Copper,Zinc-Superoxide Dismutase Protein but not mRNA Is Lower in Copper-Deficient Mice and Mice Lacking the Copper Chaperone for Superoxide Dismutase

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Cu,Zn-superoxide dismutase (SOD1) is an abundant metalloenzyme important in scavenging superoxide ions. Cu-deficient rats have lower SOD1 activity and protein, possibly because apo-SOD1 is degraded faster than holo-SOD1. Previous work with mice lacking the Cu chaperone for SOD1 (CCS) indicated a drastic loss of SOD1 activity but not protein, suggesting an accumulation of apo-SOD1. We produced dietary Cu deficiency in mice to clarify this issue. Compared with Cu-deficient rats, reduction in liver SOD1 activity and protein was much less than Cu-deficient mouse dams and offspring. However, after perinatal Cu deficiency, 4-week-old mouse pups had lower levels of SOD1 activity and protein in liver and heart, but not brain, compared with Cu-adequate controls. Reduction in brain Cu was greater than liver. In CCS –/– mice, there was severe reduction in liver, heart, and brain SOD1 activity and protein. In fact, the reduction in activity was similar to the loss of protein. Neither Cu-deficient mouse liver nor CCS –/– mouse liver had altered SOD1 mRNA levels compared with control values. These results in mice are comparable with rats and suggest a posttranscriptional mechanism for reduction of SOD1 protein when Cu is limiting in SOD1. Exp Biol Med 228:959–966, 2003

Key words: copper-deficient; rats; mice; copper,zinc-superoxide dismutase; CCS; chaperone; enzyme activity, mRNA, Western blots

Survival in an aerobic environment requires the concerted action of many enzymes that control the levels of reactive oxygen species. Cu,Zn-superoxide dismutase (SOD1) is one such enzyme that effectively limits the buildup of superoxide, the univalent reduction product of molecular oxygen (1). SOD1 is a 32-kDa homodimer containing one Cu and one Zn atom per subunit. SOD1 is present in all eukaryotes and is regulated by environmental and genetic factors.

Teleologic thinking might predict that SOD1 is regulated by cellular Cu levels because the catalytic function of SOD1 depends on Cu redox chemistry at the active site. Indeed, in baker's yeast, the SOD1 transcript is under the influence of ACE1, a Cu-binding protein transcription factor (2, 3). SOD1 protein levels are elevated when yeast are exposed to higher Cu levels and are lower when Cu chelators are introduced (4, 5). ACE1 also regulates yeast metallothionein expression (6). Mutants lacking yeast metallothionein can be protected from Cu exposure by overexpressing SOD1 (7). This implies that SOD1 is an important Cu-binding protein as well as catalyst.

One report indicated that the concentration of SOD1 mRNA was lower in liver of Cu-deficient rats (8). However, this was not confirmed for liver, heart, or brain in a similar study (9). Thus, there is little evidence that changes in cellular Cu, in higher eukaryotes, impact SOD1 transcription.

In contrast, there are several laboratories that have studied the impact of Cu deficiency on SOD1 protein levels. Four studies indicated that there was no diminution in SOD1 protein. The first report studied Cu-deficient chicks and evaluated aortal extracts (10). This was followed by studies using rats and investigating liver, lung, and red blood cells (11–13). However, four more recent reports seem to indicate the opposite, namely that SOD1 protein levels are lower in tissues from Cu-deficient rats (8, 9, 14, 15). Thus, although there is some controversy, there is recent evidence that SOD1 protein levels are reduced in Cu-

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deficient rat tissues. The reduction of SOD1 protein and activity are proportional to the degree of Cu deficiency (9). Rossi *et al.* (16) reported that liver SOD1 from Cu-deficient rats contained lower Cu and Zn contents compared with native enzyme. Perhaps the low metal content forms of SOD1 are degraded faster than native enzyme.

SOD1 requires a specific Cu chaperone (Cu chaperone for SOD1 [CCS]) to deliver Cu from CCS to the active site of SOD1 (17). Mice lacking CCS have greatly lower SOD1 activity in a number of tissues, including liver, kidney, and brain (18). These mice had normal ceruloplasmin activity, indicating that Cu delivery to other chaperone systems, namely atox1, was not affected. Interestingly, there was no alteration in SOD1 protein detected in CCS –/– mice in those studies (18).

This raised the possibility that prior observations on SOD1 in Cu-deficient rats were unique to that species. We investigated this possibility and report herein that mice, similar to rats, have lower SOD1 protein when Cu is lower in tissues, and also that CCS –/– mice do indeed have lower SOD1 protein, suggesting a common hypothesis that apo-SOD1 is degraded faster than holo-SOD1.

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, Cu deficient (–Cu) or Cu adequate (+Cu), consisting of a Cu-deficient purified diet (Teklad Laboratories, Madison, WI) and either low Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was formulated according to the AIN-76A diet and contained the following major components (grams per kilogram 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. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained 0.4 mg of Cu/kg and 43 mg of Fe/kg by chemical analysis. Offspring and dams on the Cu-deficient treatment drank deionized water, whereas Cu-adequate treatment groups drank water that contained 20 mg of 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-hr light cycle (0700–1900 hr). All protocols were approved formally by the University of Minnesota Institutional Animal Care and Use Committee.

Established models of perinatal Cu deficiency were used with slight modifications (19, 20). Pregnant rats were placed on the Cu-deficient treatment 7 days after mating. Two days after parturition, the litter size for each dam was adjusted to 10 pups. Offspring were weaned when 18 days old, placed in stainless steel cages, and maintained on the same treatment as their respective dams for an additional 5 days. A total of eight litters (five +Cu and three –Cu) were sampled. Pregnant mice were placed on treatment 19 days after mating. Male offspring were weaned at age 21 days and were placed in stainless steel cages for 1 or 2 weeks. A total of six litters (three +Cu and three –Cu) were sampled.

We also analyzed eight 2-month-old C57BL/6J mice derived from mating CCS +/– mice generated by targeted deletion of exons 1 and 2 of the CCS gene as described elsewhere (18). Littermates analyzed were of two genotypes as determined by PCR, namely CCS +/+ and CCS –/–. Mice were fed a standard Cu-adequate rodent chow and tap water and weighed 20 to 28 g before sacrifice.

Sample Collection. After light ether anesthesia, and after decapitation, trunk blood was drawn from rats and mice into heparinized microhematocrit tubes. A small aliquot was also removed for hemoglobin analysis. Plasma was obtained by centrifugation. A portion of the liver was removed, rinsed with deionized water, weighed, and processed for metal analysis. The remainder was quick-frozen in liquid nitrogen. Hearts were dissected into ventricles and atria and were quick-frozen. Brains were removed and divided into the cerebellum, medulla oblongata/pons, cerebral cortex, and the remainder (midbrain). All brain samples but the cortex were quick-frozen; cortex samples were analyzed for Cu.

Chemical Analyses. Portions of liver, cerebral cortex, and 1-g samples of diets were wet-digested with 4 ml of concentrated HNO_3 (AR select grade; Mallinckrodt, St. Louis, MO), and the residue was brought to 4.0 ml with 0.1 *M* HNO_3. Samples were then analyzed for Cu and Fe by flame atomic absorption spectroscopy (model 2380; Perkin-Elmer, Norwalk, CT). Total protein content of tissue extracts was determined by using a modified Lowry method with bovine albumin as a reference (21). Hemoglobin was determined spectrophotometrically as metcyanohemoglobin as described previously (22).

Enzyme Assays. Plasma ceruloplasmin diamine oxidase activity was determined by using *o*-dianisidine as substrate as described elsewhere (22). Activity of SOD1 was measured spectrophotometrically by monitoring inhibition of pyrogallol autooxidation, 0.04 m*M*, at 320 nm, 25°C, and pH 8.2 as described previously (22). Under these conditions, the change in absorbance is 0.02/min. Cardiac ventricles, liver, cerebellum, or midbrain tissues were homogenized for 30 sec in 9 vols of 50 m*M* potassium phosphate (pH 7.0) with a Tissumizer and microprobe (SDT-080EN; Tekmar, Cincinnati, OH). A portion of the homogenates was treated with mammalian protease inhibitor cocktail (Sigma P8340; Sigma Chemical, St. Louis, MO) and was centrifuged at 13,000*g* for 10 min. Aliquots of the supernate were used to measure protein, processed for SOD1 assays, and prepared for western immunoblots. SOD1 activities were expressed per milligram of total protein.

Preparation of SOD1 deficient in metals was accomplished by following a published protocol with minor modifications (23). Purified bovine erythrocyte superoxide dis-

mutase (Sigma S 2515; Sigma Chemical) was dissolved in water and dialyzed using a Slide-A-Lyzer 7K dialysis cassette (Pierce, Rockford, IL) against 10 m*M* EDTA, then 10 m*M* $MgCl_2$, and finally 10 m*M* sodium acetate, all at pH 3.8 and 4°C. An aliquot of apo-SOD1 was removed and the remainder was reconstituted with 20 equivalents of Cu and Zn by overnight dialysis in 10 m*M* sodium acetate, pH 4.0, followed by dialysis against 50 m*M* potassium phosphate, pH 7.8, containing 0.1 m*M* EDTA, and finally by several changes of deionized metal free water. The resulting holo-SOD1 had approximately 1.3 times the specific activity of the starting material.

Northern Hybridization. Total RNA was isolated from quick-frozen liver using RNAwiz (Ambion, Austin TX) described in detail elsewhere (24). Liver total RNA was size fractionated on 1.5% agarose denaturing gel, transferred to Nytran Plus membranes (Schleicher & Schuell, Keene, NH), and hybridized overnight with a ^{32}P random-primed 0.43-kb probe for rat Cu,Zn-SOD (3.3 ng/ml) as described previously (9). After autoradiography, membranes were stripped and rehybridized with a ^{32}P-labeled 1.2-kb probe for mouse 18S ribosomal RNA (Ambion) to verify equal loading and transfer of RNA. Images of autoradiograms were captured with the FluorChem system (Alpha Innotech, San Leandro, CA) and the density of band profiles was integrated using the manufacturer's software.

Western Blot Analyses. Protein extracts were size fractionated on 15% SDS-PAGE gels and were electroblot transferred to 0.2-μ*M* nitrocellulose membranes (Protran; Schleicher & Schuell) and processed for immunoblotting as described elsewhere (25). Membranes were stained with Ponceau S (Sigma Chemical) to ensure equal protein loading.

Immunoblotting was carried out with primary antibody diluted 1:10,000 by using rabbit anti-bovine Cu, Zn-SOD (AB 1237; Chemicon International, Temecula, CA) followed by secondary antibody (donkey anti-rabbit immunoglobulin [Ig] G conjugated to horseradish peroxidase (Amersham NA 934; Amersham Pharmacia, Piscataway, NJ) diluted 1:5,000.

Membranes were processed for chemiluminescence detection (SuperSignal; Pierce) and the images were directly captured using the FluorChem system. The size of the immunoreactive bands was estimated from regression analysis using standard peptides (Bio-Rad, Hercules, CA).

Statistical Analyses. Means, SD or SEM, were calculated. 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, Berkeley, CA).

Results

Comparison between Rat and Mouse Dams. To compare the SOD1 response between rats and mice, we first studied dams that had just finished lactating. We knew that this physiological stress increases the need for Cu compared with nonpregnant rodents (26). Rat dams maintained on the –Cu treatment for 2 weeks of gestation and 3 weeks of lactation had a 70% reduction in liver Cu levels compared with control dams on the +Cu treatment (mean ± SD, $n = 3$) (1.00 ± 0.05 μg Cu/g liver vs 3.41 ± 0.02 μg Cu/g liver). In contrast, –Cu mouse dams had only a 45% reduction in liver Cu concentration compared with the mouse dams (2.44 ± 0.91 μg Cu/g liver vs 4.47 ± 0.29 μg Cu/g liver). The –Cu mouse dams did display features consistent with Cu deficiency as their average hematocrit (26% ± 2.8%) was much lower than the +Cu mouse dams, which was 50% ± 1.5%. Hematocrit values for rat dams were not altered by Cu deficiency (–Cu, 44% ± 1.5% versus +Cu, 50% ± 1.8%). Liver SOD1 activity was 84% lower in –Cu rat dams compared with +Cu rat dams, but was not altered in –Cu mouse dams (Fig. 1A). Western blots of liver extracts demonstrated a 52% reduction in density of SOD1 in –Cu rat dams (Fig. 1B). We did not detect differences in SOD1 density in the mouse dam liver extracts.

Comparison between Weanling Male Rat and Mouse Offspring. Rat offspring (age 23 days) and mouse offspring (age 35 days) were compared for SOD1 and Cu status. Compared with +Cu rats, the liver Cu concentration in –Cu rats was 97% lower (0.55 ± 0.09 μg/g compared with 15.8 ± 2.86 μg/g). The liver Cu levels in –Cu mice was 60% lower than +Cu mice (1.54 ± 0.39 μg/g

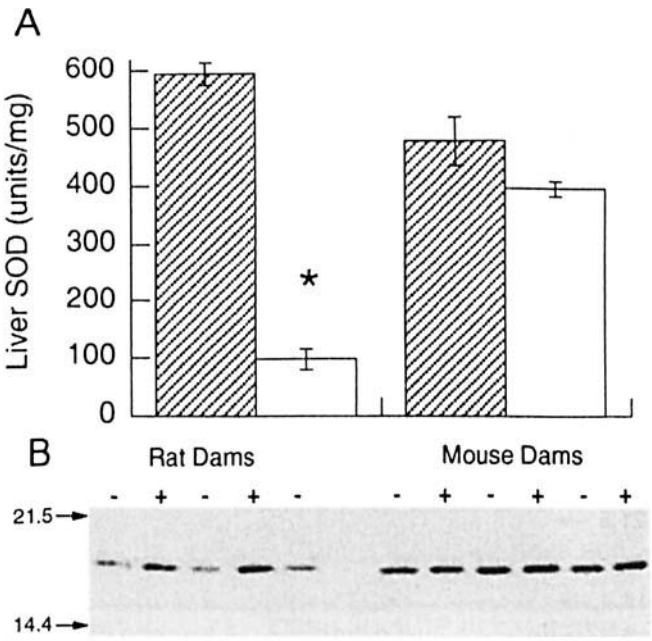

Figure 1. Comparison of Cu deficiency on liver SOD1 in rat and mouse dams. (A) SOD1 enzyme activity per milligram of liver protein was determined spectrophotometrically in liver of dams immediately after lactation. Bars represent means ± SEM ($n = 3$). Means from Cu-deficient rats, clear bars, were significantly different than means from Cu-adequate rat dams, $^*P < 0.05$. (B) SDS-PAGE and western immunoblot of total liver protein, 1 μg per lane from same liver samples as in A, was carried out using rabbit anti-bovine SOD1 antiserum (Chemicon AB 1237). Samples from Cu-adequate (+) and Cu-deficient (–) dams were compared. Numbers on left are molecular weight markers detected with Ponceau S. Cu-deficient rat dams but not mouse dams had lower density of the SOD1 band compared with Cu-adequate controls, $P < 0.05$.

vs 3.90 ± 0.28 μg/g). Liver SOD1 activity was also lower in –Cu rats and –Cu mice compared with the controls (Fig. 2A). For –Cu rats, the reduction in enzyme activity was 86% and for –Cu mice it was 34%. Immunoblot data demonstrated significant reduction in SOD1 protein in liver of both –Cu rats and –Cu mice compared with +Cu controls, 74% and 35%, respectively (Fig. 2B). In fact, the reduction in SOD1 protein and activity were similar in magnitude.

Copper Status and SOD1 in Male Mice. To extend the work in male mouse offspring, a second study was done to evaluate SOD1 in other organs. Four-week-old –Cu mice exhibited many features consistent with Cu deficiency compared with their +Cu counterparts (Table I). The –Cu mice were anemic, had cardiac hypertrophy, and decreased levels of Cu in liver and brain, 49% and 76%, respectively. Extracts of liver, heart, and brain were subjected to immunoblot analysis for SOD1 (Fig. 3). For three pairs of samples we found significant reductions in SOD1 protein for –Cu liver (25%) and –Cu heart (33%), but no alternations in brain despite a lower brain Cu content. These results were consistent with observations we made in –Cu rats.

SOD1 Evaluation and Comparison of Young Adult CCS –/– and CCS +/+ Mice. Four wild-type CCS +/+, two males and two females, and four knockout mice CCS –/–, two males and females, were sacrificed and evaluated. They were maintained on a commercial nonpurified diet with adequate Cu. There were few gender differences, thus data from the four mice of each genotype were pooled (Table II). The CCS –/– mice were not anemic, had no changes in heart weight, or changes in liver and brain Cu compared with CCS +/+ mice. There was a tendency for the liver Cu to be lower in the CCS –/– female mice (+/+, 4.75 and 4.76 μg/g vs 3.21 and 3.52 μg/g for –/–); however, overall CCS –/– mice showed no signs of liver Cu deficiency and had normal ceruloplasmin activity, 48 ± 5.5 units/l vs 51 ± 2.0 units/l for CCS +/+ mice.

Tissue extracts were prepared from liver, heart, and brain, and total protein, SOD1 activity, and SOD1 protein were determined. In agreement with previous work (18), we found major reductions in SOD1 activity in CCS –/– mice compared with CCS +/+ mice (Table III). The reductions for liver, heart, and brain were 72%, 64%, and 59%, respectively. The same extracts were subjected to immunoblot analyses (Fig. 4). We found major reductions in SOD1 protein in liver, heart, and brain of CCS –/– mice compared with CCS +/+ controls. The reduction in liver was 79%,

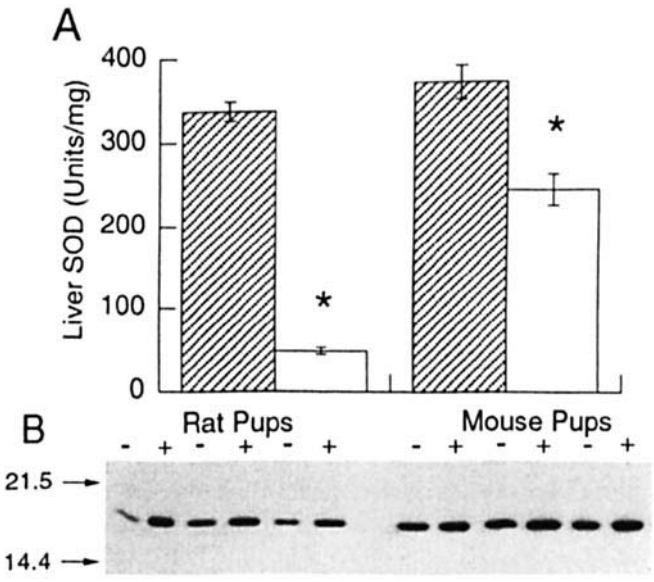

Figure 2. Comparison of Cu deficiency on liver SOD1 in rat and mouse offspring. (A) SOD1 enzyme activity per milligram of liver protein in was determined spectrophotometrically for 23-day-old male rats and 35-day-old male mice after perinatal Cu deficiency. Bars represent means ± SEM (n = 3). Means from Cu-deficient rats and mice, clear bars, were significantly different than means from Cu-adequate controls, $^*P < 0.05$. B, SDS-PAGE and western immunoblot of total liver protein, 1 μg per lane from same liver samples as in A, was carried out using rabbit anti-bovine SOD1 antiserum (Chemicon AB 1237). Samples were from Cu-adequate (+) and Cu-deficient (–) male offspring. Numbers on left are molecular weight markers detected with Ponceau S. Cu-deficient rats and mice had lower density of the SOD1 band compared with Cu-adequate controls, $P < 0.05$.

Table I. Copper Status of 28-Day-Old Male Mice after Dietary Copper Deficiency

Characteristics	Cu-adequate	Cu-deficient
Hemoglobin, g/l	117 ± 5.5	90.7 ± 3.6[a]
Heart weight, mg/g	5.94 ± 0.26	7.44 ± 0.21[a]
Liver copper, μg/g	3.93 ± 0.12	2.01 ± 0.22[a]
Brain copper, μg/g	2.82 ± 0.09	0.68 ± 0.14[a]

Note. Values are means ± SEM (n = 5) of each group. Pups were born to and nursed by Cu-deficient or Cu-adequate dams. Treatment began 2 days before parturition. Pups were maintained on the same treatment of their dams for 1 week before sacrifice. Brain and liver copper levels were determined by flame atomic absorption after wet ashing.
[a] $P < 0.01$.

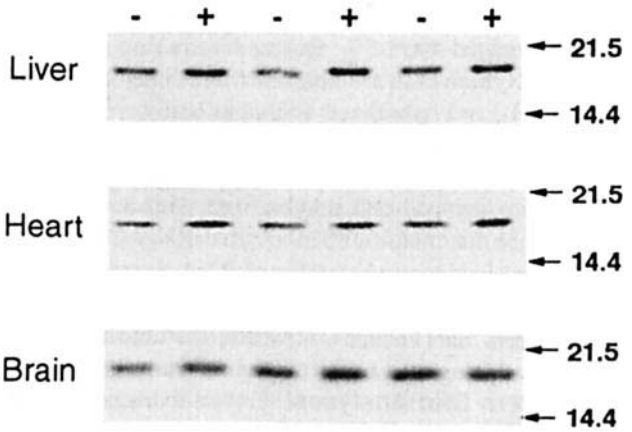

Figure 3. Impact of Cu deficiency on tissue SOD1 in 28-day-old male mice. SDS-PAGE and Western immunoblot of total protein, 1 μg per lane, from liver, heart (cardiac ventricles), and brain (midbrain), was carried out using rabbit anti-bovine SOD1 antiserum (Chemicon AB 1237). Samples from Cu-adequate (+) and Cu-deficient (–) offspring were compared. Numbers on left are molecular weight markers detected with Ponceau S. Cu-deficient mice had lower density of the SOD1 band compared with Cu-adequate controls for liver and heart, $P < 0.05$.

Table II. Copper Status of Young Adult Copper Chaperone for SOD Null Mice (CCS –/–) and Wild-Type Mice (CCS +/+)

Characteristics	CCS +/+	CCS –/–
Hemoglobin, g/l	140 ± 4.8	140 ± 3.8
Heart weight mg/g	5.87 ± 0.14	5.42 ± 0.12
Liver copper, μg/g	6.51 ± 0.93	5.30 ± 1.18
Brain copper, μg/g	2.94 ± 0.12	2.79 ± 0.11

Note. Values are means ± SEM (n = 4) per group. Each group consists of two males and two females. Liver and brain copper levels were determined by flame atomic absorption spectroscopy after wet ashing.

similar to the reduction in SOD1 activity. The reductions for heart (30%) and brain (22%) of SOD1 protein were less than reductions in enzyme activity.

For the dietary study, the 21% reduction in liver SOD1 activity in –Cu mice was also similar to reduction in SOD1 protein (Table III). For heart tissue, the reduction in SOD1 protein exceeded the apparent reduction in SOD1 enzyme activity. We did not detect differences in brain SOD1 activity or SOD1 protein between –Cu and +Cu mice.

Northern Hybridization Analyses of SOD1. To determine if the lower SOD1 protein levels observed in –Cu mice and CCS –/– mice were due to decreased protein synthesis, we evaluated steady-state SOD1 mRNA levels. Two –Cu mouse livers (Fig. 2B) with lower SOD1 protein were compared with two +Cu mice and were subjected to Northern hybridization analyses with a SOD1-specific probe. There was no apparent difference in SOD1 mRNA between the pairs of –Cu and +Cu mice (Fig. 5A). Blots were reprobed for 18S ribosomal RNA to ensure similar loading and transfer.

In a similar manner, aliquots of quick-frozen liver were used to isolate total RNA from four CCS –/– mice and four CCS +/+ mice. Liver RNA was subjected to Northern hybridization and blots were probed for SOD1 and 18S ribosomal RNA (Fig. 5B). Transcript levels of SOD1 were not different in the liver of CCS knockout mice compared with CCS wild-type mice.

Antibody Specificity. Two additional experiments were conducted to strengthen the observations concerning SOD1 protein. First, we prepared apo-SOD and holo-SOD from commercial bovine erythrocyte SOD (Sigma Chemical) following the protocol of Beem *et al.* (23). Pure erythrocyte SOD, using our assay, has a specific activity of 125,000 units/mg. The apo-SOD preparation had 40% residual activity compared with reconstituted holo-SOD. The holo-SOD activity was 165,000 units/mg. We ran an immunoblot with our commercial antibody and detected equal intensity for equal protein loading of bovine erythrocyte apo-SOD, holo-SOD, and untreated SOD (data not shown). Thus, our immunoblot detection of rodent SOD1 protein seemed independent of metal status of the protein.

We also ran Western blots using two other antibodies that detect SOD1 protein and we found qualitatively similar results to those shown using the commercial antibody in other experiments (Fig. 6). One antibody is rabbit anti-human SOD polyclonal antiserum previously used by others in the CCS –/– studies (18). The other is a commercial antibody (Santa Cruz G-19; Santa Cruz Biotechnologies, Santa Cruz, CA) made against human CCS domain II that is highly homologous to SOD1. Both of these additional antibodies demonstrate that CCS –/– mice have lower SOD1 protein than CCS +/+ mice and that –Cu rats and mice also have lower SOD1 protein than +Cu rats and mice (Fig. 6).

Discussion

Restriction of dietary nutrients such as Cu can limit the availability of needed cofactors to activate cellular enzymes. SOD1 activity was first shown to be impacted by dietary Cu deficiency in rat brain (27). This was extended to other rat organs sometime later (28). Even later SOD1 activity was shown to be lower in organs of –Cu mice (22). Only recently has the explanation for these observations been studied. In –Cu rats and, now, in –Cu mice, there is clear evidence that the reduction in SOD1 activity is due to both a lower co-factor level and a lower protein level (8, 9, 14, 15, 22). The reduction in SOD1 activity in rat and mouse tissues is less than reductions in cytochrome c oxi-

Table III. Cu,Zn-Superoxide Dismutase (SOD1) Activity and Protein Levels in Organs of Cu-adequate and Cu-deficient Mice and Copper Chaperone for SOD1 Null Mice (CCS –/–) and Wild-Type Controls (CCS +/+)

Characteristic	Cu-adequate	Cu-deficient	CCS +/+	CCS –/–
Liver SOD1, units/mg	415 ± 6.7	328 ± 22.2[a]	408 ± 15.7	115 ± 8.88[a]
Liver SOD1 protein	99.8 ± 3.6	75.2 ± 4.8[a]	100 ± 13.0	20.7 ± 3.91[a]
Heart SOD1, units/mg	111 ± 6.8	86.9 ± 3.1[a]	99.4 ± 5.65	35.5 ± 0.91[a]
Heart SOD1 protein	99.9 ± 7.5	66.7 ± 4.2[a]	100 ± 8.32	69.7 ± 6.91[a]
Brain SOD1, units/mg	225 ± 15.1	197 ± 7.2	114 ± 7.84	47.1 ± 1.75[a]
Brain SOD1 protein	99.8 ± 10.6	91.4 ± 18.8	100 ± 4.28	78.5 ± 5.67[a]

Note. Values are means ± SEM (n = 3) for the dietary study and (n = 4) for the genetic study. SOD1 activity was determined spectrophotometrically on organ homogenates and illustrated significant differences for all comparisons except brain in the dietary model. SOD1 protein was determined densitometrically from immunoblots. Mean density for control groups was set at 100 and all values for a given blot were determined relative to that density.
[a] $P < 0.05$.

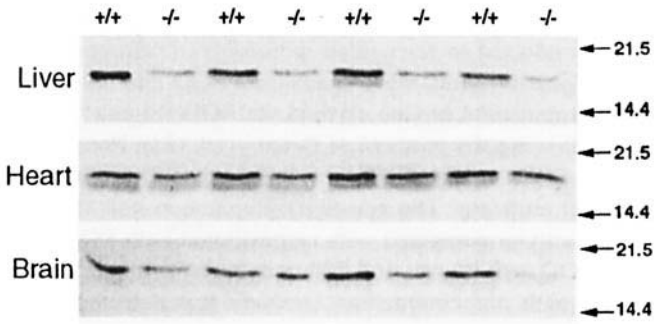

Figure 4. Impact of copper chaperone for superoxide dismutase (CCS) expression on SOD1. SDS-PAGE and Western immunoblot of total protein, 1 μg per lane, from liver, heart (cardiac ventricles), and brain (cerebellum), was carried out using rabbit anti-bovine SOD1 antiserum (Chemicon AB 1237). Samples from CCS +/+ wild-type (+/+) and CCS –/– knockout (–/–) mice were compared. Numbers on right are molecular weight markers detected with Ponceau S. CCS –/– mice had lower density of the SOD1 band compared with CCS +/+ controls for liver, heart, and brain, $P < 0.05$.

dase, another cuproenzyme, with the exception of rat liver, where SOD1 activity is especially vulnerable to dietary Cu restriction (29).

In general, in –Cu rodents, the reduction in SOD1 activity is proportional to the reduction in organ Cu (9). Results from the current experiments support this position as well, as a reduction in liver Cu was greater for –Cu rat dams

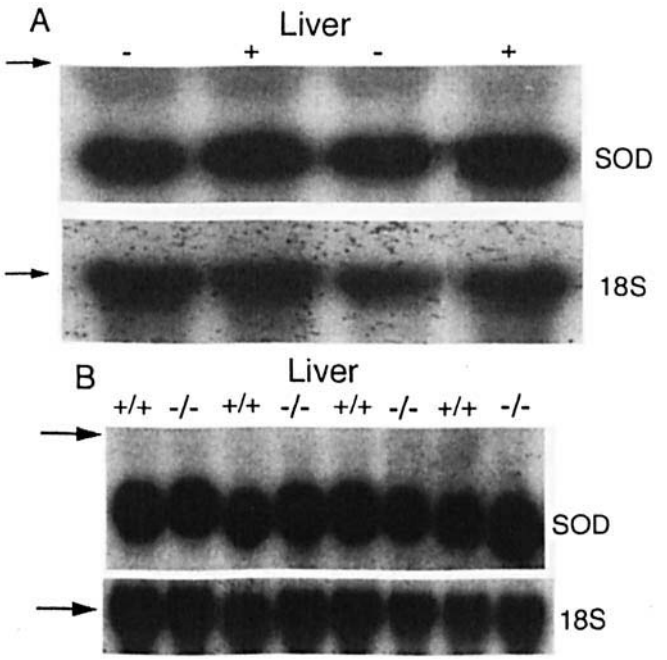

Figure 5. Northern blot hybridization analysis of mouse liver total RNA. (A) Total liver RNA was subjected to denaturing electrophoresis, capillary transfer, and binding to ^{32}P-labeled DNA probes specific for rat Cu, Zn-SOD (SOD), and murine 18S ribosomal RNA (18S). Arrows indicate migration position of 18S ribosomal RNA visualized with acridine orange. Lanes were loaded with RNA (10 μg/lane) isolated from liver of 35-day-old male mice after perinatal Cu deficiency. The SOD1/18S density ratio was determined and the values for Cu-deficient (–) mice were not different from the Cu-adequate (+) mice. (B) Same procedure as in A was applied to samples from CCS +/+ wild-type (+/+) and CCS –/– knockout (–/–) mice; no significant differences in the SOD1/18S ratio were detected.

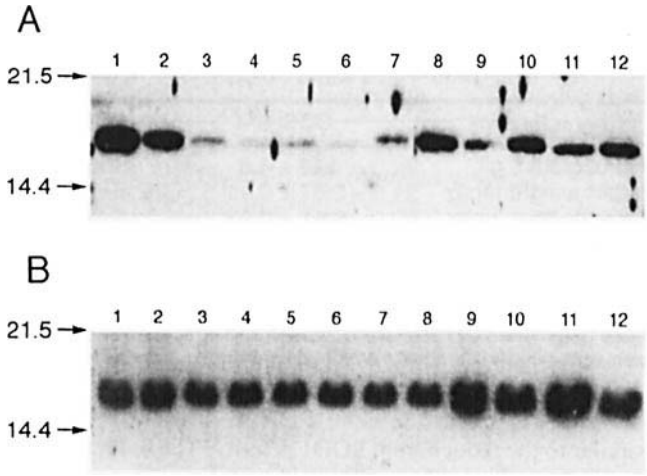

Figure 6. Alternate antibodies still detect differences in SOD1 expression. (A) SDS-PAGE and western immunoblot of total protein, 1 μg per lane, was carried out using rabbit anti-human SOD1 antiserum at 1:1000 dilution (18). Numbers on left are molecular weight markers detected with Ponceau S. Lanes 1, 3, and 5. extracts from a male CCS +/+ mouse liver, heart, and brain; lanes 2, 4, and 6, same tissues from male CCS –/– mouse; lanes 7 and 9, from –Cu rat liver; lanes 8 and 10, from +Cu rat liver; lane 11, from –Cu mouse liver; lane 12, from a +Cu mouse liver. (B) SDS-PAGE and Western immunoblot of total protein, 50 μg per lane, was carried out by using goat anti-human CCS antiserum at 1:200 dilution (Santa Cruz SC-14258). Lanes 1, 3, 5, and 7, from cerebella of 28-day-old male +Cu mice; lanes 2, 4, 6, and 8, from cerebella of 28-day-old male –Cu mice; lanes 9 and 11, from cerebella of male CCS +/+ mice; lanes 10 and 12, from cerebella of male CCS –/– mice.

and pups compared with –Cu mouse dams and pups, and a reduction in SOD1 activity was also attenuated in –Cu mice. In fact, to see the modest reductions in SOD1 protein on immunoblots in –Cu mice, the total protein load was lowered to 1 from 10 μg used previously for rats to prevent saturation and to enhance sensitivity. Brain SOD1 activity and protein was not impacted by Cu deficiency in mice in the current studies (Table III and Figs. 3 and 6B). This agrees with our previous work on mouse midbrain, although we did report modest reductions in SOD1 activity in other brain regions (20). Brain SOD1 seems especially resistant to Cu deficiency, especially after brain development is nearly complete.

The reduction in SOD1 protein in mammals appears to be a posttranscriptional mechanism as evidenced by no alteration in SOD1 mRNA in –Cu rat tissues or in –Cu mouse liver (this study) (9). This is quite different than baker's yeast in which SOD1 mRNA is transcriptionally regulated by ACE1 (2–5). Although the current studies do not address a posttranscriptional mechanism, it is possible that the reduction in steady-state SOD1 protein is due to an enhanced degradation of apo-SOD1 that is lacking Cu. Others have created SOD1 mutants and have evaluated turnover after pulse-chase experiments. For example, the Ala4Thr mutant SOD1 protein half-life is 18 vs 78 hr for wild type (30). Other studies showed that mutations not only affected SOD1-specific activity, but also stability (31). The degradation of these mutant SOD1 proteins involves the proteo-

some (32). Perhaps metal-deficient SOD1 is cleared by the same pathway. Other cuproenzymes lacking Cu are also degraded faster than holoenzymes. This appears to be true for ceruloplasmin and cytochrome c oxidase (33, 34).

One could argue that the state of Cu deficiency per se leads to accelerated cuproprotein degradation. However, this would not explain our current results in CCS –/– mice because they also exhibit low SOD1 protein but are not Cu deficient. In elegant earlier studies, it has been shown that apo-SOD1 interacts with CCS domain II to transfer Cu (35). Thus, it made perfect sense that CCS –/– mice should have lower SOD1 activity because they are lacking the catalytic co-factor (18). In agreement with those studies, we also detected major (greater than 60%) reductions in SOD1 activity in liver, heart, and brain of CCS –/– mice. However, in contrast, we also detected major reductions in SOD1 protein, whereas earlier work detected no differences (18). One explanation could be use of different antibodies. However, by using the same antibody as in those studies, we do detect a reduction in SOD1 protein in CCS –/– mice and in –Cu rodents compared with controls (Fig. 6). We believe that another factor might be the amount of protein loaded on the SDS PAGE gels, 1 μg for most of our blots vs 100 μg for the previous work (18). Further evidence suggesting less SOD1 protein in CCS –/– mice is from liver Cu data that suggest lower Cu in female CCS –/– mice compared with CCS +/+. SOD1 is a major Cu-binding protein in liver, and reduction in SOD1 protein would be consistent with reduction in Cu of the magnitude observed in these studies.

Thus, our current data suggest that low SOD1 activity in CCS –/– mice is due primarily to lower SOD1 protein rather than the existence of inactive apo-SOD1. Therefore, for both dietary Cu deficiency where co-factor level is restricted and for CCS –/– mice where co-factor donation is restricted, there appears to be lower SOD1 protein in the steady state. Both conditions support the working hypothesis that apo-SOD1 is degraded faster than holo-SOD1. Perhaps the stability of SOD1 is also dependent on the presence of CCS. However, a recent communication reported higher rather than lower CCS levels in liver and red cells of male Cu-deficient rats (36). It will be interesting to determine what the impact of SOD1 deletion has on CCS stability. Further research will be necessary to elucidate fully the determinants of SOD1 stability and metabolism.

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1. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244**:6049–6055, 1969.
2. Carri MT, Galiazzo F, Ciriolo MR, Rotilio G. Evidence for coregulation of Cu,Zn superoxide dismutase and metallothionein gene expression in yeast through transcriptional control by copper via the ACE 1 factor. FEBS Lett **278**:263–266, 1991.
3. Gralla EB, Thiele DJ, Silar P, Valentine JS. ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. Proc Natl Acad Sci U S A **88**:8558–8562, 1991.
4. Greco MA, Hrab DI, Magner W, Kosman DJ. Cu,Zn superoxide dismutase and copper deprivation and toxicity in Saccharomyces cerevisiae. J Bacteriol **172**:317–325, 1990.
5. Galiazzo F, Schiesser A, Rotilio G. Oxygen-independent induction of enzyme activities related to oxygen metabolism in yeast by copper. Biochim Biophys Acta **965**:46–51, 1988.
6. Thiele DJ. ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. Mol Cell Biol **8**:2745–2752, 1988.
7. Culotta VC, Joh HD, Lin SJ, Slekar KH, Strain J. A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J Biol Chem **270**:29991–29997, 1995.
8. Lai CC, Huang WH, Askari A, Wang Y, Sarvazyan N, Klevay LM, Chiu TH. Differential regulation of superoxide dismutase in copper-deficient rat organs. Free Radical Biol Med **16**:613–620, 1994.
9. Prohaska JR, Brokate B. Lower copper, zinc-superoxide dismutase protein but not mRNA in organs of copper-deficient rats. Arch Biochem Biophys **393**:170–176, 2001.
10. Dameron CT, Harris ED. Regulation of aortic CuZn-superoxide dismutase with copper. Effects in vivo. Biochem J **248**:663–668, 1987.
11. Chung K, Romero N, Tinker D, Keen CL, Amemiya K, Rucker R. Role of copper in the regulation and accumulation of superoxide dismutase and metallothionein in rat liver. J Nutr **118**:859–864, 1988.
12. Tran DD, Romero N, Tinker D, Rucker R. Dietary copper and the net accumulation of liver and lung superoxide dismutase. Nutr Res **9**:1161–1166, 1989.
13. DiSilvestro RA. Copper activation of superoxide dismutase in rat erythrocytes. Arch Biochem Biophys **274**:298–303, 1989.
14. Levieux A, Levieux D, Lab C. Immunoquantitation of rat erythrocyte superoxide dismutase: its use in copper deficiency. Free Radical Biol Med **11**:589–595, 1991.
15. Rossi L, Ciriolo MR, Marchese E, De Martino A, Giorgi M, Rotilio G. Differential decrease of copper content and of copper binding to superoxide dismutase in liver, heart and brain of copper-deficient rats. Biochem Biophys Res Commun **203**:1028–1034, 1994.
16. Rossi L, Marchese E, De Martino A, Rotilio G, Ciriolo MR. Purification of a fully metal-depleted Cu, Zn superoxide dismutase from copper-deficient rat liver. Biometals **10**:257–262, 1997.
17. Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD. The copper chaperone for superoxide dismutase. J Biol Chem **272**:23469–23472, 1997.
18. Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartnikas TB, Culotta VC, Price DL, Rothstein J, Gitlin JD. Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc Natl Acad Sci U S A **97**:2886–2891, 2000.
19. Prohaska JR, Bailey WR. Regional specificity in alterations of rat brain copper and catecholamines following perinatal copper deficiency. J Neurochem **63**:1551–1557, 1994.
20. Prohaska JR, Bailey WR. Persistent regional changes in brain copper, cuproenzymes and catecholamines following perinatal copper deficiency in mice. J Nutr **123**:1226–1234, 1993.
21. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87**:206–210, 1978.
22. Prohaska JR. Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. J Nutr **113**:2048–2058, 1983.
23. Beem KM, Rich WE, Rajagopalan KV. Total reconstitution of copper-zinc superoxide dismutase. J Biol Chem **249**:7298–7305, 1974.
24. Prohaska JR, Brokate B. Copper deficiency alters rat dopamine β-monooxygenase mRNA and activity. J Nutr **129**:2147–2153, 1999.
25. Prohaska JR, Brokate B. Dietary copper deficiency alters protein levels of rat dopamine-β-monooxygenase and tyrosine monooxygenase. Exp Biol Med **226**:199–207, 2001.
26. Prohaska JR, Brokate B. The timing of perinatal copper deficiency in mice influences offspring survival. J Nutr **132**:3142–3145, 2002.

27. Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: a possible model for Menkes' steely-hair disease. J Neurochem **23:**91–98, 1974.
28. Paynter DI, Moir RJ, Underwood EJ. Changes in activity of the Cu-Zn superoxide dismutase enzyme in tissues of the rat with changes in dietary copper. J Nutr **109:**1570–1576, 1979.
29. Prohaska JR. Changes in Cu,Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper- deficient mice and rats. J Nutr **121:**355–363, 1991.
30. Nakano R, Inuzuka T, Kikugawa K, Takahashi H, Sakimura K, Fujii J, Taniguchi N, Tsuji S. Instability of mutant Cu/Zn superoxide dismutase (Ala4Thr) associated with familial amyotrophic lateral sclerosis. Neurosci Lett **211:**129–131, 1996.
31. Borchelt DR, Lee MK, Slunt HS, Guarnieri M, Xu ZS, Wong PC, Brown RH Jr, Price DL, Sisodia SS, Cleveland DW. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. Proc Natl Acad Sci U S A **91:**8292–8296, 1994.
32. Hoffman EK, Wilcox HM, Scott RW, Siman R. Proteasome inhibition enhances the stability of mouse Cu/Zn superoxide dismutase with mutations linked to familial amyotrophic lateral sclerosis. J Neurol Sci **139:**15–20, 1996.
33. Holtzman NA, Gaumnitz BM. Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. J Biol Chem **245:**2354–2358, 1970.
34. Medeiros DM, Shiry L, Sâmelman T. Cardiac nuclear encoded cytochrome c oxidase subunits are decreased with copper restriction but not iron restriction: gene expression, protein synthesis and heat shock protein aspects. Comp Biochem Physiol A Physiol **117:**77–87, 1997.
35. Schmidt PJ, Kunst C, Culotta VC. Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1. J Biol Chem **275:**33771–33776, 2000.
36. Bertinato J, Iskandar M, L'Abbe MR. Copper deficiency induces the upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats. J Nutr **133:**28–31, 2003.