Genes & Nutrition Vol. 1, No. 1, pp. 51-60, 2006
ISSN 1555-8932 print, Copyright © 2006 by New Century Health Publishers, LLC
www.newcenturyhealthpublishers.com
All rights of reproduction in any form reserved

VARIABLE RESPONSE OF SELECTED CUPROPROTEINS IN RAT CHOROID PLEXUS AND CEREBELLUM FOLLOWING PERINATAL COPPER DEFICIENCY

Anna A. Gybina and Joseph R. Prohaska

Department of Biochemistry and Molecular Biology, University of Minnesota Medical School, Duluth, MN 55812

[Received February 10, 2006; Accepted March 16, 2006]

ABSTRACT: *Recent immunohistochemical characterization of the copper transport protein, Ctr1, reported enriched levels in mouse choroid plexus, and enhancement by copper deficiency. To extend and confirm this, experiments were conducted with Holtzman rats. Following perinatal copper deficiency there was an 80% reduction in brain copper of 24-27 day old copper-deficient (Cu-) rat pups compared to copper-adequate (Cu+) controls. Choroid plexus immunoblot analysis with rabbit anti-hCtr1 demonstrated a 50% higher Ctr1 protein expression in Cu- samples. However, levels of copper chaperone for superoxide dismutase (CCS) were unchanged, suggesting that Ctr1 buffers the choroid plexus against copper deficiency, since CCS normally is much higher in Cu- tissues. There were 13% lower levels of cytochrome c oxidase subunit IV (COX IV) detected in Cu- choroid plexus. In contrast, in cerebellum of Cu- rats CCS was 2-fold higher and COX IV 1.7-fold lower than Cu+ rats consistent with severe copper deficiency. Brain mitochondria from Cu- rats had severe reductions in COX IV content and CCO activity and modest but significant elevations in CCS and reductions in Cu, Zn-superoxide dismutase. COX IV may be a more sensitive marker for copper deficiency than CCS and may prove useful to assess copper status.*

KEY WORDS: Brain, CCS, Copper, Copper-deficient, COX IV, Ctr1, Rat

Corresponding author: Prof. Joseph R. Prohaska, Department of Biochemistry and Molecular Biology, University of Minnesota Medical School Duluth, 1035 University Drive, Duluth, MN 55812, USA; FAX: 218-726-8014; E-mail: jprohask@d.umn.edu

INTRODUCTION

Many essential micronutrients are required to ensure that proper transcription and translation occur so that accurate, normal, and robust development can proceed. One such essential micronutrient is the transition metal copper (Cu). Although Cu may not play a direct role in mammalian replication, two labs independently and simultaneously created a null mouse lacking the copper transporter, Ctr1, and showed that -/- mice died during gestation (Kuo et al., 2001; Lee et al., 2001). Brain Cu and the activity of two brain Cu-dependent enzymes (cuproenzymes), cytochrome *c* oxidase (CCO) and Cu, Zn-superoxide dismutase (SOD1) were impacted even in heterozygote (Ctr1 +/-) mice (Lee et al., 2001). This reconfirmed older dietary data suggesting the importance of Cu for normal mammalian development. Mammals contain about a dozen well-characterized proteins that require Cu for biological activity. It is surmised that the biological need for Cu is the function of one or more of these cuproenzymes (Failla et al., 2001).

Copper can redox cycle between cupric and cuprous states; the latter is potentially toxic as an electron donor to peroxides. Thus, Cu homeostasis is critical for cell development and survival but excessive levels are not desirable. There is no storage pool of cellular Cu, although some have argued that the small cysteine rich protein, metallothionein, can serve this function when Cu is limiting (Suzuki et al., 2002). Three processes could potentially be regulated to control homeostasis: absorption, excretion, and utilization.

Which genes and gene products control Cu absorption? It is assumed that Ctr1 is important as the enterocyte plasma membrane translocator. Recent data suggest that duodenal enterocytes from Cu-deficient suckling mice have increased Ctr1 in the apical membrane perhaps to capture the limiting luminal Cu (Kuo et al., 2006). For basolateral Cu efflux the protein ATP7A is necessary as deletion of this protein in humans causes Menkes syndrome and results in high intestinal Cu and peripheral Cu deficiency (Danks et al., 1972). It is likely that the combination of Ctr1/ATP7A is required for all cellular apical to basolateral Cu transport. The late Arturo Leone and colleagues have used a contemporary differential display/proteome approach to evaluate intestines from rats fed Cu limiting diets (Marzullo et al., 2004; Tosco et al., 2005). Though these studies did not reveal new insights into Cu absorption homeostasis they should provide stimulus for further metabolomic work as copper and iron differentially regulated several interesting enzymes.

Regulation of excretion when Cu is limiting was studied in

liver of rats using differential display (Wang et al., 1996). Upregulation of several genes was detected, including ferritin that might have been a secondary effect of the hepatic iron overload phenotype of Cu-deficiency. There were no detectable changes in the mRNA of hepatic genes involved in biliary excretion: Ctr1, ATP7B, Murr1, or ceruloplasmin (Cp). However, that work preceded discovery of most of these genes. Nevertheless, there is no experimental support for transcriptional regulation of these genes in response to Cu limitation (Gitlin et al., 1992; Schaefer et al., 1999; Lee et al., 2000).

Utilization of Cu requires transport from the portal blood, through the liver, and across the apropos cellular barriers such as endothelial cells lining vessels. The identity of the plasma Cu transporter is unknown but is not Cp, the most abundant plasma cuproprotein (Meyer et al., 2001). Recent immunohistochemical data suggest that Ctr1 may be a necessary protein for Cu utilization (Kuo et al., 2006). It is also clear that three copper chaperone proteins are required to insert Cu into apo-proteins in cellular secretory pathways (atox1), into cytoplasmic SOD1 (CCS), and into mitochondria for CCO (Cox 17) (Prohaska and Gybina, 2004). The levels of the cuproenzymes, the Cu-transporters, or the Cu chaperones could impact utilization and, therefore, biochemical function of Cu. It will be interesting to learn if changes in these classes of Cu binding proteins might be useful to assess Cu status when cellular Cu is altered by nutritional or genetic factors.

Recently we showed that the choroid plexus of mouse brain was a tissue enriched in Ctr1 protein (Kuo et al., 2006). Importantly, the intensity of staining for Ctr1 was greater in Cu-deficient suckling mice. The purpose of the current studies was to confirm and extend this finding by determining if Ctr1 protein was higher in choroid plexus of Cu-deficient rats. Another purpose was to see if this putative change in Ctr1 protected this "brain" region from Cu deficiency. We used two sensitive markers that respond to changes in Cu status, copper chaperone for superoxide dismutase (CCS) and subunit four of CCO (COX IV) (Medeiros et al., 1993; Prohaska et al., 2003a).

METHODS AND MATERIALS

Animal care and diets

Holtzman sperm-positive rats, purchased commercially (Harlan Sprague Dawley, Indianapolis, IN), received one of two dietary treatments, Cu-deficient or Cu-adequate, consisting of a Cu-deficient modified AIN-76A diet (Teklad Laboratories, Madison, WI) and either low Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet contained 0.34 mg Cu/kg and 48 mg Fe/kg by chemical analysis. This diet contains approximately one-tenth of the optimal dietary Cu level for rats. Offspring and dams on the Cu-deficient treatment drank deionized water, whereas Cu-adequate treatment groups drank water that contained 20 mg Cu/L by adding $CuSO_4$ to the drinking water. Rats were given free access to diet and drinking water.

Pregnant dams were placed on the Cu-deficient treatment either 7 (experiments 1,2, and 4) or 14 days (experiment 3) after they were identified as sperm-positive. Two days following parturition litter size was adjusted to ten pups. Offspring in the perinatal model were sampled at postnatal age 20-27. A total of 26 litters (12 Cu-adequate and 14 Cu-deficient) were studied in a set of four separate experiments. All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (0700-1900-h). The University of Minnesota Animal Care Committee formally approved all protocols.

Rats were killed by decapitation, and brains were removed, weighed and processed for biochemical analysis. Brains were dissected on a chilled glass plate following established guidelines (Glowinski and Iversen, 1966). Depending on the experiment, the cerebellum, the cerebral cortex and choroid plexus were studied independently.

Biochemical analyses

Hemoglobin was determined spectrophotometrically as metcyanhemoglobin. Plasma was obtained following centrifugation of heparinized blood, and the activity of the cuproprotein ceruloplasmin (EC 1.16.3.1) was measured by following oxidation of ortho-dianisidine at 37°C (Lehmann et al., 1974). Protein levels were measured by a modified Lowry procedure using bovine albumin as reference (Markwell et al., 1978).

Copper analyses

Brain tissue and 1-g portions of diet were wet-digested with 4 mL of concentrated HNO_3 (Trace Metal Grade, Fischer Scientific, Pittsburg, PA) and the residue was brought to 4.0 mL with 0.1 mol/L HNO_3. Samples were then analyzed for total Cu and Fe by flame AAS (Perkin Elmer model 1100B). The method was checked with a certified bovine liver standard (1577, U.S. National Bureau of Standards and Technology, Gaithersburg, MD). Our copper estimate was 99.3 ± 0.91% of that certified.

Western immunoblot sample preparation

Western immunoblot analysis was performed on total protein extracts from cerebellum. Tissues were minced and gently homogenized with four volumes of ice-cold 0.32 mol/L sucrose (pH 7.0) using a chilled Potter-Elvehjem homogenizer. Four full strokes were used to homogenize to maintain subcellular organelle integrity. Protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, MO) were added to samples. Total protein extracts were prepared by treating an aliquot of tissue homogenate with 5 g/L Triton X-100. Samples were then further homogenized, vortexed, sonicated for 10 s, and centrifuged at 14,000 x g for 15 min at 4°C. Supernatant fluid, containing both soluble and membrane protein, was stored at –80°C until later analysis.

Choroid plexi were dissected under microscope and homogenized in 0.5 mL tubes with a custom made ground glass homogenizer in 35 µL buffer containing 0.055 mol/L Tris-HCl (pH 6.8), 45 g/L SDS, 90 g/L glycerol, 6g/L Triton-X 100, 0.025 mol/L DTT and 5 µL protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). Homogenates were sonicated for 75 sec and boiled for 5 minutes. Equal volume aliquots of homogenates were mixed with loading buffer containing 20 g/L SDS and 0.14

mol/L 2-mercaptoethanol and analyzed on a 15% SDS-PAGE gel. Loading was evaluated by Ponceau S staining and immunoblot analysis with actin as loading control.

Kidneys from a single adult mouse were extracted and membrane and cytosolic extracts were prepared by the following method. Tissues were minced and gently homogenized with nine volumes of cold buffer (0.3 mol/L sucrose, 0.02 mol/L potassium phosphate (pH 7.0), 0.1 mmo/L PMSF) using a chilled Potter-Elvehjem homogenizer. Four full strokes were used to homogenize to maintain subcellular organelle integrity. Homogenates were centrifuged at 400 x g for 5 min at 4° C, and the resulting supernatant was spun at 100,000 x g for 2 hr. The cytoplasm was saved and the membrane pellet solubilized in a buffer containing 0.06 mol/L Tris-HCl (pH 6.8), 50 g/L SDS, and 100 g/L glycerol. Aliquots of protein were boiled in loading buffer containing 20 g/L SDS and 0.14 mol/L 2-mercaptoethanol.

Whole brain mitochondrial isolation was carried out as previously described (Gybina and Prohaska, 2003). CCO and SOD1 activities were determined spectrophotometrically as described previously (Prohaska, 1991). Cerebellar CCO assays in Experiment 2 were conducted after homogenates were frozen for 7 months.

WESTERN IMMUNOBLOTS

Western blotting analysis was performed by size fractionation of proteins on SDS-PAGE gels and electroblot transfer to 0.2 mM nitrocellulose membranes (Protran, Schleicher & Schuell). Membranes were stained with Ponceau S (Sigma, St. Louis, MO) to verify equal loading of protein and then used in immunoblotting as described elsewhere (Prohaska and Brokate, 2001b). Some membranes were reprobed after incubation of membranes with buffer containing 2-mercaptoethanol and SDS at 55°C for 30 min.

Copper transport protein (Ctr1) was detected using a rabbit antibody against the intracellular loop of human Ctr1, residues 99-116, that was developed at Duke University and generously provided by Dr. Dennis Thiele. This affinity purified anti-hCtr1 antibody was used at a 1:2,500 dilution. Actin was probed using mouse monoclonal anti-actin antibody (MAB1501, Chemicon International, Temecula, CA) at a 1:10,000 dilution. Copper chaperone for superoxide dismutase (CCS) was evaluated using affinity purified rabbit anti-hCCS from antiserum commercially produced and characterized previously at a 1:1000 dilution (West and Prohaska, 2004). Cu, Zn superoxide dismutase (SOD1) was detected using a rabbit polyclonal anti-SOD 1 antibody (AB1237, Chemicon International, Temecula, CA) at a 1:5,000 dilution. Cytochrome c oxidase subunit IV (COX IV) and Complex I (39 kDa subunit) protein levels were analyzed using mouse monoclonal anti-COX IV, at 1:4000 dilution, and mouse monoclonal anti-Complex I (39 kDa subunit specific), at 0.5 µg/mL, (A-21348 and A-21344, Molecular Probes, Eugene, OR). Secondary species specific antibodies were diluted 1:5000.

SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) was used to detect selected proteins. Chemiluminescence was captured using high speed blue X-ray film (Lake Superior X Ray Inc., Duluth, MN) and densitometry was carried out 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 ANALYSIS

Specific mean comparisons were tested by Student's *t* test, a = 0.05, after variance equality was established. Data were analyzed using statistical software (Statview 4.5, Abacus Concepts, Berkeley, CA or Microsoft Excel).

RESULTS

Copper status of the rats in experiments 1-2

Following 5 weeks of Cu depletion, 2 during gestation and 3 during lactation, weanling pups at postnatal age 24 (P24) displayed signs consistent with severe Cu deficiency reported previously (Table 1) (Gybina and Prohaska, 2003). Compared to Cu-adequate pups, Cu-deficient pups for both Experiments 1 and 2 were smaller and had pronounced cardiac hypertrophy. The Cu-deficient pups were anemic and had 87% and 83% reduction in concentration of total Cu as determined by atomic absorption spectroscopy. There were also profound and similar reductions in the activity of cerebellar cytochrome *c* oxidase (CCO) in Cu-deficient pups, in Experiment 1 a 83% reduction and in Experiment 2 a 73% reduction. Though there were some subtle differences in the two experiments the Cu status of both Cu-deficient groups was similar and indicated major differences from Cu-adequate controls. Plasma ceruloplasmin activity was determined for all rats in these experiments. Activity in Cu-deficient rats was not detectable compared to a mean ± SEM of 86.8 ± 11.6 units/L for the Cu-adequate rats combining all four experiments.

Table 1. Characteristics of 24 day old male rats following perinatal copper deficiency

	Experiment 1		Experiment 2	
Characteristics	Cu-adequate	Cu-deficient	Cu-adequate	Cu-deficient
Body weight, g	88.7 ± 1.1	45.1 ± 1.5*	80.8 ± 2.4	57.0 ± 5.6*
Heart/Body, mg/g	5.1 ± 0.2	16.0 ± 1.2*	5.6 ± 0.4	12.5 ± 1.7*
Hemoglobin, g/L	102 ± 3.0	53.8 ± 3.5*	87.7 ± 2.7	67.4 ± 5.6*
Brain Cu, nmol/g	35.0 ± 1.1	4.54 ± 1.1*	38.3 ± 1.3	6.62 ± 0.43*
Cerebellar CCO, units/mg	0.75 ± 0.06	0.13 ± 0.02*	0.33 ± 0.03	0.09 ± 0.03*

Values are means ± SEM (n=4). Rats were born to and nursed by Cu-deficient or Cu-adequate dams. Treatment began two weeks prior to parturition. Pups were maintained on the same treatment as their dams until killing. Means were tested by unpaired Student's t-test. Compared to Cu-adequate rats, Cu-deficient rats had significantly different values, * $P < 0.01$

Ctr1 antibody characterization and Ctr1 protein in rat choroid plexus.

Affinity purified rabbit anti-hCtr1 antibody directed against the intracellular loop of Ctr1 was characterized by incubation with a membrane containing several protein extracts: adult mouse kidney membrane and cytosolic proteins, and total protein extracts from mouse embryonic fibroblast (MEF) cells which either expressed wild-type (+/+) or were null (-/-) for Ctr1 (Lee et al., 2002a) (Fig 1A). Wild type MEF cells showed a prominent Ctr1 band with a mobility equivalent to approximately 35 kDa, which was conspicuously absent in the lane loaded with extract from null cells. Wild-type cells also exhibited minor bands unspecific to Ctr1 protein that were present in the -/- cell lane as well. Ctr1 membrane location was consistent with our immunoblot analysis that demonstrated that the most prominent 35 kDa band in the mouse kidney membrane protein lane was absent from the cytosolic protein lane. However, when compared to Ctr1 +/+ fibroblast cells, the putative kidney membrane protein Ctr1 band differed in quality by appearing thicker, perhaps consisting of more than one band, and spanning a wider molecular weight range. Furthermore, other bands of various molecular weights appeared in both the kidney membrane and cytosolic lanes that were not present in the MEF cell lanes.

Ctr1 antibody analysis of rat choroid plexus total protein extract showed only one prominent band at approximately 30 kDa (Fig 1B). We believe this to be Ctr1, and densitometry analysis of choroid plexi from P24 male rats revealed that there was approximately 50% higher abundance of Ctr1 in Cu-deficient extracts than Cu-adequate extracts. Ctr1 densities were expressed relative to actin. This technique is used by others but was especially important in these samples since small sample size prevented a separate protein assay prior to loading the gel. The mean ± SEM (n=4) Ctr1/actin raw density values for Cu-deficient rats was 0.85 ± 0.14 compared to 0.56 ± 0.11 for Cu-adequate rats, $P < 0.05$. Density of actin was not impacted by dietary Cu deficiency.

Figure 1. Characterization of rabbit anti-hCtr1 antibody in various tissues

A. Protein samples were fractionated on a 12% SDS-PAGE gels and transferred to nitrocellulose membrane. Adult male mouse kidney (KY) membranes and cytoplasm, 30 µg protein per lane, and mouse embryonic fibroblast cells either Ctr1+/+ or Ctr1-/-, 40 µg per lane were compared. Large arrows indicate molecular weight standards (kDa) and the small arrow indicates position of Ctr1. B. Immunoblot of choroid plexus tissue from Cu-adequate (Cu+) and Cu-deficient (Cu-) male P24 rats incubated with hCtr1 antibody. Protein samples were fractionated on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. Arrow indicates the location of the purported Ctr1 band. C. Immunoblot analysis of Ctr1 and actin in choroid plexus of 8 P24 male Cu-adequate (+) or Cu-deficient (-) rats, Experiment 2. Equal volumes of total choroid plexus homogenate were loaded per lane. Protein loading was evaluated by Ponceau S staining and by actin immunoblot analysis. No difference was detected in density of actin due to dietary treatment. Bars represent relative density/mean copper-adequate (+Cu) density ± SEM. Mean +Cu density was set at 1.0 for both Ctr1 and actin. The Ctr1/actin ratio was 51% higher in choroid plexus from Cu- than Cu+ rats, ($P < 0.05$).

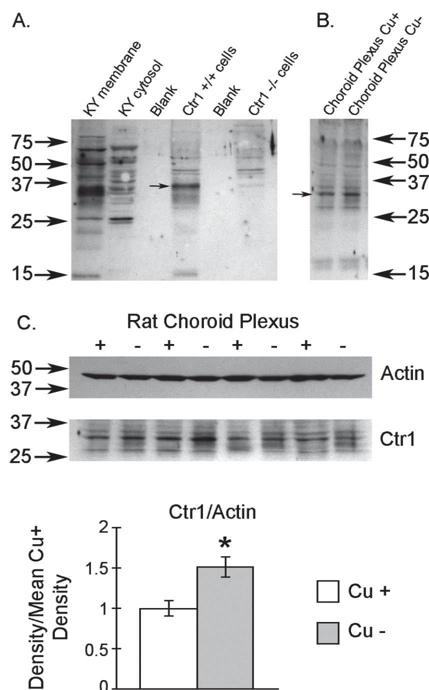
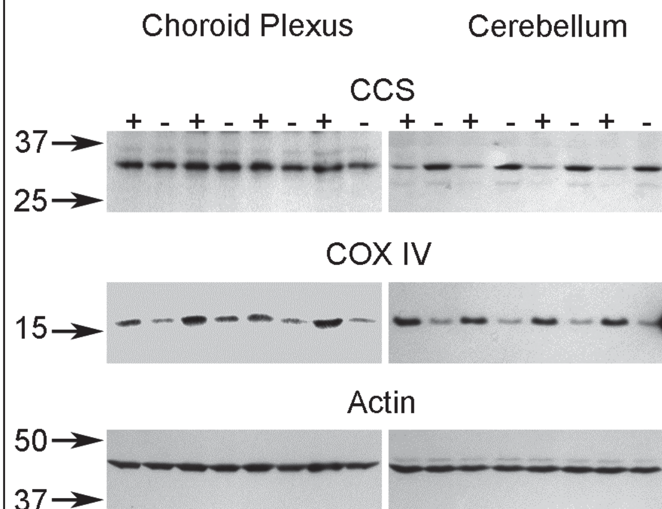


Figure 2. Immunoblot analysis of selected proteins in two brain areas, choroid plexus and cerebellum, of Cu-adequate (+) or Cu-deficient (-) P24 male rat pups

Equal volumes of total choroid plexus homogenate, Experiment 2, were loaded per lane and proteins were fractionated on 15% SDS PAGE gels. The membrane was sequentially reprobed for actin, to evaluate protein load and transfer, and CCS and COX IV, to evaluate Cu status. A similar gel was run using 50 µg of cerebellar protein per lane from P24 male rats, Experiment 1. In choroid plexus no statistical difference was detected in levels of CCS or the CCS/actin ratio ($P > 0.05$) due to Cu deficiency. In contrast, COX IV levels and the COX IV/actin ratio was significantly lower ($P < 0.05$) in choroid plexus from Cu-deficient compared to Cu-adequate rats. In cerebellum, CCS and the CCS/actin ratio were significantly higher ($P < 0.05$) in Cu-deficient extracts , whereas the COX IV and COX IV/actin ratios were significantly lower in Cu-deficient compared to Cu-adequate extracts ($P < 0.05$). No statistical difference between dietary treatment groups was detected in levels of actin for either choroid plexus or cerebellum.



Copper deficiency markers in rat choroid plexus and cerebellum

Cu deficiency markers were assessed in choroid plexus for Experiment 2 since this area of the brain was not studied previously. This tissue was compared to cerebellum from gender and age-matched rats, Experiment 1. The degree of brain Cu deficiency between the two experiments was determined similar based on reduction of cerebellar CCO activity and brain Cu (Table 1).

CCS protein levels have previously been reported to be a sensitive marker for copper deficiency (Bertinato and L'Abbe, 2003; Prohaska et al., 2003a). Somewhat surprising, no significant difference in CCS protein levels or CCS/actin ratio was detected in total protein extracts from choroid plexus of P24 male rats suggesting this tissue was not "Cu-deficient" (Fig.2). However, COX IV subunit analyses of the same choroid plexus blot suggested otherwise. There was a modest but significant reduction in density of this CCO subunit in extracts from Cu-deficient pups compared to controls $P < 0.05$. The COX IV/actin ratio in Cu-adequate rats was 3.38 ± 0.31 compared to 2.93 ± 0.19 for Cu-deficient, a modest 13% higher value in Cu-adequate rats.

In contrast to choroid plexus, there was a marked increase of CCS protein levels in cerebellum of Cu-deficient rats compared to controls (Fig.2). Density of the CCS band relative to actin was nearly two-fold higher. The CCS/actin levels of Cu-deficient rats was 1.54 ± 0.24 compared to levels of 0.52 ± 0.08 in Cu-adequate rats. Also changes in CCO, a 1.7-fold higher abundance of COX IV in Cu-adequate compared to Cu-deficient cerebella, suggested a more severe Cu deficiency. The COX IV/actin ratio was 0.48 ± 0.06 for Cu-adequate rats compared to 0.18 ± 0.06 for Cu-deficient rats. Density of actin was not impacted by dietary Cu deficiency. The reduction in cerebellar CCO enzyme activity of Cu-deficient rats (Experiment 1, Table 1), 83%, was similar to reduction in cerebellar COX IV protein level detected by immunoblot (Fig. 2), 62%. Together these data strongly suggest that the cerebellum is more Cu deficient than choroid plexus based both on CCS and COX IV content (Fig. 2).

Cu status of the rats used for mitochondrial isolation

The striking reduction in CCO subunit four, COX IV, in cerebellar total protein extracts prompted further studies on brain mitochondria, the source of CCO. Two sets of animals were studied. Male rats in Experiment 3 that were subjected to 4 weeks of Cu depletion via dams (1 week of gestation and 3 weeks of lactation) displayed signs of Cu deficiency similar to those in Experiments 1 and 2 (Table 2), although growth impairment was blunted somewhat. Compared to Cu-adequate males the Cu-deficient males were smaller, anemic, and had 81% reduction in total Cu in cerebella. There was also 67% reduction in brain mitochondrial CCO activity in Cu-deficient samples compared to Cu-adequate samples. As expected, the specific activity of CCO in mitochondria was higher than that in cerebellar homogenates (Tables 1 and 2). Mitochondria from these rat brains were used in immunoblot studies of COX IV and mitochondrial Complex 1.

Female offspring, P26, obtained in the same manner as male offspring in Experiments 1 and 2 were also studied, Experiment 4 (Table 2). Compared to Cu-adequate female rats Cu-deficient rats were smaller, anemic, and had an 80% reduction in brain mitochondrial CCO activity. These data suggest that both male and female weanling pups derived from Cu-deficient dams and used for the mitochondrial immunoblot studies were indeed Cu deficient. Mitochondria from rats in Experiment 4 were used for immunoblot studies of CCS and Cu, Zn-superoxide dismutase (SOD1).

Table 2. Characteristics of 24-27 day old rats used for mitochondrial isolation following perinatal copper deficiency

	Exp. 3 Males		Exp. 4 Females	
Characteristics	Cu-adequate	Cu-deficient	Cu-adequate	Cu-deficient
Body weight, g	99.7 ± 3.8	81.1 ± 4.9*	70.5 ± 1.7	49.4 ± 5.75*
Hemoglobin, g/L	103 ± 3.4	60.3 ± 5.8*	108 ± 3.6	76.2 ± 4.7*
Cerebellar Cu, nmol/g	36.7 ± 0.85	6.94 ± 0.77*	ND	ND
Mito CCO, units/mg	2.26 ± 0.38	0.74 ± 0.20*	1.75 ± 0.03	0.35 ± 0.04*

Values are means ± SEM, (n=8 Exp. 3 or n=4 Exp. 4). Rats were born to and nursed by Cu-deficient or Cu-adequate dams. Treatment began one week prior to parturition for Exp. 3 and two weeks prior to parturition for Exp. 4. Pups were maintained on the same treatment as their dams until killing. Two adequate and two deficient rats were killed on four consecutive days in Experiment 3 and all four adequate and four deficient pups on one day P26 in Experiment 4. Cerebellar Cu was not determined (ND) in Experiment 4.
Means were tested by unpaired Student's t-test. Compared to Cu-adequate rats, Cu-deficient rats had significantly different mean values, * $P < 0.05$.

Copper deficiency impacts copper proteins in purified rat brain mitochondria

Immunoblots of brain mitochondrial proteins from rats in Experiments 3 and 4 extended results obtained in whole brain and regional tissue extracts. Alterations in all four mitochondrial proteins probed were detected (Fig. 3).

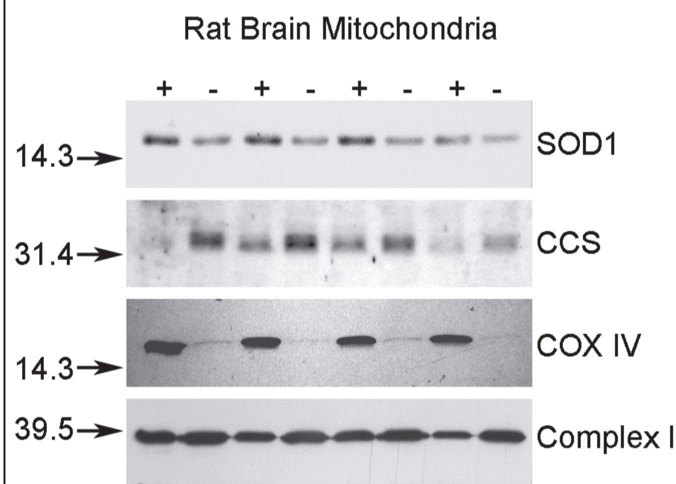
SOD1 is reported to be located in the mitochondrial intermembrane space in addition to the cytoplasm. Immunoblot analysis of the mitochondria (Experiment 4) showed a 20% reduction in density of SOD1 protein in Cu-deficient rat brain. Cu-adequate values were 1.31 ± 0.02 compared to Cu-deficient values 1.06 ± 0.05, $P < 0.05$. Although our immunoblot data was not designed to establish location, our Western blot data was corroborated by SOD enzyme activity that was detected in our mitochondrial samples. Importantly, our analysis of SOD1 activity

in purified brain mitochondria (Experiment 4) indicated that activity in Cu-deficient samples was reduced by 30%, $P < 0.01$. Activity, units/mg protein, in Cu-adequate rats was 43.8 ± 6.1 compared to 30.2 ± 4.2 in Cu-deficient rats.

The same mitochondrial extracts were used to prepare a separate membrane that was probed for CCS content (Fig. 3). Our results indicate that CCS was also detected in purified brain mitochondria. We believe this is not due to cytoplasmic contamination but have not rigorously examined that point. Importantly, the Cu-deficient phenotype for CCS was evident in these mitochondrial samples. CCS protein levels were 25% higher in Cu-deficient mitochondria, 1.04 ± 0.06, compared to Cu-adequate brain mitochondria, 0.83 ± 0.05, $P < 0.05$.

Figure 3. Immunoblot analysis of SOD1, CCS, COX IV, and Complex I in purified rat brain mitochondria from copper adequate (+) and copper deficient (-) P24-P27 rat male and female pups.

Brain mitochondria were isolated according to an established method (Lee et al., 1993). Protein loads per lane for SOD1, CCS, COX IV, and the 39 kDa subunit of Complex I blots were 5, 15, 10, and 10 µg respectively. Compared to brain mitochondria from Cu-adequate rats mitochondria from Cu-deficient rats had higher levels of CCS and Complex I subunit and lower levels of SOD1 and markedly lower levels of COX IV, $P < 0.05$.



Purified brain mitochondria from Cu-deficient male rats (Experiment 3) showed a drastic 85% reduction in COX IV protein (8.85 ± 0.53 in Cu-adequate samples compared to 1.32 ± 0.07 for Cu-deficient samples, $P < 0.01$). This is similar to the major reduction in CCO activity analyzed in the same set of mitochondria (Table 2). This reduction in COX IV protein, a component of mitochondrial Complex IV, was not reflected by other components of the electron transport chain. In fact there were 16% higher levels of mitochondrial Complex I protein 39 kDa subunit in Cu-deficient brain samples compared to Cu-adequate mitochondria when this membrane was stripped and reprobed (Fig. 3). Density values in Cu-adequate preparations were 6.61 ± 0.63 compared to 8.27 ± 0.23 for Cu-deficient samples, $P < 0.05$.

DISCUSSION

The affinity purified rabbit anti-hCtr1 detected a prominent band in extracts from mouse fibroblasts expressing wild-type protein (+/+) with a size consistent with a monomeric and glycosylated protein. This antibody also yielded a similar, slightly smaller, size band in tissue from rat choroid plexus. Mammalian Ctr1 proteins (human, mouse, and rat) are all greater than 90% homologous with each other and contain 190, 196, and 187 amino acids, respectively. There is an N-linked asparagine glycosylation site in the N-terminus extracellular domain (Klomp et al., 2002). Although the current antibody detected only one major band in the fibroblast cell extract, it was less specific in the detection of Ctr1 in mouse kidney. For some reason, it has been very difficult to produce antibody against mammalian Ctr1 that works well with immunoblots. The current antibody was produced against hCtr1 residues 99-116, whereas previous data was generated with antibody against residues 93-113 (Kelleher and Lonnerdal, 2003) or against residues 95-114 (Kuo et al., 2006). One would predict similar immunoblot results but this is not true. It is useful, perhaps essential, to have tissue from null mice to carefully interpret the results of tissue specific Ctr1 expression since the protein not only is subject to posttranslational modification by glycosylation but can also form oligomers (Eisses and Kaplan, 2002; Lee et al., 2002b).

The finding of a modest increase in Ctr1 in the choroid plexus of Cu-deficient rats is consistent with recent work in mice (Kuo et al., 2006). In those studies, using antibody directed at a slightly different sequence, increased Ctr1 was detected in choroid plexus of P16 Cu-deficient mice by immunohistochemistry and in membrane preparations from whole brain by immunoblot. These data, however, do not resolve the issue clearly as to whether the staining data reflects more protein or a change in intracellular localization. Studies in cell culture indicate that Ctr1 moves from the plasma membrane to vesicular compartment when exposed to Cu (Petris et al., 2003). This observation has been challenged by similar experiments in the same cell type (Eisses et al., 2005).

The enhanced Ctr1 staining/expression in choroid plexus of Cu-deficient mice and rats, published and this current data, suggests an attempt to sequester limiting Cu from the cerebral spinal fluid. Results from the current study strongly support the idea that the choroid plexus is "spared" from Cu deficiency. The choroid plexus from Cu-deficient rats is the first tissue that has not shown a robust enhancement in CCS protein (Prohaska et al., 2003a). Previously, our lab showed that CCS was elevated in brain of suckling mice that had no change in SOD1 activity, suggesting that CCS responds even in marginal Cu deficiency (Prohaska et al., 2003a). Also, in the current experiments the cerebella of similar Cu-deficient rats displayed two-fold higher CCS in Cu-deficient tissue. Importantly, the content of CCO subunit four, COX IV, although "significantly" lower, 13%, in choroid plexus of Cu-deficient rats in these studies, was much lower, 62%, in cerebellum of Cu-deficient rats.

Another observation consistent with functional consequences of higher Ctr1 expression following dietary Cu limitation is the

marginal impact of Cu deficiency on the kidney. Similar to the choroid plexus both kidney tubules and duodenal enterocytes had higher Ctr1 staining in P16 Cu-deficient mice (Kuo et al., 2006). This may explain why that of seven rat organs examined following dietary Cu deficiency, the decrease in CCO activity (reflecting altered copper status) was least in kidney, even less than brain (Prohaska, 1991).

Current studies also suggest that COX IV measurement may be a useful tool to assess Cu status. The COX IV content of choroid plexus and cerebellum in the present studies are consistent with previous mitochondrial difference spectra data from brain suggesting loss of CCO (Prohaska and Wells, 1975). Several other groups have reported lower COX IV protein in heart tissue from Cu deficient rats in various dietary models (Medeiros et al., 1993; Rossi et al., 1998; Johnson and Brown-Borg, 2006). Interestingly, at least in heart, there is good evidence for reduction in several other COX subunits following Cu deficiency (Chao et al., 1994; Zeng et al., 2006). Together these data suggest the stability or perhaps assembly of CCO subunits is dependent on adequate Cu.

Our analysis of purified brain mitochondria demonstrate a striking reduction in COX IV protein similar to the reduction in CCO activity. Immnoblot data also detected SOD1 in purified brain mitochondria. Consistent with data from whole brain, there was a significant reduction in SOD1 protein in samples from Cu-deficient rats. Although specific activity of SOD1 was about 40% lower in purified mitochondria than what is measured in total brain homogenates, reflecting that SOD1 is not as abundant in mitochondria as it is in the cytoplasm, our results suggest that SOD1 is also located in rat brain mitochondria. Elegant work by others has more convincingly shown that this is also true for rat liver (Okado-Matsumoto and Fridovich, 2001).

We have recently suggested that another useful way to assess Cu status is to measure the increase in the ratio of CCS/SOD1 in blood (West and Prohaska, 2004). Following Cu deficiency in rodents there is an increase in CCS protein and a decrease in SOD1 protein in many organs (Prohaska and Brokate, 2001a; Bertinato et al., 2003; Prohaska et al., 2003a; Prohaska et al., 2003b). Our current studies show that this is evident even in purified brain mitochondria from Cu-deficient rats. Thus, the approach of using CCS/SOD1 seems reasonable. Using COX IV for Cu assessment of blood would be limited to white cells compared to red or white cells for the CCS/SOD1 approach, since erythrocytes do not possess mitochondria. Use of immunoblots for CCO subunit quantification would have an advantage over enzyme activity because the CCO assay is tedious and hard to standardize.

Plasma Cu and Cp measurements have been used extensively to assess Cu status and set dietary reference intakes (Trumbo et al., 2001). These two variables reflect the same thing and are subject to change without dietary Cu influences because Cp is an acute phase protein. We have evaluated the use of another plasma enzyme, peptidylglycine á-amidating monooxygenase (PAM), as a potential marker (Peterson and Prohaska, 1999). Compared to Cp, PAM is also abundant in plasma, but not impacted by lactation or inflammation (Prohaska and Broderius, 2006). PAM like Cp is affected by age. Further research is needed to identify the best blood markers of copper status.

The discovery that brain CCO (COX IV) protein levels were lower following Cu deficiency is consistent with enzyme activity data and heme *a* content (Prohaska and Wells, 1975). This pattern, lower cuproenzyme protein following Cu deficiency, has been observed previously for SOD1 and PAM (Prohaska and Brokate, 2001a; Prohaska et al., 2003b; Prohaska et al., 2005). Recently, the protein levels of the intestinal ferroxidase, hephaestin, were also reported lower following Cu depletion both in cell culture and in rats (Nittis and Gitlin, 2004; Reeves et al., 2005). Perhaps the apo-proteins lacking Cu are degraded faster. In the case of SOD1 and PAM it is not due to impaired synthesis due to lower mRNA (Prohaska and Brokate, 2001a; Prohaska et al., 2003b; Prohaska et al., 2005). COX IV mRNA has not been evaluated in Cu-deficient brain tissue.

The higher brain CCS level and higher brain Ctr1 level are also not due to enhanced steady state levels of mRNA (Lee et al., 2000; Prohaska et al., 2003a). Studies in cell culture suggest the degradation of CCS is slower when Cu is limiting (Bertinato and L'Abbe, 2003). Perhaps this mechanism exists in vivo for CCS but has not been tested.

Additional studies will be required to determine the impact, if any, of Cu deficiency on transcription of genes involved in copper homeostasis and/or function. Likewise, further work will be needed to study post-transcriptional regulation by Cu of cuproproteins.

ACKNOWLEDGEMENTS

Support for this research was provided by NIH grant HD 39708. The skillful technical assistance of Margaret Broderius and Joshua Pyatskowit is appreciated. The affinity purified rabbit anti-hCtr1 and extracts from mouse fibroblasts were kind gifts of Dr. Dennis Thiele, Duke University.

REFERENCES

Bertinato J. and L'Abbe M. R. (2003) Copper modulates the degradation of copper chaperone for Cu,Zn superoxide dismutase by the 26 S proteosome. *Journal of Biological Chemistry* **278**, 35071-35078.

Bertinato J., Iskandar M. and L'Abbe M. R. (2003) Copper deficiency induces the upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats. *Journal of Nutrition* **133**, 28-31.

Chao J. C., Medeiros D. M., Davidson J. and Shiry L. (1994) Low levels of ATP synthase and cytochrome c oxidase subunit peptide from hearts of copper-deficient rats are not altered by the administration of dimethyl sulfoxide. *Journal of Nutrition* **124**, 789-803.

Danks D. M., Campbell P. E., Stevens B. J., Mayne V. and Cartwright E. (1972) Menkes's kinky hair syndrome. An inherited defect in copper absorption with widespread effects. *Pediatrics* **50**, 188-201.

Eisses J. F. and Kaplan J. H. (2002) Molecular characterization of hCTR1, the human copper uptake protein. *Journal of Biological Chemistry* **277**, 29162-29171.

Eisses J. F., Chi Y. and Kaplan J. H. (2005) Stable plasma membrane levels of hCTR1 mediate cellular copper uptake. *Journal of Biological Chemistry* **280**, 9635-9639.

Failla M. L., Johnson M. A. and Prohaska J. R. (2001) Copper. In: Bowman B. A. and Russell R. M., Eds), Present Knowledge in Nutrition, Eighth Edition (Washington, DC: ILSI Press), pp 373-383.

Gitlin J. D., Schroeder J. J., Lee-Ambrose L. M. and Cousins R. J. (1992) Mechanisms of caeruloplasmin biosynthesis in normal and copper- deficient rats. *Biochemical Journal* **282**, 835-839.

Glowinski J. and Iversen L. L. (1966) Regional studies of catecholamines in the rat brain. *Journal of Neurochemistry* **13**, 655-669.

Gybina A. A. and Prohaska J. R. (2003) Increased rat brain cytochrome c correlates with degree of perinatal copper deficiency rather than apoptosis. *Journal of Nutrition* **133**, 3361-3368.

Johnson W. T. and Brown-Borg H. M. (2006) Cardiac cytochrome-c oxidase deficiency occurs during late postnatal development in progeny of copper-deficient rats. *Experimental Biology and Medicine* **231**, 172-180.

Kelleher S. L. and Lonnerdal B. (2003) Marginal maternal Zn intake in rats alters mammary gland Cu transporter levels and milk Cu concentration and affects neonatal Cu metabolism. *Journal of Nutrition* **133**, 2141-2148.

Klomp A. E., Tops B. B., Van Denberg I. E., Berger R. and Klomp L. W. (2002) Biochemical characterization and subcellular localization of human copper transporter 1 (hCTR1). *Biochemical Journal* **364**, 497-505.

Kuo Y. M., Zhou B., Cosco D. and Gitschier J. (2001) The copper transporter CTR1 provides an essential function in mammalian embryonic development. *Proceedings National Academy of Sciences USA* **98**, 6836-6841.

Kuo Y. M., Gybina A. A., Pyatskowit J. W., Gitschier J. and Prohaska J. R. (2006) Copper transport protein (ctr1) levels in mice are tissue specific and dependent on copper status. *Journal of Nutrition* **136**, 21-26.

Lee C. P., Sciamanna M. and Peterson P. L. (1993) Intact rat brain mitochondria from a single animal: Preparation and properties. *Methods in Toxicology* **2**, 41-50.

Lee J., Prohaska J. R. and Thiele D. J. (2001) Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proceedings National Academy of Sciences USA* **98**, 6842-6847.

Lee J., Petris M. J. and Thiele D. J. (2002a) Characterization of mouse embryonic cells deficient in the ctr1 high affinity copper transporter. Identification of a Ctr1-independent copper transport system. *Journal of Biological Chemistry* **277**, 40253-40259.

Lee J., Pena M. M., Nose Y. and Thiele D. J. (2002b) Biochemical characterization of the human copper transporter Ctr1. *Journal of Biological Chemistry* **277**, 4380-4387.

Lee J., Prohaska J. R., Dagenais S. L., Glover T. W. and Thiele D. J. (2000) Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* **254**, 87-96.

Lehmann H. P., Schosinsky K. H. and Beeler M. F. (1974) Standardization of serum ceruloplasmin concentrations in international enzyme units with *o*-dianisidine dihydrochloride as substrate. *Clinical Chemistry* **20**, 1564-1567.

Markwell M. A., Haas S. M., Bieber L. L. and Tolbert N. E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**, 206-210.

Marzullo L., Tosco A., Capone R., Andersen H. S., Capasso A. and Leone A. (2004) Identification of dietary copper- and iron-regulated genes in rat intestine. *Gene* **338**, 225-233.

Medeiros D. M., Davidson J. and Jenkins J. E. (1993) A unified perspective on copper deficiency and cardiomyopathy. *Proceedings of the Society for Experimental Biology and Medicine* **203**, 262-273.

Meyer L. A., Durley A. P., Prohaska J. R. and Harris Z. L. (2001) Copper transport and metabolism are normal in aceruloplasminemic mice. *Journal of Biological Chemistry* **276**, 36857-36861.

Nittis T. and Gitlin J. D. (2004) Role of copper in the proteo some-mediated degradation of the multicopper oxidase hephaestin. *Journal of Biological Chemistry* **279**, 25696-25702.

Okado-Matsumoto A. and Fridovich I. (2001) Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu, Zn-SOD in mitochondria. *Journal of Biological Chemistry* **276**, 38388-38393.

Peterson D. J. and Prohaska J. R. (1999) Evaluation of rat white blood cell and plasma petidylglycine a-amidating monooxygenase activity as indicators of copper status. *Nutrition Research* **19**, 1041-1047.

Petris M. J., Smith K., Lee J. and Thiele D. J. (2003) Copper-stimulated endocytosis and degradation of the human

copper transporter, hCtr1. *Journal of Biological Chemistry* **278**, 9639-9646.

Prohaska J. R. (1991) Changes in Cu,Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper- deficient mice and rats. *Journal of Nutrition* **121**, 355-363.

Prohaska J. R. and Wells W. W. (1975) Copper deficiency in the developing rat brain: evidence for abnormal mitochondria. *Journal of Neurochemistry* **25**, 221-228.

Prohaska J. R. and Brokate B. (2001a) Lower copper, zinc-superoxide dismutase protein but not mRNA in organs of copper-deficient rats. *Archives of Biochemistry and Biophysics* **393**, 170-176.

Prohaska J. R. and Brokate B. (2001b) Dietary copper deficiency alters protein levels of rat dopamine-β-monooxygenase and tyrosine monooxygenase. *Experimental Biology and Medicine* **226**, 199-207.

Prohaska J. R. and Gybina A. A. (2004) Intracellular copper transport in mammals. *Journal of Nutrition* **134**, 1003-1006.

Prohaska J. R. and Broderius M. (2006) Plasma peptidylglycine alpha-amidating monooxygenase (PAM) and ceruloplasmin are affected by age and copper-status in rats and mice. *Comparative Biochemistry and Physiology Part B* **143**, 360-366.

Prohaska J. R., Broderius M. and Brokate B. (2003a) Metallochaperone for Cu,Zn-superoxide dismutase (CCS) protein but not mRNA is higher in organs from copper-deficient mice and rats. *Archives of Biochemistry and Biophysics* **417**, 227-234.

Prohaska J. R., Geissler J., Brokate B. and Broderius M. (2003b) Copper,Zinc-superoxide dismutase protein but not mRNA is lower in copper-deficient mice and mice lacking the copper chaperone for superoxide dismutase. *Experimental Biology and Medicine* **228**, 959-966.

Prohaska J. R., Gybina A. A., Broderius M. and Brokate B. (2005) Peptidylglycine-alpha-amidating monooxygenase activity and protein are lower in copper-deficient rats and suckling copper-deficient mice. *Archives of Biochemistry and Biophysics* **434**, 212-220.

Reeves P. G., Demars L. C., Johnson W. T. and Lukaski H. C. (2005) Dietary copper deficiency reduces iron absorption and duodenal enterocyte hephaestin protein in male and female rats. *Journal of Nutrition* **135**, 92-98.

Rossi L., Lippe G., Marchese E., De Martino A., Mavelli I., Rotilio G. and Ciriolo M. R. (1998) Decrease of cytochrome c oxidase protein in heart mitochondria of copper-deficient rats. *Biometals* **11**, 207-212.

Schaefer M., Hopkins R. G., Failla M. L. and Gitlin J. D. (1999) Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. *American Journal of Physiology* **276**, G639-G646.

Suzuki K. T., Someya A., Komada Y. and Ogra Y. (2002) Roles of metallothionein in copper homeostasis: responses to Cu-deficient diets in mice. *Journal of Inorganic Biochemistry* **88**, 173-182.

Tosco A., Siciliano R. A., Cacace G., Mazzeo M. F., Capone R., Malorni A., Leone A. and Marzullo L. (2005) Dietary effects of copper and iron deficiency on rat intestine: a differential display proteome analysis. *Journal Proteome Research* **4**, 1781-1788.

Trumbo P., Yates A. A., Schlicker S. and Poos M. (2001) Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal American Dietetic Association* **101**, 294-301.

Wang Y. R., Wu J. Y., Reaves S. K. and Lei K. Y. (1996) Enhanced expression of hepatic genes in copper-deficient rats detected by the messenger RNA differential display method. *Journal of Nutrition* **126**, 1772-1781.

West E. C. and Prohaska J. R. (2004) Cu,Zn-superoxide dismutase is lower and copper chaperone CCS is higher in erythrocytes of copper-deficient rats and mice. *Experimental Biology and Medicine* **229**, 756-764.

Zeng H., Saari J. T. and Dahlen G. M. (2006) Copper deficiency increases fibulin-5 (DANCE/EVEC) but decreases cytochrome C oxidase VIb subunit expression in rat heart. *Journal of Inorganic Biochemistry* **100**, 186-191.