**Mitochondrial and sarcoplasmic protein changes in hearts from copper deficient rats: Upregulation of PGC1-α transcript and protein as a cause for mitochondrial biogenesis in copper deficiency**

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KEY WORDS: proteinomics, copper deficiency, rats, PGC-1α, metabolic enzymes.

Running title: PGC1-α protein and transcript is up-regulated in copper-deficient hearts

This article was published as:

Medeiros DM, Jiang Y, Klaahsen D, Lin D. 2009. Mitochondrial and sarcoplasmic protein changes in hearts from copper-deficient rats: up-regulation of PGC-1α transcript and protein as a cause for mitochondrial biogenesis in copper deficiency. Journal of Nutritional Biochemistry 20: 823-830.

**Abstract**

Changes in mitochondrial and sarcoplasmic proteins using proteinomics and western blotting in hearts from copper deficient rats were explored in this study. Also, key enzymes that are involved in cardiac energy metabolism via glycolysis and fatty acid oxidation and related transcription factors were determined. Rats were fed one of two diets: a copper adequate diet containing 6 mg Cu/kg diet or a diet with less than 1 mg Cu/kg diet for 5 weeks. Copper deficiency was confirmed by low liver copper levels, decreased hematocrit levels, and cardiac hypertrophy. Proteinomic data revealed that of the more than 50 proteins identified from the mitochondrial fraction of heart tissue, 6 were significantly down regulated and 9 were upregulated. The proteins that were decreased were beta enolase 3, carbonic anhydrase 2, aldose reductase1, glutathione peroxidase, muscle creatine kinase, and mitochondrial aconitase 2. The proteins that were up-regulated were isocitrate dehydrogenase, dihydrolipoamide dehydrogenase, transferrin, subunit d of ATP synthase, transthyretin, preproapolipoprotein A-1, GRP 75, alpha-B crystalline, and heat shock protein alpha. Follow-up western blots on rate limiting enzymes in glycolysis (phosphofructose kinase), fatty acid oxidation (medium chain acyl dehydrogenase, peroxisome proliferator-actvator receptor-α or PPARα), and gluconeogenesis (phosphoenolpyruvate carboxykinase), did not reveal changes in metabolic enzymes. However, a significant increase in peroxisome proliferator-activated receptor-$\gamma$ coactivator (PGC)-1α protein, as well as the transcript, which increased 2.5 fold were observed. It would appear that increased mitochondrial biogenesis known to

occur in copper deficiency hearts is caused by an increase expression in the master regulator of mitochondrial biogenesis, PGC-1α.

**Introduction**

Hearts from copper-deficient animals develop cardiac hypertrophy whereby the area occupied by mitochondria is greatly enhanced. The phenotype is similar to cardiac mitochondrial disorders with respect to the appearance of myocytes. Not only are there more mitochondria, they often appear fragmented and vacuoles are prominent. However, while some studies suggest that signs of heart failure appear (1,2 ) these signs are modest compared to other rodent heart disease models. Many animal models of heart failure have demonstrated for instance that atrial natreuretic factor (ANF) is dramatically up-regulated and the isomyosins revert back to the fetal stage (3). While such signs occur in hearts from copper-deficient rats, they are modest in comparison to these other models of cardiac failure. One possibility is that copper-deficient rats often succumb to aneurysms because lysyl oxidase activity, a cuproenzyme, is significantly impaired and results in diminished cross-linking of adjacent collagen molecules to enhance strength. Thus the animals succumb prior to later stages of heart failure.

Maintaining ATP levels in a normal range is important for the failing heart to remain functional. To maintain ATP levels the failing heart may shift from one type of energy substrate to another to maintain ATP levels. The preferred energy substrate in a healthy heart is fatty acids. In heart failure fatty acids may not be oxidized to the same extent as a healthy heart (4). In heart failure glucose utilization or anaerobic metabolism increases and aerobic metabolism decreases, again resembling the fetal heart. Whether or not such a shift in energy occurs in copper deficient hearts has not been studied. Since copper deficient hearts exhibit mitochondrial pathology, it is not unreasonable to ask

whether there is a potential change in substrate preference. Such as shift would readily be apparent in a number of enzymes that control energy metabolism. Furthermore, recent evidence suggests that PGC-1α, a master regulator of mitochondrial biogenesis, may also be altered in heart disease states (5). Again, we would expect that this transcriptional factor could be up-regulated in copper-deficiency.

In heart failure, multiple enzymes and thereby gene programs could be changing. In addition, many times a disease state will lead to secondary changes unrelated to the primary causal trigger. This is a problem in studying nutrient deficits upon organ function as that many proteins may change, some in direct consequence to a lack of the specific nutrient under study, and others due to secondary effects. This would suggest many other proteins and enzymes could be altered.

The central question posed here is whether or not there were changes in enzymes involved with energy pathways that changed in response to copper deficiency, and whether the master regulator of mitochondrial biogenesis, PGC-1α is up-regulated. To answer these questions we used proteinomic techniques initially to obtain a more global perspective upon potential non-myofibrillar protein changes due to copper deficiency and followed up these techniques with western blotting of specific target enzymes. Our study reports on the robust up-regulation of PGC-1α in hearts from copper-deficient rats with only minimal changes in enzymes involved in cardiac energy metabolism. Also, we report on changes in other proteins from copper-deficient hearts not previously reported in the literature.

## **Materials and Methods**

*Animals and design*

Sixteen male weanling Long-Evans rats were purchased from Charles River, Inc. (Boston, MA) and divided into two treatment groups. One group of rats were fed a diet with adequate copper (CuA group; n=8) and the other group were fed a diet with no added copper (CuD group, n=8). The diets were purchased from Research Diets (New Brunswick, NJ). Animals were fed their respective diets for 5 weeks, which followed the recommendations of the American Institute of Nutrition, consisting of (g/g diet by weight) 0.50 sucrose, 0.20 casein, 0.15 cornstarch, and 0.05 corn oil as energy sources (6). The CuA group received copper in the form of cupric carbonate at 6.0 µmol Cu/kg diet. The CuD group received feed with no added copper. To verify copper composition diets were digested with nitric acid and analyzed using flame atomic absorption spectrophotometry (Perkin Elmer Model 5000, Norwalk, CT) after the termination of the study. Analysis revealed that the CuA diet contained 5.31 µg Cu/g $\pm$ 0.457 and the CuD group 0.66 µg Cu/g $\pm$ 0.130. The Institutional Laboratory Animal Care and Use Committee at Kansas State University approved the protocol for this study.

Rats were singly housed in stainless steel cages in a controlled environment with a 12-hour light: dark cycle at a constant room temperature. All animals had free access to deionized-distilled water and food throughout the study. After 5 weeks each rat was anesthetized with an intravenous injection of thiobutabarbital sodium (Inactin, 100 mg/kg body wt; Research Biochemicals International, Natick, MA). The thoracic cavities were opened by midline incision and a small sample of blood was obtained by cardiac puncture and placed in a heparinized tube for hematocrit determination. Hearts were removed and

placed into in liquid nitrogen . Heart tissue was then stored at - 80°C until processed for RNA extraction. Livers were removed from all rats and placed on ice and frozen at -20 $^{o}$C for subsequent determination of liver Cu levels.

*Hematocrit assay and liver copper determination.*

Heparinized blood was transferred to microhematocrit tubes and centrifuged in a microcapillary centrifuge for 2 minutes. Hematocrit was determined as the percentage of space occupied by packed red blood cells. Liver was analyzed for copper by digesting approximately 1 g of liver in 10 mL of nitric acid prepared for trace element analysis. After digestion samples were diluted up to 10 mL with deionized-distilled water. Bovine liver standard from the National Bureau was analyzed to verify methods. Copper levels were measured by flame atomic absorption spectrophtometry, (Perkin-Elmer Model No, 5000, Norwalk, CT).

*Sample preparation for proteinomic analysis*

The protein lysis were purified by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules, CA) and reconstituted in protein sample buffer (7M urea, 2M thiourea, 4% CHAPS, 30mM Tris, pH=8.5). The protein concentration was determined by the protein RC DC Protein Assay (Bio-Rad, Hercules, CA). The internal standard was composed by pooling equal amount of proteins from both copper deficient and adequate rats. Sample labeling with cyanine minimal dyes was carried out according to the manufacture's instruction (GE Healthcare, Piscataway, NJ). The ratio of protein to Cydye is 1 µg protein: 25pmol Cydye. An internal standard was always added to each CuA and CuD sample labeled with Cy2 fluorescent dye (yellow). The samples were labeled with Cy3 fluorescent dye

(green) and Cy5 fluorescent (red) alternatively. That is half of the copper deficient samples received Cy3 and the other half Cy5, and the same thing were done with the copper adequate rats. Each gel had added a copper adequate and copper deficient sample with different dyes to reduced variation between gels. The excitation and emission wavelengths for each dye were per the manufacturer's suggestions (GE Healthcare).

*2D-Electrophoresis*

Two series of gels were developed to identify and quantify proteins. To quantify proteins the protein mixture containing 25 µg labeled internal standard and 25 µg labeled protein from each treatment group were mixed with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 140mM DTT and 2% ampholytes pH 3-10) and used to dehydrate IPG (pH 3 to 10) strips for 14 hours. Separately another series of gels containing 250 µg unlabelled protein was sued to identify individual proteins spots on the gel. The isoelectric focusing was carried on a PROTEAN IEF Cell following manufacture's instruction (Bio-Rad, Hercules, CA). Following IEF focusing, the strips were equilibrated in 4 ml of equilibration solution I (50 mM TRIS-HCl, 6 M urea, 30% glycerol, 2% SDS, 2.5% DTT, pH=8.8) for 10 minutes and then in 4 ml of equilibration solution II (50 mM TRIS-HCl, 6 M urea, 30% glycerol, 2% SDS, 2% idoacetamide, pH=8.8) for 10 minutes. SDS-PAGE was conducted using a precast 8-20% gradient gel. Electrophoresis condition was set at 200V for 60 -70 minutes until the dye front reached the bottom of the gel.

After running, the gels with Cy dye labeled proteins were scanned using a Typhoon 9410 scanner (GE Healthcare,NJ) with a resolution of 50 µm. Spot detection was performed on the gel images using the DeCyder 6.5 software. Before the matching

process, up to 20 landmark protein spots were defined on the gel. After matching, the cycle of reviewing and confirming the matches and re-matching was repeated manually until no new level 1 mismatches were found as per the manufacturer's protocol (DeCyder 2D Software, Version 6.5, GE Healthcare, NJ). The difference between the CuA and CuD groups were analyzed by t-test, which is provided by Decyder 6.5.

*Protein identification*

For the non-labeled gels, the spots of interest were excised and subjected to in gel digestion using trypsin (Sigma, St Louis, MO). The gels of non labeled protein were stained with Commassie blue for protein spot identification. The digested peptide were analyzed on a MALDI TOF/RTOF instrument (Bruker, CA) using α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) as matrix. Peak annotation was carried out automatically using software provided by the instrument manufacturer. The m/z-lists were submitted to an online database (MASCOT) to search the NCBI protein sequence database. This technique was used only to identify the protein spots and not quantify the results.

*Western blotting*

*Antibodies*. PFK, β-actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PFK host was goat anti-human and β-actin was mouse anti-human. The secondary antibodies were goat anti-mouse IgG1, goat anti-rabbit IgG, and donkey anti-goat IgG. PEPCK was purchased from Cayman Chemicals (Ann Arbor, Michigan) and was rabbit anti-human. PGC1α was purchased from Norvus

Biologicals (Littleton, CO) and was mouse anti-human. MCAD and PPARα were provided as a generous gift from the laboratory of Dr. Dan Kelly, Washington University School of Medicine (St. Louis, MO) and were rabbit anti-human.

*Electrophoresis*. Non-myofibrillar proteins consists of mitochondria and sarcoplasm in heart tissue. The myofibrils were separated from the non-myofibrillar protein by homogenizing 0.2 g left ventricular heart tissue in 2 mL of 0.1 mol/L KCl in 1.5% Triton X-100, followed by centrifugation for 20 min at 1100 x g. The supernatant was used for SDS-PAGE and Western blotting. About 80 μg protein per well were loaded in 4-20 % precast polyacrylamide mini gel (Pierce, Rockford, IL. Gels were run in BupH Tris-HEPES-SDS running buffer (Pierce, Rockford, IL) for 45 min at constant 150 V. Proteins were then transferred to nitrocellulose membrane (0.2 micro meter)using Bio-Rad (Richmond, CA) semi-dry transfer cells at 20 V for 30 min. Towbin transfer buffer was always freshly prepared. Transfer efficiency was confirmed by Ponceau S stain (Sigma, St Louis, MO). Then, membranes were blocked in 5 % non-fat milk (in water) for 1 hr. After blocking, membranes were blotted with primary antibodies overnight at 4 C with rocking and then secondary antibodies for additional 1 hr at room temperature with rocking. Membranes were washed with TDN washing buffer thoroughly. Finally immobilon Western HRP substrate luminol reagents (Millipore, Billerica, MA) were used to develop the specific bands using a Fluorchem 8800 imaging system (Alpha Innotech, San Leandro, CA). Eight rat hearts from each treatment group were analyzed.

*Real-time PCR*

PGC-1α gene expression in rat hearts was determined by using randomly-primed rat heart cDNA as template and a Taqman® Gene Expression Assay for PGC-1α mRNA (ABI #Rn00676177; 900 nM each primer, 250 nM probe). 18S rRNA was used for normalization and the comparative (ΔΔCt) method was found suitable to calculate gene fold change. The PGC-1α primers used were sense: CACAACCGCAGTCGCAAC; anti-sense: TGGCTTTATGAGGAGGAGTCG. Four rats hearts from each treatment group were analyzed.

*Statistical analysis*

A student t-test was used to determine significant differences at a $p \leq 0.05$ using a two tailed test. For the proteinomic data, the ratio of signal intensity of the CuD group to the CuA group was calculated by first normalizing the signal intensities to the internal standard labeled with Cy 2, and then performing a t-test on the normalized data by diet copper treatment. The absolute levels of proteins in each treatment group were not determined as our goal was to determine relative expression of proteins in the CuD group relative to the control CuA group using the Cy dye techniques described above.

**Results**

The results suggest that rats fed the copper deficient diet showed signs of copper-deficiency (Table 1). Final body weight, liver copper concentration, and hematocrit were significantly lower in the copper-deficient group compared to the adequate copper group; whereas heart weight and heart:body weight was significantly higher in the copper-

deficient group. There was more variation in hematocrit data among the CuD group with a low value of 15 in one rat to a high of 40, thus a larger standard error.

Over 50 protein spots were identified (Tables 2 and 3, Figure 1). Fifteen proteins were either up-regulated (n=9) and or downregulated (n=6) significantly ($p \leq 0.05$; Table 2) in the copper-deficient group compared to the copper adequate group. Among the proteins that were decreased were beta enolase 3, carbonic anhydrase 2, aldose reductase1, glutathione peroxidase, muscle creatine kinase, and mitochondrial aconitase 2. The proteins that were up-regulated were isocitrate dehydrogenase, dihydrolipoamide dehydrogenase, transferrin, subunit d of ATP synthase, transthyretin, preproapolipoprotein A-1, alpha-B crystalline, and heat shock protein alpha.

Western blots did not demonstrated differences by treatment for PPARα, medium chain acyl dehydrogenase, phosphofructokinase, and phospho enol pyruvate carboxykinase (Figure 2). For PGC-1α the protein was increased (Figure 2) and the transcript revealed a 2.5 fold increase in hearts from copper deficient rats (Figure 3).

## Discussion

Many of the proteins that changed in the proteinomic analysis are stress proteins that may change when an organ is damaged. Here the changes in the proteins could be secondary to copper deficiency and more likely explained by a weakened heart. For example, alpha-B crystalline is a heat shock protein and increases in heart muscle in response to stress and in ischemia-reperfusion (7, 8). This protein has been shown to increase in congestive heart failure in dogs (8). A decrease in beta enolase 3 is thought to be linked to beneficial changes in contractile properties occurring during cardiac

hypertrophy (9). Carbonic anhydrase 2 can decrease in failing hearts (10) The increase in dihydrolipomide dehydrogenase is a mitochondrial enzyme essential for energy metabolism as it is a part of pyruvate dehydrogenase (11). The decrease in aldose reductase is thought to be useful to attenuate maladaptive heart responses to injury (12). We report here that muscle creatine kinase is decreased and that the sarcomeric isoform (sometimes referred to as mitochondrial) approached statistical significance (P = 0.055, Table 2). This enzyme is involved in synthesis of creatine phosphate from ATP, and/or cleavage of the phosphate group from creatine to liberate energy. Creatine kinase can decrease in heart failure (4, 13) and this may suggest that copper-deficient rate hearts are in early heart failure. However, these data are at odds with a previous report by our group that creatine phosphate is elevated in hearts from copper deficient rats (14), which would suggest that this enzyme would be elevated. On-the-other-hand, the increased in creatine phosphate in hearts from copper deficient rats may be due to a decrease in creatine kinase.

An increase in chain A transthretin accelerates atherosclerosis and copper-deficient rats do have increase blood cholesterol which could suggest some initiation of this process occurring (15). On the other hand, aconitase 2, an iron regulating protein, was down downregulated. This is likely due to the fact that in copper-deficiency, iron is trapped in the liver and other organs have decreased iron, including the heart (16). A decrease is aconitase 2 would decrease ferritin which binds iron for storage and increase transferrin receptors to increase organ uptake of iron.

Here we also report an increase is subunit d of mitochondrial ATP synthase. In previous studies we have suggested an impaired function of ATP synthase (14, 17). ATP

synthase consists of two domains called $F_o$ and an $F_1$. The $F_o$ portion is embedded in the mitochondrial membrane and the $F_1$ subunit is above the membrane. Each of these domains are composed of several peptides. Subunit d is part of the $F_o$ domain. Here the increase is subunit d may most likely be due to increased number of mitochondria rather than a specific alteration in function. The β subunits of ATP synthase had a non-significant decreased trend and this is the catalytic portion of ATP synthase and is part of the $F_1$ subunit. This presumably could lead to decreased ATP formation. However, we have demonstrated earlier (14) that ATP levels in copper-deficiency are unchanged relative to copper adequate rat hearts. While there may be a decrease in the enzyme activity, the decrease does not appear to be physiologically relevant and it is widely accepted that cardiac ATP levels is a very poor predictor of cardiac health and function.

We could not detect any key rate limiting metabolic enzymes nor proteins involved with mitochondrial biogenesis using the proteinomic techniques described here. Therefore we followed this part of the study using western blotting and probe for key regulatory enzymes that are likely to change in heart disease. We probed for proteins that were rate limiting for glycolysis, aerobic metabolism, and gluconeogenesis, and determined the levels of PGC-1 alpha, the master controller of mitochondrial biogenesis. Our results demonstrated that PGC-1 alpha is significantly up-regulated in copper deficient hearts. There were no differences in phosphofructose kinase 1, phosphoenolpyruvate carboxykinase, or medium chain acyl dehydrogenase. It appears that in copper-deficiency, mitochondrial biogenesis is switched on, but there is no shift in substrate utilization as would be expected in compromised hearts. In a previous study we

reported that MCAD mRNA transcripts were elevated in hearts from copper deficient rats (18) which is in contrast to the protein levels reported here.

Previously we have shown that other mitochondrial biogenic transcription factors downstream from PGC-1 alpha are upregulated. Specifically NRF-1 and NRF-2 are upregulated early in the onset of copper deficiency (19) and mitochondrial transcription factor A shows a robust increase in hearts of copper deficient rats. Peroxisomal proliferating activating receptor- γ coactivator (PGC-1), as the master regulator of mitochondrial biogenesis and its interaction with mtTFA, NRF-1 and NRF-2 continues to be the subject of ongoing investigations. Spiegelman's lab originally discovered this transcription factor and its ability to induce the production of mitochondria in brown adipose tissue (20). There are various isoforms of PGC-1 which constitutes a family: PGC-1α, PGC-1β, and PGC-1-related coactivator. Both PGC-1α and PGC1-β have high expression in tissues rich in mitochondria. Unlike some other transcription factors, PGC-1α does not have any response elements, meaning it does not bind to a DNA promoter directly. Rather it acts via a protein-protein interaction but it does not have enzymatic activity. PGC-1α is more likely involved in the recruitment of other transcription factors that allow the chromatin to be remodeled. (21). Transfection of PGC-1 into $C_2C_{12}$ cells by Wu et al. (22) and into myocytes by Lehman et al. (23) all resulted in indices of mitochondrial biogenesis in terms of mitochondrial protein, transcripts and mitochondrial volume densities of the cells. PGC-1 may act as a coactivator of NRF-1 (22), which then is thought to bind to the promoter of mtTFA to initiate the concomitant upregulation of both mitochondrial and nuclear encoded proteins in a coordinated fashion. Another set of transcription factors are also needed to initiate mitochondrial biogenesis. Mitochondrial

Transcription Specificity factors (TFB1M and TFB2M). There are recognition sites within the promoters for NRF1 and NRF2 for these two mitochondrial transcription factors. It is also reported that PGC1-α will upregulate these two transcription factors. Upregulation of mtTFA augments mitochondrial biogenesis with these other transcription factors (24).

What signals are sent to heart cells that trigger PGC1-α upregulation? A strong candidate for this is the known production of nitric oxide in hearts of copper deficient hearts. Saari et al. (25) demonstrated that hearts from copper deficient rats have increased endothelial NO synthase and inducible NOS protein expressions, as measured by Western blot analysis. Cardiac NOS activity, as measured by conversion of (3)H-arginine to (3)H-citrulline, was 130% higher in Cu-deficient than in Cu-adequate rats. Furthermore, NFkB activation as determined by the p65 subunit was higher in hearts from copper deficient rats. This is significant as NFkB is a transcription factor inducible NOS. The role of NFkB was shown to robustly upregulated in a recent paper our lab published (26). Nisoli et al (27) reported that nitric oxide triggered mitochondrial biogenesis and that PGC1-α mediated this effect in a variety of cell types. Also important is the NO will bind with the copper-dependent enzyme, cytochrome c oxidase to decrease respiration (28); an enzyme that is already decreased in activity and peptide levels in copper deficiency (17, 28). We propose that nitric oxide is a candidate for the upregulation of PGC1-α that leads to mitochondrial biogenesis in hearts of copper-deficient rats.

**Acknowledgements**

Publication contribution number from K-State Research and Extension is 09-016-J. Supported in part from funds for USDA multi-state project number W1002: Nutrient bioavailability: phytonutrients and beyond.

Proteinomic analysis was made possible by a Kansas State University Targeted Excellence Award entitled “Functional Genomics Consortium”. National Science Foundation Research Instrumentation Grant Number 0521587 funded a portion of the Biotechnology Core and Proteinomics facility.

The authors wish to acknowledge the contribution of Dr. Yasuaki Hiromasa of the Biotechnology Core and Proteinomics facility for his help with the proteinomic analysis.

**Figure legends**

**Figure 1.** A representative gel image with CyDye staining. Each sample had an internal standard added (cy2). In this particular image, a copper deficient rat was labeled with cy3 (showing green) and a copper adequate rat was labeled with cy5 (showing red). The samples were run in the same 1st dimension on IGP strips and the 2nd dimension on SDS-PAGE. The above image shows the overlap of the 3 sample of the gel image( internal standard, copper deficient and copper adequate samples). Green spots indicate higher protein level for copper-deficient and red spots indicate higher expression for that protein for the copper adequate rat. Areas of white indicate that the levels of internal standard, copper deficient protein, and copper adequate proteins were similar in quantity.

**Figure 2.** Representative western blot signals and mean intensity of proteins studied: medium chain acyl dehydrogenase (MCAD), phosphofructokinase-1 (PFK-1), phosphoenol pyruvate carboxykinase (PEPCK), PPARα, PGC1α, and β-actin. Data are mean $\pm$ standard error (n=8 in each treatment group mean). CuA=copper adequate diet, CuD= copper deficient diet.

**Figure 3.** Real-time qPCR showed a 2.54 fold increase of PGC-1α mRNA expression in copper deficient rat hearts (*p=0.001). CuA=copper adequate diet, CuD= copper deficient diet. (N=4 for each treatment group)

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