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ScienceDirect
Journal of Nutritional Biochemistry 18 (2007) 719–726
Journal of Nutritional Biochemistry

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 Nfκb1

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Received 31 August 2006; received in revised form 14 November 2006; accepted 20 November 2006

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 peroxisomal-like proliferating related coactivator 1α (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 5 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 nuclear factor κB1 (Nfκb1) in the copper-deficient treatment animals, compared to the control group, and this was confirmed by real time quantitative polymerase chain reaction. The results of this study merit the further investigation of the role of reactive oxidative species with regard to Nfκb1 in the copper deficient rat heart.
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Keywords: Copper deficiency; Heart; Rat; Mitochondrial Nfκb1; Ppargc1a

1. 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 mitochondrial 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 coactivator peroxisomal-like proliferating related coactivator 1α (Ppargc1a).

Ppargc1a was first identified as a coactivator 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

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doi:10.1016/j.jnutbio.2006.11.011

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 adenosine triphosphate (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 coactivator 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 is being overexpressed 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 Ppargc1a 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 estrogen related receptor-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.

2. Methods and materials

2.1. Animals and diets

Eight male Long–Evan rats were obtained 3 days post weaning. Animals were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). 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 four 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 (BW) and composition. Diets were obtained from Research Diets (New Brunswick, NJ, USA). 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, USA) 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- h 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 weight; Research Biochemicals International, Natick, MA, USA). 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°C for subsequent determination of liver Cu-Zn superoxide dismutase (SOD) activity.

2.2. Hematocrit assay

Heparinized blood was transferred to microhematocrit tubes and centrifuged in a microcapillary centrifuge for 2 min. Hematocrit was determined as the percentage of space occupied by packed red blood cells.

2.3. Liver SOD assay

Liver that was collected from the rats at the termination of the study was used to determine liver SOD enzyme

Table 1
Customized array of genes constructed for study by functional category of protein product

Functional category	Gene	GenBank number
Substrate utilization	Medium chain acyl dehydrogenase	NM_016986
	Long-chain acyl dehydrogenase	NM_012819
	PPAR-α	NM_013196
	PPAR-δ	NM_013141
	PPAR-γ	NM_013124
	Retinoic acid receptor-alpha	NM_031528
	Retinoic acid receptor-gamma	XM_217064
	Phosphoenolpyruvate carboxykinase	NM_198780
	Carnitine palmitoyltransferase	NM_013200
	Insulin-like growth factor b3	NM_012588
Mitochondrial biogenesis	Ppargc1a	NM_031347
	Tfam	NM_031326
	Nuclear respiratory factor-1	XM_231566.
	Nuclear respiratory factor-2	XM_575228
Electron transport	Oligomycin sensitivity conferring protein	NM_138883
	Inhibitor of ATP hydrolysis by the mitochondrial A complex	NM_012915
	Cytochrome C oxidase subunit II	NC_001665
	Cytochrome C oxidase subunit IV	NM_017202
	ATP synthase beta subunit	M19044
	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	NM_139106
Cardiac Proteins	Troponin C, cardiac/slow skeletal	XM_214266
	Troponin I, cardiac	NM_017144
	Troponin T2, cardiac	NM_012676
	Myoglobin	NM_021588
	Cardiotrophin 1	NM_017129
	Natriuretic peptide precursor type A	NM_012612
Connective Tissue	Collagen alpha1 type I	Z78279
	Collagen, type III, alpha 1	NM_032085
	alpha-3 type IV collagen	XM_343607
	Fibronectin 1	NM_019143
	Elastin	NM_012722
Apoptosis and cell cycle	NFκB, p105 subunit	XM_342346
	BAX	NM_017059
	BCL2	NM_016993
	Caspase 8	NM_022277
	Caspase 9	NM_031632
	Cyclin-dependent kinase 6	AF352168
	O-6-methylguanine DNA methyltransferase	NM_012861
	E2F transcription factor 1	XM_230765
	Early growth response 1	NM_012551
	Kirsten rat sarcoma viral oncogene homolog 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 SCO_2)	NM_005138
	COX17 homolog, cytochrome C oxidase assembly protein (yeast) (Cox17)	NM_053540
	Copper chaperone for SOD	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

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.

2.4. Customized array development

One custom array (SuperArray Biosciences) was provided for each sample for a total of eight 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 (Table 1). Genes that were determined to be targets of interest were complied into a list containing the National Center for Biotechnology Information gene bank number, unigene and gene symbol and sent to SuperArray, which manufactured the custom array layout according to the predetermined gene list. One hundred twenty genes were printed on each array.

2.5. 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°C. The other half of the heart was snap-frozen in liquid nitrogen and also stored at −80°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 buffer or the small pieces of heart tissue in RNAlater were directly homogenized in lysis buffer with a rotor/stator-type homogenizer for 30 s 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.

2.6. 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). Briefly, primers were annealed to total RNA at 70°C for 10 min, followed by cDNA synthesis for 50 min at 42°C on a thermocycler. In vitro transcription of biotinylated (Biotin-16-UTP, Roche Applied Sciences) cRNA targets was performed for 9 h at 37°C. The cRNA was then column-purified and eluted in 10 mM Tris, quantified on a Nanodrop spectrophotometer and 2 μg of each biotin-labeled cRNA target was used for hybridization to eight customized oligo arrays.

2.7. cRNA target hybridization and detection

Arrays were prehybridized at 60°C for 2 h 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/hybridization bottle). Two micrograms of each cRNA target per array was added to the array tubes and allowed to hybridize overnight at 60°C (SuperArray Biosciences). The next day, post-hybridization washes were performed at 60°C with prewarmed 2× SSC/1% sodium dodecyl sulfate (SDS) and a high stringency wash with 0.1× 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 min under a FluorChem 8800 Imaging System (Alpha Innotec, San Leandro, CA, USA). The remaining arrays were left in detection buffer G until exposed, in pairs. Captured array images were uploaded into GEArray Suite Analysis (SuperArray Biosciences) for analysis. Once uploaded into GEArray Suite, the arrays were normalized using interquartile normalization and background was subtracted. 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.

2.8. Real-time quantitative polymerase chain reaction

In a second study, a more sensitive method of mRNA detection was used to further investigate differential gene expression during copper deficiency. Real-time quantitative polymerase chain reaction (qPCR) was used to determine fold changes in nuclear factor κB1 (Nfκb1) (p105 subunit) using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The Assay ID number for Nfκb1 is Rn01399583_m1, and a final concentration of 900 nm for each primer and a 250-nm probe were used. Gene expression assays have a FAM reporter dye at the 5′ end of the Taqman minor groove binding probe and a nonfluorescent quencher at the 3′ end of the probe. Rat heart cDNA (n=3) was reverse-transcribed from total RNA using random hexamer priming (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA, USA) 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. To ensure the cDNA samples were normalized on an equal basis, the 18S rRNA primer was diluted 1:6 in 10 mM Tris and amplified using 1 μl of cDNA sample, so the 18S Ct values were closer to the Ct range of the genes of interest (i.e., 18S Ct=23). Next, the cDNA samples were diluted according to the highest 18S Ct value (or lowest concentration), and fold changes were calculated for each gene when the 18S Ct values between samples were all within 10%. The polymerase chain reaction (PCR) reactions were run on an ABI 7000 Prism Detection System (Applied Biosystems) in a 20-μl reaction volume on a 96-well plate (in triplicates), using the following cycling conditions: initial steps (one cycle): 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

2.9. Statistical analysis

Because of nonparametric 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 .05. Results were reported to be a significant trend using a P value of .10.

3. Results

Final heart weights (HWs) between the two groups indicated that the CuD rats had a lower mean BW ($P \leq .0001$), a higher mean HW ($P \leq .0001$), and the final HW:BW measurements indicated that the CuD rats had a significantly higher ratio ($P \leq .05$) (Table 2). Analysis of the hematocrit assay and liver Cu,Zn SOD assay demonstrated

Table 2
Indices of copper deficiency in male Long–Evans rats fed a 5-week CuD

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

Values expressed as means±S.E.
* $P \leq .05$.
** $P \leq .001$.
*** $P \leq .0001$.

that there was a significant decrease of the means in the CuD group ($P\leq.05$ and .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 ($P\leq.05$) and that had a fold increase or decrease of at least 50% (Table 3). A second set of results are presented where genes were statistically significant by treatment ($P\leq.05$) but did not have a fold change of >50% or <50% (Table 3). A third set of data is presented in which there was a significant trend in data between the two treatment groups, indicated by $P\leq.05$–.10 (Table 4).

Genes that demonstrated a significant difference ($P\leq.05$) are shown in Table 3. Genes that were different and had a fold increase or decrease of at least 50% included: Nfκb1, 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 ($P\leq.05$), 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 3. These genes were Kirsten rat sarcoma viral oncogene homolog 2 (active), insulin-like growth factor binding protein, caspase 8, elastin and cyclin-dependent kinase 6.

Genes that demonstrated a trend ($P=.05$–0.10) and displayed a fold change induction of >50% or <50%, as compared to the controls, are shown in Table 4. 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.

Since the array demonstrated an increase in Nfκb1 expression, we decided to use real-time qPCR to confirm this finding. This transcription factor gene was found to be increased in expression by at least 4.6 times (using $2^{-\Delta CT}$) and as much as 5.3 times using the comparative method (or $2^{-\Delta\Delta CT}$), which includes normalization with 18S rRNA.

Table 3
Genes that showed a significant difference between the CuD treatment and the CuA control groups with a *P* value <.05, as determined by the F-rank *t* test

Observed gene	Fold change
Nfκb1	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 homolog 2 (active)	1.37**
Insulin-like growth factor b3	1.27*
Caspase 8	1.14*
Elastin	1.11*
Cyclin-dependent kinase 6	0.73*

Control value is equal to 1.0.
* $P\leq.05$.
** $P\leq.01$.

Table 4
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 $P=.05$–.010, as determined by the F-ranked *t* test

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

Control is equal to 1.0.
* $P=.05$–.10.

4. 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]. The decrease in body weight among the copper-deficient rats has been shown not to alter the increased mitochondria of the heart in copper deficiency as demonstrated by pair-feeding [5]. Thus, any decrease in energy intake by copper-deficient rats in not likely to impact mitochondria biogenesis.

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 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 Tfam and Nrf1 or 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 5 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 and Medeiros [23] did implicate that these findings may be due to a transient change that 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. There may be several reasons for this. First, the sensitivity of the custom arrays were relatively low ($<10^3$ dynamic range), and were used more as a screening tool to detect overall changes in multiple genes. Second, the cRNA probes used for the arrays are biased towards the 3′ ends of mRNA transcripts, because they are oligo-dT-primed in reverse transcription. This 3′ bias limits the number of alternatively spliced transcripts the array could detect. Since the Taqman assays are randomly primed, have no positional bias within the gene, are very sensitive ($>10^5$) and do not detect genomic DNA or highly homologous genes, they were used to detect changes in Nfκb1 to confirm our findings in the oligo arrays.

The various PPAR isoforms regulate fatty acid metabolism in various tissues including the heart. A coactivator is needed for the PPAR isoforms to function as promoters. The transcriptional coactivator that was identified as the regulating factor within the heart was Ppargc1a. [8]. Further studies within the Kelly lab at Washington University (St. Louis, MO, USA) found that Ppargc1a was directly linked to PPAR-α [24] and appeared to increase its activity and function with regards to mitochondrial biogenesis control [11,25]. Similar studies also demonstrated that forced overexpression 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 qPCR 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 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 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. Furthermore, we did not observe any increase in genes that encode for enzymes involved in glucose utilization or a decrease in genes involved with fatty acid metabolism. We would have expected changes in these directions based on cardiac hypertrophy that leads to heart failure. One reason why this may not have been observed is that the hearts may not have been in failure but simply hypertrophied. Heart failure may be considered a secondary effect, and in our study, we were likely examining the primary effect of copper deficiency and cardiac hypertrophy.

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]. We can assume, based on studies from our lab and others, that there was increased mitochondria volume density in hearts from copper-deficient rats due to this universal observation.

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 (hepatic nuclear factor 4) and MEF2 (myocyte enhancer factor 2). Nfκb1 has also been suggested to be a key regulator of a number of enzymes. Nfκb1 is a major element of the cellular growth and inflammatory and apoptotic pathways [28]. We found significant up-regulation 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. 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 insulin-like growth factor b3, Kristen rat sarcoma viral oncogene homolog 2 and myelocytomatosis (both oncogenes, also involved in cell survival), early growth response 1 and E2F transcription factor 1. Interestingly, cyclin-

dependent kinase 6, which enhances the cell cycle, was down-regulated in copper-deficient rats.

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 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 nuclear factor κB (NFκB) expression is related to copper status [31]. In our study, the antioxidant enzyme Cu,Zn SOD was decreased suggesting that the potential for greater free radical production was present. 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 Nfκb1 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, as is observed in many forms of cardiac hypertrophy that lead to heart failure. To our knowledge, there have not 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 meet energy demands. The role of Nfκb1 in copper deficiency and cardiac hypertrophy may be a novel line of inquiry.

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