EVALUATION OF ERYTHROCYTE COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS) AS A BIOMARKER FOR MARGINAL COPPER DEFICIENCY

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Abstract

Copper is an essential dietary trace metal responsible for the functioning of at least 12 mammalian enzymes. Despite current increases in cases of copper deficient humans, a sensitive, reliable, and easily evaluated copper deficiency biomarker has yet to be identified. Multiple studies using rodent models were used to evaluate currently accepted blood biomarker proteins through Western blotting and activity assays. In the first study, male postweanling Sprague Dawley rats were fed copper deficient (CuD) or copper adequate (CuA) diets and deionized water and sampled after one, two, and four weeks. A group fed an iron deficient diet (FeD) was also included and sampled after two weeks. Lastly, after the four weeks a repletion study was performed for two weeks with samples taken after a total of six weeks of study. Superoxide dismutase (Sod1) and ceruloplasmin (Cp) were influenced by CuD and FeD diets, while the copper chaperone for superoxide dismutase (CCS) and the ratio of CCS/Sod1 were influenced only by CuD diet and after just one week. Blood cuproenzymes from CuD repleted rats normalized after two weeks on the CuA diet. Next, for two weeks male postweanling Sprague Dawley rats were fed CuA or CuD diets and compared with a group of marginally copper deficient (CuM) rats whose water was copper supplemented. Although differences between the CuD and CuM groups versus the CuA groups were identified, only CCS showed statistically different results between all groups, allowing for the identification of marginally copper deficient individuals. Further, there was high negative statistical correlation between liver copper and erythrocyte CCS.

For all mouse trials, Swiss Webster mice were fed either CuD or CuA diets for five weeks. In one experiment, postweanling mice were housed in solid bottom cages and thus had access to their feces. In another postweanling mice were housed in stainless steal mesh bottom cages. CuD mice from solid bottom cages were found to be less deficient, likely due to coprophagia. These animals showed no reduction in plasma Cp or Sod1 expression, but did have higher CCS expression. Lastly, adult Swiss Webster mice were house in stainless steal mesh bottom cages and then evaluated for copper deficiency. CuD adult mice had no changes in Cp activity, liver copper levels, hemoglobin quantities, or Sod1 and Cp expression, but they did have a significantly elevated expression of CCS.

These rodent model results indicated that CCS maybe an excellent biomarker for marginal copper deficiency in human diagnosis due to CCS's reliability and sensitivity to variable copper status.

Table of Contents

List of Tables

List of Figures

Thesis Organization

Chapter 1 of this thesis is an introduction to the importance of nutritional copper states and the current biomarkers for the assessment of copper deficiency with an emphasis on the copper chaperone for superoxide dismutase. Chapter 2 is a manuscript that has been published by the journal *Nutrition Research* (Lassi, K.C., Prohaska, J.R. (2010) Nutr. Res. 31:698-706). Chapter 3 is a manuscript that has been accepted by The Journal of Nutrition for publication. Chapter 4 is an expanded discussion focusing on the implications of the rodent model studies and suggestions for future research.

CHAPTER 1

Copper is an essential dietary trace metal that is responsible for normal functioning of approximately 12 mammalian enzymes (Prohaska 2006). Once copper is ingested it is absorbed in the intestines, shuttled into the blood, and then distributed through out all tissues in the body with large quantities collecting in the liver (Linder *et al.* 1998). The uses for copper within mammals is diverse and includes antioxidant defense, melanin synthesis, catecholamine synthesis, tissue cross linking, neuropeptide and peptide hormone processing, and iron homeostasis (Prohaska 2006). Mammals that do not have adequate amounts of copper generally experience physical symptoms in the form of spastic gait (commonly referred swayback), sensory ataxia, and peripheral neuropathy due to demyelination in the central nervous system (Kumar *et al.* 2004b, Jensen *et al.* 1958, Bennetts & Chapman 1937). The hematological effects of copper deficiency include sideroblastic anemia, leukopenia and neutropenia (Dunlap *et al.* 1974). Copper exists in two forms under physiological conditions, Cu^{2+} and Cu^{1+}, and cells use copper in single-electron-transfer reactions. However, copper's useful redox properties also make it highly toxic as it can catalyze the production of hydroxyl radicals that can damage major biomolecules like proteins, DNA, and lipids (Halliwell & Gutteridge 1985).

The recommended dietary allowance (RDA) of copper, 0.9 mg for adults, is usually met though a normal diet, although some subgroups of the population have difficulty obtaining or maintaining adequate copper levels for normal functioning. The amount of copper required for pregnant and lactating women may need to be increased to meet the

needs of the mother and growing child (Prohaska & Brokate 2002). People ingesting excessive amounts of zinc through zinc containing denture cream, cold medications, or pica (such as coins) have disrupted copper absorption (Kumar & Jazieh 2001). Excess zinc ingestion induces the expression of the copper chelating protein metallothionein, which has a greater affinity for copper than zinc. Metallothionein remains bound to the copper molecules until they are sloughed off in the feces preventing adequate absorption (Cousins 1985, Broun *et al.* 1990). Absorptive disorders due to gastrointestinal tract inflammation or bariatric surgery can also lead to copper deficiency when RDA levels are not increased (Juhasz-Pocsine *et al.* 2007, Kumar *et al.* 2004a). Incidence of copper deficiency in the American population is estimated to increase as the popularity of bariatric surgery increases to reduce the escalating rates of obesity and diabetes mellitus type II. The severity of these diseases have been shown to be reduced through these surgeries (Trus *et al.* 2005, Santry *et al.* 2005). Also, as the general age of the American people increases due to baby boomer age progression, the population will also be using and consuming more zinc laden denture cream which could also lead to larger populations of copper deficient people (Iskandar *et al.* 2005, Nations *et al.* 2008, Afrin 2010). Currently there are no accurate and reliable methods for the identification of moderate copper deficiency in humans (Bertinato & Zouzoulas 2009). These possible increases in activities that have been shown to be correlated with copper deficiency make a reliable and sensitive biomarker for copper deficiency even more necessary.

Currently, copper deficiency is detected most often through ceruloplasmin (Cp) activity from plasma or the measurement of copper in plasma Cp activity is used in

human measurements due to its ease of collection through a blood sample and apparent sensitivity to copper deficiency. Plasma copper is largely reflected in plasma Cp as about 90 to 95 percent of plasma copper is bound to this protein (Harvey *et al.* 2009). However, plasma Cp is an acute phase protein whose abundance can vary depending on the individual's age or sex, illness, localized and general acute or chronic inflammation, pregnancy, arthritis, drug use, mental disorders, and stress (Reiser *et al.* 1985, Johnson & Prohaska 2000, Ganaraja *et al.* 2004, Bielli & Calabrese 2002, Montagna *et al.* 1994). Cp is thus limited in its reliability as a copper biomarker. Although liver copper levels are an accurate and reliable way to diagnose copper deficiency it is a needlessly invasive procedure and is thus unfit for routine human analyses.

Other copper biomarkers that have previously been evaluated are included in Table 1-1, the most promising of which have been copper, zinc superoxide dismutase (Sod1), the chaperone for copper, zinc superoxide dismutase (CCS), and the ratio of CCS to Sod1. Sod1 protein quantity in erythrocytes is lower in copper deficient animals, but reduction is modest even in severely deficient animals (Broderius & Prohaska 2009). This characteristic makes the use of this protein unsuitable for human diagnosis as it is rare for humans to be severely copper deficient. Erythrocyte CCS not only shows marked increases in copper deficient animals, but its quantities are also reduced in copper overloaded animals; this makes CCS not only a good biomarker for copper deficiency, but for copper status in general (Bertinato *et al.* 2010). CCS has been previously studied in rats, mice, and cows following severe copper deficiency. Human CCS responses have only been studied following supplementation (Suazo *et al.* 2008). CCS's high

augmentation of expression in severely deficient animals implies that it may also respond adequately to moderate, more human like copper deficiency. There is only a single research communication on this topic with one sample analyzed (Bertinato *et al.* 2003). Thus investigation of the response of red cell CCS following marginal copper deficiency seems warranted.

CCS is a homodimetric protein with 33 kDa subunits found mainly in the cytoplasm that functions as a metallochaperone (Rothstein *et al.* 1999, Casareno *et al.* 1998). CCS helps to transport copper molecules safely to the correct protein, superoxide dismutase (Sod1). Sod1 then catalyzes superoxide breakdown to hydrogen peroxide. CCS is made of three domains: the N-terminal or copper binding site, the central domain that is highly similar to and interacts directly with Sod1, and the C-terminus which is highly conserved among species and also binds copper (Schmidt *et al.* 1999). CCS and its analogs have been identified in animals, plants, fungi, and prokaryotes including archea illustrating the widespread ubiquity of this protein and the importance of copper throughout biological kingdoms (Robinson & Winge 2010). Lys7 (the yeast analog of mammalian CCS), was first discovered in yeast as the transporter of the copper ion to activate superoxide dismutase. Yeast strains that lacked Lys7 exhibited the same behavior as Sod1 knockout strains (Culotta *et al.* 1997). Recent studies in mice have also suggested a link between lowered CCS and increased likelihood of the development of Alzheimer's disease. Although the exact method of alteration is not known, Gray *et al.* (2010) found a link between the decreased expression of CCS and an increase in amyloid-β production at the BACE1 site.

CCS is coded for by a single gene on chromosome 11 and is ubiquitously expressed in all human tissues. CCS is highly specific to and exhibits a large proprtion of structural homology with its target protein Sod1 (Schmidt et al. 1999). Human CCS is 85% identical to rat and 87% to mouse CCS, but only 26% identical to its homolog in yeast (Hiromura *et al.* 2000, Bartnikas *et al.* 2000, Wright *et al.* 2011). In animals, CCS levels have been shown to be consistently inversely related to copper levels, but the mechanism for its quantity flux is not fully known (Bertinato *et al.* 2003). CCS quantities and activity by copper is regulated post-transcriptionally. Monitored degradation rates of CCS in human and rat cultured cells has shown that increasing levels of copper result in a faster turnover rate for CCS and decreased copper resulted a resistance to degradation by CCS. It has been theorized that the binding of copper to CCS results in conformational changes that make the protein less stable and therefore more likely to be degraded by the 26 S proteosome (Bertinato & L'Abbe 2003). Brady et al. (2010) also support the idea that CCS quantities are regulated by post-translational proteosome degradation, but they theorized varying ubiquitination by the X-linked inhibitor of apoptosis (XIAP) of CCS in relation to copper levels determines CCS quantities.

Currently, very few CCS studies have been done on adult animals, moderately deficient animals, or human populations. The studies in this thesis focus upon moderate deficiency in order to advance the study of biomarkers for copper deficiency that could be applied to human diagnosis. Major topics researched included the timing of copper deficiency symptoms in blood, the reversibility of copper deficiency alterations to blood, differences between marginal copper deficiency in rats and mice, and response of CCS to

adult copper deficiency.

Table 1-1. Currently used copper biomarkers for copper deficiency

Biomarker	**Notes**	**Reference(s)**
Ceruloplasmin (Cp) activity	Acute phase protein that binds about 95% of serum Cu.	(Holmberg & Laurell 1947, Bielli & Calabrese 2002)
Erythrocyte superoxide dismutase (Sod1)	Levels influenced by diet (unrelated to Cu). Does not exhibit sensitive responses.	(Feillet-Coudray *et al.* 2000, Reiser *et al.* 1985)
Platelet cytochrome C oxidase (CCO)	Found to have a highly variable response to CuD, may not be adequate for marginal copper deficiency.	(Milne 1998)
Plasma diamine oxidase (DAO)	Sensitive reduction to marginal copper deficiency but influenced by inflammation.	(Kehoe *et al.* 2000, DiSilvestro *et al.* 1997, Luk *et al.* 1980)
Lysyl oxidase	Quantity is influenced by Cu deficiency and excess, variable expression throughout different tissues, invasive skin biopsy.	(Rucker *et al.* 1996, Harris 1976)
Peptidylglycine a-amidating mono-oxy- genase (PAM)	Less responsive to external non-Cu related factors than Cp, sensitive to marginal copper deficiency. Difficult to assay.	(Prohaska *et al.* 1997)
Erythrocyte copper chaperone for superoxide dismutase (CCS)	Sensitive, has not been studied in humans.	(Bertinato & L'Abbe 2003, West & Prohaska 2004, Suazo et al. 2008))
Liver copper	Accurate and sensitive in models, highly invasive and unsuitable for human diagnosis	(Prohaska 2006)

CHAPTER 2
Rapid alteration in rat red blood cell copper chaperone for superoxide dismutase (CCS) following marginal copper deficiency and repletion

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Running title: Marginal copper deficiency alters rat erythrocyte CCS and Sod1

List of Abbreviations:

AAS: flame atomic absorption spectroscopy

CCS: copper chaperone for superoxide dismutase

Sod1: copper, zinc superoxide dismutase

Cp: ceruloplasmin

CuD: copper-deficient

CuM: copper-marginal

CuA: copper-adequate

ABSTRACT

There is an increased incidence of copper deficiency in humans. A sensitive and reliable blood biomarker may reveal additional cases of less severe deficiency. In the first experiment, weanling male Sprague Dawley rats were offered a copper deficient diet (CuD) for 4 weeks and samples were evaluated after 1, 2, and 4 weeks and compared to copper adequate (CuA) controls. Also, iron deficient rats were included for comparison following 2 weeks of depletion. Red blood cell and plasma cuproenzymes were evaluated through Western blot analysis or enzyme assay. Superoxide dismutase (Sod1) and ceruloplasmin (Cp) protein were found to be altered by both iron and copper deficiency, while the copper chaperone for superoxide dismutase (CCS) and the CCS/Sod1 ratio were found to only be altered only in CuD rats; and importantly, after only 1 week of treatment. Following 4 weeks of CuD depletion, 2 weeks on CuA diet restored cuproenzyme levels to control values. In a second experiment, marginal copper deficient (CuM) rats were compared to CuA and CuD rats after 2 weeks of treatment. Sod1, Cp, and CCS/Sod1 abundances were lower in CuM and CuD groups compared to CuA rats, but there was no statistical difference between the CuM and CuD groups. However, CCS was statistically different between all groups and the abundance highly correlated with liver copper concentration. These results indicate that red cell CCS may be an excellent candidate for diagnosis of rapid and marginal copper deficiency.

Key words: copper-deficient; superoxide dismutase; copper chaperone for superoxide dismutase; red blood cell, biomarker.

INTRODUCTION

Copper is an essential dietary component that plays an important role in many biological processes. Copper is necessary for the correct functioning of many biochemical reactions and is the cofactor for approximately twelve mammalian enzymes (cuproenzymes) (Prohaska 2006). Copper is known to be necessary to achieve normal hemoglobin levels thus preventing anemia as reviewed recently (Fox 2003). Currently, the recommended dietary allowance (RDA) for copper is 0.9 mg for an adult. This RDA for copper is usually met through a normal diet, and because of this, copper deficiency (CuD) is often not recognized as a public health issue. Subsequently, the biomarkers that indicate the onset of marginal copper deficiency have not been fully characterized.

However, some subgroups have consistently demonstrated problems obtaining adequate copper in their diet for normal functioning. For example, the copper requirement for pregnant and lactating women may be higher than currently set, and if not met, could lead to pediatric copper deficiency (Fox 2003). Absorptive disorders due to inflammation or gastrointestinal surgeries may lead to copper deficiency (Prohaska & Brokate 2002, Juhasz-Pocsine *et al.* 2007). Excessive zinc ingestion can lead to copper deficiency (Halfdanarson *et al.* 2008, Kumar & Jazieh 2001, Nations *et al.* 2008). Current and projected rates of bariatric surgeries to deal with obesity and diabetes mellitus type 2, may result in a larger proportion of the population becoming copper deficient (Kumar et al. 2004a, Tan *et al.* 2006).

Therefore, diagnosis of marginal copper deficiency is critical, and a reliable biomarker should be identified. Traditionally, the level of copper in plasma has been

quantified directly or through the measurement of the activity of the cuproenzyme ceruloplasmin (Cp), which generally binds about 95% of plasma copper (Harvey *et al.* 2009). However, Cp is an acute-phase reactant. Its abundance can also vary depending upon age, sex, arthritis, myocardial complications, pregnancy, and drug use (Mendez *et al.* 2004, Ganaraja *et al.* 2004, Gruys *et al.* 2005). Because of the impact of these factors on Cp abundance, it limits Cp as a biomarker to diagnose marginal copper deficiency.

Other possible biomarkers for diagnosing moderate copper deficiency have been proposed. One studied by many is erythrocyte copper-zinc superoxide dismutase (Sod1) (Bohnenkamp & Weser 1976, Andrewartha & Caple 1980, Okahata *et al.* 1980). Sod1 protein activity and concentrations have been shown to be lower in multiple rodent tissues including blood following copper deficiency. However, changes in Sod1 activity and protein concentrations are often modest following even severe copper deficiency (Prohaska & Brokate 2001b). Another candidate biomarker is the copper chaperone for Sod1 (CCS). CCS protein levels are higher in copper deficient rodent tissues (Bertinato et al. 2003, Prohaska *et al.* 2003a). CCS has shown promise in previous studies as a possible biomarker for marginal copper deficiency, but it was only evaluated after six weeks of dietary treatment (Bertinato et al. 2003). CCS has been evaluated in a single human supplementation study, but not following copper deprivation (Suazo et al. 2008).

CCS abundance has been shown to increase in erythrocytes following copper deficiency. In fact, the ratio in blood of CCS/Sod1 has been proposed as an even better biomarker than either protein by itself (West & Prohaska 2004). Although CCS has shown promise as a possible biomarker, the majority of studies conducted to evaluate this

have been done on severely copper deficient rodents. The purpose of current studies was to determine if CCS and Sod1 could be used for reliably diagnosing marginal copper deficiency. A secondary goal was to determine how soon changes in CCS and/or Sod1 could be detected following dietary copper deficiency restriction. Thirdly, how fast would putative changes disappear following dietary copper repletion.

MATERIALS AND METHODS

Experimental Diets and Animals

Weanling (postnatal day 21, P21) male Sprague Dawley rats were purchased from Charles River, weights upon arrival averaged 53 g. Rats in Experiment 1 (Exp. 1) were given deionized water to drink and were fed either a copper-deficient (CuD), iron deficient (FeD), or copper-adequate (CuA) modified AIN-93G diet (Teklad, Madison, WI) (Bastian *et al.* 2010). The modified AIN-93G CuD diet contained 0.46 mg Cu/kg and 78 mg Fe/kg, the FeD diet contained 7.2 mg Cu/kg and 5.3 Fe/kg, and the CuA diet contained 8.73 mg Cu/kg and 80.3 mg Fe/kg. Exp. 1 rats were sacrificed at intervals of 1 week (P28), 2 weeks (P35), and 4 weeks (P49). After the initial CuD diet was consumed for 4 weeks, the CuD animals were repleted with CuA diet for 2 weeks (P63). FeD rats were only evaluated at P35.

Rats in Experiment 2 (Exp. 2) were divided into three groups: copper-adequate group were fed CuA diet and deionized water, copper-deficient group were fed CuD diet and deionized water, and copper-marginal rats (CuM) were fed CuD diet and were supplemented with either 360 or 830 μg Cu/L in deionized drinking water. Data from

rats in the two CuM groups were pooled because of similar characteristics and Cu intake. Based on results in Exp. 1, Exp. 2 rats were sacrificed after 2 weeks of treatment (P35). All rats were maintained at 24 °C with 55% relative humidity on a 12-h light cycle (0700-1900 h). All protocols were approved formally by the University of Minnesota Animal Care and Use Committee. All rats were housed in stainless steel cages, weighed weekly, anesthetized with an intraperitoneal ketamine/xylazine injection, and killed by cardiac puncture.

Isolation of Erythrocytes

Rat blood was collected by cardiac puncture. Blood collection needles were rinsed with the anticoagulant acid citrate dextrose (ACD) consisting of trisodium citrate dihydrate (22 g/L), citric acid monohydrate (8 g/L), and dextrose (24.5 g/L). Collected whole blood was mixed with ACD at a ratio of 1 mL blood to 0.16 mL ACD. Blood was centrifuged at 160 x g for 20 minutes at 25 °C, the supernatant (plasma) was removed and stored at -20 °C. The resulting red blood cell pellet was centrifuged again at 1000 x g at 4 °C for 5 minutes and the pellet was washed twice with an equal volume of phosphate buffered saline. Red blood cells were lysed as previously described (Broderius & Prohaska 2009). An aliquot of red blood cell lysate was treated with 0.4 volumes of chloroform/ethanol (15:25, v/v) to remove hemoglobin which is known to interfere with Sod1 detection in red blood cells (West & Prohaska 2004).

Biochemical Analyses

Liver was removed, rinsed with deionized water, and a portion was processed for metal analysis. The remainder was quick-frozen in liquid nitrogen. Tissue and diet were

wet-digested with 4 ml of 8M HNO3 (Trace Metal grade, Fisher Scientific, Pittsburgh, PA) and the residue was suspended in 0.1 M HNO3 and analyzed for copper and iron by flame atomic absorption spectroscopy (AAS) (Model AA240 FS, Varian Walnut Creek, CA). Accuracy of the method was checked by analysis of NBS primary standard, bovine liver. Total protein content of plasma was determined by using a modified Lowry method with bovine albumin as a reference (Markwell *et al.* 1978, Prohaska 1983). Hemoglobin was determined spectrophotometrically as metcyanohemoglobin (Prohaska 1983). Plasma ceruloplasmin diamine oxidase activity was determined using o-dianisidine as substrate as described elsewhere (Prohaska 1991).

Immunoblot Protocols

The following proteins were targeted for detection in Western immunoblots: Sod1, CCS, glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Cp, and transferrin (Tf). Blood proteins were boiled with SDS Laemmli sample buffer for 5 min at 95 °C, were size fractionated on either 8% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gels, electroblot transferred to 0.2 μM nitrocellulose membranes (Protran; Schuell, Keene, NH), and Ponceau S (Sigma-Aldrich Co, St, Louis, MO) stained to ensure equal protein loading. Detailed Western immunoblotting protocols are provided elsewhere (Prohaska & Brokate 2001a).

To detect Sod1, membranes were probed with rabbit antibovine Cu, Zn-SOD (AB 1237; Chemicon International Inc, Temecula, CA) diluted to 1:5000. For CCS detection, affinity-purified rabbit antihuman CCS primary antibody diluted to 1:1000 was used, as previously characterized (West & Prohaska 2004). Membranes that used Gapdh as a

loading control were reprobed with mouse antirabbit Gapdh (MAB374, Millipore, Billerica, MA) diluted to 1:1000. Goat antirat ceruloplasmin (Innovative Research, Novi, MI) diluted 1:4000 to was used to probe for rat Cp. Membranes with plasma used transferrin (Tf) as a loading control were reprobed with goat antihuman transferrin (Sigma-Aldrich Co, St, Louis, MO) diluted to 1:3000. Appropriate secondary antibodies were used. Immunoreactivity was detected via chemiluminescence with SuperSignal (Themo Scientific, Rockford, IL) and image captures and densitometry were performed on the FluorChem system (Alpha Innotech, San Leandro, CA).

Statistical Analyses

Means and SEM were calculated. Variance equality was evaluated using the F-test or Barlett's test. Significant differences were evaluated at $\alpha = 0.05$. For Exp. 1, data were analyzed by factorial ANOVA (diet x time). Significant interactions terms were evaluated by Tukey's test. For P35 data, with FeD rats, one way ANOVA and Tukey's test was used to compare mean differences between the three groups. Student's unpaired two-tailed t-test was used when comparing P63 CuA and CuD repleted rats in Exp. 1. One way ANOVA and Tukey's test was used to compare mean differences for Exp. 2. Correlation coefficients were also calculated for Exp. 2 data. Calculations were performed using Microsoft Excel™ or KalidaGraph (Synergy Software, Reading PA). For comparative purposes immunoblot data were normalized. A value of 1.0 was assigned to the mean pixel density of CuA samples then both CuA and CuD individual densities were recalculated before graphing. Some other data was normalized for graphical presentation.

RESULTS

Biochemical Characteristics of Experimental Rats

Thirty male Sprague Dawley rats were utilized in Exp. 1. On average these rats had a body mass of 53 g at the onset of the study (P21). Following 4 weeks of copper deficiency there was no significant impact of diet on body weight, however, as expected there was a strong impact of time on the body weight due to normal growth of the adolescent rats ($P<0.01$). Following 4 weeks of dietary treatment the mean body weight of the six rats at that time point group was 289 ± 9.0 g. Thus, the rats in this study on the new modified AIN-93G diet grew well and appeared healthy throughout the experimental protocol.

Liver metals are often used to evaluate copper status of rodents on purified diets, thus liver copper and iron were determined over time on the animals used in Exp.1 by atomic absorption spectroscopy (AAS) (Figure 2-1). Liver copper was rapidly depleted in these growing rats fed the CuD diet, even by one week on the diet and continued to decrease throughout the course of the study. After 4 weeks on the diet, CuD rats were offered copper adequate diet for 2 weeks, results indicated a rapid restoration of liver copper levels in these repleted rats. Previous versions of the AIN-76A and 93G diets usually result in an accumulation of liver iron in copper deficient rats. However the new modified AIN-93G diet with extra iron resulted in liver iron levels that were not different due to dietary treatment across the time course of the experiment. Two weeks of copper repletion had no impact on liver iron (Figure 2-1). Thus, based on liver metal data, the

new AIN-93G diet resulted in copper deficiency based on the rapid decrease in liver copper concentrations, however, unlike previous studies, liver iron was not augmented in the CuD rats in these studies.

Anemia is a common feature of dietary copper deficiency, thus hemoglobin measurements were performed on each animal in Exp.1. Results indicate that there was a significant impact of the CuD diet on hemoglobin levels over the 4 weeks of copper depletion (Figure 2-1). Two weeks of copper repletion was sufficient to restore this deprivation back to normal CuA levels. Plasma iron was also impacted by copper deficiency over the time course of depletion. There were no significant interactions in terms of time on the diet for plasma iron. Plasma iron concentration also responded rapidly to repletion, and after 2 weeks levels were not significantly different than values in CuA rats. Thus, the imposition of postnatal copper deficiency in male Sprague Dawley rats on this protocol produced animals that were marginally anemic with lower liver copper and plasma iron concentrations but normal liver iron levels.

In order to ensure that the impact of copper deficiency on the biomarkers to be studied was unique and not similar to another trace metal deficiency that impacts blood, i.e. iron deficiency, three additional male rats were studied after a two-week dietary depletion protocol (Figure 2-1). The same measurements made in CuA and CuD rats were determined in the P35 iron deficient (FeD) male rats. Iron deficiency over this acute period of time did not significantly impact body weight as assessed by one-way ANOVA (data not shown). However, the FeD rats had significantly higher liver copper levels, a highly significant lower hemoglobin concentration, and greatly diminished liver iron and

plasma iron levels consistent with frank iron deficiency.

Western Immunoblot Analysis of Blood Following Copper or Iron Deficiency

Plasma and red blood cells were analyzed for a number of potential biomarkers throughout the study and an example illustrated following 2 weeks of copper or iron deficiency (Figure 2-2). Erythrocyte Sod1 was impacted by both dietary copper deficiency and iron deficiency. On average the FeD Sod1 density was higher than either CuA or CuD rats, however the density for Sod1 was lower in CuD compared to CuA rats following 2 weeks of treatment (Figure 2-2A) $P<0.05$. In contrast, CCS density was not impacted by iron deficiency while it was clearly augmented in the three CuD rats. Calculation of the CCS/Sod1 ratio, used previously as a potential biomarker for copper status, was only augmented in the CuD rats. Iron deficiency had no impact on the erythrocyte CCS/Sod1 ratio. Gapdh abundance was not impacted by either dietary copper deficiency or iron deficiency.

Following 2 weeks of copper or iron deficiency, the abundance of two plasma glycoproteins was evaluated (Figure 2-2B). Copper deficiency greatly diminished the abundance of plasma ceruloplasmin (Cp), and there was also a significant, yet smaller, diminution in the density of Cp in the FeD compared to CuA rats ($P<0.05$). There was also a significant augmentation in plasma transferrin (Tf) levels in the FeD group compared to CuA or CuD (Figure 2-2B), however Tf was not impacted by copper deficiency after 2 weeks of dietary copper deficiency. The results shown in Figure 2-2 were confirmed by replicate immunoblots (data not shown).

Evaluation of Erythrocyte CCS and Sod1 as Biomarkers Following Dietary Copper

Deficiency and Repletion

Immunoblots were also performed on erythrocyte lystate extracts either with or without organic solvent treatment to determine the abundance of the CCS and Sod1 proteins following 1, 2, or 4 weeks of dietary copper deficiency and 2 weeks of copper repletion (Figure 2-3). There was a rapid and sustained augmentation in the abundance in CCS as early as 1 week following dietary copper deficiency. Similarly there was a rapid diminution in the abundance of erythrocyte Sod1. The ratio of CCS/Sod1 was significantly augmented at each time point evaluated during the four-week depletion period. Throughout the experiment (depletion and repletion) no changes in abundance of Gapdh were detected. This protein was used a loading control for the CCS blots because they were not treated with an organic solvent. It was not possible to use a loading control on the Sod1 blots as samples were pretreated with organic solvents.

Following 2 weeks of copper repletion, mean values for CCS, Sod1, and CCS/Sod1 ratio were compared by Student's t-test and found to be equivalent between repleted and CuA control rats, despite the appearance of an incomplete recovery ($P>0.05$) (Figure 2-3).

Impact of Dietary Copper Deficiency on Plasma Biomarkers

The plasma diamine oxidase of Cp was evaluated during depletion and following repletion from dietary copper deficiency (Figure 2-4). There was a rapid and near total loss of enzyme activity as early as 1 week of dietary copper deficiency. Activity rebounded to CuA levels after 2 weeks of repletion. There was also a significant effect of time and a significant interaction in evaluating enzyme activity of Cp. CuA rats at 1 week

were different from the rats at 2 or 4 weeks, but the CuA at 2 and 4 weeks were not different. The activity of Cp was also evaluated following two weeks of iron deficiency. In contrast to the densitometry data (Figure 2-2), Cp activity of the iron deficient rats was not different than CuA rats, but much higher than the CuD rats. Mean Cp activity of the iron deficient (FeD) rats was 78.2 ± 7.3 units/L.

Following dietary copper deficiency, the relative density of plasma Cp was greatly diminished in as early as 1 week, this persisted throughout the depletion period (Figure 2-4). Following 2 weeks of repletion, CuD rats Cp density was actually significantly higher than CuA rats ($P<0.05$). Assessment of Tf as a loading control on the immunoblots for this data revealed that there was no effect of CuD diet or repletion on Tf abundance (data not shown).

Biochemical Characteristics of Marginally Deficient Rats

To confirm and extend the results of Exp. 1, 12 male Sprague Dawley rats were divided into three treatment groups (one CuA rat died of natural causes shortly into the study). A 2 week depletion time was used and marginal copper-deficient (CuM) was used to compare with CuD and CuA rats. Thus, there were CuD (N=3), CuM (N=6) and CuA (N=2) rats studied. At the beginning of the dietary trial, rats at P22 had an average body weight of 47.5 g. One-way ANOVA was used to evaluate the impact of diet on the three groups after 2 weeks of treatment. There was no significant impact of diet on body weight or hemoglobin level in this experiment (Figure 2-5). Also, liver iron and plasma iron were not altered (data not shown). Liver copper levels were statistically different between all groups with the copper deficient group (CuD) having the lowest levels, as

expected ($P<0.05$).

Impact of Marginal Dietary Copper Deficiency on Erythrocyte and Plasma Biomarkers

Erythrocyte biomarkers, CCS and Sod1, were evaluated on lysate extracts either with or without organic solvent treatment. Sod1 was statistically lower in CuD and CuM rats as compared to CuA, but there was no difference between CuD and CuM rats (Figure 2-6). CCS expression was statistically different between all groups with CuD animals exhibiting the highest abundance (Figure 2-6). Gradation of CCS expression was highly correlated with the concentration of liver copper ($r=0.91$, $P<0.01$). The ratio of CCS/Sod1 did not show a difference between CuD and CuM, but both groups had a statistically higher ratio compared to CuA ($P<0.05$). Gapdh, the loading control for CCS, was equivalent between treatment groups.

Density of Cp and diamine oxidase of Cp were both statistically lower in CuD and CuM groups compared to CuA rats ($P<0.05$), but there was no difference between the two copper restricted groups, similar to Sod1 and CCS/Sod1 in red cells (Figure 2-5). The plasma loading control, Tf, was not different among the three groups (data not shown). Correlations to liver copper concentrations were also examined for additional biomarkers (Sod1, CCS/Sod1, Cp abundance, and Cp activity). Only Cp abundance was highly significantly ($P<0.01$) correlated with liver copper concentration ($r=0.81$) similar to erythrocyte CCS abundance.

DISCUSSION

Although prior to this study, the ratio of red blood cell CCS/Sod1 was shown to be higher in CuD rats, the timing of the alteration was not known . This study indicates that the ratio of CCS/Sod1 in CuD rat red blood cells is significantly altered as soon as 1 week following dietary treatment (Exp. 1, Figure 2-3). In the follow-up study (Exp. 2), in rats that were not severely CuD (not even anemic) there was a robust correlation between red cell CCS quantity and copper status. This correlation was not detected for Sod1 or the CCS/Sod1 ratio in red blood cells. CCS abundance in blood was highly negatively correlated with liver copper quantity. Liver copper reduction is often regarded as one of the most accurate ways to diagnose copper deficiency. Thus, red cell CCS abundance seems to be an excellent candidate as a copper status biomarker. Others have also commented on the potential of CCS as a copper biomarker (Bertinato & Zouzoulas 2009, Harvey & McArdle 2008).

CCS was first reported to be augmented in the blood of rats in 2003 (Bertinato et al. 2003). Concurrently, CCS was shown to be augmented not only in CuD rats, but also in CuD mice and in many tissues besides blood (Prohaska et al. 2003a). Subsequently it was demonstrated that since copper deficiency was also associated with a major reduction in Sod1, that the ratio of CCS/Sod1 in blood might even be a stronger biomarker (Prohaska & Brokate 2001b, West & Prohaska 2004). Current studies extend and support those original observations in rats and mice by suggesting that CCS in blood changes very rapidly in response to copper deficiency and can occur in marginal copper deficiency thus providing a more broad-spectrum biomarker than previously thought. Furthermore it appears that iron deficiency does not have an impact on red blood cell

CCS abundance. Additional support for CCS as a biomarker has recently been demonstrated in cattle (Hepburn *et al.* 2009). Additional studies have shown that rat CCS is augmented following high dietary zinc exposure, extending the use of CCS as a potential biomarker for zinc toxicity in humans and subsequent copper deficiency (Iskandar *et al.* 2005). Furthermore, use of CCS as a biomarker in humans has been evaluated. In those studies, CCS mRNA was reported as a useful tool to follow copper status in humans (Suazo et al. 2008). Further recent studies have shown that high levels of copper in the diet can suppress red blood cell CCS (Bertinato *et al.* 2010). This suggests that CCS may be a good biomarker for all doses of copper, being augmented when copper deficiency is expressed and suppressed when copper overload is present. This dose response is one of the characteristics of a good biomarker.

Changes observed in the current experiments in CuD rats were observed in animals in which the dietary iron was augmented compared to prior studies. Liver iron augmentation, a hallmark of dietary copper deficiency in rats, was not observed. This is similar to results with younger rats and rat dams (Bastian *et al.* 2011). CuD rats still displayed lower plasma iron and signs consistent with copper deficiency; however, augmentation in dietary iron to these rapidly growing rats attenuated the degree of anemia observed in the animals. In fact, in Exp. 2 the CuD rats were not statistically anemic as evaluated by hemoglobin levels, yet they clearly showed a demonstrable augmentation in red blood cell CCS. This argues that even in marginal copper deficiency, without anemia, CCS is a good biomarker for copper status.

Since clinically anemia would be evaluated primarily by hemoglobin levels, and

anemia is commonly thought to be caused by dietary iron rather than copper deficiency, it was important to address this concern as well. Preliminary results indicate that red cell CCS is not impacted by severe iron deficiency. FeD rats in these experiments were indeed severely anemic and had low plasma iron, but did not demonstrate changes in red cell CCS. However, current experiments did detect a significant reduction in Cp protein but not activity, and a significant augmentation in red cell Sod1 protein compared to levels in CuA rats. This suggests that CCS is less impacted by dietary iron deficiency than Cp or Sod1.

Other biomarkers and other cell types beside erythrocytres have been evaluated for potential to reflect copper status. In both of the current experiments (similar to CCS data) Cp protein concentrations were also significantly impacted by copper status. In Exp. 1 CuD rats exhibited significantly lower concentrations within 1 week of CuD diet onset and CuD rats in Exp. 2 had Cp levels that correlated positively with copper concentrations in the liver. In spite of these positive results, Cp may still not be an ideal biomarker due to its variable expression and acute phase response. Fluxes in this protein due to illness, inflammation, stress, and other non-copper deficiency related physiological factors make Cp potentially unreliable and limits Cp as a sole biomarker for human diagnosis of copper deficiency. Sod1 levels also rapidly responded to CuD in the current studies but were not as strongly correlated with liver copper as were CCS and Cp. A number of other cuproenzymes have been considered previously as reviewed in detail elsewhere (Harvey et al. 2009). That review concluded that plasma copper was the most reliable indicator studied thus far. Since plasma copper tracks with Cp expression

limitations to its utility are the same as for Cp. Several status indicators are likely superior to a sole biomarker.

Choice of plasma, a cell from blood, a buccal swipe, a skin biopsy, or a urinary biomarker are other considerations. Our lab evaluated platelet and white blood cell CCS and Sod1 in young rat pups and their mothers (Broderius & Prohaska 2009). Ease of purifying red cells and the robust expression of CCS and or Sod1 makes erythrocytes a good choice. Other cells may have utility under certain circumstances. For example, cytochrome c oxidase subunits are greatly reduced by copper deficiency but would only be detected in cells with mitochondria, thus eliminating red cells for this cuproenzyme.

The mechanisms for changes in CCS and Sod1 protein in rat red cells is not fully understood. Copper limitation does not appear to impact mRNA concentrations for these two proteins suggesting that altered transcription is not a likely cause for alterations in protein level (Prohaska & Brokate 2001b, Prohaska et al. 2003a). It is possible that Sod1 is reduced in CuD animals due to lower stability when the protein is not loaded with copper. However, the rate of Sod1 turnover in red cells has not been correlated with the reduction in copper. CCS augmentation in cells low in copper has been associated with slower ubiquitin-dependent turnover of CCS (Bertinato & L'Abbe 2003). This process may involve another copper binding protein XIAP (X-linked inhibitor of apoptosis) (Brady *et al.* 2010). It is not clear if this putative post-translational change is occurring in reticulocytes or mature red cells. Regardless of the mechanism, age and species must be considered in evaluating current model data. Rat circulating rat red blood cell have a life span of about 60 days, half that of humans, thus changes due to copper limitation may be

slower to detect in humans than rats (Ganzoni *et al.* 1971). Further rats in the current studies were growing and new blood volumes were continually being added to accommodate new circulatory needs. Because of these factors, early detection of moderate copper deficiency using CCS as a biomarker will be challenging in adult humans. However, current data obtained on the blood of copper repleted rats suggests that a CCS analysis taken before and after copper supplementation will be useful using each subject as there own control. Thus, CCS has great potential as a biomarker for copper status in humans.

ACKNOWLEDGEMENTS

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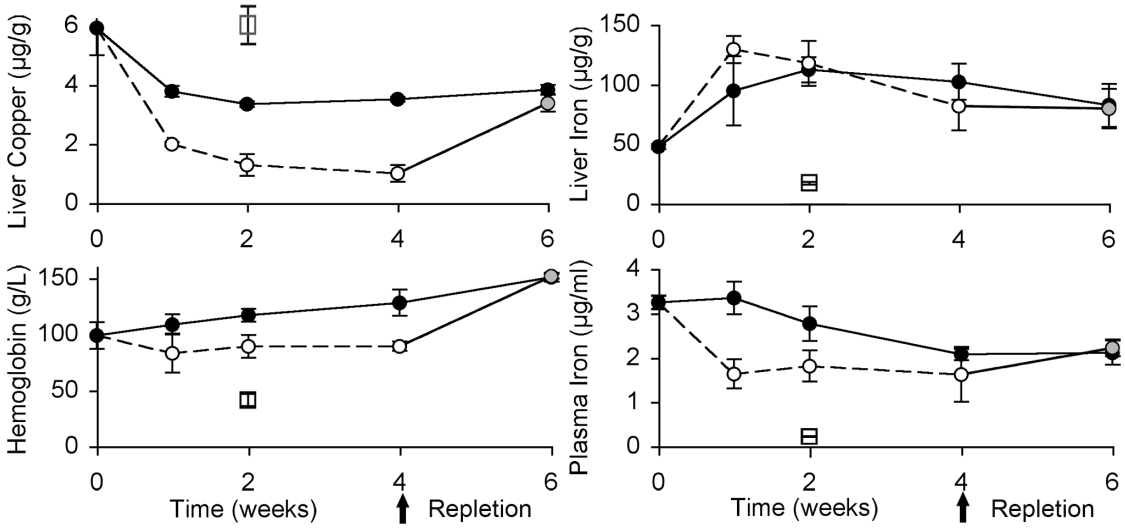
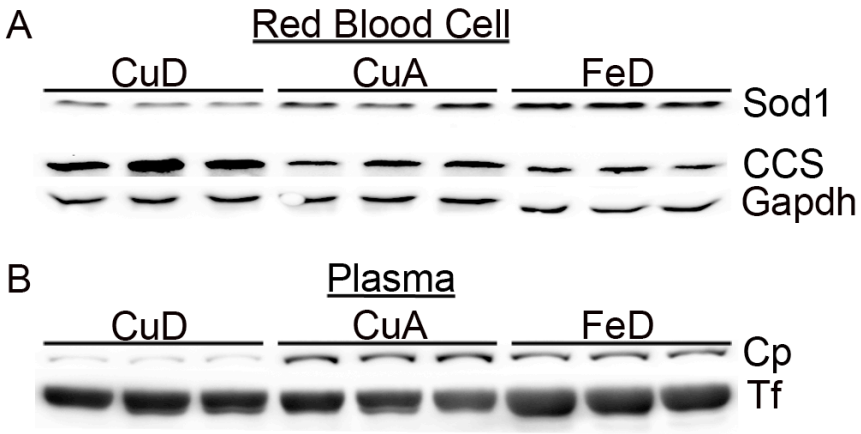
Figure 2-1. Impact of copper deficient (CuD) and iron deficient (FeD) diets on liver metals, hemoglobin, and plasma iron concentration



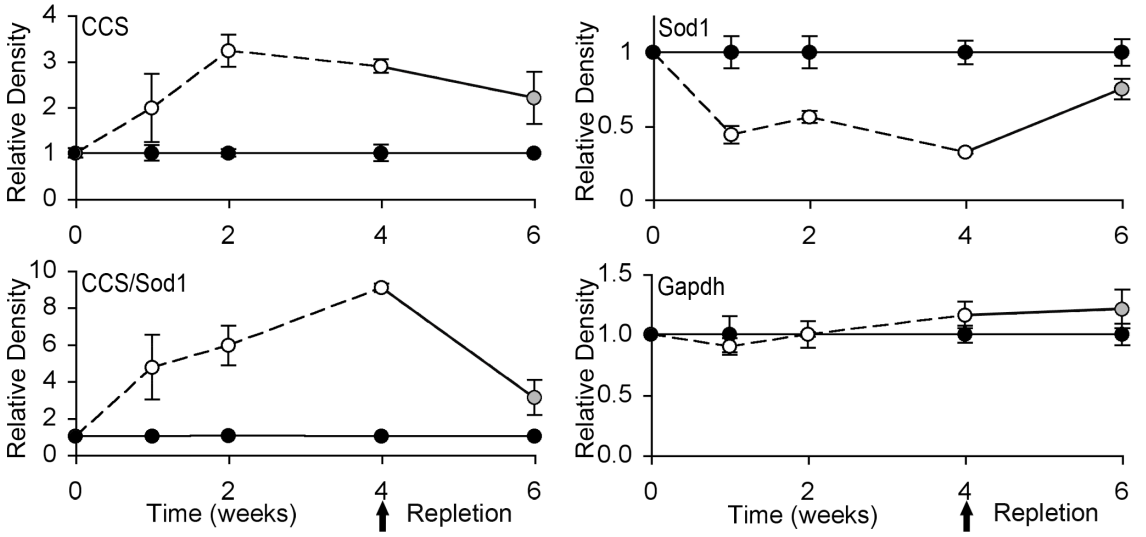
Means ± SEM are shown. Liver copper concentration of CuD rats (open circles) was lower throughout the dietary trial and returned to CuA (closed circles) levels upon repletion (shaded circles). FeD rats (open squares) exhibited significantly higher levels of liver copper compared to CuD and CuA rats. Liver iron was not significantly impacted by CuD diet at anytime in the dietary trials, but liver iron was significantly lower in FeD rats, as expected. Hemoglobin abundance was reduced at all time points during copper depletion. Upon repletion, CuD rats expressed normal hemoglobin levels. FeD rats had significantly lower expression of hemoglobin as compared to CuD and FeD rats. Plasma iron abundance was significantly lower during copper depletion, but returned to normal levels after repletion. FeD rats had a significantly lower level of plasma iron as compared to CuD and CuA rats.

Figure 2-2. Blood protein levels of P35 male rats following copper or iron deficiency



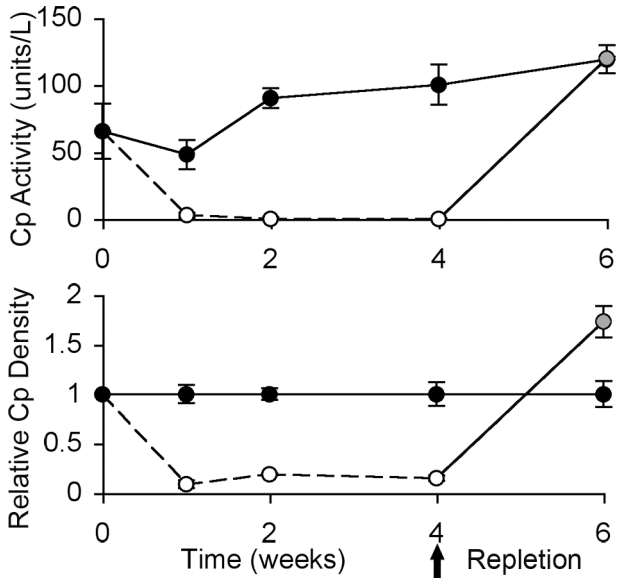
A. Following organic solvent extraction, 25 µg of red blood cell lysate protein was subjected to Western immunoblot techniques using 15% SDS PAGE denaturing gel electrophoresis and probed with rabbit anti-bovine Cu, Zn-superoxide dismutase (Sod1). On a separate gel, 31 µg of red blood cell lysate was subjected to immunoblot techniques using a 15% SDS PAGE gel and was probed with affinity purified rabbit anti-human copper chaperone for superoxide dismutase (CCS), then reprobed with rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as a loading control. B. Plasma extracts (15 µg protein) were subjected to immunoblot techniques using 8% SDS PAGE denaturing gels and probed with goat anti-rat ceruloplasmin (Cp), stripped, and reprobed with goat anti-human transferrin (Tf) to evaluate loading.

Figure 2-3. Impact of time of CuD diet on red blood cell proteins



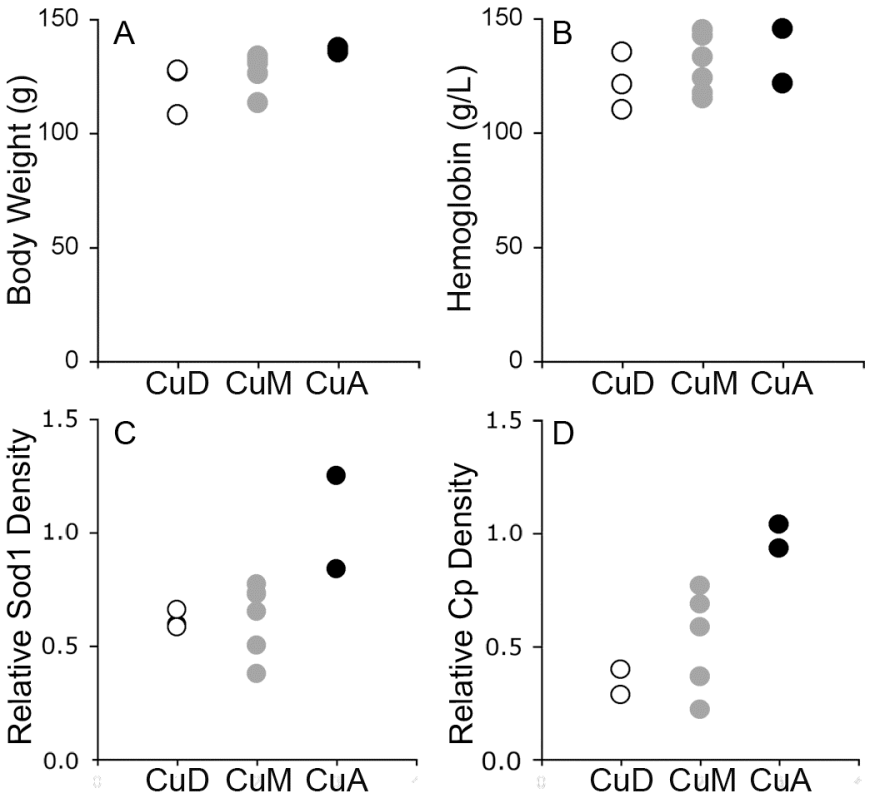
Relative expression of copper chaperone for superoxide dismutase (CCS) and superoxide dismutase (Sod1) were evaluated after immunoblot densitometry measurements. Means ± SEM are shown. Compared to CuA rats (solid circles), CuD rats (open circles) had significantly higher expression of CCS, lower expression of Sod1, and higher CCS/Sod1 ratios throughout the depletion period. Repleted rats (shaded circles) demonstrated no significant differential expression for CCS, Sod1, or the CCS/Sod1 ratio compared to CuA rats ($P>0.05$). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was not impacted by diet or time. Arrows indicate onset of repletion.

Figure 2-4. Impact of CuD diet on ceruloplasmin (Cp) abundance and activity in rat plasma



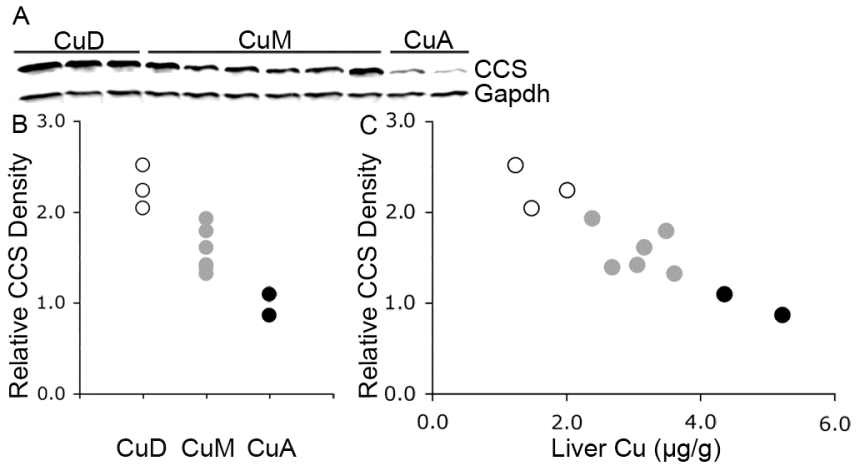
CuD rats (open circles) had significantly lower Cp activity than CuA rats (closed circles). Repleted rats (shaded circles) did not show differential activity compared to CuA rats. Relative expression plasma Cp density was significantly lower in CuD rats throughout the depletion. Cp density increased in repleted rats to a level significantly higher than CuA rats ($P<0.05$). Transferrin (Tf) level, used as a loading control, was not impacted by diet or time (not shown).

Figure 2-5. Impact of variable dietary copper on body weight, hemoglobin, erythrocyte superoxide dismutase (Sod1), and plasma ceruloplasmin (Cp) density.



Rats on the copper marginal (CuM) diet (shaded circles) did not show significant differences from rats on CuA (closed circles) or CuD (open circles) diets for any of the four variables.

Figure 2-6. Response of copper chaperone for superoxide dismutase (CCS) to marginal copper deficiency



A. Red blood cell lysate (31 µg) was subjected to immunoblot techniques using a 15% SDS PAGE gel and was probed with affinity purified rabbit anti-human CCS, then reprobed with rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as a loading control. B. Relative red blood cell CCS density in CuD (open circles), CuM (shaded circles), and CuA (closed circles) rats was determined. CCS expression was statistically different among all dietary groups, with CuD having the highest expression. C. Relative CCS density was highly correlated with liver copper abundance ($r=0.91$, $P<0.05$).

CHAPTER 3

Augmentation of erythrocyte copper chaperone for superoxide dismutase (CCS) following marginal copper deficiency in adult and post weanling mice

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List of Abbreviations:

AAS: flame atomic absorption spectroscopy

CCS: copper chaperone for superoxide dismutase

Sod1: copper, zinc superoxide dismutase

Cp: ceruloplasmin

CuA: copper-adequate

CuD: copper-deficient

ABSTRACT

A sensitive and reliable biomarker has yet to have been identified for marginal copper deficiency in humans. Four experiments were devised to induce marginal copper deficiency through copper deficient (CuD) diets (5 weeks for mice and 4 weeks for rats). In experiments 1 and 2, male post weanling mice were raised in either solid bottom plastic cages (Exp. 1) or stainless steel hanging cages (Exp.2). Adult mice and post weanling rats were also studied using hanging cages. CuD rats exhibited the most severe changes in biomarkers due to copper limitation, including major reductions in plasma ceruloplasmin (Cp) and erythrocyte superoxide dismutase (Sod1) and augmentation in copper chaperone for Sod1 (CCS). CuD mice in Exp. 2 were more deficient than CuD mice in Exp. 1, likely due to coprophagia differences. In fact CuD mice in Exp. 1 had unaltered Sod1 or Cp levels. Importantly though, these marginally deficient mice and CuD adult mice, that had no changes in Cp activity or liver copper level, had robust augmentation of CCS. Erythrocyte CCS was the only consistent biomarker to change in copper deficiency for all dietary groups suggesting that CCS may be an excellent biomarker for human diagnosis of marginal copper deficiency.

Key words: copper-deficient; superoxide dismutase; copper chaperone for superoxide dismutase; ceruloplasmin; red blood cell; biomarker; mice; rats.

INTRODUCTION

Copper is one of several micronutrients essential for development and function of mammals. While copper is only required for approximately 12 mammalian enzymes, it has a widespread impact on active functioning of most biological systems (Prohaska & Broderius 2006). In humans dietary intake of copper is thought to adequately match the needs of most healthy adults. However, there are an increasing number of cases describing adult copper deficiency in the population. Some of the explanations for this include the rising use of bariatric surgery to offset the negative aspects of obesity in America (Gletsu-Miller *et al.* 2011). There are also increasing incidents of copper deficiency due to zinc overload due to the improper use of dental creams as well as isolated cases of abnormal absorption of copper following intestinal surgery (Kumar et al. 2004a). A recent review of the current use of copper status indicators for humans concluded that the primary useful marker was plasma copper (Harvey et al. 2009). Plasma copper is largely reflected in the protein ceruloplasmin representing approximately 95 percent of total copper. However, ceruloplasmin is an acute phase protein and thus the increase incidence of inflammation associated with chronic disease would confound the use of ceruloplasmin as a biomarker. Several other recent reviews suggest there is a great need for developing a biomarker to assess copper status in humans.

One of the biomarkers discussed is the copper chaperone for superoxide dismutase (CCS) (Bertinato & Zouzoulas 2009, Harvey & McArdle 2008). CCS was first discovered in 1997 as a protein necessary for the metallation of copper zinc superoxide

dismutase in yeast (Culotta *et al.* 1997). Shortly thereafter CCS was shown to be essential for superoxide dismutase (Sod1) activation in mammals through generation of a CCS null mouse (Wong *et al.* 2000). Human CCS is coded for on chromosome 11, whereas mouse CCS is located on chromosome 19; however, the mouse protein is 87% identical to the human (Bartnikas *et al.* 2000). Much of the CCS biomarker evaluation has been conducted on experimental rats. Rat CCS, like the mouse protein, is 87% identical to the human (Hiromura *et al.* 2000). Thus, it is hypothesized that model studies on CCS in rodents may be useful for clinical studies in humans. In 2003, two concurrent publications suggested that CCS maybe a useful biomarker in mammals for copper status. Both of these studies reported that there was an augmentation in CCS abundance following dietary copper deficiency (Bertinato et al. 2003, Prohaska et al. 2003a).

Most of the model work that has been done assessing CCS as a biomarker has used dietary copper deficiency and overload using rats as models (Bertinato et al. 2010, Broderius & Prohaska 2009). For CCS to be a useful biomarker for humans it is necessary to demonstrate that the protein can be altered by marginal copper deficiency. Although this was previously shown in a single rat study, in the initial experiments, little work has been done subsequently to evaluate this issue. CCS is augmented in copper deficient mouse pups and dams following dietary copper deficiency (Prohaska et al. 2003a, West & Prohaska 2004). However these animals were severely copper deficient. It is known that dietary copper deficiency in rats is usually more severe in rats than in mice, for example following weaning, copper deficient rats typically show a growth stunting after approximately four weeks on a purified diet low in copper (Prohaska &

Heller 1982). However the exact same nutritional deprivation (same diet and time frame) does not impair growth of post weanling mice (Prohaska & Lukasewycz 1989).

The purpose of the current experiments was to test the hypothesis that CCS would be altered following marginal copper deficiency, further supporting the idea that CCS is a potential useful biomarker for humans. Several studies were conducted to compare marginal copper deficiency in mice. First, two different dietary methods were used to induce copper deficiency in post weanling mice. One in which the mice were kept on corn cob shavings in a sold bottom cage with access to their fecal material. Second, these mice were compared to mice reared in hanging stainless steel cages. A third experiment was conducted using adult mice. In all three mouse experiments copper deficiency was induced by using exactly the same diet and period of copper deprivation. These mouse studies were compared to a post weanling copper deficient rat model. Collectively these studies showed blood CCS is the best biomarker for marginal copper deficiency.

MATERIALS AND METHODS

Animal Care and Diets

Weanling (postnatal day 21, P21) male Sprague Dawley rats, P21 male Swiss Webster mice, and adult male retired breeder Swiss Webster mice were purchased from Charles River. All rodents were given deionized water to drink and were fed either a copper-deficient (CuD) or copper-adequate (CuA) modified AIN-93G diet (Teklad Labratories). The AIN-93G diet contained 0.46 mg Cu/kg and 78.3 mg Fe/g (CuD) or 8.73 mg Cu/kg and 80.3 mg Fe/g (CuA) (Bastian et al. 2010). Rats were sacrificed after

four weeks of treatment (P49). Mice in Experiments 1 and 2 (Exp. 1 and Exp. 2) were kept on treatment for five weeks and killed at P56. Exp. 1 mice were housed in "shoebox cages" with corn cob bedding and Exp. 2 mice were housed in hanging stainless steel (mesh bottom) cages. Adult mice in Exp. 3 were also reared in hanging cages for five weeks. All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (0700-1900 h). All protocols were formally approved by the University of Minnesota Institutional Animal Care and Use Committee. Rats were anesthetized with an intraperitoneal ketamine/xylazine injection and killed by cardiac puncture. All mice were lightly anesthetized and killed by decapitation.

Biochemical Methods

Activity of plasma ceruloplasmin (Cp) was determined by measuring oxidation of o-dianisidine at 37° C as described elsewhere (Prohaska 1991). Diets and selected tissues were wet digested in HNO3 (Trace Metal grade; Fisher Scientific, Pittsburgh, PA) and analyzed for copper and iron content by flame atomic absorption spectroscopy (Model AA240 FS, Varian Walnut Creek, CA). Plasma iron was also measured by flame AAS as described previously (Pyatskowit & Prohaska 2008). Hemoglobin was measured spectrophotometrically as metcyanohemoglobin. (Prohaska 1991).

Blood Collection and Processing

Rat blood was collected by cardiac puncture. Blood collection needles were rinsed with the anticoagulant acid citrate dextrose (ACD) consisting of trisodium citrate dihydrate (22 g/L), citric acid monohydrate (8 g/L), and dextrose (24.5 g/L). Mouse blood was collected dropwise after decapitation. Collected whole blood was mixed with

ACD at a ratio of 1 mL to 0.16 mL. Blood was spun at 160 x g for 20 minutes at 25 °C, the supernatant (plasma) was removed and stored at -20 °C. The resulting red blood cell pellet was spun at 1000 x g at 4 °C for 5 min and the pellet was washed twice with an equal volume of phosphate buffered saline. Red blood cells were lysed as previously described (Broderius & Prohaska 2009). An aliquot of red blood cell lysate was treated with 0.4 volumes of chloroform/ethanol (15:25, v/v) to extract hemoglobin as the protein Sod1 has similar mobility (West & Prohaska 2004).

Immunoblot Protocols

The following proteins were targeted for detection in Western immunoblots: Sod1, CCS, glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Cp, and transferrin (Tf). Membrane proteins were boiled with SDS Laemmli sample buffer for 5 min at 95 °C, were size fractionated on either 8% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gels, electroblot transferred to 0.2 μM nitrocellulose membranes (Protran; Schuell, Keene, NH), and Ponceau S (Sigma-Aldrich Co) stained to ensure equal protein loading. Full Western immunoblotting protocols are provided elsewhere (Prohaska & Brokate 2001a).

To detect Sod1, membranes were probed with rabbit anti-bovine Cu, Zn-SOD (AB 1237; Chemicon International Inc, Temecula, CA) diluted to 1:5000. For CCS detection, affinity-purified rabbit anti-human CCS primary antibody diluted to 1:1000 was used, as previously characterized (West & Prohaska 2004). Membranes that used Gapdh as a loading control were reprobed with mouse anti-rabbit Gapdh (Millipore, Billerica, MA) diluted to 1:1000. Goat anti-rat ceruloplasmin (Innovative Research, Novi, MI) diluted

1:4000 to was used to probe for rat Cp. Goat anti-human Cp antiserum (Sigma C0911) was diluted 1:1000 and used to probe mouse Cp. Membranes with plasma used transferrin (Tf) as a loading control were reprobed with goat anti-human transferrin (Sigma-Aldrich Co) diluted to 1:3000. Appropriate secondary antibodies were used. Immunoreactivity through chemiluminescence was detected with SuperSignal (Themo Scientific, Rockford, IL) and image captures and densitometry were performed on the FluorChem system (Alpha Innotech, San Leandro, CA).

Statistical Analysis

Means and SEM were calculated for all data. Student's unpaired two-tailed t-test was used for comparison of data between the two dietary treatments (α=0.05). For comparative purposes data in mouse Exps. 1 and 2 were compared by one-way ANOVA and Tukey's test. Data were processed using Microsoft Excel^{TM} or KalidaGraph (Synergy Software, Reading PA).

RESULTS

Evaluation of Copper Deficiency in Post Weanling Mice

Weanling outbred albino mice were placed on CuD dietary treatment for five weeks using two independent methods of rearing to create a spectrum of deficiency characteristics (Table 3-1, Figure 3-1). There was a great deal of variability between the results for Exp. 1 and Exp. 2. Body weight and plasma iron concentration were not impacted by dietary copper deficiency although there were some minor differences in values between Exps. 1 and 2 (Table 3-1). However, CuD Swiss Webster mice housed in

wire bottom stainless steel cages exhibited cardiac hypertrophy and lower hematocrit than the other three groups of mice. Interestingly, CuD mice in both experiments demonstrated similar lower brain copper concentrations, reductions of 35% and 38% for Exps.1 and 2 respectively (Table 3-1).

Importantly, CuD mice in Exp. 1 did not have a significant reduction in liver copper, often thought of as the best indicator of copper status (Figure 3-1). Further support for mild copper deficiency in CuD mice from Exp. 1 can be seen in the failure to develop augmented liver iron and anemia, in contrast to the CuD mice in Exp. 2. Cp activity was different between CuA and CuD mice in both experiments, although CuD mice in Exp. 2 had a more pronounced reduction in activity (97%) than CuD mice in Exp. 1 (67%), consistent with a very marginal degree of copper deficiency in Exp. 1 as compared to Exp. 2.

Immunoblot Evaluation of Blood Following Post Weanling Copper Deficiency in Mice

Selected copper biomarkers were compared by immunoblot technology and densitometry was evaluated by one way ANOVA in erythrocyte extracts and plasma from CuA and CuD mice in Exps. 1 and 2 (Figure 3-2). Abundance of erythrocyte Sod1 was lower only in CuD mice from Exp. 2, consistent with other markers mentioned above. Similarly, plasma Cp abundance was lower in CuD mice than CuA mice in Exp. 2 but not in Exp. 1. Loading controls Gapdh and Tf were not impacted by diet or rearing. Importantly, CCS levels were augmented in CuD mice compared to CuA mice in both experiments though the degree of augmentation, for Exp. 1, 2.1 fold, was slightly less

than the augmentation for Exp. 2, 2.9 fold. Also the CCS/Sod1 evaluation was higher in CuD than CuA mice from Exp. 2 but not Exp. 1. These results suggest that erythrocyte CCS abundance, by itself, may be the most sensitive copper status biomarker in mice.

Comparison to Dietary Copper Deficiency in Rats

In order to compare marginal copper deficiency in mice to rats, weanling male Sprague Dawley rats were placed on the CuA or CuD treatment for four weeks (one less week than the mouse trials). Growth was not impaired by the CuD treatment but CuD rats had a 30% reduction in hemoglobin concentration and exhibited cardiac hypertrophy, $P < 0.05$, similar to the CuD mice in Exp. 2. However, the P49 CuD rats had no detectable Cp activity and a 72% reduction in liver copper. In contrast, the CuD mice in Exp. 2 had only a 32% reduction in liver copper (Figure 3-1). Thus, the CuD rats at P49 were as copper deficient or more so than the P56 CuD mice in Exp.2.

Erythrocyte and plasma samples from a group of CuA and CuD rats were subjected to the same immunoblot technology as mice (Figure 3-3). CuD rats demonstrated dramatic differences from CuA rats in blood protein biomarkers Sod1, CCS, and Cp. Sod1 was lower, CCS higher, the CCS/Sod1 higher, and Cp lower in CuD compared to CuA rats, $P < 0.01$.

Induction of Copper Deficiency in Adult Mice

To extend the studies on CCS evaluation as a biomarker, another marginal copper deficiency experiment was designed, Exp.3. Adult retired mouse breeders were placed on the two dietary treatment protocols using the same strain, diets and time of deprivation as the post weanling mouse experiments. The adult CuD mice exhibited signs of very

marginal copper (Table 3-2). In fact the CuD mice were not anemic and had no reduction in liver copper. Interestingly, and similar to Exps. 1 and 2, there was a significant 16% reduction in brain copper. Neither brain iron or zinc was impacted by dietary copper deficiency (data not shown). Cp activity was not statistically different between CuA and CuD mice suggesting no evidence of copper deficiency. Liver iron level was also not impacted in Exp. 3 in contrast to the robust augmentation in Exp. 2 (Figure 3-1).

Surprisingly however, analysis of blood biomarkers indicated similar results to the marginally CuD mice in Exp. 1 (Figure 3-4). CCS showed a significant augmentation in CuD compared to CuA adult mice, $P<0.05$. Neither Sod1 or Cp levels were impacted in adult CuD mice.

DISCUSSION

Based on the collective results of these three experiments with mice, erythrocyte CCS protein expression appears to be a strong biomarker candidate for assessing copper status compared to other previously tested techniques such as the ratio of CCS to Sod1 protein, Cp activity, Cp protein level, and even liver copper concentration. Although erythrocyte CCS, liver copper and Cp activity were all significantly altered in both of the juvenile mouse CuD studies, Cp quantity, as evaluated through Western blotting was not. In the adult mouse study, only CCS quantity was significantly altered by copper deficiency; even plasma copper, evaluated by Cp activity was not. This was a surprising result and validates the potential of blood CCS protein level as biomarker of marginal copper deficiency in humans.

CCS has been studied previously in other model systems including rats, mice, and cattle (Bertinato et al. 2003, Hepburn et al. 2009, Prohaska et al. 2003a). These studies consistently demonstrate significant increases in CCS quantity following severe dietary copper deficiency. CCS was augmented in liver, brain, heart, kidney in addition to blood of copper deficient rats and mice (Prohaska et al. 2003a). CCS was also shown to be augmented in other cells from blood of copper deficient rats including white blood cells and platelets (Broderius & Prohaska 2009). That study also demonstrated that erythrocytes appeared to have a high content of CCS and a robust response to severe copper deficiency. Current studies now demonstrate erythrocyte CCS augmentation in marginally deficient young and adult mice. The discovery that CCS is a reliable copper status marker in marginally deficient adult mice is crucial to the argument that CCS will be a useful biomarker for copper status in adult humans. Most cases of human adult copper deficiency are in older people and they are only marginally deficient in copper. Blood CCS protein has yet to be evaluated in humans with copper deficiency. One study did report a decrease in CCS mRNA level in peripheral blood mononuclear cells following a copper supplementation trial (Suazo et al. 2008). Further research will be necessary to demonstrate that erythrocyte CCS is a good biomarker of copper status in humans since the CCS response may be species specific.

For example, in the current studies comparing CuD male post weanling rats and mice in Exp. 2 (the most severe case of copper deficiency) it was clear the CCS augmentation in erythrocyte CCS was similar but other features of copper status were not. CuD rats showed greater magnitude of difference between CuA controls for anemia,

cardiac hypertrophy, and Cp activity than CuD mice. As both of these species were juveniles, P21 at the onset, it is possible that the greater magnitude of copper deficiency signs expressed by rats is due to the greater growth between species. Rats grew 4.5 fold in the four week treatment period whereas mice grew 1.7-fold. This growth differential would also be reflected in the greater relative blood volume produced under limited copper conditions. This might be responsible for the more pronounced reductions in red cell Sod1 and plasma Cp in CuD rats compared to mice.

One exception to the magnitude of alterations by copper deficiency between rats and mice was hepatic iron level. Hepatic iron was augmented in CuD mice (Exp. 2) but not rats. Augmentation of hepatic iron is a feature often associated with copper deficiency in rats but has been absent since using the new modified AIN-93G diet with added dietary iron as in the current studies (Bastian et al. 2010, Bastian et al. 2011). Differences in response to copper limited diet, illustrated by hepatic iron augmentation, emphasize the need to verify biomarker responses in the species intended.

Although the focus of the current experiments was on the evaluation of copper deficiency biomarkers, results obtained also highlighted the importance of animal husbandry practice in trace element research. Mice that were housed in the stainless steel wire bottom cages were more copper deficient than the CuD mice housed in solid bottom plastic cages. Exp. 2 CuD mice exhibited significant differences in liver copper reduction, liver iron augmentation, detectable anemia (hematocrit and hemoglobin), reduction in Sod1 level, and lower Cp expression compared to CuD mice in Exp. 1. Likely these differences are due to the fact that mice in hanging cages had limited access

to feces (Exp. 2) compared to mice in plastic bottom cages that could engage in coprophagia (Exp. 1). Importantly both groups of CuD mice demonstrated a significant increase in erythrocyte CCS abundance. These studies also confirm that it is necessary to separate fecal material from rodents when conducting micronutrient deficiency studies.

One of the interesting results of these studies was the observation that brain copper levels were lower following copper restriction in the CuD mice. This was true for both experiments with juvenile mice and surprisingly in CuD adult mice. Recall, these older CuD mice had no change in liver copper concentration, a marker most often altered by dietary copper restriction. Brain zinc and iron concentration were not impacted by copper deficiency in any of the three mouse studies suggesting the copper effect was specific. Normally brain copper turns over very slowly following copper restriction, at least in rats (Levenson & Janghorbani 1994). Perhaps mice have a more labile brain copper pool. Additional research will be needed to clarify these observations.

Although liver copper is usually considered the most accurate method for assessing copper status, liver biopsies for human copper assessment are excessive and warranted only in unusual cases. Thus, the need for an easily measured copper biomarker from blood, urine, or another easily sampled tissue is needed. In these studies of very marginal copper deficiency post weanling CuD mice showed statistical differences in copper biomarkers CCS, Cp activity, and liver copper, while the CuD adult mice showed only a statistical difference in CCS and not the "gold standard" biomarkers Cp activity or liver copper level. Cp or plasma copper is commonly used in human assessment of copper status and is easily obtained through a blood sample (Harvey et al. 2009). However,

plasma copper is highly variable as levels can be altered due to stress, illness, infection, and other physiological afflictions because most of the plasma copper is bound to Cp. Current and previous studies suggest that erythrocyte CCS maybe the best candidate for copper deficiency biomarker due to its consistency and ease of analysis. Recent work with rats assessing the impact of copper supplementation and showing a reduction in red cell CCS after consuming a high dietary copper diet further extends the possibility of CCS as a biomarker for a wide range of copper exposures (Bertinato et al. 2010).

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Table 3-1. Characteristics of eight week old male mice following copper deficiency

	Exp. 1		Exp. 2	
Characteristic	CuA	CuD	CuA	CuD
Body Weight (g)	37.8 ± 1.3[a]	34.5 ± 0.7[a,b]	31.5 ± 0.8[b]	30.7 ± 0.9[b]
Hematocrit (%)	45.7 ± 0.3[a]	45.1 ± 0.9[a]	46.0 ± 0.6[a]	32.6 ± 6.4[b]
Heart/BW (mg/g)	4.50 ± 0.16[a]	4.80 ± 0.22[a,b]	4.83 ± 0.3[a,b]	6.86 ± 1.23[b]
Plasma Fe (μg/ml)	3.52 ± 0.37[a]	2.32 ± 0.26[a,b]	2.50 ± 0.19[a,b]	2.21 ± 0.31[b]
Brain Cu (μg/g)	4.18 ± 0.08[a]	2.70 ± 0.17[b]	4.07 ± 0.13[a]	2.52 ± 0.10[b]

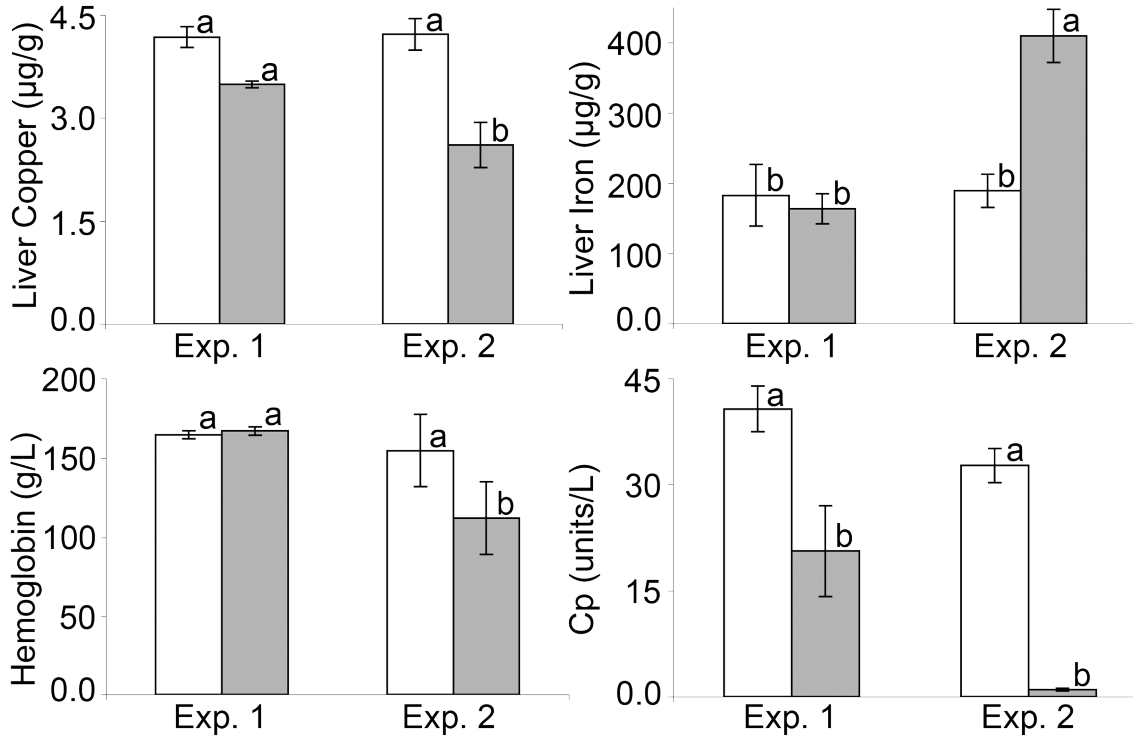
Values are means ± SEM (n=5). Mice were fed copper-adequate (CuA) or copper-deficient (CuD) diets for five weeks post weanling. Mice in Exp. 1 were reared in plastic cages with bedding and in Exp. 2 in stainless steel hanging cages. Data were analyzed by one way ANOVA and Tukey's test. Means with unlike superscripts are different, $P < 0.05$.

Table 3-2. Characteristics of adult male mice following dietary copper deficiency

Characteristic	CuA	CuD
Body Weight (g)	38.0 ± 0.8	43.0 ± 1.7 *
Plasma Fe (μg/ml)	2.23 ± 0.18	2.75 ± 0.34
Brain Cu (μg/g)	6.08 ± 0.14	5.12 ± 0.07 *
Liver Cu (μg/g)	4.04 ± 0.14	3.56 ± 0.45
Hemoglobin (g/L)	155 ± 1.1	153 ± 8.6

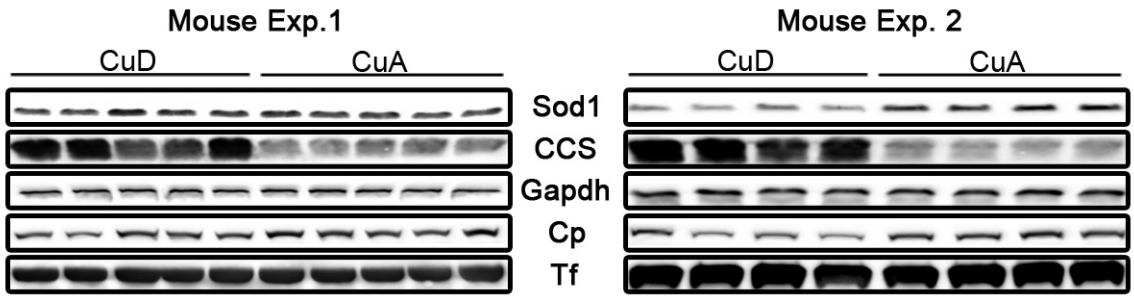
Values are means ± SEM (n=5). Mice were fed copper-adequate (CuA) or copper-deficient (CuD) diets for five weeks. Means were compared by Student's t-test, an asterisk indicates a significant difference, $P < 0.05$.

Figure 3-1. Impact of five weeks of dietary copper deficiency on selected characteristics of post weanling male mice reared by two methods



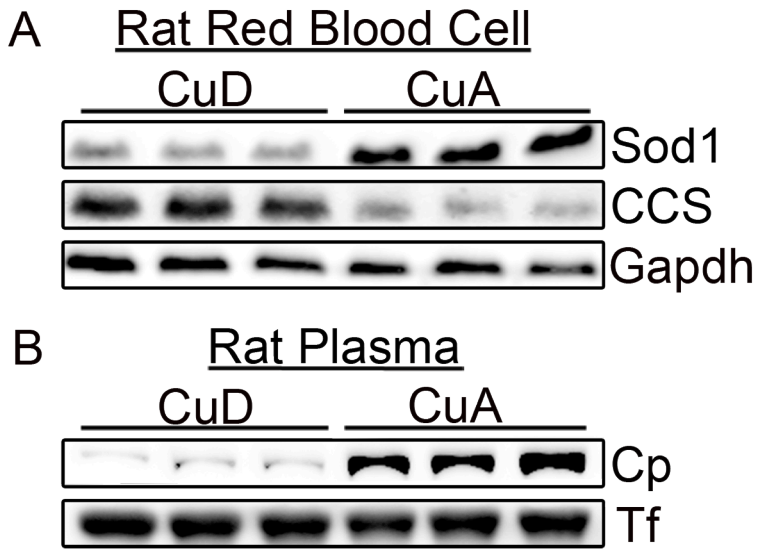
Mice were housed in solid bottom plastic shoe box cages with corn cob bedding (Exp. 1) or in stainless steel mesh bottom hanging cages (Exp. 2). Data (means ± SEM) from copper deficient (CuD) mice (shaded columns) were compared to copper adequate (CuA) (open columns) and analyzed by one-way ANOVA and Tukey's test. Means with unlike superscripts are different, $P < 0.05$.

Figure 3-2. Blood protein expression levels of male post weanling copper deficient (CuD) and copper adequate (CuA) mice following five weeks of treatment



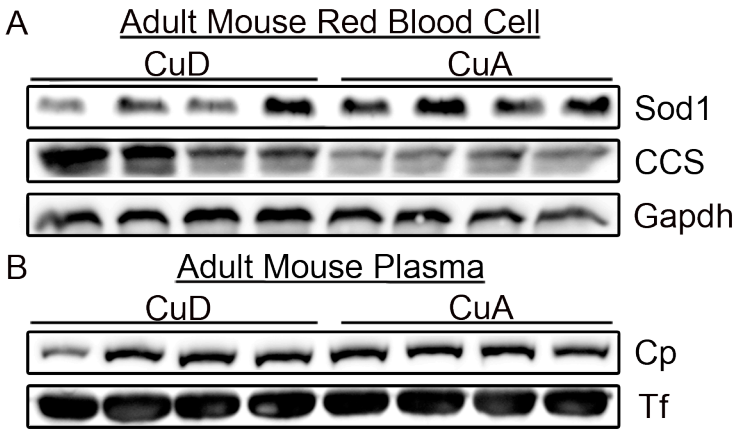
Mice were housed in solid bottom plastic shoebox cages with corn cob bedding (Exp. 1) or in stainless steel mesh bottom hanging cages (Exp. 2). After organic solvent extraction, 25 µg of red blood cell lysate protein was subjected to Western immunoblot techniques using 15% SDS PAGE denaturing gel electrophoresis and probed with rabbit anti-bovine Cu, Zn-superoxide dismutase (Sod1). On a separate 15% SDS PAGE gel, 31 µg of red blood cell lysate was subjected to immunoblot techniques and probed with affinity purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and reprobed with rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for evaluation of equal loading. Mouse plasma proteins, 15 µg, were subjected to immunoblot techniques using 8% SDS PAGE denaturing gels. Plasma gels were probed with goat antihuman ceruloplasmin (Cp) and reprobed with goat antihuman transferrin (Tf) for evaluation of equal loading. Data were analyzed by one-way ANOVA. Sod1 and Cp levels were only lower in CuD mice of Exp. 2. Compared to CuA controls or CuD mice from Exp. 1. CCS levels were augmented in CuD mice from both experiments, $P < 0.05$.

Figure 3-3. Blood protein expression levels of P49 male rats following four weeks of copper deficiency



After organic solvent extraction, 25 µg of red blood cell lysate protein was subjected to Western immunoblot techniques using 15% SDS PAGE denaturing gel electrophoresis and probed with rabbit anti-bovine Cu, Zn-superoxide dismutase (Sod1). On a separate 15% SDS PAGE gel, 31 µg of red blood cell lysate was subjected to immunoblot techniques and probed with affinity purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and reprobed with rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for evaluation of equal loading. Rat plasma, 15 µg protein, were subjected to immunoblot techniques using 8% SDS PAGE denaturing gels. Plasma gels were probed first with goat anti-rat ceruloplasmin (Cp) and reprobed with goat anti-human transferrin (Tf) for evaluation of equal loading. Compared to CuA rats CuD rats had 68% lower Sod1, 85% lower Cp, and 4.8-fold higher CCS levels, $P < 0.01$.

Figure 3-4. Blood protein expression levels of adult male copper deficient (CuD) and copper adequate (CuA) mice following five weeks of treatment



After organic solvent extraction, 25 µg of red blood cell lysate protein was subjected to Western immunoblot techniques using 15% SDS PAGE denaturing gel electrophoresis and probed with rabbit anti-bovine Cu, Zn-superoxide dismutase (Sod1). On a separate 15% SDS PAGE gel, 31 µg of red blood cell lysate was subjected to immunoblot techniques and probed with affinity purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and reprobed with rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (Gapdh) for evaluation of equal loading. Mouse plasma proteins, 15 µg, were subjected to immunoblot techniques using 8% SDS PAGE denaturing gels. Plasma gels were probed with goat anti-human ceruloplasmin (Cp) and reprobed with goat anti-human transferrin (Tf) for evaluation of equal loading. The only significant change was a 60% augmentation in CCS level in CuD compared to CuA mice, $P < 0.05$.

CHAPTER 4

This collection of research supports that CCS is the most reliable and sensitive biomarker for rodent models and showed the limits of currently accepted and used methods for diagnosis. Even between species, CCS was consistently altered by very marginal copper deficiency even when no other indicators were present including anemia, liver copper, and Cp activity. The support for CCS being a key biomarker for copper deficiency was furthered though its high correlation to liver copper levels, which are often referred to as the "gold standard" for measuring copper status within mammals. To a lesser extent, this body of knowledge showed that juvenile rats, likely due to a greater amount of growth during the dietary trial, show a greater degree of deficiency symptoms in a shorter time period. The mice trials contained groups that showed very marginal copper deficiency with some trials showing only changes in CCS between CuA and CuD mice.

CCS levels have been shown to only vary with copper deficiency at the post-translational level. That is, no changes in steady state CCS RNA have been detected between CuD and CuA rodents (Prohaska *et al.* 2003b). Currently, there are two likely causes for changes in CCS quantities in cells: instability of CCS when copper binds and varying ubiquitination as related to copper concentrations. It has been suggested that when copper binds to CCS the protein changes to an unstable form and it is then more likely to be degraded by proteosomes. When copper is concentrated within the cell more CCS would be in unstable confirmations and thus more CCS would be destroyed, while low copper levels would lead to low CCS copper binding and thus higher overall levels

of CCS within the cell (Bertinato & L'Abbe 2003, Caruano-Yzermans *et al.* 2006). It has also been suggested that XIAP ubiqitination of CCS regulates CCS levels within a cell. XIAP is a copper binding protein and is also a E3 ubiquitin ligase. When copper is present in a cell CCS can deliver copper atoms to Sod1 and XIAP. CCS favors interactions with Sod1, but when copper levels rise and Sod1 is saturated with copper, the CCS molecules will shuttle copper to XIAP. Brady *et al.* (2010) theorized that the binding of a copper molecule then activates XIAP to ubiquitinate CCS to mark it for destruction by the proteosome and thus maintain homeostatic levels of copper within a cell. Both of these suggested mechanisms follow the empirical data that increases in copper decrease CCS abundance and decreases in copper increase CCS abundance (Brady et al. 2010).

In the context of these studies, CCS was investigated solely as a biomarker for copper status. However, within a cell CCS is involved in many biological processes. The first discovered, and what is thought to be the primary function, of CCS is the shuttling of free copper atoms to apo-Sod1 (Culotta et al. 1997). CCS may also play a role in Alzheimer's disease. Through an unknown mechanism, CCS regulates the activity of the amyloid-β protein precursor cleaving enzyme (BACE1) reducing its activity and thus reducing the amount of amyloid-β plaques formed. BACE1 is a copper binding protein known to interact with CCS. Recently, it was demonstrated that decreases in CCS through siRNA technology and cells from CCS knockout mice significantly increased levels of intracellular and secreted amyloid-β (Gray et al. 2010). Also CCS plays a role in the activation of hypoxia-induced transcription factor 1 (HIF-1α), although the exact

mechanism is unknown activity (Feng *et al.* 2009, Jiang *et al.* 2007). CCS is thought to help shuttle copper into the nucleus where it is then incorporated into and stabilizes HIF-1α. Also, it appears that CCS is required for copper interaction with HIF-1α as HIF-1 and CCS have been co-immunoprecipitated and CCS silencing suppresses HIF-1 transcription. Thus, these roles for CCS and copper delivery are involved in many aspects of human metabolism (antioxidant defense, neuronal homeostasis, and angiogenesis) known to be impacted by altered copper status.

Although copper deficiency is rare, it is projected that the number of cases of copper deficiency will closely follow current and projected increases in bariatric surgeries and zinc consumption (Iskandar et al. 2005, Varela 2011, Seidell & Flegal 1997). Also, humans that are diagnosed as copper deficient are rarely severely deficient and usually of advanced age. Current research in the area of copper deficiency biomarkers has focused on the identification of molecules that are altered, with dietary trials usually conducted on young (growing) and severely deficient animals. Without a biomarker that can successfully identify adults with marginal copper deficiency, the breadth of how many people are affected by this cannot properly be identified. If a proper copper deficiency biomarker can be identified, the number of people known to be deficient will likely increase.

It is likely that humans in general may not show the same robust change in this or any other blood biomarker as human blood cells have a much longer half-life than either of the rodent models studied. It was suggested that rats became more copper deficient, as compared to mice, because of their large magnitude of growth during development and

thus their greater increase in blood volume and number of cells in the absence of copper. Humans, having about twice the red blood cell half-life are then more likely to attain a noticeable level of copper deficiency more slowly and may maintain at levels that are not distinguishable from humans that are at low-to-normal levels. As CCS was identified as the most reliable sensitive biomarker though out the five rodent studies included in this report, suggested future work would also focus on this protein in humans. Other beneficial work would concentrate on other methods for sample collection. Blood was focused upon for this work due to ease of collection, but buccal cells, urine, fecal samples, and skin biopsies may also contain sensitive biomarkers for accurate diagnosis of copper deficiency, though not necessarily CCS.

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