Differential expression of genes involved with apoptosis, cell cycle, connective tissue proteins, fuel substrate utilization, inflammation, and mitochondrial biogenesis in copper-deficient rat hearts: implication of a role for Nfkb1.

Kristen Ricklefs

Darcey Klaahsen

Denis M Medeiros[1]

Department of Human Nutrition

213 Justin Hall

Kansas State University

Manhattan, Kansas 66506

Medeiros@ksu.edu

Phone: 785-532-0150

FAX: 785-532-3132

[1]To whom correspondence should be addressed

ABSTRACT

We hypothesized that the increase in mitochondrial proliferation in hearts from copper-deficient rats is due to an increase in expression of the transcriptional factor Ppargc1a, which regulates transcriptional activity for many of the genes that encode for mitochondrial proteins. In addition to several transcriptional factors implicated in mitochondrial biogenesis, we also looked at a number of genes involved in cell cycle regulation and fuel substrate utilization. Long-Evans rats were placed on either a copper-adequate (n=4) or copper-deficient (n=4) diet 3 days post-weaning and remained on the diet for five weeks; their copper deficiency status was confirmed using previously established assays. Custom oligo arrays spotted with genes pertinent to mitochondrial biogenesis were hybridized with cRNA probes synthesized from the collected heart tissue. Chemiluminescent array images from both groups were analyzed for gene spot intensities and differential gene expression. Our results did not demonstrate any significant increase in Ppargc1a or its implicated targets, as we had predicted. However, consistent with previous data, an up-regulation of genes that encode for collagen type 3, fibronectin and elastin were found. Interestingly, there was also a significant increase in the expression of the transcriptional factor Nfκb1 in the copper-deficient treatment animals compared to the control group. The results of this study merit the further investigation of the role of ROS with regard to Nfkb1 in the copper deficient rat heart.

KEY WORDS: Copper-deficiency, heart, rat, mitochondrial Nfκb1, Ppargc1a

INTRODUCTION

Copper has been established as a necessary component needed for normal cardiovascular growth and function. Characteristics of the copper deficient heart include the development of concentric cardiac hypertrophy (1,2), weaknesses within the cardiac wall structure that can lead to ventricular aneurysms (2-4), and increased mitochondria proliferation (2, 5-7). Often the mitochondria have disrupted membrane microstructure and accumulation of lipid droplets (2).

The mechanisms that control mitochondrial biogenesis in the copper-deficient heart are not yet clear. A number of transcriptional factors have been implicated as components in the mitochondria biogenesis regulatory pathway. These transcriptional factors that have been identified as of yet include, nuclear respiratory factor 1 and 2 (Nrf1, Nr2f) and mitochondria transcription factor A (Tfam). It has also been shown that all of these transcriptional factors require functional activity and binding of another key regulatory receptor in order to exert their own activity, the transcriptional co-activator peroxisomal-like proliferating related co-activator 1- α (Ppargc1a).

Ppargc1a was first identified as a co-activator needed for functional activity of the family of peroxisomal proliferating activator receptors (PPARs) (8). Three PPAR isoforms have been identified and all are related to fatty acid metabolism and storage. The two main isoforms that are found in the heart include PPAR- α and PPAR-δ/β. PPAR- α has been associated with regulation of the fatty acid oxidation pathway, while the isoform PPAR- δ/β has been linked to fatty acid storage and transport (9).

In the normal adult heart long chain fatty acids are the preferred source of fuel for energy metabolism. This is due to the high energy demands of the heart and the

efficiency by which fatty acids can provide adequate amounts of ATP, the main source of fuel used by the body. In the failing heart there is a shift in substrate utilization from that of primarily lipid oxidation to glucose as the main source of fuel. This shift in energy metabolism mimics substrate utilization within the fetal heart and previous studies have shown that there is an onset and up-regulation of fetal genes being expressed (10). The increase in mitochondria proliferation and biogenesis has been established as a primary disease state of some forms of cardiac hypertrophy, however, it remains unclear in regards to abnormal lipid oxidation as to whether or not the shift in energy metabolism occurs prior to states of cardiac hypertrophy and the development of heart failure or whether it is caused by these disease states. It is also uncertain if the same regulatory components that function to regulate mitochondrial proliferation and biogenesis are also factors in mitochondrial lipid metabolism. It has been implicated that the PPAR- α and PPAR-δ/β transcriptional factors have a role in both mitochondria biogenesis and lipid metabolism. This suggests that its co-activator Ppargc1a, which is a known regulator of both factors for proliferation and growth of the mitochondria as well as proper function, is acting to coordinate this series of events in a concerted fashion and thereby has been deemed the "master-regulator" of the mitochondria (9, 11).

The purpose of this study is to determine whether gene expression level of Ppargc1a are being over-expressed in the copper-deficient rat model heart as well as any other possible gene regulation. We propose that there will be a significant up-regulation of Ppargc1a which would be reflective of the increase in mitochondria proliferation and biogenesis. Other transcriptional factors downstream from PGC1-α should also be up-regulated according to our experimental hypothesis such as Nrf1, NrF2, and Tfam. We

also propose that the targets of Ppargc1a that are involved in lipid oxidation, PPAR- α, PPAR-δ/β, and ERR- α may be down-regulated, which would be reflective of the dynamic shift in fuel substrate selection. Moreover those genes that are involved in glycolysis should be up-regulated in copper-deficiency and the lipid oxidation genes should be down-regulated if there is a shift in substrate utilization during copper-deficiency as proposed. To determine if the genes involved both in mitochondrial biogenesis and proliferation were regulated in concert with one another we used customized oligo gene arrays in which multiple genes could be analyzed simultaneously. Other genes involved with apoptosis, cell cycle, connective tissue metabolism and inflammation were also studied as variables of interest.

METHODS AND MATERIALS

Animals and Diets

Eight male Long-Evan rats were obtained 3 days post-weanling. Animals were purchased from Harlan Sprague Dawley (Indianapolis, IN). Initial weights were recorded at the beginning of the study and were recorded once a week for the duration of the study. Animals were assigned into two groups of 4 rats each and placed upon either a copper-adequate diet (CuA) or a copper-deficient diet (CuD). Groups were assigned based upon similar weights in order to eliminate any bias of final heart body weight and composition. Diets were obtained from Research Diets, Inc. (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 (12). The control groups (CuA) received copper in the form of cupric carbonate at 94.5 μmol Cu/kg diet. The experimental groups (CuD) 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. 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, weighed, cut into two equal parts, with half placed into RNAlater (Ambion) and the other half frozen 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-Zn SOD activity.

Hematocrit Assay

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 Superoxide Dismutase (SOD) assay

Liver that was collected from the rats at the termination of the study was used to determine liver SOD enzyme activity. One gram of frozen liver tissue from each rat was individually homogenized and prepared according to a standard liver SOD protocol (13, 14). Enzyme activity was analyzed using spectrophotometry.

Customized array development.

One custom array (SuperArray Biosciences Corporation) was provided for each sample for a total of 8 arrays. The custom array layout contained a variety of selected genes, including genes that were involved with substrate utilization, enzymes, energy

metabolism, cell cycle regulation, apoptosis, and mitochondria biogenesis. Genes that were determined to be targets of interest were complied into a list containing the NCBI gene bank number, unigene, and gene symbol and sent to SuperArray, which manufactured the custom array layout according to the pre-determined gene list. One hundred and twenty genes were printed on each array.

Total RNA Extraction

The hearts of each animal were weighed and cut in half: one-half of the heart was placed in 10V RNAlater (Ambion), cut into small pieces (0.5 cm in the smallest dimension), and stored at $^{-}80\ ^{o}$ C. The other half of the heart was snap frozen in liquid nitrogen and also stored at $^{-}80\ ^{o}$ C. Total RNA from rat hearts was isolated using the RNeasy® Protect Midi Kit (Qiagen), following the manufacturer's recommendations for heart tissue. Heart tissue (100-150 mg) was either ground in liquid nitrogen and homogenized in lysis butter or the small pieces of heart tissue in RNAlater were directly homogenized in lysis buffer with a rotor/stator-type homogenizer for 30 seconds on high. Proteinase K digestion of the lysate was included to get rid of contaminating proteins and to facilitate complete homogenization. The remaining RNA extraction steps were followed as stated in the manufacturer's protocol. Total RNA was eluted from the columns, quantitated on a Nanodrop spectrophotometer, and aliquoted for further applications. In addition, the quality of the total RNA (~250 ng) was assessed on an Agilent 2100 Bioanalyzer Nanochip for subsequent steps and determined to be pure and of high-quality.

Linear Amplification and Labeling of cRNA Targets for Oligo GEArray®Hybridization

One microgram of each total RNA sample was used for linear RNA amplification using the TrueLabeling-AMP 2.0 Kit (SuperArray Biosciences Corporation). Briefly, primers were annealed to total RNA at 70 ^{o}C for 10 minutes, followed by cDNA synthesis for 50 min at 42 ^{o}C on a thermocycler. *In vitro* transcription of biotinylated (Biotin-16-UTP, Roche Applied Sciences) cRNA targets was performed for 9 hours at 37 ^{o}C. The cRNA was then column-purified and eluted in 10 mM Tris, quantified on a Nanodrop spectrophotometer, and two micrograms of each biotin labeled cRNA target was used for hybridization to eight customized oligo arrays.

cRNA target hybridization and detection

Arrays were prehybridized at 60 ^{o}C for two hours with 2 ml hybridization buffer in small hybridization tubes (provided) which were placed in two larger glass hybridization bottles in a hybridization oven (five array tubes/hyb bottle). Two micrograms of each cRNA target per array was added to the array tubes and allowed to hybridize overnight at 60 ^{o}C (SuperArray Biosciences Corp). The next day, post-hybe washes were performed at 60 ^{o}C with prewarmed 2XSSC/1%SDS and a high stringency wash with 0.1X SSC/0.5%SDS. Arrays were blocked at room temperature and then bound to alkaline phosphatase (AP) -conjugated streptavidin at 1:8000. After AP labeling, the arrays were washed in detection solutions and exposed with 1:1 CDP-Star:Buffer G in pairs (one copper deficient array/one control array per five exposures). Each pair of arrays was exposed for 15 minutes under a FluorChem™ 8800 Imaging

System (Alpha Innotec Corporation, San Leandro, CA). The remaining arrays were left in detection buffer G until exposed, in pairs. Captured array images were uploaded into GEArray Suite Analysis (SuperArray Biosciences Corp) for analysis. Once uploaded into GEArray Suite, the arrays were normalized using inter-quartile normalization and background was substracted. The copper- deficient array spots were compared to the control array spots to determine fold change differences in gene expression as determined by spot intensities/ratios.

Real-time qPCR

In a second study, real-time quantitative PCR was used to determine fold changes in Nfkb1 (p105 subunit) and PGC-1 alpha using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA). The Assay ID number for Nfkb1was Rn01399583_m1 and a final concentration of 900nm each primer and 250 nm probe was used. The PGC-1alpha Assay ID number was Rn00676177_g1 and primer and probes was also used at 900nm of each primer and 250 nm probe final concentration. Both gene expression assays had a FAM reporter dye at the 5'end of the Taqman MGB probe and a nonfluorescent quencher at the 3'end of the probe. Rat heart cDNA was reverse transcribed from total RNA using random hexamer priming (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA), as 18S eukaryotic rRNA (VIC-MGB labeled) was used as an endogenous control for relative quantitation and to determine fold change by the ΔΔCT (comparative) method. All cDNA samples were adjusted to the highest 18S Ct value (or sample with the lowest concentration). The PCR reaction was run on an ABI 7000 Prism Detection System (Applied Biosystems) using

the following cycling conditions: 15 seconds@ 95^{o}C and 1 min @60^{o}C (40 cycles). A semi-log regression curve of ΔCT vs. input amount of cDNA template was plotted to ensure the efficiency of the target gene (Nfkb1 or PGC-1 alpha) and reference gene (18S rRNA) were approximately equal (or slope ≤0.1) and that ΔΔCt calculations were valid.

Statistical Analysis

Due to non-parametric data, the F-rank protected T-test was employed as the statistical means of analysis. Total binding intensity (pixels) was averaged for each group and analyzed. Results were reported as significant using a p-value of 0.05. Results were reported to be a significant trend using a p-value of $p< 0.10$.

RESULTS

Final heart weights between the two groups indicated that the CuD rats had a lower mean body weight ($p \leq 0.0001$), a higher mean heart weight ($p \leq 0.0001$), and the final heart weight to body weight measurements (HW:BW) indicated that the CuD rats had a significantly higher ratio ($p \leq 0.05$) (Table 1). Analysis of the hematocrit assay and liver Cu,Zn SOD assay demonstrated that there was a significant decrease of the means in the CuD group ($p \leq 0.05$ and 0.001, respectively).

Gene expression results from this study are presented by degree of significance of fold change. In one series of results we reported those genes that were significantly different from one another by treatment ($p \leq 0.05$) and that had a fold increase or decrease of at least 50% (Table 2). A second set of results are presented where genes were statistically significant by treatment ($p \leq 0.05$) but did not have a fold change of greater or less than 50% (Table 2). A third set of data is presented in which there was a significant trend in data between the two treatment groups, indicted by $p \leq 0.05$-0.10 (Table 3).

Genes that demonstrated a significant difference ($p \leq 0.05$) are shown in Table 2. Genes that were different and had a fold increase or decrease of at least 50% included: nuclear factor κ B1, collagen type 3a1, early growth response 1, E2F transcription factor 1, myelocytomatosis viral oncogene homolog, matrix metalloprotidase 12, carnitine palmitoyltransferase, and O-6-methylguanine DNA methyltransferase. In addition, genes that demonstrated a significant difference ($p \leq 0.05$) as determined by the F-rank test but did not display a fold change induction of greater or less than 50% as compared to the controls are also shown in Table 2. These genes were Kirsten rat sarcoma viral oncogene

homologue 2 (active), insulin-like growth factor binding protein, caspase 8, elastin, and cyclin-dependent kinase 6.

Genes that demonstrated a trend (p=0.05-0.10) and displayed a fold change induction of greater or less than 50% as compared to the controls are shown in Table 3. Observed genes that met both of these criteria include interleukin 2, cyclin-dependent kinase inhibitor 2b (Cdkn2b), fibronectin 1, DNA-inducible transcript 3, and caspase 9.

DISCUSSION

Previous studies have shown that rats fed a copper-deficient diet demonstrate decreased copper-dependent enzyme activity (15), impaired mitochondrial respiration (16), abnormal membrane pathology (3), and increased mitochondrial biogenesis (17). The copper-deficient rat model displays characteristics in the changes and pathology of the heart structure that mimics pressure overload (18).

Consistent with this altered state, analysis of our data showed that there was a significant increase in the connective tissues elastin and collagen type 3a transcripts, which is a primary collagen type found in the cardiac tissue. These connective tissues have previously been represented to be up-regulated in the copper-deficient heart (2, 19). It was also shown in this study that the connective tissue fibronectin showed a trend towards being significantly up-regulated (20, 21), which is reflective of our findings regarding copper-deficiency and enzyme activity, in addition to previously reported data (15).

In recent studies much attention has been directed towards the increase in mitochondrial biogenesis within the heart and the regulating factors governing this pathway. Targets of these studies have included the transcriptional factors mitochondrial transcription factor A (Tfam), Nrf1, Nr2f, PPAR-α, and Ppargc1a and their interactions as possible signaling pathways that are responsible for the demonstrated pathologies. Previous hypotheses suggested the interactions between mtTFA and Nrf1, Nr2f as a possible mechanism for enhancing mitochondrial biogenesis within the copper-deficient

heart. Most notably Mao et al. (22, 23) found that rats fed a CuD diet for five weeks displayed significantly higher amounts of Tfam proteins expressed within the CuD hearts, however there was no change in the expression of Nrf1 or Nr2f. These findings have not shown to be consistent from study to study, and Mao *et al.,* (23) did implicate that these findings may be due to a transient change that is occurs in a time-dependent manner within a failing heart, with the up-regulation of Nrf1 and Nr2f occurring early in the development of heart failure and returning to normal or near normal levels after the initial change (23). Our current findings did not demonstrate any significant difference among the treatment and control groups for any of the three transcriptional factors involved in mitochondrial biogenesis.

The various PPAR isoforms regulate fatty acid metabolism in various tissues including the heart. A co-activator is needed for the PPAR isoforms to function as promoters. The transcriptional co-activator that was identified as the regulating factor within the heart was peroxisomal proliferator activated-receptor 1-alpha (Ppargc1a). (8). Further studies within the Kelly lab at Washington University (St. Louis, MO) found that PGC-1-α was directly linked to PPAR-alpha (24) and appeared to increase its activity and function with regards to mitochondrial biogenesis control (11, 25). Similar studies also demonstrated that forced over-expression of Ppargc1a in vivo resulted in an enhanced expression within cardiomyocytes and increased mitochondria gene transcriptional levels within the cardiac tissue (24). Several studies have now reported that Ppargc1a directly targets Nrf1, Nr2f, Tfam, and the PPAR family and appears to enhance transcriptional activity. Our findings were not consistent with these previous reports. While we did observe an increased fold change in Ppargc1a within the CuD treatment group, it did not

prove to be significant. Further studies in our lab using real-time quantitative PCR have not yet shown clear, consistent changes in Ppargc1a expression.

The preferred energy substrate of the heart is fatty acids. Hearts that display cardiac hypertrophy with heart failure demonstrate a profound decrease in lipid oxidation and increase glucose utilization, which mimics energy metabolism within the fetal heart (10). As mentioned above, genes that encode for mitochondrial fatty acid metabolism are regulated by the peroxisome-proliferating activated receptors (PPARs) a family of transcriptional factors, which in addition to being linked to fatty acid metabolism, have been suggested to play a key role in mitochondrial biogenesis. There are three isoforms of PPARs, all of which are induced by fatty acids and exert their function by binding to the retinoic receptor (RXR) as well as a repeating binding sequence motif (26). PPAR-α and more recently the isoform PPAR-δ have been implicated as the main isoforms present in cardiac as well as brown adipose tissue and are responsible for targeting the promoter region of key mitochondrial enzymes to regulate cardiac energy metabolism. PPAR-α is suggested to be the main isoform responsible for fat metabolism within the heart and has been shown to be involved in the majority of processes governing fatty acid catabolism. None of these genes appeared to be differentially expressed in our study.

Our results indicated that there was a significant increase in the translocation protein carnitine palmytioltransferase 1 (CPT-1) which is associated with fatty acid metabolism and functions to shuttle the fatty acids across the mitochondrial membrane to the electron transport chain. This up-regulation of CPT-1 that we observed may be explained by the increase in total mitochondria within the heart and not to an increase in

free fatty acid mobilization. This finding is consistent with prior studies related to the copper-deficient heart and cardiac hypertrophy (1, 27).

A novel finding reported in this study in regards to the copper-deficient rat model and heart failure was the up-regulation of Nfκb1. Our preliminary studies using real time PCR supports this finding for Nfκb1. Nfκb1 is a transcriptional factor that has been implicated as a regulator of multiple factors such as Ppargc1a, PPARs, Nrf1, Nr2f, HNF4, and MEF2. Nfκb1 has also been suggested to be a key regulator of a number of enzymes. Nbκb1 is a major element of the cellular growth, inflammatory, and apoptotic pathways (28). We found significant upregulation in the mRNA expression levels for a variety of known genes associated with cell survival and DNA repair such as caspase 9 and O-6-methylguanine-DNA-methyltransferase enzyme, respectively. We also found an up-regulation trend in the anti-apoptotic/cell survival genes Mdm2, which function to inhibit p53 growth suppression, BCL2 and Gadd45 both of which may mediate a delay in G2 to M cell cycle progression or may induce DNA repair. Also, we report that data collected and analyzed from the copper-deficient hearts displayed a decrease that was near significant in the apoptotic genes caspase 8. There was also an observed increase in a number of genes that are related to cell growth and proliferation such as Igfb3, Kras2 and Myc (both oncogenes, also involved in cell survival), Erg1, E2F1, and Vegf.

Nfκb1 has recently received attention as a component of the pathway governing cardiac hypertrophy and subsequent heart failure signaling. One of the major ways that Nfκb1 is induced is via reactive oxidative species (ROS). ROS are a by-product of the oxidative/phosphorylation process within the mitochondria. Copper-deficient rats

produce more reactive oxygen species and results in lipid peroxidation in the heart (29, 30) and one report suggests that NFkB expression is related to copper status (31). In copper-deficiency a possible increase in glucose utilization could lead to a decrease in the membrane efficiency to produce adequate energy to meet metabolic demands. This further leads to an increase in the electrons retained in the electron transport chain and may lead to an increase in ROS formation. Other studies have shown that activation of Nbκb1 is required for the development of cardiac hypertrophy *in vivo* (32) as well as needed for hypertrophic growth of ventricular cardiomyocytes in culture (32, 33). Studies using aortic banding-induced cardiac hypertrophy have demonstrated increase expression levels of Nfκb1 (32), while studies that have used NFκB inhibition decreases the hypertrophic response of cardiac myocytes.

Finally, the negative observations reported here on genes involved with glucose and fatty acid oxidation may simply mean that there is no shift in substrate utilization. To our knowledge, there have been any reported studies on fatty acid and/or glucose oxidation in the heart during copper-deficiency. The increased number of mitochondria observed in copper-deficiency may be sufficient enough to met energy demands. The role of Nfκb1 in copper-deficiency and cardiac hypertrophy may b a novel line of inquiry.

Functional category	Gene	GenBank number
Substrate utilization	-Medium chain acyl dehydrogenase -Long chain acyl dehydrogenase -PPAR-alpha -PPAR-delta -PPAR-gamma -Retinoic acid receptor-alpha -Retinoic acid receptor-gamma -phosphoenolpyruvate carboxykinase -Carnitine palmitoyltransferase -Insulin-like growth factor b3	NM_016986 NM_012819 NM_013196 NM_013141 NM_013124 NM_031528 XM_217064 NM_198780 NM_013200 NM_012588
Mitochondrial biogenesis	-PGC-1alpha -mitochondrial transcription factor A - -Nuclear respiratory factor-1 -Nuclear respiratory factor-2	NM_031347 NM_031326 XM_231566. XM_575228
Electron transport	-oligomycin sensitivity conferring protein -inhibitor of ATP hydrolysis by the mitochondrial A complex -cytochrome C oxidase subunit II -cytochrome C oxidase subunit IV -ATP synthase beta subunit -ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	NM_138883 NM_012915 NC_001665 NM_017202 M19044 NM_139106
Cardiac Proteins	-troponin C, cardiac/slow skeletal -troponin I, cardiac -troponin T2, cardiac -myoglobin -cardiotrophin 1 -natriuretic peptide precursor type A	XM_214266 NM_017144 NM_012676 NM_021588 NM_017129 NM_012612
Connective Tissue	-collagen alpha1 type I -collagen, type III, alpha 1 -alpha-3 type IV collagen -fibronectin 1 -elastin	Z78279 NM_032085 XM_343607 NM_019143 NM_012722
Apoptosis and cell cycle	-nuclear factor kappa B, p105 subunit -BAX -BCL2 -Caspase 8 -Caspase 9	XM_342346 NM_017059 NM_016993 NM_022277 NM_031632

	-Cyclin Dependent kinase 6	AF352168
Apoptosis and cell cycle (con't)	-O-6-methylguanine DNA methyltransferase	NM_012861
	-E2F transcription factor 1	XM_230765
	-Early growth response 1	NM_012551
	-Kirsten rat sarcoma viral oncogene homologue 2 (active)	NM_031515
	-cyclin-dependent kinase inhibitor 2B	NM_130812
	-Myelocytomatosis viral oncogene homolog	NM_012603
	-DNA-inducible transcript 3	NM_024134
Copper chaperone proteins	-SCO cytochrome oxidase deficient homolog 1 (yeast)	NM_004589
	-endothelial growth factor 1 (77% similar to human SCO2)	NM_005138
	-COX17 homolog, cytochrome c oxidase assembly protein (yeast) (Cox17)	NM_053540
	-copper chaperone for superoxide dismutase	NM_053425
	-ATX1 (antioxidant protein 1) homolog 1 (yeast)	NM_053359
	-estrogen- related receptor, alpha	NM_001008511
	-superoxide dismutase 1	NM_017050
Other	-Matrix Metalloprotidase 12 (proteolytic enzyme)	NM_053963
	-Interleukin 2 (Immune response)	NM_053836
Endogenous controls*	-Glyceraldehyde-3-phosphate dehydrogenase	NM_017008
	-Aldolase A	NM_012495
	-Lactate dehydrogenase A	NM_017025
	-Ribosomal protein L32	NM_013226

Table 1. Indices of copper deficiency in male Long Evans rats fed a 5 week copper deficient (CuD) diet. Values expressed as means ± standard error (S.E.).

Variable	Control (CuA) Mean ± S.E.	Copper-deficient (CuD) Mean ±S.E.
Final Heart Weight (g)***	1.23 ± 0.048	1.60 ± 0.198
Body Weight (g)***	296.72 ± 11.5	166.02 ± 8.7
HW:BW ratio*	0.0060 ± 0.002	0.0041 ± 0.0011
Hematocrit*	41.3 ± 2.1	31.2 ± 3.7
Liver Cu,Zn SOD (U/g wet wt)**	10278.80 ± 8.123	2746.4 ± 11.475

*** $p \leq 0.0001$
** $p \leq 0.001$
* $p \leq 0.05$

Table 2. Genes that showed a significant difference between the CuD treatment and the CuA control groups with a p-Value < 0.05, as determined by the F-rank t-test. Control value is equal to 1.0.

Observed Gene	**Fold Change***
Nuclear factor kappa b 1	2.43*
Collagen type 3a1	2.17*
Early growth response 1	1.77*
E2F transcription factor 1	1.65*
Myelocytomatosis viral oncogene homolog	1.56*
Matrix Metalloprotidase 12	1.52*
Carnitine palmitoyltransferase	1.52*
O-6-methylguanine DNA-methyltransferase	1.51*
Kirsten rat sarcoma viral oncogene homologue 2 (active)	1.37**
Insulin-like growth factor b3	1.27*
Caspase 8	1.14*
Elastin	1.11*
Cyclin Dependent kinase 6	0.73*

* $p \leq 0.05$

** $p \leq 0.01$

Table 3. Genes within the CuD treatment group that were observed to have a fold change 50% greater or less than the expressed control group. These genes were found to demonstrate a trend towards significance, at P = 0.05-0.010, as determined by the F-ranked t-test. Control is equal to 1.0.

Observed Gene	**Fold Change CuD vs. CuA***
Interleukin 2	2.97
Cdkn2b	2.24
Fibronectin 1	2.17
DNA-inducible transcript 3	1.88
Caspase 9	1.79

*p = 0.05 – 0.10

REFERENCES

1. Medeiros DM, Shiry L, Samelman T. Cardiac nuclear encoded cytochrome c oxidase subunits are decreased with copper restriction but not iron restriction: Gene expression, protein synthesis and heat shock protein aspects. Comp Biochem Physiol A Physiol. 1997; 117:77-87.

2. Davidson J, Medeiros DM, Hamlin RL. Cardiac ultrastructural and electrophysiological abnormalities in postweanling copper restricted and repleted rats in the absence of hypertrophy. J. Nutr. 1992; 122:1566-1575.

3. Medeiros DM, Jennings D. Role of copper in mitochondrial biogenesis via interaction with ATP synthase and cytochrome c oxidase. J Bioenerg Biomembr. 2002 34:389-395.

4. Dawson R, Milne G, Williams RB. Changes in the collagen of rat heart in copper-deficiency-induced cardiac hypertrophy. Cardiovasc Res. 1982;16:559-565.

5. Chao JC, Medeiros DM, Davidson J, Shiry L. Low levels of ATP synthase and cytochrome c oxidase subunit peptide from hearts of copper-deficient rats are not altered by the administration of dimethyl sulfoxide. J Nutr. 1994; 124:789-803.

6. Goffart S, von Kleist-Retzow JC, Wiesner RJ. Regulation of mitochondrial proliferation in the heart: Power-plant failure contributes to cardiac failure in hypertrophy. Cardiovasc Res. 200; 64:198-207.

7. Goodman JR, Warshaw JB, Dallman PR. Cardiac hypertrophy in rats with iron and copper deficiency: Quantitative contribution of mitochondrial enlargement. Pediatr Res. 1970; 4:244-256.

8. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998; 92: 829-839.

9. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Cell 1999; 4:611-617

10. Lehman JJ, Kelly DP. Transcriptional activation of energy metabolic switches in the developing and hypertrophied heart. Clin Exp Pharmacol Physiol.. 2002; 29: 339-345.

11. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 2004; 18: 357-368.

12. American Institute of Nutrition. Second report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 1980; 110: 1726.

13. Markland S, Markland G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assy for superoxide dismutase. Eur. J. Biochem. 1974; 47: 469-474.

14. Prohaska JR. Changes in growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase to dietary or genetic copper deficiency in mice. J. Nutr. 1983; 113: 2048-2058.

15. Prohaska JR. Development of copper deficiency in neonatal mice. J Nutr Biochem. 1990; 1:415-419.

16. Chao JCJ, Medeiros DM, Altschuld RA, Hohl CM. Cardiac nucleotide levels and mitochondrial respiration in copper-deficient rats. Comp. Biochem. Physiol. 1993; 104A:163-168.

17. Mao S, Medeiros DM, Hamlin RL. Marginal copper and high fat diet produce alterations in electrocardiograms and cardiac ultrastructure in male rats. Nutrition. 1999; 15:890-898.

18. Wildman REC, Medeiros DM, McCoy E. Cardiac changes with dietary copper, iron or selenium restriction: organelle and basal laminae aberrations, decreased ventricular function and altered gross morphometry. 1995; J. Tr. El. Exp. Med. 8:11-27.

19. Medeiros DM, Davidson J, Jenkins JE. A unified perspective on copper deficiency and cardiomyopathy. Proc Soc Exp Biol Med. 1993; 203:262-273.

20. Liao Z, Allred J, Keen CL, McCune SA, Rucker RB, Medeiros DM. Copper deficiency alters isomyosin types and levels of laminin, fibronectin and cytochrome c oxidase subunits from rat hearts. Comp Biochem Physiol B Biochem Mol Biol. 1995; 111:61-67.

21. Liao Z, Medeiros DM, McCune SA, Prochaska LJ. Cardiac levels of fibronectin, laminin, isomyosins, and cytochrome c oxidase of weanling rats are more vulnerable to copper deficiency than those of postweanling rats. J Nutr Biochem. 1995; 6:385-391.

22. Mao, S, Leone TC, Kelly DP, Medeiros. Mitochondrial Transcription Factor A is increased but expression of ATP synthase β subunit and medium-chain acyl-CoA dehydrogenase genes are decreased in hearts from copper-deficient rats. J. Nutr. 2000; 130: 2143-2150.

23. Mao S, Medeiros DM. 1991. Nuclear respiratory factors 1 and 2 are upregulated in hearts from copper-deficient rats. Biol. Trace El. Res. 2001; 83: 57-68.

24. Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP. Peroxisome proliferator-activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis. J. Clin. Invest. 2000; 106: 847-856.

25. Huss JM, Kelly DP. Mitochondrial energy metabolism in heart failure: A question of balance. J Clin Invest. 2005; 115: 547-55.

26. Huss JM, Kelly DP. Nuclear receptor signaling and cardiac energetics. Circ Res. 2004; 95:568-578.

27. Medeiros DM, Bagby D, Ovecka G. Myofibrillar, mitochondrial and valvular morphological alterations in cardiac hypertrophy among copper deficient rats. J. Nutr. 1991; 121:815-824.

28. Nakano H, Nakajima A, Sakon-Komasawa S, Piao JH, Xue X, Okumura K. Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. Cell Death Differ. 2006; 13: 730-737.

29. Bureau I, Guenx E, Mazur A, Rock E, Rousell AM, Rayssiguier Y. Female Rats Are Protected against Oxidative Stress during Copper Deficiency. J. Am. Coll. Nutr. 2003; 22: 239-246.

30. Cockell KA, Belonje B. The carbonyl content of specific plasma proteins is decreased by dietary copper deficiency in rats. J. Nutr. 2002; 132: 2514-2518.

31. Kudrin AV, Trace elements in regulation of NF-kappaB activity. J. Trace Elem. Med. Biol. 2000; 14:129-142.

32. Li Y, Ha T, Gao X, Kelly J, Williams DL, Browder IW, Kao RL, LiC. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. Am. J. Physiol. Heart Circ. Physiol. 2004; 287: H1712-1720.

33. Planavila A, Laguna JC, Vazquez-Carrera M. Nuclear factor-kappaB activation leads to down-regulation of fatty acid oxidation during cardiac hypertrophy. J. Biol. Chem. 2005; 280: 17464-17471.