and glutathione transferase (29).

Specificity of the antisera used in these experiments was also demonstrated for CCS in that a signal for the CCS was not observed in mice in which this gene had been deleted. This was important to establish, because other bands were present when using immune serum that were not CCS, because they were not absent in the CCS –/– mice. Our data show that there is a robust increase in CCS protein in erythrocyte extracts of copper-deficient rats. This agrees with the previous observation of Bertinato et al. (17). Furthermore, this increase in CCS was observed even when the extracts were corrected for hemoglobin concentration. Also, the elevation in CCS was observed in mice as well as in rats, extending the CCS observation to another mammalian species. Red cell data confirm other recent work that has shown CCS is elevated following copper deficiency in both rats and mice in liver, heart, and brain (16). Bertinato et al. reported higher CCS levels in rat liver and blood after marginal copper deficiency (17). Current studies also show that the CCS:SOD ratio is also very sensitive to copper status and may be a useful way to identify marginal copper deficiency in humans because small changes in either of the two amplify the ratio. This method may be useful as a clinical marker for the evaluation of marginal copper status because, currently, a well-established, consistent marker is not available.

Our previous work suggested that the decreased concentration of SOD protein in cells from copper-deficient rodents and from CCS –/– mice was perhaps due to the instability of SOD1 that was not fully loaded with copper (14, 15). Thus, increased turnover of SOD1 in the state of copper deficiency could explain those observations. However, a recent publication by Bartnikas and Gitlin has challenged this thinking (30). In those studies it was shown that the turnover of SOD protein was not dependent on copper. Turnover of SOD was the same in cells from CCS +/+ mice as in CCS –/– mice. In contrast, our data demonstrated major reductions in SOD protein in CCS –/– mice compared with that of wild-type mice (15). Thus, the issue of the decrease in SOD protein remains somewhat of an enigma. Our previous results have shown that the mRNA for SOD1 protein is not affected by copper deficiency in either rats or mice (14, 15). Furthermore, current data in the erythrocyte also challenge the turnover theory of SOD1 in copper deficiency because red cell protein degradation is minimal, yet clear evidence for a decreased content of SOD protein was presented (31). Perhaps the changes in SOD protein levels occur during maturation of the reticulocyte population. Elucidation of the biochemical mechanism for the reduction in SOD protein accompanying copper deficiency will require further research.

Likewise, the observation of elevated CCS in erythrocytes is somewhat puzzling. Bertinatto and L'Abbe have recently reported that the elevation in CCS in cell culture studies is due to the stability of the protein when cellular copper is reduced (32). CCS is normally degraded by the 26S proteosome pathway. Again, the slow turnover of proteins, if any, in the erythrocyte would not explain an elevation in CCS (31). Like SOD, the mRNA for CCS is not altered by copper deficiency in mice or rats (16, 32). Thus, the CCS elevation is not likely due to enhanced synthesis. Further research will be necessary to identify the etiology for enhanced red cell CCS and depressed red cell SOD.

Erythrocyte CCS and SOD protein alterations during copper deficiency have the potential to be useful markers to reflect dietary copper intake. The mechanisms for their alterations, however, remain to be elucidated.

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