Copper Deficiency Induces the Upregulation of the Copper Chaperone for Cu/Zn Superoxide Dismutase in Weanling Male Rats


ABSTRACT The most commonly used indices for determining copper deficiency in humans are reduced serum/plasma copper concentration and decreased activity of ceruloplasmin and Cu/Zn superoxide dismutase (SOD1). However, these indicators are influenced by many factors unrelated to copper status and lack the sensitivity required to detect marginal deficiency, limiting their usefulness in many situations. In vivo, the insertion of copper into SOD1 is dependent on the copper chaperone for SOD1 (CCS). In this study, we explored the possibility that the expression level of CCS may reflect copper status and thus serve as a useful marker of copper nutriture. Weanling male Wistar rats were fed either a normal (5.3 mg Cu/kg diet), moderately deficient (0.84 mg Cu/kg diet) or deficient (0.34 mg Cu/kg diet) copper diet for 6 wk. Rats fed moderate and deficient diets showed differences (P < 0.05) in several hematological measurements, indicating varying degrees of copper deficiency in these groups. Copper-deficient rats had reduced (P < 0.05) liver and erythrocyte SOD1 activity and body weight. Western blot analysis revealed a dose-dependent increase (P < 0.05) in CCS expression in liver and erythrocytes of copper-deficient rats. We report CCS protein level as a novel marker for assessing copper status. J. Nutr. 133: 28–31, 2003.

KEY WORDS: • copper status • copper chaperone • Cu/Zn superoxide dismutase • expression level • rats

Many symptoms related to copper deficiency result from decreased activity of copper-requiring enzymes attributed to the absence of metal. Abnormalities associated with copper deficiency include anemia, neurological damage, hypercholesterolemia, cardiomyopathy, osteoporosis and impaired immune function (1).

Despite the scarcity of copper in the environment, overt copper deficiency is rare in humans but does present a concern in specialized situations. Deficiency has been observed in individuals with restricted diets and in premature infants fed cow’s milk formulas (2–5). Severe deficiency is often seen in Menkes disease, a genetic disorder characterized by a defect in copper efflux from various tissues (6). Chronic ingestion of large quantities of zinc reduces the efficiency of copper absorption and has been reported to cause deficiency (7). Also, individuals suffering from malnutrition and severe malabsorption syndromes as well as patients undergoing certain chelation therapies are at increased risk of deficiency.

Copper deficiency induces a number of enzymatic and biochemical changes (8–13), although an ideal indicator of copper status is still lacking. The most frequently used indices for determining copper deficiency in humans are decreased plasma/serum copper concentration and ceruloplasmin protein level and activity. However, these variables fluctuate with age and sex (14) and are altered by a wide range of common conditions unrelated to copper nutriture, including pregnancy, oral contraceptive use, estrogen therapy, inflammation and infection (15–17).

Decreased activity of Cu/Zn superoxide dismutase (SOD1) is also a well-established indicator of low copper stores (10,18). In contrast to copper and ceruloplasmin measurements, SOD1 activity does not seem to be affected by age, sex and hormone supplements (14,15), although activity increases under conditions of oxidative stress (19) and in diseases such as alcoholism (20) and Down’s syndrome (21).

With a plethora of factors influencing the usual measures of copper status, assessment of total-body copper level with a single indicator can result in misdiagnosis. Furthermore, commonly used markers lack the sensitivity needed to detect marginal deficiency, a condition that may be more prevalent than previously realized (17). Therefore, there is still a pressing need for specific and sensitive indicators that will provide an accurate estimation of copper nutriture.

In vivo, insertion of copper into SOD1 largely depends on the copper chaperone for SOD1 (CCS) (22). Mammalian CCS is a homodimer of ~30-kDa subunits and is the largest of the copper metallochaperones. When copper is abundant, CCS is expressed at low concentrations (23), consistent with its catalytic role in activating SOD1. Here, we report a dose-dependent upregulation of CCS protein in tissues of rats fed

1 Publication no. 568 of the Bureau of Nutritional Sciences. This work was presented in part at the 44th Annual Meeting of the Canadian Federation of Biological Societies, Montreal, Canada, June 12–16, 2002 [Bertinato, J., Iskandar, M. & L’Abbe, M. R. (2002) Upregulation of the copper chaperone for SOD1 in rats fed low copper diets. F004 (abs.).]

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low copper diets. Our data indicate that CCS protein level can be used as a marker for evaluating copper status.

MATERIALS AND METHODS

Animals and test diets. The experimental protocol was approved by the institutional Animal Care Committee of the Health Products and Food Branch of Health Canada. Rats were maintained in accordance with the guidelines of the Canadian Council on Animal Care. Weanling (21-d-old) male Wistar rats (Charles River Canada, St. Constant, Canada) were randomly assigned to one of three groups (n = 10/group) and housed individually in wire-bottom stainless steel cages in a climate-controlled room maintained at 22 ± 1°C and 60% relative humidity with a 12-h day/night cycle. Rats had free access to one of three test diets4 (24) and demineralized drinking water. Body weights were measured weekly. After 6 wk, rats were killed by exsanguination while under 3% isoflurane anesthesia. Blood was immediately withdrawn from the abdominal aorta into EDTA or heparinized tubes for hematological measurements and determination of SOD1 activity, respectively. Liver, brain and heart were extracted and immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Test diets were prepared by adding appropriate amounts of copper (cupric carbonate) from a 5 mg Cu/g corn starch mix to the diets. Diets were dry ashed and dissolved in 3 mol/L HCl, and the copper contents were determined by flame atomic absorption spectrophotometry (Perkin–Elmer 5100 PC; Perkin Elmer Cetus Instruments, Norwalk, CT). Copper normal (Cu-N), moderately deficient (Cu-M) and deficient (Cu-D) diets contained 5.3 ± 0.06, 0.84 ± 0.03 and 0.34 ± 0.01 mg Cu/kg diet, respectively. Accuracy of analytical measurements was verified using NIST (Gaithersburg, MD)-certified reference material.

Hematological measurements. Complete blood cell count was measured using a Coulter Counter S-PLUS IV system (Coulter Cytomtery, Hialeah, FL) on whole blood samples.

Western blotting. Liver extracts were prepared by homogenizing with a glass homogenizer in ice-cold 0.2% (v/v) Triton X-100 and centrifuging (10,000 × g, 10 min) to remove insoluble material. Total protein concentration was determined using the BCA method (25). Proteins (50 μg per lane) were resolved over 4–12% or 4–20% Tris–glycine gradient gels (Invitrogen, Burlington, Canada) under denaturing and reducing conditions and electroblotted onto PVDF membranes (Invitrogen). Blots were blocked in TBS-Tween [20 mmol/L Tris, 500 mmol/L NaCl, 0.1% Tween 20 (v/v), pH 7.5] supplemented with 5% (wt/v) nonfat dry milk (BioRad, Hercules, CA) at room temperature (RT) for 1 h. Membranes were probed with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to SOD1 and H-ras (H-235) at final concentrations of 1, 4 and 2 mlg, respectively, for 24 h at 4°C in blocking solution. Membranes were washed (5 × 10 min) with TBS-Tween and then incubated with an anti-goat (0.08 mg/L) or anti-rabbit (0.02 mg/L) HRP-conjugated secondary antibody (Santa Cruz Biotechnology) in blocking solution for 2 h at RT. Blots were washed as above and proteins were detected by chemiluminescence with ECL Western Blotting Detection Reagents (Amersham Biosciences, Baie d’Urfe, Canada) and exposure to Hyperfilm ECL (Amersham Biosciences). Erythrocytes were isolated by centrifugation (13,000 × g, 3 min) of whole blood collected in heparin tubes, washed with ice-cold saline solution (150 mmol/L NaCl) and lysed with glutathione reagent (10 mmol/L KH2PO4/K2HPO4, 2 mmol/L glutathione, pH 7.0). Extracts were normalized to hemoglobin (100 μg per lane). Hemoglobin concentration was determined with Drabkin hemoglobin reagent using human hemoglobin (Sigma, Oakville, Canada) as a reference standard. Film was scanned using a Hewlett–Packard Scanjet ADF scanner (Hewlett–Packard, Palo Alto, CA) and densitometry was performed using Scion Image software (Scion Corporation, Frederick, MD). Band intensities were determined at exposures within the linear response range of the film.

SOD1 assay. SOD1 activity was measured by the cytochrome c reduction assay, which follows absorbance at 550 nm essentially as described (26). A flux of O2⋅− was generated by the aerobic action of xanthine oxidase on xanthine. SOD1 activity was quantified in terms of its ability to inhibit the reduction of cytochrome c by O2⋅−. Tissue extracts were prepared as described for Western blotting. SOD1 activity was determined on the chloroform/ethanol (3:5, v/v) extract. Samples and standards were plated in a 96-well plate and absorbance at 550 nm was determined in triplicate using a SPECTRAMax PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical analysis. Statistical analyses were performed using Statistica 6.0 software (StatSoft, Tulsa, OK). Data were analyzed by one-way ANOVA followed by Fisher’s least significant difference test and reported as mean ± SD. Differences were considered significant at P < 0.05.

RESULTS

Copper deficiency in Cu-M and Cu-D rats was confirmed by measuring liver and erythrocyte SOD1 activities. Activity was significantly (P < 0.05) reduced in both liver and erythrocytes of Cu-M and Cu-D rats compared to Cu-N rats (Table 1). Brain SOD1 activity was less affected by feeding low copper diets because only Cu-D rats showed a significant decrease (Table 1). Also, body weights declined with decreasing amounts of copper in the diet (Table 1).

Hematological measurements of mean corpuscular volume, mean corpuscular hemoglobin and red cell distribution width indicated varying degrees of copper deficiency–induced anemia in Cu-M and Cu-D rats, in that values differed (P < 0.05) between the groups (Table 2). In addition, more pronounced copper deficiency in the Cu-D group led to death of 4/10 rats compared to only 1/10 rats in the Cu-M group attributed to left ventricular heart lesions within wk 6 of the study (data not shown).

A polyclonal antibody (G-19) against an internal region of human CCS, which is highly homologous to SOD1, was used to examine expression of CCS in rat tissues. Western blot of liver proteins using this antibody gave two bands of ~33 and 18 kDa (Fig. 1A), the expected molecular weight of CCS and SOD1, respectively. Probing the same extract with a SOD1-4 Values are means ± SD. Differences were considered significant at P < 0.05.

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diet group</th>
<th>Cu-N</th>
<th>Cu-M</th>
<th>Cu-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver SOD1 activity, U/mg protein</td>
<td>82.2 ± 24a</td>
<td>29.5 ± 9.3b</td>
<td>22.4 ± 8.9b</td>
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<td>Erythrocyte SOD1 activity, U/mg HB</td>
<td>44.7 ± 7.4a</td>
<td>14.4 ± 2.1c</td>
<td>22.5 ± 2.8b</td>
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<tr>
<td>Brain SOD1 activity, U/mg protein</td>
<td>16.4 ± 3.6a</td>
<td>14.0 ± 1.9ab</td>
<td>12.8 ± 2.1b</td>
<td></td>
</tr>
<tr>
<td>Body weight, wk 6, g</td>
<td>371 ± 18a</td>
<td>339 ± 19b</td>
<td>289 ± 32c</td>
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</tbody>
</table>

1 Values in a row with different letters differ, P < 0.05.
2 Test diets: Cu-N, normal copper; Cu-M, moderately copper deficient; Cu-D, copper deficient.
3 Values are means ± SD, n = 6.
4 Values are means ± SD, n = 10, 9 and 6 for Cu-N, Cu-M and Cu-D, respectively.

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specific antibody (FL-154) gave only the 18-kDa band, confirming cross-reactivity of antibody G-19 with SOD1 (Fig. 1A). A single 18-kDa band with the SOD1-specific antibody, even after prolonged exposure (data not shown), excludes the possibility that the 33-kDa band detected with antibody G-19 is a slower migrating form of SOD1.

To test whether CCS expression was affected by copper status, CCS protein levels in livers from rats representing the three diet groups were analyzed by Western blot (Fig. 1B). In Cu-N rats, CCS was expressed at low levels and showed little variation in expression between rats (Fig. 1B, C). CCS was markedly upregulated in moderately deficient rats and even more dramatically elevated in rats fed the Cu-D diet (Fig. 1B, C). Concomitant with increased CCS expression, SOD1 protein levels modestly decreased with copper deprivation (Fig. 1B) as previously reported (27,28). Liver β-tubulin expression was unaffected by the test diets and showed equal protein loading.

Quantification of liver CCS immunoreactive protein revealed a 2.8- and 9.6-fold increase in Cu-M and Cu-D rats, respectively, compared to Cu-N rats (Fig. 1C). CCS protein was increased to comparable levels in erythrocytes (Cu-M, 3.8-fold; Cu-D, 7.2-fold) (Fig. 1C). Note that, even though copper-restricted rats displayed signs of anemia, the relative change in CCS expression reported for Cu-M and Cu-D rats was only minimally influenced by normalizing the protein extracts to hemoglobin, given that the MCHC measurements were slightly lower (P < 0.05) only for Cu-D rats (Table 2). CCS expression was also elevated in heart of Cu-D rats, albeit to a lesser degree than that in liver or erythrocytes, consistent with a less significant decrease in SOD1 activity (data not shown).

Low copper diets reduced liver SOD1 activity from 82.2 to 29.5 and 22.4 U/mg protein for Cu-M and Cu-D rats, respectively (Table 1). However, activity was not significantly (P > 0.05) lower in Cu-D compared to Cu-M rats. Moreover, because erythrocyte SOD1 activity was normalized to hemoglobin, activity was higher in Cu-D than in Cu-M rats (Table 1). In contrast, both liver and erythrocyte CCS expression levels differed (P < 0.05) among all three diet groups (Fig. 1C). Also, the fold increase in CCS protein for moderate and deficient rats (Fig. 1C) was larger than the fold decrease in SOD1 activity (liver: Cu-M, 1.8; Cu-D, 2.7; erythrocytes: Cu-M, 2.1; Cu-D, 1.0). Together, these results demonstrate a strong dose-dependent upregulation of CCS protein in tissues depleted in copper.

### DISCUSSION

In the cell, where the concentration of free copper is extremely low, copper chaperones facilitate insertion of copper into specific target proteins. However, the mechanism by which chaperones obtain and deliver copper to their target when copper is limiting is poorly understood. CCS was identified as the chaperone essential for incorporation of copper into SOD1 (29). In this study, we examined the expression of CCS in rats fed low copper diets and found that CCS is sharply upregulated in a dose-dependent manner with increasing severity of copper deficiency.

Activity of many cuproenzymes decreases with copper deficiency because of the lack of metal. Therefore, under such conditions there is likely to be a competition between chaperones and other copper-binding proteins for the limited supply of copper. The findings reported here demonstrate that the cell responds to low copper levels by increasing CCS expression. It is likely that the larger quantity of CCS molecules available to scavenge for copper increases the efficiency of copper transfer to SOD1. This result would be consistent with a role for CCS in prioritizing the distribution of copper when it is scarce. Whether other copper chaperones behave in a similar way to CCS remains to be verified.

There are several mechanisms that can account for the increased expression of CCS induced by copper deficiency. CCS may be upregulated at the transcriptional level through actions of a transcription factor that is sensitive to changes in copper status. Alternatively, CCS may be upregulated by a posttranslational mechanism. For instance, copper-loaded CCS may be degraded faster than apoCCS.

Because CCS can interact with SOD1 in the absence of copper (30,31), it is possible that the increase in CCS expression under low copper conditions results from formation of a stable intermediary complex between apoCCS and apoSOD1. This complex may persist until CCS is able to acquire copper and insert it into SOD1. Because SOD1 is much more abundant than CCS (23), a small fraction of the total SOD1 stabilizing CCS in a complex would be sufficient to produce large fold increases in CCS.

The data from this study show that CCS protein level can better distinguish between rats fed normal, moderately deficient or deficient copper diets than SOD1 activity. Further, because the decrease in SOD1 protein follows closely the decrease in SOD1 activity, CCS is also a better indicator of copper deficiency than SOD1 protein level. In fact, the change in SOD1 protein level is less than the change in SOD1 activity as some apoSOD1 accumulates (28). Studies are cur-

### TABLE 2

<table>
<thead>
<tr>
<th>Diet group</th>
<th>ERCS (10^12/L)</th>
<th>HCT (g/L)</th>
<th>Hb (g/L)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/L)</th>
<th>RDW (%)</th>
<th>PLT (10^9/L)</th>
<th>MPV (fl)</th>
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</thead>
<tbody>
<tr>
<td>Cu-N</td>
<td>7.0 ± 0.2a</td>
<td>0.40 ± 0.02a</td>
<td>138.0 ± 5.6a</td>
<td>58.0 ± 2.3a</td>
<td>19.8 ± 0.8a</td>
<td>341.7 ± 2.8a</td>
<td>12.4 ± 0.7c</td>
<td>1088 ± 144a</td>
<td>6.3 ± 0.2b</td>
</tr>
<tr>
<td>Cu-M</td>
<td>7.1 ± 0.6a</td>
<td>0.39 ± 0.03a</td>
<td>128.4 ± 12.9a</td>
<td>53.6 ± 3.7b</td>
<td>18.0 ± 1.4b</td>
<td>363.4 ± 5.3a</td>
<td>18.2 ± 4.3b</td>
<td>1175 ± 133a</td>
<td>6.7 ± 0.4a</td>
</tr>
<tr>
<td>Cu-D</td>
<td>4.6 ± 1.2b</td>
<td>0.23 ± 0.06b</td>
<td>70.5 ± 18.2b</td>
<td>47.9 ± 0.8c</td>
<td>15.3 ± 0.5c</td>
<td>318.5 ± 9.3b</td>
<td>25.0 ± 1.1a</td>
<td>1221 ± 189a</td>
<td>6.5 ± 0.4ab</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, Cu-N, n = 10; Cu-M, n = 9; Cu-D, n = 6. Values in a column with different letters differ, P < 0.05.
2 Test diet abbreviations are as indicated in Table 1.
Currently under way to determine the suitability of CCS for diagnosing marginal copper deficiency in humans and as an indicator for setting minimum copper requirements.

ACKNOWLEDGMENTS

We are grateful to Judy Edgar for the hematological assessments and Jocelyn Souligny for preparing the test diets. We also thank the staff of the Animal Resources Division, Health Canada for care of the animals.

LITERATURE CITED


FIGURE 1 Characterization of antibody G-19 (A) used to determine the effect of dietary copper on CCS expression in liver of individual rats (B) and mean expression of CCS in liver and erythrocytes (C). (A) Western blots of the same liver extract using antibodies against CCS (G-19) or SOD1 (FL-154). Positions of BioRad Kaledioscope molecular weight standards are indicated to the right. (B) Western blot of extracts from livers of rats fed normal (N15, N9, N3), moderately deficient (M13, M11, M17) or deficient (D21, D16, D29) copper diets. The membrane was sequentially probed with antibodies against SOD1, CCS and β-tubulin. A short and long exposure is shown for CCS. (C) Liver and erythrocyte CCS immunoreactive protein from rats fed normal, moderately deficient or deficient copper diets was quantified by densitometric analysis of band intensity and presented graphically as arbitrary units. Relative expression levels are indicated at the bottom. A representative Western blot showing CCS expression in erythrocytes of rats N9, M17 and D16 is depicted within the graph. Bars represent means ± SD (n = 4/group). For each tissue, values with different letters differ (P < 0.05).