SELECT CARDIAC COPPER CHAPERONE PROTEINS ARE UP-REGULATED BY DIETARY COPPER DEFICIENCY

by

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Abstract

Copper deficiency has been linked with many health problems, among them cardiac hypertrophy. Because of its potential for causing oxidative damage, copper within the cell must be bound to chaperone proteins. In this thesis, we examined the role of dietary copper deficiency in the regulation of select copper chaperone proteins in cardiac tissue of rats. Sixteen weanling male Long-Evans rats were randomized into treatment groups, one group receiving a copper deficient diet (< 1 mg Cu/kg diet) and one group receiving a diet containing adequate copper (6 mg Cu/kg diet) for 5 weeks. Rats were sacrificed and a small blood sample was removed to determine hematocrit. Also, heart and liver tissues were removed for subsequent analysis. Rats fed the copper deficient diet had lower body weights but greater heart weights and heart:body weight. Hematocrit levels and liver copper concentrations were markedly decreased in copper deficient rats. These variables indicated that the copper deficient diet did in fact induce a copper deficiency in these animals. Non-myofibrillar proteins from the hearts were removed and separated by SDS-PAGE. Western Blotting was used to determine the concentrations of CTR1, CCS, Cox17, SCO1, Cox1 and Cox4. No changes were observed in the concentrations of CTR1 and Cox17. CCS and SCO1 were up-regulated as a result of copper deficiency, while Cox1 and Cox4 were both down-regulated. However, use of another antibody against Cox subunits suggested that only the nuclear encoded subunits including subunit IV were decreased, but not subunits I and II. These data provide new insight into the cardiac hypertrophy observed in copper deficiency, which suggests that select chaperone proteins may be up-regulated by a dietary copper deficiency.

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CHAPTER 1 - Introduction

Copper is an essential micronutrient. This mineral functions in such diverse processes as infant growth, bone strength, host defense mechanisms, iron transport, red and white blood cell maturation, myocardial contractility, brain development, and cholesterol and glucose metabolism. Copper is indispensible to the formation of collagen and elastin, as well as the electron transport chain in the mitochondria. Currently the DRI for copper is 0.9 mg/day.

Copper deficiency was first noted in cattle exhibiting what was known as "falling disease" (1), due to copper deficiency in the grazing land. It was first documented in humans in malnourished Peruvian children (2) who exhibited anemia, neutropenia, and scurvy-like bone abnormalities. Because pregnancy increases the requirements for many nutrients, copper deficiency may be acquired prenatally if the mother does not increase her copper intake during the pregnancy. Genetic disorders can also produce copper deficiency. Menkes' disease involves a defect in ATP 7A, discussed later, and causes inhibited absorption of copper from the small intestine. This leads to copper deficiency, and produces signs such as slow infant growth, pili torti, and infant death (3). Other symptoms of copper deficiency include depigmentation of skin and hair, kinkiness of hair, connective tissue abnormalities, and neurological damage.

Copper deficiency has also been linked with the development of cardiac hypertrophy. This hypertrophy is concentric, rather than the eccentric hypertrophy normally seen accompanying anemia. Medeiros *et al.* (4) concluded that this hypertrophy was due primarily to increase in mitochondria numbers. It has been suggested that the increase of mitochondria observed in copper-deficient cardiac hypertrophy may be an attempt to increase the mitochondrial surface area and therefore increase the uptake of copper and oxygen (5). It is of interest to note that with mitochondrial increase and proliferation in brain tissue of copper-deficient animals, an

accompanying increase in ATP production is not observed (6). Even when hypertrophy is not present, copper-deficient animals exhibit ultrastructural changes in the cardiac tissue and abnormal electrocardiograms (5).

Much of the essentiality of copper can be ascribed to its role as a cofactor for several enzymes within the cell. Copper is incorporated into Cu-Zn superoxide dismutase (Cu-Zn SOD), an enzyme which functions in the scavenging of free radicals and the prevention of oxidative stress. It is also a key component of two ATPases, ATP 7A and ATP 7B. These ATPases function in the absorption and excretion of copper into and out of cells, respectively. Defects in these enzymes lead to the genetic disorders Menkes' Disease (ATP 7A) and Wilson's Disease (ATP 7B). Copper is required for the functioning of cytochrome C oxidase (CCO). This enzyme is the Complex IV of the mitochondrial electron transport chain, and therefore is crucial to normal energy metabolism. Finally, copper is also needed by lysyl oxidase, an enzyme involved in the formation of collagen. This requirement may account for some of the bone and connective tissue abnormalities seen in copper deficient models.

Copper exists in two oxidation states, Cu^{1+} and Cu^{2+}. As such, like most metals, it can act as a pro-oxidant within the cell. Copper is therefore incorporated into various chaperone proteins, and this prevents oxidative damage to the cell. Copper is transported into the cell by the protein CTR1 (Figure 1). From this protein, the mineral can take any of several routes. Metallothionein can bind copper, like several other minerals, and transport it throughout the body or in some cases store it to a certain degree. The copper chaperone for superoxide dismutase (CCS) transports copper to Cu-Zn SOD. Atox-1 ferries copper to ATP 7B in the trans-Golgi network. From the trans-Golgi network, copper can be excreted from the cell directly or via binding with ceruloplasmin. Cox 17 carries copper to the mitochondria and into the intermembrane space. Once in the mitochondria, Sco 1 and Sco 2 function in transporting copper to, and incorporating it into, the CCO complex (reviewed by 7).

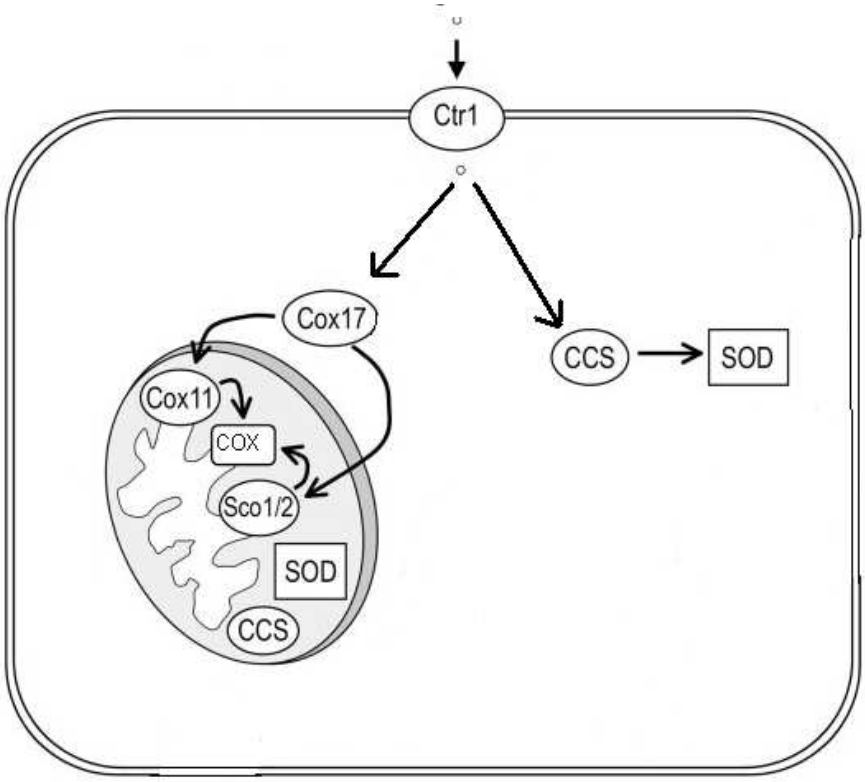

Figure 1. Copper movement within a cell. Modified from Prohaska & Gybina 2004 (7).

The mechanisms of copper deficiency's contribution to cardiac hypertrophy are not well understood. The Medeiros lab has done previous work investigating the role of copper deficiency in the development of cardiomyopathy. Particularly of interest have been the behaviors of the above mentioned copper chaperone proteins in the mitochondria of hypertrophied hearts from copper deficient animals. In the present study, copper deficiency was induced to investigate its effect on the concentrations of Cox17, SCO1, subunit 1 of CCO (Cox1), and CTR1. Also studied were CCS and subunit 4 of CCO (Cox4). We examined the heart tissue of copper deficient rats to determine whether these proteins were up- or down-regulated, or unchanged, by dietary copper deficiency.

CHAPTER 2 – Literature Review

Copper Intake Recommendations

The most well-known form of dietary recommendation, particularly for micronutrients, is the recommended daily allowance, or RDA. Initially established in 1941 by the Food and Nutrition Board of the US Institute of Medicine, the RDAs are updated periodically to reflect more current research. The RDAs were formulated initially to provide guidelines for the planning of food supplies for population groups (8). The RDA system has largely been replaced by the use of dietary reference intakes, or DRIs, except in nutrition labeling. The DRIs represent the most current set of recommendations put forth by the Food and Nutrition Board. Under the broad DRI system, several recommendations exist for a given nutrient, including the estimated average requirement, the RDA, the adequate intake value, and the tolerable upper intake limit.

As of 1998, an RDA for copper had not been established. Klevay argued at this time that such a recommendation should be made in light of the severe damage that copper deficiency can induce. A majority of the population likely consumes only marginal levels of copper on a regular basis, and so a mild deficiency may be quite common, according to Klevay's hypothesis (9). In 2001, the Food and Nutrition Board issued a DRI for copper of 200-900 μg/day, increased to 1000-1300 μg/day in pregnancy or lactation (10). The commonly cited DRI for copper is 0.9 mg/day according to the latest Food and Nutrition Board recommendation (11).

Signs of Copper Deficiency

Copper deficiency in humans is thought to be rare, but it has occurred. One good example of a functional copper deficiency is observed in patients presenting with Menkes' syndrome. This genetic disorder is caused by a mutation of ATP7A, resulting in failure in copper absorption from the intestine. Danks (3) describes the symptoms of this disease at some length. Briefly, severe mental

retardation, drowsiness, lethargy, hypothermia, convulsions, retarded growth, pallor, pili torti, and bone abnormalities are among the most striking clinical symptoms of this disorder. Similarly, total parenteral nutrition has been shown to lead to deficiency in copper and other micronutrients. In such cases, nutrient formulas given to patients were devoid of copper prior to knowing of its essentiality. In a case described by Karpel and Peden (12), the patient developed anemia, neutropenia, and bone abnormalities. Bone abnormalities have also been reported in copper-deficient rats (13). Neutropenia and anemia were also noted by Dunlap *et al.* (14). Cordano reviewed studies of copper deficient children and infants and noted that neutropenia was the earliest sign of the deficiency; also noted were hypoceruloplasminemia and serum copper concentrations of <0.45 mg/L (15). Cardiac hypertrophy has also been associated with copper deficiency in scores of studies on rodents and other animals such as pigs (4, 16-23).

Copper Deficiency and Cardiovascular Disease

According to the World Health Organization (24), ischemic or coronary heart disease is the leading cause of death worldwide. Klevay (25) presented the copper-zinc theory of ischemic heart disease in humans in 1975, suggesting that an imbalance in copper and zinc concentrations may play a key role in the development of this disease. In support of this, similarities have been noted between human patients with ischemic heart disease and laboratory rats with induced copper deficiency (26). Among these similarities are hypercholesterolemia; arterial damage; myocardial fibrosis, rupture, hypertrophy, and copper deficiency; death; abnormal electrocardiogram readings, including bundle branch block and ST abnormalities; hyperuricemia; and abnormal blood clotting.

In rats, copper deficiency increases total plasma concentrations of cholesterol and triglycerides, as well as increasing the pool sizes for both these nutrients. Marginal copper status produces similar results, though these are not as drastic as those seen in overt deficiency (20, 27). Interestingly, this increase in cholesterol level has been attributed to an increase in plasma HDL

cholesterol; however, in some studies copper deficiency shows no increase in HDL protein, VLDL + LDL cholesterol, or VLDL + LDL protein, and the previously noted increase in triglyceride levels is also not observed (28). The increase in HDL cholesterol seen here was due to an increase in apolipoprotein A-I, associated with HDL cholesterol; these data suggest an alteration in HDL cholesterol metabolism may be partially responsible for the hypercholesterolemia observed in copper deficient models. VLDL + LDL fractions from copper deficient rats are also more prone to oxidation *in vitro*, suggesting a role for copper deficiency in the generation of atherosclerosis (29).

Medeiros *et al*. has consistently reported that hearts from copper deficient rats are hypertrophied (4, 16-22). Heart tissue from copper deficient rats exhibits increased mitochondrial area, mitochondrial proliferation, vacuolization, inclusion of glycogen granules and fat droplets, and poor arrangement of cristae, as well as an apparent disorganization of myofibrils (4). However, a proliferation of myofibrils may also contribute to cardiac hypertrophy in copper deficient animals as well. As the degree of cardiac hypertrophy increases in rats, the mitochondria:myofibril may decrease. However, Mao *et al.* proved that in copper deficient myocytes the increase in absolute cell size is due to an absolute increase in mitochondria with no change in myofibrils (30). Elsherif *et al.* demonstrated that copper deficient mouse hearts are hypertrophic, including vacuoles containing lipid droplets and increased collagen formation (23). Also noted are a decrease in systolic blood pressure, an increase in diastolic pressure, and stiffness of the left ventricle.

Copper deficient animals exhibit ventricular aneurysm, hemothorax, pleural effusion, and hemopericardium, as well as electrocardiogram abnormalities (5, 31). These electrocardiogram abnormalities occur in 80% of animals studied, and include depressed and inverted ST segments, bundle branch block, bradycardia, and enhanced R wave amplitudes (5, 31, 32). A lack of difference between QRS axes from copper deficient and copper adequate animals suggests that the cardiac hypertrophy may not be strictly right- or left-ventricular (32).

Collagen abnormalities have also been noted in copper deficient animals. Copper deficient swine exhibit increased levels of type III collagen in cardiac tissue, and decreased levels of collagen cross-linking. Type III collagen is associated with increased compliance and elasticity of tissue; however, the hearts studied do not exhibit decreased stiffness (33).

It is interesting to note that male rats appear more susceptible to copper deficiency-induced myocardial abnormalities than female rats. Male rats exhibit decreased ventricular function and decreased relative heart rate, whereas female rats fail to show these decreases and even present increased relative heart rates (16). Davidson *et al*. (5) notes that copper deficiency during different life stages may cause cardiac damage without the classic signs of copper deficiency (such as cardiac hypertrophy or anemia), and this damage may not be reversible by means of copper repletion.

Copper Deficiency and Cuproenzymes

Many of the signs of copper deficiency may be attributable to copper's essential role for several key enzymes within the cell. Copper is required for the function of cytochrome C oxidase, the terminal step in the mitochondrial electron transport chain and the production of ATP. Similarly, copper-zinc superoxide dismutase requires the presence of copper to function in the scavenging of superoxide radicals. Copper is also essential to the functioning of glutathione peroxidase, dopamine β-monooxygenase, glutathione transferase, peptidylglycine α-amidating monooxygenase, and two copper-dependent ATPases.

In both mice and rats, cytochrome C oxidase activity has been shown to decrease with copper deficiency in such varied tissues as brain, spleen, thymus, intestine, heart, liver, and kidney (34-38). Prohaska and Bailey (39) demonstrate reduced cytochrome C oxidase activity in all brain regions of deficient animals, and copper repletion for one month fails to rescue this condition. However, Paynter *et al.* (40) show reduced cytochrome C oxidase activity in all tissues except lung and brain, and have found that copper repletion leads to slow recovery of enzyme activity. The Cox4 subunit of

this enzyme has also been shown to decrease in copper deficiency (38, 41-43). Copper deficiency may decrease not only the activity but also the protein level of Cu-Zn superoxide dismutase. Protein levels have been shown to be reduced in copper deficient rats (38, 44). Cu-Zn superoxide dismutase activity in chicken aorta tissue increases during the first four days of chick development, then levels off in control chicks; however enzyme activity steadily declines to non-detectable levels in chicks fed a copper deficient diet. This decline is reversed by administering copper to deficient animals. Contrary to the findings noted above, superoxide dismutase is present, but nonfunctional, which suggests that the limiting factor is copper, not the protein (45). Interestingly, inflammation in copper adequate rats decreases Cu-Zn superoxide dismutase activity but does not impact protein levels; however, protein levels are decreased in copper deficiency (46). Superoxide dismutase activity has been depressed by copper deficiency in the livers, kidneys, brain, heart, small intestine, spleen, and thymus of both mice and rats (34-37). However, Paynter *et al.* demonstrate a reduction of enzyme activity in all tissues except brain and muscle (40). Copper repletion in that study led to rapid increases in superoxide dismutase activity in liver and heart, and a slower recovery in red blood cells. Notably, males and females may react to copper deficiency differently in terms of superoxide dismutase activity. Copper deficient females exhibit decreased superoxide dismutase activity in the medulla pons; however, males exhibit enzyme inactivity in all brain regions except the midbrain. These depressions are rescued by copper repletion (39).

Copper deficient mice and rats exhibit decreased glutathione peroxidase activity in liver and plasma (36). Copper deficient rats exhibit unaltered glutathione transferase activity in liver; copper deficient mice exhibit reduced glutathione transferase activity when deficiency is induced during suckling but not when deficiency is induced during the postweanling period (36). Copper deficient rats also exhibit decreased peptidylglycine α-amidating monooxygenase activity in heart and midbrain (37). Dopamine β-monooxygenase activity in midbrain of copper deficient rats is higher

and in heart is lower; repletion fails to restore these activities to control levels (37). Similarly, Gross and Prohaska have found decreased dopamine β-monooxygenase activity in heart tissue of copper deficient mice, and note that norepinephrine turnover in cardiac tissue is increased by copper deficiency (47).

Copper Deficiency and Copper Chaperone Proteins

Like most metals, copper can act as a pro-oxidant in the cell and can thus cause oxidative damage. Therefore it is often bound to various chaperone proteins which carry it throughout the cell. CTR1 is the copper transporter, by means of which copper enters the cell. From CTR1 copper can be transported to superoxide dismutase by the copper chaperone for superoxide dismutase, CCS. Alternatively, it may be bound by Atox 1 for transport to the trans-Golgi network and efflux from the cell via ATP7B, or similarly bound to metallothionein. Cox17 can bind to copper and transport it to the mitochondria, where it is carried to the electron transport chain by SCO1 and SCO2 (7).

Expression of CTR1 stimulates copper uptake in a time-dependent manner; through CTR1 $^{+/-}$ mouse crosses, no CTR1 $^{-/-}$ offspring are produced, and dietary copper supplementation of mothers does not rescue these embryos. Thus, there is no alternate system for copper uptake if CTR1 is absent in mice. Ectoderm and mesoderm are poorly developed in CTR1 $^{-/-}$ embryos; closure of neural tube, diminished mesenchyme cell formation, and failure of embryos to turn are observed in these animals (48). Similarly, CTR1 $^{-/-}$ embryos only exist to 9.5 days of embryonic development. No gastrulation is observed, with sparse endoderm and no mesoderm present in these embryos (49). CTR1 heterozygotes are phenotypically identical to wild-type mice, though brain copper levels are decreased by approximately 50% (49). CTR1 activity in rat liver and choroid plexus decrease when copper concentration is high in the nucleus, though copper distribution to lysosomes continues. CTR1 transcription is lower during low ceruloplasmin activity (50). The choroid plexus of copper deficient rats presents a 50% higher abundance of CTR1 (38). Copper toxicity can result in

embryonic death similar to that seen in lack of copper influx. Atox1 $^{-/-}$ mice show decreased physical activity and increased mortality by postnatal day 3; skin is lax; peripartum hemorrhaging occurrs. Perinatal mortality is 45%, and surviving -/- mice are growth-retarded, hypothermic, and hypopigmented. Less copper is transported into -/- embryos; increased copper accumulation in Atox1 $^{-/-}$ cells due to impaired efflux appears to cause these changes (51).

CCS $^{-/-}$ mice show reduced superoxide dismutase activity (10-20% of normal in all tissues, except 30% of normal in liver) but similar levels of superoxide dismutase protein to control littermates. ^{64}Cu-labeled superoxide dismutase does not appear in CCS $^{-/-}$ mice, and these animals exhibit an increased sensitivity to neural damage and decreased fertility (52). Copper deficiency appears to elevate CCS levels. Copper deficient rat livers and erythrocytes show increased levels of this protein even with mild deficiency, and CCS levels are also elevated in cardiac tissue, though less so (44). However, no difference is observed in CCS levels of the choroid plexus, though an increase is observed in the cerebellum and in purified brain mitochondria (38).

SCO1 and SCO2 are both required for the delivery and insertion of copper at the same stage of cytochrome C oxidase assembly. The Cox17 subunit of cytochrome C oxidase interacts with SCO2 in delivery of copper in the mitochondria; overexpression of Cox17 rescues cytochrome C oxidase activity in SCO2 mutant cells (53). Overexpressing SCO1 in *SCO2* mutants, and vice versa, further depresses cytochrome C oxidase assembly, rather than rescuing enzyme function. This suggests that the roles of these two proteins are nonoverlapping and essential, but related (53, 54). However, the roles are not yet entirely understood. Mutations in the genes for these chaperones, in particular SCO2, have been observed in humans. These mutations are fatal in infancy, due to severe respiratory impairment caused by deficiency in cytochrome C oxidase activity. Such mutations produce cardiac hypertrophy similar to that seen in dietary copper deficiency, in addition to cardiaoencephalomyopathy as seen in Leigh's syndrome (54, 55).

As with SCO1 and SCO2, the impact of copper deficiency upon most copper chaperones is relatively unknown. The only chaperone which has been studied in depth in relation to dietary copper deficiency is CCS. In this thesis, we present data revealing that dietary copper deficiency increases the expression of select copper chaperone proteins.

CHAPTER 3 – Materials & Methods

Animals and Diets

Sixteen male weanling Long-Evans rats were acquired from Charles River Laboratories (Lee's Summit, MO). Animals were housed singly in wire-bottom stainless steel cages and were randomized into two groups such that the mean initial body weight for each group was not significantly different. One group of 8 rats was given free access to deionized distilled water and a semi-purified diet with no added copper, and one group of 8 was given free access to deionized distilled water and a semi-purified diet with added copper. Diets were acquired from Research Diets (New Brunswick, NJ), and the composition was based on recommendations of the American Institute of Nutrition (AIN 1980). The nutrients consisted of (g/g diet by weight) 0.50 sucrose, 0.20 casein, 0.15 cornstarch, and 0.05 corn oil as energy sources. The copper adequate diet was formulated to contain 6.0 µg Cu/g diet and the copper deficient diet less than 1.0 µg Cu/g diet. The copper adequate diet contained copper in the form of cupric carbonate. To verify copper content of the diets, each diet was digested with trace element grade nitric acid and analyzed by flame atomic absorption spectrophotometry (Perkin Elmer model 5000, Norwalk CT). Analysis revealed that the copper adequate diet had 6.25 µg Cu/g diet, and the copper deficient diet had 0.69 µg Cu/g diet. The Institutional Laboratory Animal Care and Use Committee at Kansas State University approved the study protocol.

Cages and glassware were sterilized once per week (Schlyer Machine, model CBW1026KD), with water changed or refreshed as needed and food refilled regularly. Body weights were determined for animals once per week. After 5 wk. in a treatment group, animals were anesthetized using sodium pentobarbithol (100 mg/kg body weight). A midline incision was made with surgical tools and the thoracic cavity exposed. Blood was extracted from the ventricle of the heart with an 18-gauge needle and the blood was placed into heparinized tubes for hematocrit determination.

Hearts were removed, rinsed in 0.85% NaCl, and weighed, before being flash-frozen with liquid N_2 for later evaluation. A sample of liver tissue was also removed from each animal and placed on ice for later evaluation.

Cardiac Tissue Processing

Cardiac tissue samples weighing 0.2 g were processed to separate the non-myofibrillar fraction of the tissue for analysis of copper chaperone proteins. Each 0.2 g sample was homogenized in 2 mL 0.1M KCl + 0.15% Triton X. The samples were centrifuged in an Eppendorf 5415R centrifuge at 4˚C for 20 minutes at 1086Xg. The supernatant for each tissue sample was aliquoted into 8 - 100 μL samples, and remaining supernatant, pellet, and aliquots were stored at -80˚C.

Western Blotting

Prior to analysis, each supernatant sample was sonicated using a sonic dismembrator (Fisher Scientific model #100). 100 μL 2X SDS was added to each 100 μL aliquot, and the aliquots were boiled for 3 minutes to denature proteins. 4-20% gradient gels (Pierce Precise Protein gels, 12-well; #25224) were loaded as follows: Chemiluminescent Blue Ranger molecular weight marker was loaded in the first well of each gel (Pierce #26651). 10 μL of sample was loaded into each subsequent well, and gels were run for 45 minutes at 100V for approximately 1 hr in BuPH Tris-HEPES SDS running buffer (Pierce #28398).

Gels and nitrocellulose membranes (Trans-Blot transfer medium, 0.2 μm; BioRad #162-0112) were equilibrated in 25 mM Tris, 192 mM glycine, and 20% methanol buffer for 20 minutes (Sigma Tris-Glycine Buffer 10X concentrate), and protein fractions were transferred to the membranes using a BioRad Trans-Blot SD semi-dry transfer cell at 20V, for 30 min. Membranes were blocked using 5% milk for 1 hr.

Membranes were probed with primary antibodies overnight at 4˚C with agitation, as follows. For CCS, rabbit anti-CCS was used at a 1:200 dilution (Santa Cruz #sc-20141). Rabbit anti-CTR1

(Santa Cruz #sc-66847) at a 1:200 dilution was used to probe for CTR1. SCO1 was probed for with goat anti-SCO1 (Santa Cruz #sc-49108) at 1:100 dilution. Mouse anti-Cox4 (Santa Cruz #sc-58348) was used to probe for Cox4 at a dilution of 1:200. Cox1 was visualized using MitoSciences mouse anti-Cox1 (MS404) at a dilution of 1:1000. Sample preparation for Cox17 analysis was slightly different from the other proteins examined, due to the tendency of this protein to trimerize. A new 2X SDS loading buffer was prepared using 300 mM DTT and 2% SDS, and samples were boiled for 15 minutes to denature trimers of the Cox17 protein. The primary antibody used for this protein was raised in rabbits by Dennis Winge (University of Utah), and was used at a 1:1000 dilution. Additionally, all CCO proteins were visualized using a polyclonal antibody raised in rabbits, at a dilution of 1:500. This antibody was received from Lawrence Prochaska of Wright State University (Dayton, OH). Finally, β-actin was visualized using mouse anti-actin from Abcam (#ab6276) at a dilution of 1:1000.

Following the overnight probe with primary antibodies, membranes were rinsed in TDN (58.5 g NaCl, 7.45 g EDTA, 12.1 g Tris, per L) for approximately 5 minutes, after which they were probed with secondary antibodies 1:1000 at room temperature with agitation for approximately 1 hour. These were goat anti-rabbit, donkey anti-goat, and goat anti-mouse (Santa Cruz #sc-2030, sc-2033, and sc-2031, respectively). After probing with secondary antibodies, membranes were rinsed in TDN for 1.5 hr, changing the solution every 15 minutes.

Membranes were developed using either a 1:1 chemiluminescent substrate (Thermo Scientific #34080) or a maximum sensitivity chemiluminescent substrate (Pierce #34095). Images were produced using a multi-image light cabinet from Alpha Innotech (San Leandro, CA).

Hematocrit Determination

Heparinized blood was transferred to microhematocrit tubes and centrifuged in a microcapillary centrifuge for two minutes. Hematocrit was determined as the percentage of space occupied by packed red blood cells.

Analysis of Liver Copper

1g samples of liver tissue were digested in 10 mL HNO_3 overnight. Samples were boiled and volume reduced to approximately 1 mL. These were then diluted to 10 mL with deionized distilled water and flame atomic absorption spectrophotometry was used to determine copper content of liver tissue (Perkin-Elmer 5000 atomic absorption spectrophotometer). Samples were measured against 1 and 5 ppm copper nitrate standards prepared from a 1000 ppm certified standard (Fisher Scientific #sc-194-500).

Statistical Analysis

Unpaired 2-tailed Student's T-tests were used to analyze the data to determine differences in means at a p-value of 0.05 or less.

CHAPTER 4 – Results

Of the 16 rats that started the study, 15 rats completed the protocol, with one rat from the copper adequate diet group euthanized due to a dental issue which caused the rat not to eat. Results suggested that rats fed the copper deficient diet were indeed copper deficient as revealed by several indicators (Table 1). Final body weight, hematocrit, and liver copper levels were significantly ($p \leq 0.01$) decreased in rats fed the copper deficient diet compared to those fed the copper adequate diet. On the other hand, final heart weight and heart:body weight were significantly ($p \leq 0.01$) increased in rats fed the copper deficient diet compared to those fed the copper adequate diet.

Neither CTR 1 nor Cox17 were significantly up- or down-regulated by dietary copper deficiency (Figures 2, 4). However, several other copper-related proteins did exhibit regulation based on dietary deficiency. Concentrations of CCS (Figure 3) and SCO1 (Figure 5) both increased significantly in copper deficient heart tissue ($p \leq 0.01$ and $p \leq 0.05$, respectively), while Cox1 and Cox4 were both decreased in deficiency ($p \leq 0.001$and $p \leq 0.001$) (Figures 6, 7). This is in contrast to results obtained with the CCO polyclonal antibody, where subunit 2 did not differ by treatment but subunit 4 decreased in copper deficient rats (data not shown).

An interesting banding pattern was revealed on the membranes used to visualize SCO1 (Figure 5). While SCO1 signal increased in the copper deficient lanes, another band appeared near the bottom of the membrane in the lanes with samples from copper-adequate animals. While this band remains unidentified, it may be a breakdown product and warrants further investigation.

Table 1. Copper deficiency incidators (Mean ± SE)

Variable	CuA	CuD
Final Body Weight (g)**	337 ± 7.23	287 ± 11.6
Final Heart Weight (g)**	1.53 ± 0.051	2.25 ± 0.157
Heart:Body Weight ($*10^{-3}$)**	5.44 ± 0.170	8.05 ± 0.800
Hematocrit (%)**	43 ± 0.8	32 ± 2.6
Liver Copper (µg Cu/g wet weight)**	4.13 ± 0.069	0.7 ± 0.092

** $p \leq 0.01$ CuA = copper adequate CuD = copper deficient

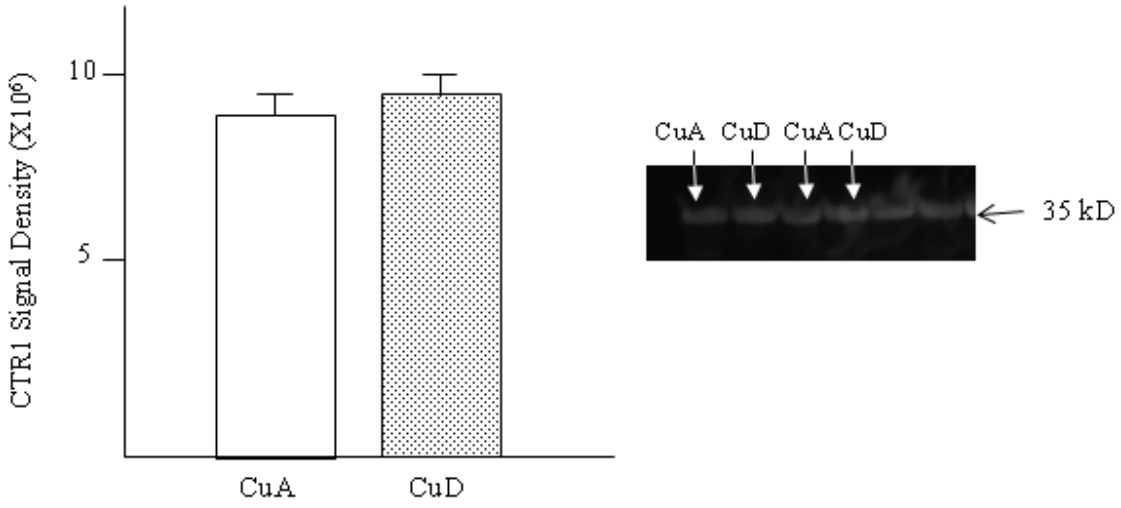

Figure 2. CTR1 Signal density (Mean ± SE).

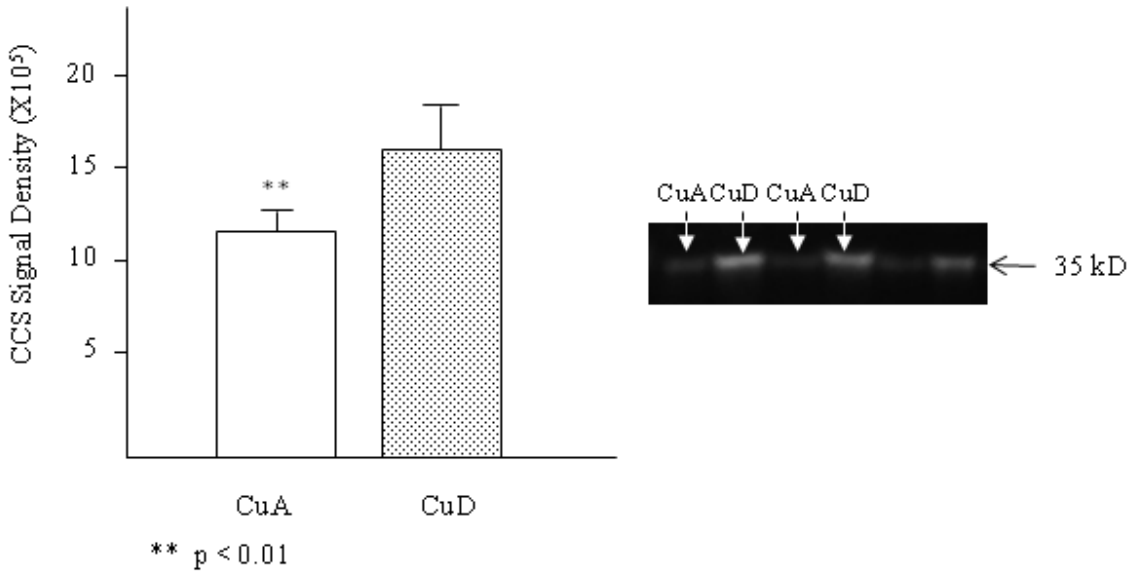

Figure 3. CCS Signal density (Mean ± SE).

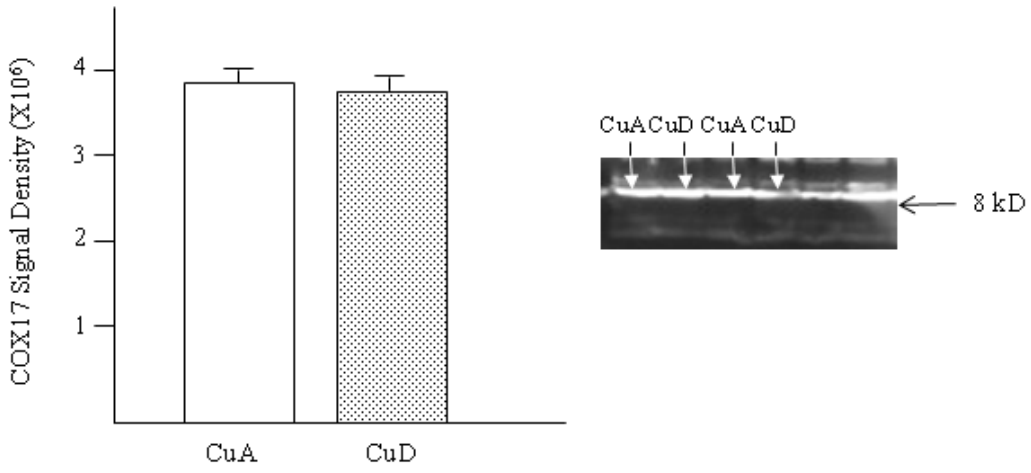

Figure 4. Cox17 Signal density (Mean ± SE).

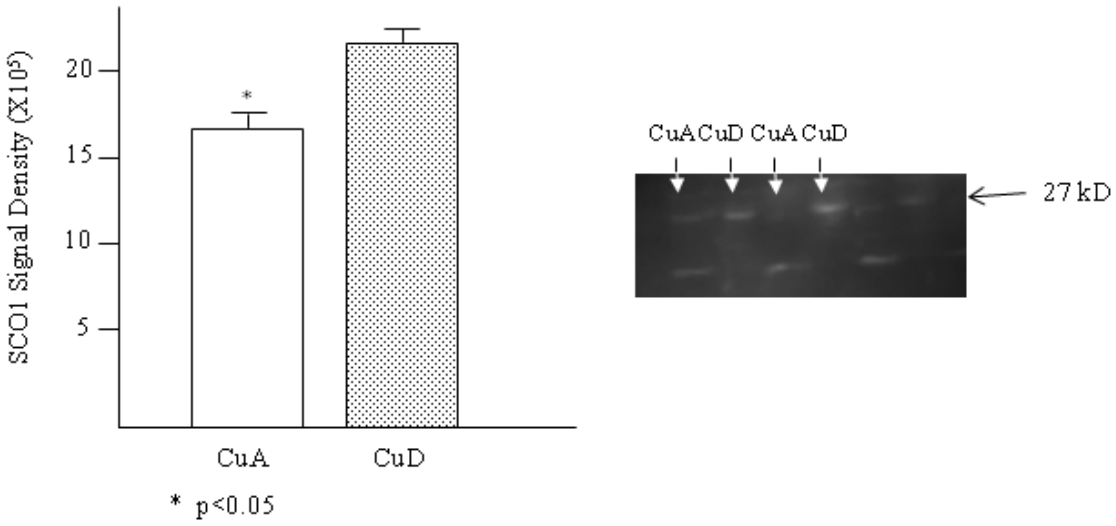

Figure 5. SCO1 Signal density (Mean ±SE).

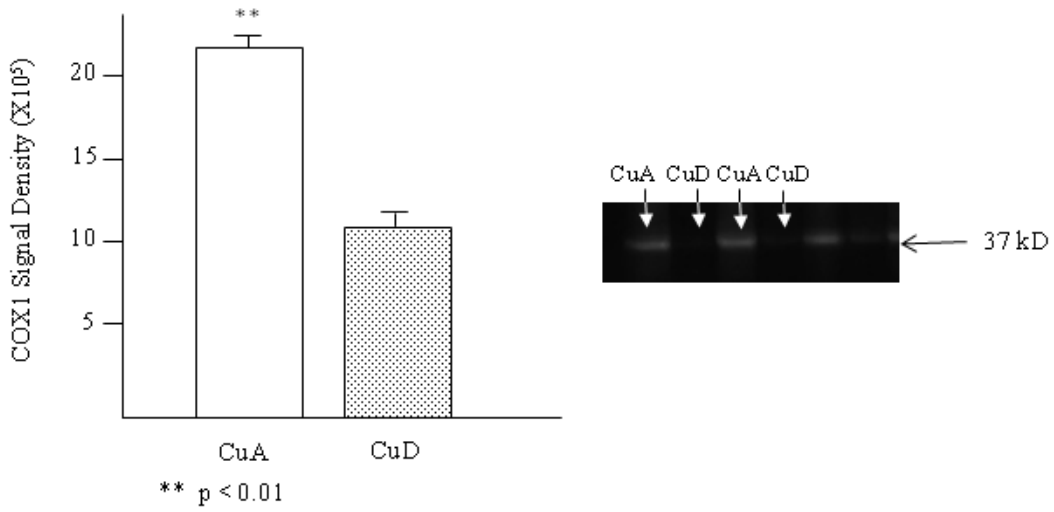

Figure 6. Cox1 Signal density (Mean ± SE).

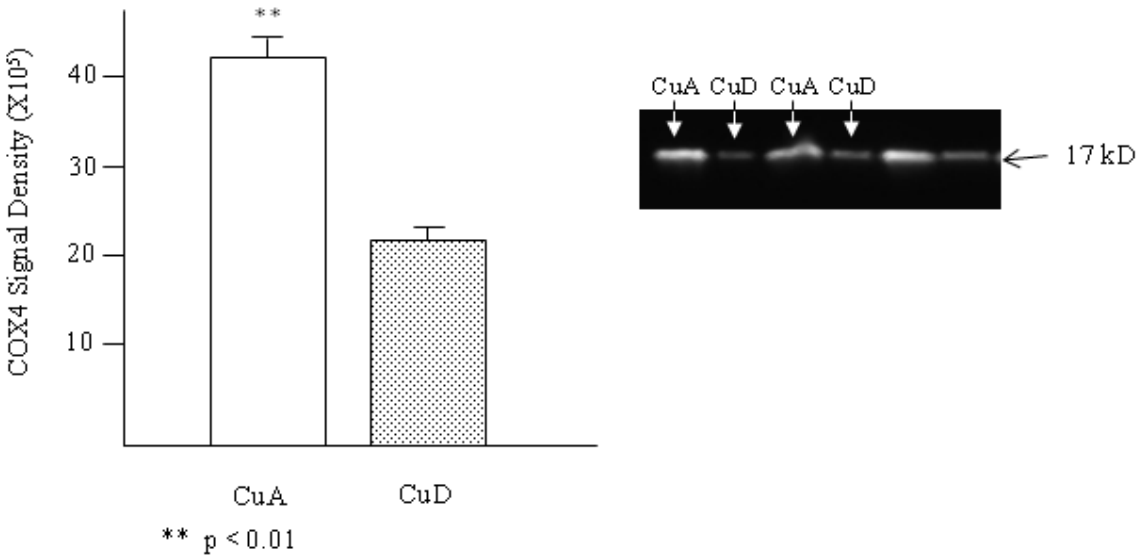

Figure 7. Cox4 Signal density (Mean ± SE).

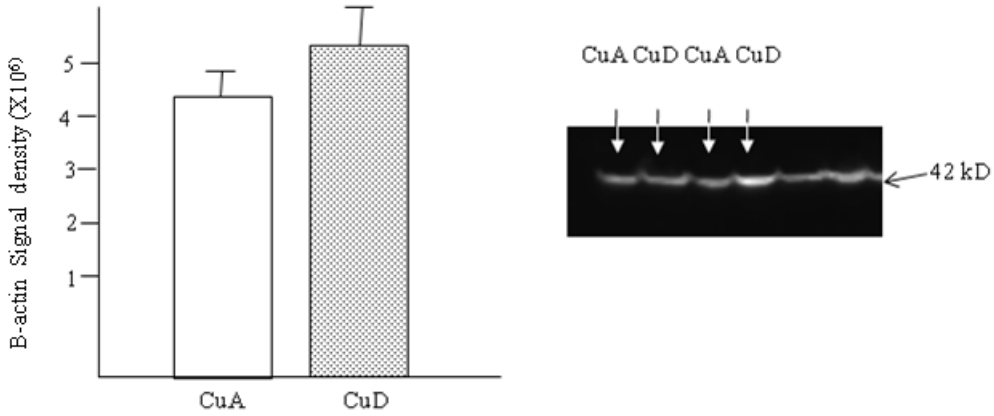

Figure 8. β-actin Signal density (Mean ± SE).

CHAPTER 5 – Discussion

Consistent with the work of others, the copper deficient rats in the current study exhibited cardiac hypertrophy, decreased hematocrit, and decreased body weight (4, 16-22). Our work demonstrated that the concentration of CCS increases with dietary copper deficiency, in agreement with the work of Bertinato *et al*. (44). However, we were also able to demonstrate that SCO1 is up-regulated in copper deficient rats, and subunits 1 and 4 of cytochrome C oxidase are down-regulated, but no change in subunit 1 was observed using a polyclonal antibody. While we did not observe a change in levels of CTR1 or Cox17, our data lends itself to a partial explanation of the mitochondrial proliferation observed in the cardiac hypertrophy of dietary copper deficiency.

The role of copper in maintaining cardiovascular health is not new. Klevay long ago reported that rats fed a diet lacking in copper develop high blood cholesterol levels and cardiac hypertrophy (56-58). Others have since verified this work (32, 59-61) The Medeiros lab continued to study the issue of cardiac hypertrophy as affected by a dietary copper deficiency (4, 5, 62). In several studies that group reported that the type of hypertrophy was concentric in nature, meaning the ventricular and atrial walls were thickened, interventricular septum was thickened, and the chamber volume was decreased. This is notable as up to that time, many in the field ascribed the hypertrophy in copper deficiency to anemia occurring with copper deficiency. However, anemia leads to an eccentric hypertrophy where the heart is larger and the chambers dilated. Furthermore, the Medeiros lab reported that cardiac hypertrophy occurred before anemia, putting an end to the argument that anemia was the causative agent in cardiac hypertrophy during copper deficiency (4, 16). An odd feature of the cardiac hypertrophy seen in copper deficiency is that concentric hypertrophy is associated with hypertension, but these rats have hypotension; thus we do have a discrepancy in this condition (16, 63). On the other hand, hearts from copper deficient rats have a dramatic increase in mitochondrial mass and it may be this feature that leads to the concentric nature of the hypertrohpy. Increased

mitochondrial biogenesis without a concomitant increase in myofibril mass will nonetheless lead to tissue hypertrophy.

The mitochondria in the copper deficient hearts appear to be vacuolated and the cristae do not appear to be in their normal parallel array. Many myofibrils appear to be distorted due to the mass of mitochondria appearing to push against the myofibrils (5). Electrocardiograms are abnormal, with decreased polarization and enhanced re-polarization time. Most notably the QRS complex is widened and there is notching in this complex (16, 31, 64). The peak to peak height of the RS components is significantly enhanced in the copper deficient rat heart, which may be another sign of cardiac hypertrophy. Finally it has been reported that lipid droplets accumulate in the myocardium (5) and the basement membrane is disrupted as well (65).

Mitochondrial biogenesis in the copper deficient rat heart has been studied and data show that certain genes which initiate mitochondrial biogenesis are up-regulated (22). For example, mitochondrial transcription factor A is known to increase the expression of nuclear DNA and mitochondrial DNA that encodes protein that becomes part of the organelle (22). Two other factors, nuclear regulatory factors 1 and 2, are up-regulated in copper deficiency and are known enhancers of mitochondrial protein production (66). Finally, recently the master regulator of mitochondrial biogenesis, peroxisomal proliferator activated receptor co-activator α (PGC1-α), has shown increased mRNA and protein levels during copper deficiency (67). Despite all these changes, ATP levels in copper deficient rat hearts are similar to those in copper adequate rat hearts (68).

Given that mitochondria play such a fundamental role in the pathogenesis of the cardiac hypertrophy observed in copper deficiency, as well as the known role Cu-Zn SOD has in organs, the next logical step was to investigate the chaperone proteins involved in the synthesis of mitochondrial cytochrome C oxidase and Cu-Zn SOD along with other chaperone proteins that bind copper and prevent it from reacting with other cell structures. Knowing whether dietary copper can regulate the

amount of these proteins may help unlock some of the mechanisms behind the pathophysiology in this model.

Results have previously been inconsistent concerning the effect of dietary copper deficiency on CCS concentration. While Bertinato *et al*. (44) observed an increase in CCS in erythrocyte and liver, Gybina and Prohaska (38) observed mixed results in various brain tissues. Our lab demonstrated an increase in the concentration of this chaperone protein during dietary copper deficiency. However, the activity of Cu-Zn SOD has previously shown marked decrease in several tissues (34- 37, 46, 40). This decrease in activity may be due to a decrease in concentration of SOD (38, 44). Alternatively, Dameron and Harris found that the protein was present but non-functional in copper deficiency (45). This finding suggests that the presence of copper may be limiting in SOD activity. When copper supply to a cell is limited, an increase in CCS such as we observed may be a compensation mechanism to maximize the copper supplied to the SOD enzyme.

The increase seen here in the concentration of SCO1 during copper deficiency may be a similar compensation. Since copper is required for the functioning of cytochrome C oxidase, and SCO1 is partially responsible for the insertion of copper into this enzyme, limited copper supply may signal an increase in Sco1 to facilitate increased copper utilization by cytochrome C oxidase. However, Cox17 also participates in making copper available to this enzyme, and we did not observe an increase in this chaperone protein. It is also possible that the increase in SCO1 rescued cytochrome C oxidase activity to such an extent that a concomitant increase in Cox17 was not necessary for cellular function. So while cellular compensation for lack of copper is a possible explanation for the increases observed in CCS and SCO1, more work is needed to confirm this hypothesis.

Cytochrome C oxidase activity has been observed to decrease overall in varied tissues during copper deficiency (34-38). In particular, the Cox4 subunit shows a marked decrease under copper deficient conditions (38, 41-43). Our results confirm this reduction in Cox4. We also observed a

reduction in Cox1 concentration with copper deficiency. Previously, however, Medeiros *et al.* have demonstrated decreases in the nuclear-encoded subunits of cytochrome C oxidase but not in the mitochondrial-encoded subunits including Cox1 (41,42). Indeed, a polyclonal antibody showed little or no change in Cox1 levels in this study, while a monoclonal antibody showed decreased levels of the protein. Whether the decrease in these two subunits only partially explains the decreased activity of the enzyme remains unknown, and further study is required to understand the mechanisms involved in the loss of enzyme activity.

Interestingly, we did not observe a change in the concentration of CTR1 with copper deficiency. It would seem beneficial for a cell to up-regulate this protein in order to maximize copper import to the cell. However, it has been demonstrated that mRNA levels for CTR1 do not change in response to dietary copper deficiency (69). Also of note is the unidentified band observed in the copper-adequate lanes of the SCO1 Western blots in the current study. The identity of this protein remains unknown, but it seems possible that it may be a breakdown product of SCO1 which is not present in copper deficient cells, where this protein is increased. The increase in SCO1 concentration observed may be due more to decreased breakdown of excess protein than to increased protein synthesis. Further study is warranted to ascertain the identity of this protein or product, and to determine the mechanism involved in the increased SCO1 concentration.

In light of these data, it seems possible that the mitochondrial proliferation observed in copper deficient cardiac hypertrophy is an effort to compensate for decreased cytochrome C oxidase activity and consequent decreased ATP production. Despite the great proliferation of mitochondria and the consequent increase in mitochondrial area and protein, an increase in ATP production is not observed in copper deficient hypertrophic hearts. It may be that ATP production by any given mitochondrion is limited by decreased cytochrome C oxidase activity. The increase in mitochondria observed in copper deficiency, and the hypertrophy this increase may cause, could be a mechanism to overcome decreased ATP production by individual mitochondria. If a decrease in the concentration

of subunits 1 and 4 of cytochrome C oxidase is sufficient to render it less active, such a hypothesis seems plausible. The observed increase in SCO1 concentration may also be a compensatory mechanism in response to a copper deficiency-induced decrease in cytochrome C oxidase activity.

In summary, the observed increase in SCO1 and CCS concentrations in cardiac tissue from copper deficient rats may be compensatory mechanisms for lack of cellular copper. The decrease reported here in subunits 1 and 4 of cytochrome C oxidase may hinder the activity of this enzyme in such a way that mitochondrial proliferation takes place in an effort to increase energy production. However, more study is needed to understand the mechanisms underlying the observed changes in protein concentration.

CHAPTER 6 – Summary

Copper deficiency has been linked with many health problems, among them cardiac hypertrophy. The cardiac hypertrophy caused by dietary copper deficiency is uniquely concentric, with mitochondrial proliferation and abnormalities. Because of its potential for causing oxidative damage, copper within the cell must be bound to chaperone proteins. While the large-scale physiological impact of dietary copper deficiency is recognized, little is known about the impact of such deficiency on these chaperone proteins. In this thesis, we examined the role of dietary copper deficiency in the regulation of select copper chaperone proteins in cardiac tissue in rats.

Male Long-Evans rats were randomized into treatment groups, one group receiving a copper deficient diet and one group receiving a diet containing adequate copper. Rats were sacrificed after 5 weeks of treatment, and heart tissue was analyzed. Western blotting was used to determine the concentrations of various copper chaperone proteins and copper-containing proteins in the heart tissue samples. Proteins studied include CTR1, CCS, Cox17, SCO1, Cox1 and Cox4.

Copper deficient rats exhibited decreased body weight, hematocrit, and liver copper, and increased heart weight and heart:body weight. No change was observed in the concentrations of CTR1 or Cox17. CCS and SCO1 were both up-regulated as a result of copper deficiency, while Cox1 and Cox4 were both down-regulated when using monoclonal antibodies; Cox1 did not change using a polyclonal antibody. The up-regulation of CCS and Sco1 might be compensatory mechanisms aimed toward providing more copper to cuproenzymes such as Cu-Zn SOD and cytochrome C oxidase. The downregulation of the two CCO subunits may indicate reduced transcription due to lack of copper. Our lab is the first to demonstrate an increase in SCO1 and CCS in hearts from copper deficient rats. Further study is warranted to fully understand these novel findings.

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Appendix A – IACUC Protocol Approval

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TO: Denis Medeiros
Human Nutrition
213 Justin

Protocol Number: 2660

FROM: Sally Olson, Chair
Institutional Animal Care and Use Committee

DATE OF APPROVAL: March 17, 2008
DATE OF EXPIRATION: March 17, 2011

RE: Approval of Animal Care and Use Protocol Entitled, "Chaperone proteins as affected by copper deficiency."

The Institutional Animal Care and Use Committee (IACUC) for Kansas State University has reviewed the protocol identified above and has approved it for three years from the date of this memo. During the period of approval, the protocol will be subject to annual monitoring, which may include the examination of records connected with the project. Announced post-approval monitoring (PAM) will be performed during the course of this approval period by a member of the University Research Compliance Office staff. Changes in the protocol affecting the care or use of animals must be reviewed by the IACUC prior to implementation. Unanticipated problems related to the humane care or use of animals must be reported to the IACUC immediately.

It is important that your animal care and use project is consistent with submissions to funding/contract entities. It is your responsibility to initiate notification procedures to any funding/contract entity of any changes in your project that affects the use of animals.