Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 823 – 830
Journal of Nutritional Biochemistry

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☆

Denis M. Medeiros*, Yu Jiang, Darcey Klaahsen, Dingbo Lin

Department of Human Nutrition, Kansas State University, Manhattan, KS 66506, USA

Received 1 July 2008; received in revised form 1 August 2008; accepted 5 August 2008

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, six were significantly down-regulated and nine were up-regulated. The proteins that were decreased were beta enolase 3, carbonic anhydrase 2, aldose reductase 1, 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-γ coactivator (PGC)-1α protein, as well as the transcript, which increased 2.5-fold, was observed. It would appear that increased mitochondrial biogenesis known to occur in copper deficiency hearts is caused by an increased expression in the master regulator of mitochondrial biogenesis, PGC-1α.
© 2009 Elsevier Inc. All rights reserved.

Keywords: Proteinomics; Copper deficiency; Rats; PGC-1α; Metabolic enzymes

1. Introduction

Hearts from copper-deficient (CuD) animals develop cardiac hypertrophy whereby the area occupied by the 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 is dramatically up-regulated and the isomyosins revert back to the fetal stage [3]. While such signs occur in hearts from CuD rats, they are modest in comparison to these other models of cardiac failure. One possibility is that CuD 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

☆ 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.

* Corresponding author. Tel.: +1 785 532 0150; fax: +1 785 532 3132.
E-mail address: medeiros@ksu.edu (D.M. Medeiros).

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.08.001

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 CuD hearts has not been studied. Since CuD hearts exhibit mitochondrial pathology, it is not unreasonable to ask whether there is a potential change in substrate preference. Such a 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 nonmyofibrillar 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 CuD rats with only minimal changes in enzymes involved in cardiac energy metabolism. Also, we report on changes in other proteins from CuD hearts not previously reported in the literature.

2. Materials and methods

2.1. Animals and design

Sixteen male weanling Long-Evans rats were purchased from Charles River (Boston, MA, USA) 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, USA). Animals were fed their respective diets for 5 weeks, which followed the recommendations of the American Institute of Nutrition, consisting of (grams per gram of 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 μg 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, USA) after the termination of the study. Analysis revealed that the CuA diet contained 5.31 μg Cu/g±0.457 and the CuD group 0.66 μg Cu/g ±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-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 and placed 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 levels.

2.2. Hematocrit assay and liver copper determination

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. 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 spectrophotometry (Perkin-Elmer Model No. 5000, Norwalk, CT, USA).

2.3. Sample preparation for proteinomic analysis

The protein lysis was purified by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules, CA, USA) and reconstituted in protein sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH=8.5). The protein concentration was determined by the protein RC DC Protein Assay (Bio-Rad). The internal standard was composed by pooling an equal amount of proteins from both CuD and CuA rats. Sample labeling with cyanine minimal dyes was carried out according to the manufacturer's instruction (GE Healthcare, Piscataway, NJ, USA). The ratio of protein to Cy dye is 1 μg protein to 25 pmol Cy dye. 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 CuD samples received Cy3 and the other half Cy5, and the same thing was done with the CuA rats. Each gel had added a CuA and CuD sample with different dyes to reduce variation between gels. The excitation and emission wavelengths for each dye were per the manufacturer's suggestions (GE Healthcare).

2.4. Two-dimensional 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 was mixed with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 140 mM DTT and 2% ampholytes, pH=3–10) and used to dehydrate IPG (pH=3–10) strips for 14 h. Separately, another series of gels containing 250 μg unlabeled protein was used to identify individual protein spots on the gel. The isoelectric focusing was carried on a PROTEAN IEF Cell following the manufacturer's instruction (Bio-Rad). 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 min 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 min. SDS-PAGE was conducted using a precast 8–20% gradient gel. Electrophoresis condition was set at 200 V for 60–70 min 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) with a resolution of 50 μm. Spot detection was performed on the gel images using the DeCyder 2D Software (version 6.5, GE Healthcare). 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 rematching was repeated manually until no new Level 1 mismatches were found as per the manufacturer's protocol (GE Healthcare). The difference between the CuA and CuD groups was analyzed by *t* test, which is provided by Decyder 2D Software.

2.5. Protein identification

For the nonlabeled gels, the spots of interest were excised and subjected to gel digestion using trypsin (Sigma, St Louis, MO, USA). The gels of nonlabeled protein were stained with Commassie blue for protein spot identification. The digested peptide was analyzed on a MALDI TOF/RTOF instrument (Bruker, California) using α-cyano-4-hydroxycinnamic acid (Sigma) 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.

2.6. Western blotting

2.6.1. Antibodies

PFK, β-actin and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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, MI) and was rabbit anti-human. PGC-1α was purchased from Norvus Biologicals (Littleton, CO, USA) and was mouse anti-human. Medium chain acyl dehydrogenase (MCAD) and PPARα were provided as a generous gift from the laboratory of Dr. Dan Kelly, Washington University School of Medicine (St. Louis, MO, USA) and were rabbit anti-human.

2.6.2. Electrophoresis

Nonmyofibrillar proteins consist of mitochondria and sarcoplasm in heart tissue. The myofibrils were separated from the nonmyofibrillar protein by homogenizing 0.2 g of 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×*g*. The supernatant was used for SDS-PAGE and Western blotting. About 80 μg of protein per well was loaded in 4–20% precast polyacrylamide mini gel (Pierce, Rockford, IL, USA). Gels were run in BupH Tris-HEPES-SDS running buffer (Pierce) for 45 min at constant 150 V. Proteins were then transferred to nitrocellulose membrane (0.2 μm) using Bio-Rad (Richmond, CA, USA) 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). Then, membranes were blocked in 5% nonfat milk (in water) for 1 h. After blocking, membranes were blotted with primary antibodies overnight at 4°C with rocking and then secondary antibodies for an additional 1 h at room temperature with rocking. Membranes were washed with TDN washing buffer thoroughly. Finally, immobilon Western HRP substrate luminol reagents (Millipore, Billerica, MA, USA) were used to develop the specific bands using the Fluorchem 8800 imaging system (Alpha Innotech, San Leandro, CA, USA). Eight rat hearts from each treatment group were analyzed.

2.7. Real-time polymerase chain reaction

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 rat hearts from each treatment group were analyzed.

2.8. Statistical analysis

A Student's *t* test was used to determine significant differences at the $P \leq .05$ level 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.

3. Results

The results suggest that rats fed the CuD diet showed signs of copper deficiency (Table 1). Final body weight, liver copper concentration and hematocrit were significantly lower in the CuD group than in the CuA group, whereas heart weight and heart/body weight were significantly higher in the CuD 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, Fig. 1). Fifteen proteins were either up-regulated (n=9) and or down-regulated (n=6) significantly ($P \leq .05$; Table 2) in the CuD group compared to the CuA group. Among the proteins that were decreased were beta enolase 3, carbonic anhydrase 2, aldose reductase 1, 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 demonstrate differences by treatment for PPARα, medium chain acyl dehydrogenase, phosphofructokinase and phosphoenolpyruvate carboxykinase (Fig. 2). For PGC-1α, the protein was increased (Fig. 2) and the transcript revealed a 2.5-fold increase in hearts from CuD rats (Fig. 3).

Table 1
Final body weight, heart weight, heart/body weight, hematocrit and liver copper concentrations in rats fed CuA and CuD diets

Variable	CuA (n=8)	CuD (n=8)
Final body weight (g)*	307±7	263±17
Heart weight (g)***	1.36±0.02	2.31±0.18
Heart/body weight ($\times 10^{-3}$)**	4.85±0.14	9.30±1.33
Hematocrit (%)**	42.6±0.5	30.9±2.8
Liver Cu (μg/g)***	4.10±0.09	0.45±0.12

Values are shown as mean±S.E.
* $P \leq .05$.
** $P \leq .01$.
*** $P \leq .001$.

4. 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=.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 CuD rat 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 CuD rats [14], which would suggest that this enzyme would be elevated. On the other hand, the increase in creatine phosphate in hearts from CuD rats may be due to a decrease in creatine kinase.

An increase in chain A transthretin accelerates atherosclerosis, and CuD rats do have increased blood cholesterol which could suggest some initiation of this process occurring [15]. On the other hand, aconitase 2, an iron-regulating protein, was down down-regulated. 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 in 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 in 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_0 and F_1. The F_0 portion is embedded in the mitochondrial membrane and the F_1 subunit is above the membrane. Each of these domains is composed of several peptides. Subunit d is part of the F_0 domain. Here the increase in subunit d may most likely be due to the increased number of mitochondria rather than to a

Table 2
Protein spots that are significantly changed (DIGE analysis) in CuD compared to CuA[a]

NCBI no.	*t* test	Ratio (CuD/CuA)	Name of the protein	
gi|126723393	0.0079	−1.63	Beta enolase 3	Down
gi|9506445	0.034	−1.5	Carbonic anhydrase 2	Down
gi|6978491	0.00016	−1.26	Aldehyde reductase 1	Down
gi|145275165	0.026	−1.21	Glutathione peroxidase	Down
gi|6671762	0.0028	−1.18	Creatine kinase; muscle	Down
gi|40538860	0.0053	−1.18	Aconitase 2, mitochondrial	Down
gi|16758446	0.035	1.2	Isocitrate dehydrogenase 3 (NAD+) alpha	Up
gi|40786469	0.011	1.24	Dihydrolipoamide dehydrogenase	Up
gi|1000439	0.0024	1.29	GRP 75 (*Rattus* sp.)	Up
gi|220904	0.0062	1.35	Subunit d of mitochondrial H-ATP synthase	Up
gi|3212532	0.027	1.35	Chain A; rat transthyretin	Up
gi|55747	0.0052	1.39	Preproapolipoprotein A-I	Up
gi|57580	0.012	1.48	Alpha-B crystallin (*Rattus norvegicus*)	Up
gi|61556986	0.0051	1.6	Transferrin	Up
gi|20302069	0.037	1.77	Heat shock protein alpha	Up

[a] Ratios are based on eight CuD and eight CuA rats.

Table 3
Protein spots that were identified but were not significantly different by copper treatment ($P>.05$)[a]

NCBI no.	*t* test	Ratio (CuD/CuA)	Name of the protein
gi|125313	0.055	−1.09	Creatine kinase; sarcomeric mitochondrial precursor
gi|1374715	0.093	−1.16	ATP synthase beta unit
gi|8393418	0.2	−1.11	Glyceraldehyde 3-phosphate dehydrogenase
pi|6981146	0.2	1.11	Isocitrate dehydrogenase B
gi|203055	0.26	−1.14	ATP synthase alpha subunit
gi|13162363	0.29	1.09	Heart fatty acid binding protein
gi|61889092	0.31	1.12	Adenylate kinase
gi|51259441	0.32	1.12	LOC498909 protein
gi|8394432	0.35	−1.06	Peroxiredoxin 2
gi|28933457	0.36	−1.11	Glutathione S-transferase; mu 2
gi|40538860	0.36	−1.07	Anconitase 2; mitochondrial
gi|6981146	0.4	−1.05	Lactate dehyrogenase B
gi|16757994	0.42	−1.07	Pyruvate kinase; muscle
gi|8394331	0.44	1.08	Superoxidase dismutase
gi|13162363	0.45	1.12	Heart fatty acid binding protein
gi|203474	0.46	1.1	Creatine kinase
gi|56961640	0.46	1.13	Protein-L-isoaspartate
gi|109466092	0.5	−1.05	Similar to 3-oxoacid CoA
gi|20304123	0.54	1.04	3-Mercaptopyruvate sulfurtransferase
gi|2392291	0.57	−1.03	Chain a; 2-enoyl-CoA hydratase
gi|6981362	0.58	1.06	Lysophospholipase 1
gi|38512111	0.62	−1.03	Tpi1 protein
gi|56090293	0.62	1.05	Pyruvate dehydrogenase (lipoamide) beta
gi|157819619	0.64	1.09	Biliverdin reductase B
gi|57527204	0.65	−1.03	Alpha B-ETF
gi|51948412	0.69	−1.07	Electron-transfer flavoprotein
gi|13162363	0.71	1.04	Fatty acid binding protein 3
gi|56541238	0.72	−1.02	Fumarate hydratase 1
gi|400269	0.72	−1.02	Methylmalonate-semialdehyde dehydrogenase
gi|127167	0.77	1.01	Myosin regulatory light chain 2
gi|25742763	0.77	−1.03	Heat shock 70-kDa protein 5
gi|33468857	0.83	−1.01	Histidine triad nucleotide binding protein
gi|51948412	0.88	1.01	Electron-transfer flavoprotein
gi|9506411	0.91	1	ATP synthase; H+ transporting; mitochondrial F0 complex; subunit d
gi|83939410	0.94	1.01	Phosphatidylethanolamine binding protein
gi|27689717	0.96	1	Similar to *N*-acetylglutamate synthase isoform 1
gi|55742725	0.97	1.01	Dihydrolipoamide S-succinate

[a] Ratios are based on eight CuD and eight CuA rats.

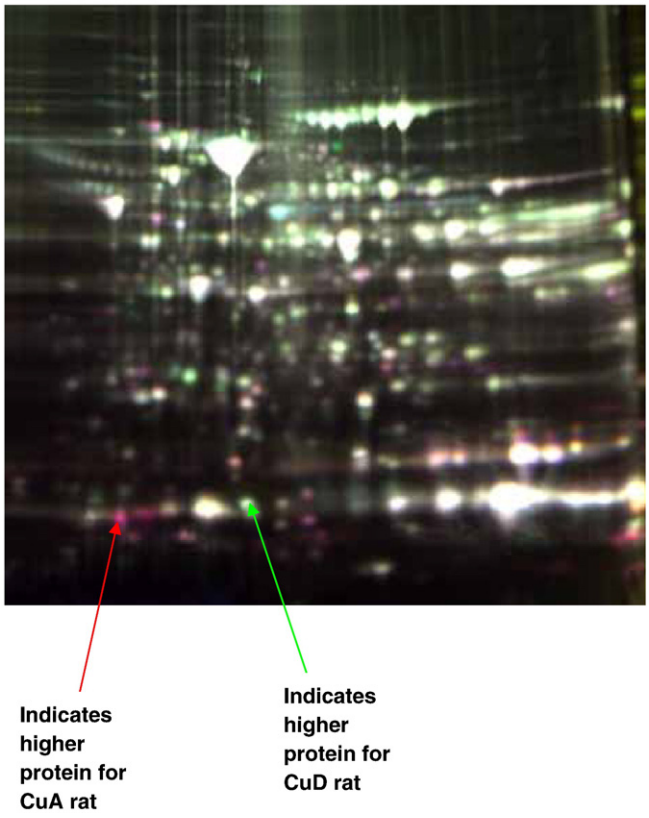

Fig. 1. A representative gel image with Cy dye staining. Each sample had an internal standard added (cy2). In this particular image, a CuD rat was labeled with cy3 (showing green) and a CuA rat was labeled with cy5 (showing red). The samples were run in the same first dimension on IGP strips and the second dimension on SDS-PAGE. The above image shows the overlap of the three samples of the gel image (internal standard, CuD and CuA samples). Green spots indicate higher protein level for CuD and red spots indicate higher expression for that protein for the CuA rat. Areas of white indicate that the levels of internal standard, CuD protein and CuA proteins were similar in quantity.

specific alteration in function. The β subunits of ATP synthase had a nonsignificant 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 CuA 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 are 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-1α, the master controller of mitochondrial biogenesis. Our results demonstrated that PGC-1α is significantly up-regulated in CuD 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 CuD 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-1α are up-regulated. Specifically, NRF-1 and NRF-2 are up-regulated early in the onset of copper deficiency [19], and mitochondrial transcription factor A shows a robust increase in hearts of CuD 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. The lab of Puigserver et al. [20] originally discovered this transcription factor and its ability to induce the production of mitochondria in brown adipose tissue. There are various isoforms of PGC-1 which constitutes a family: PGC-1α, PGC-1β and PGC-1-related coactivator. Both PGC-1α and PGC-1β 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 is then thought to bind to the promoter of mtTFA to initiate the concomitant up-regulation 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 PGC-1α will up-regulate these two transcription factors. Up-regulation of mtTFA augments mitochondrial biogenesis with these other transcription factors [24].

What signals are sent to heart cells that trigger PGC-1α up-regulation? A strong candidate for this is the known production of nitric oxide in hearts of CuD hearts. Saari et al. [25] demonstrated that hearts from CuD rats have increased endothelial NO synthase and inducible

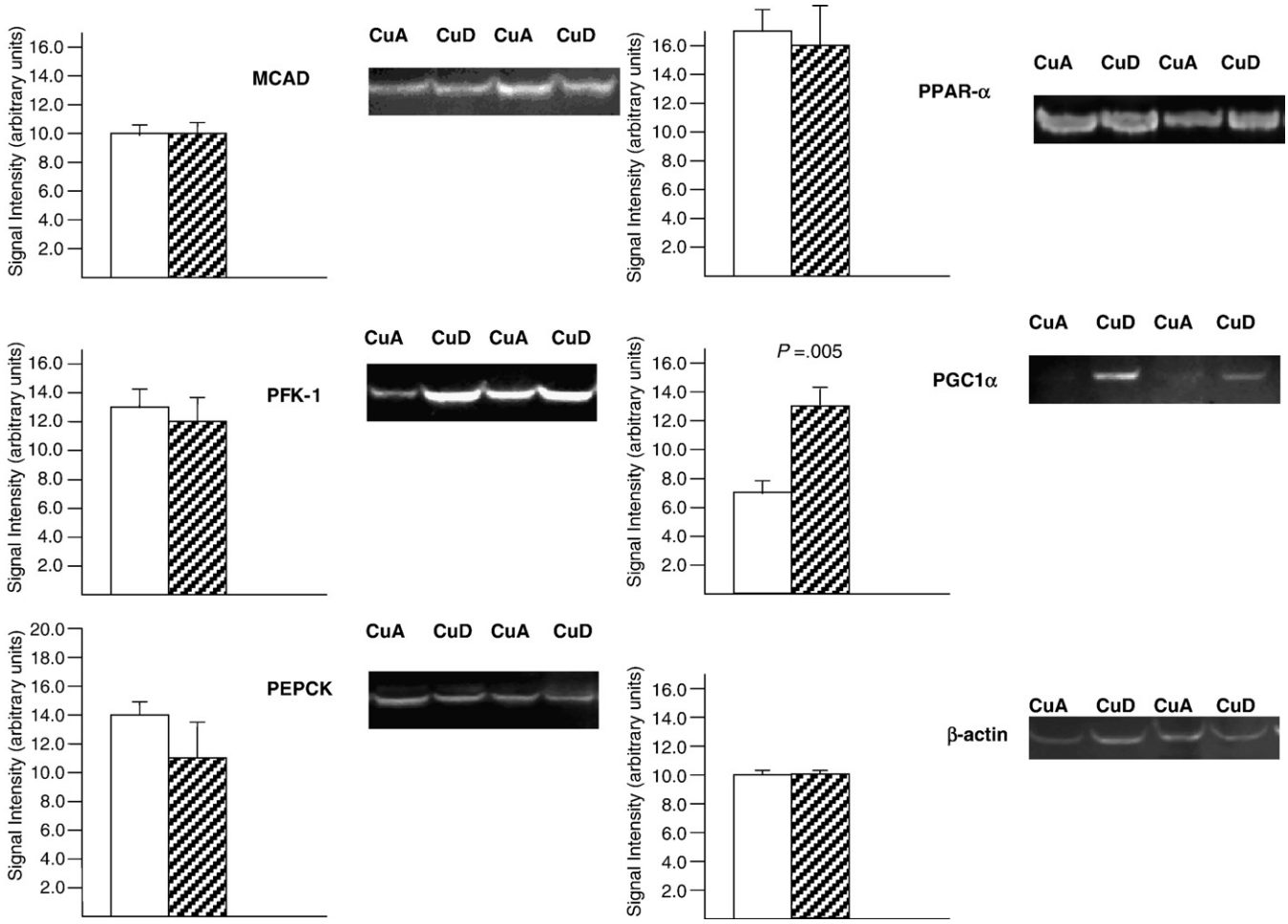

Fig. 2. Representative Western blot signals and mean intensity of proteins studied: medium-chain-acyl-dehydrogenase (MCAD), phosphofructokinase-1 (PFK-1), phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator-activator receptor-α (PPARα), peroxisome proliferator-activated receptor-gamma coactivator (PGC1-γ), and β-actin. Data are mean±S.E. (n=8 in each treatment group mean). CuA=copper-adequate diet, CuD=copper-deficient diet. Open bars indicate CuA; Hatched bars indicate CuD treatments.

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 CuD than in CuA rats. Furthermore, NFkB activation as determined by the p65 subunit was higher in hearts from CuD rats. This is significant as NFkB is a transcription factor inducible NOS. The role of NFkB was shown to be robustly up-regulated in a recent paper our lab published [26]. Nisoli et al. [27] reported that nitric oxide triggered mitochondrial biogenesis and that PGC-1α mediated this effect in a variety of cell types. Also important is that 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 [17,29]. We propose that nitric oxide is a candidate for the up-regulation of PGC-1α that leads to mitochondrial biogenesis in hearts of CuD rats.

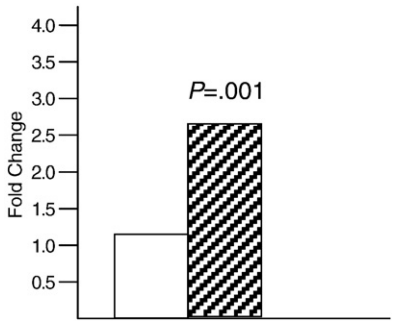

Fig. 3. Real-time qPCR showed a 2.54-fold increase of PGC-1α mRNA expression in copper-deficient rat hearts (*P=.001). CuA=copper-adequate diet, CuD=copper-deficient diet (n=4 for each treatment group). Open bars indicate CuA; Hatched bars indicate CuD treatments.

Acknowledgments

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.

References

[1] Elsherif L, Ortines RV, Saari JT, Kang YJ. Congestive heart failure in copper-deficient mice. Exp Biol Med 2003;228:811–7.

[2] 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 1995;111B:61–7.

[3] Lehman JJ, Kelly DP. Gene regulatory mechanisms governing energy metabolism during cardiac hypertrophic growth. Heart Fail Rev 2002;7:175–85.

[4] Neubauer S. The failing heart — an engine out of fuel: mechanisms of disease. N Engl J Med 2007;356:1140–51.

[5] Duncan JG, Fong JL, Medeiros DM, Finck BN, Kelly DP. Insulin-resistant heart exhibits a mitochondrial biogenic response driven by the peroxisome proliferator-activated receptor-alpha/PGC-1alpha gene regulatory pathway. Circulation 2007;115:909–17.

[6] American Institute of Nutrition. Second report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J Nutr 1980;107:1726.

[7] Jin JK, Whittaker R, Glassy MS, Barlow SB, Gottlieb RA, Glembotski CC. Localization of phosphorylated alpha B-crystallin to heart mitochondria during ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2008;294:H337–44.

[8] Dohke T, Wada A, Isono T, Fujii M, Yamamoto T, Tsutamoto T, et al. Proteomic analysis reveals significant alternations of cardiac small heat shock protein expression in congestive heart failure. J Card Fail 2006;12:77–84.

[9] Keller A, Rouzeau JD, Farhadian F, Wisnewsky C, Marotte F, Lamandé N, et al. Differential expression of alpha- and beta-enolase genes during rat heart development and hypertrophy. Am J Physiol 1995;269(6 Pt 2):H1843–51.

[10] Dzeja PP, Redfield MM, Burnett JC, Terzic A. Failing energetics in failing hearts. Curr Cardiol Rep 2000;2:212–7.

[11] Ramana KV, Willis MS, White MD, Horton JW, DiMaio JM, Srivastava D, et al. Endotoxin-induced cardiomyopathy and systemic inflammation in mice is prevented by aldose reductase inhibition. Circulation 2006;114:1838–46.

[12] Iwata K, Nishinaka T, Matsuno K, Kakehi T, Katsuyama M, Ibi M, et al. The activity of aldose reductase is elevated in diabetic mouse heart. J Pharmacol Sci 2007;103:408–16.

[13] Lygate CA, Fischer A, Sebag-Montefiore L, Wallis J, ten Hove M, Neubauer S. The creatine kinase energy transport system in the failing mouse heart. J Mol Cell Cardiol 2007;42:1129–36.

[14] Chao JCJ, Medeiros DM, Altschuld RA, Hohl CM. Cardiac nucleotide levels and mitochondrial respiration in copper-deficient rats. Comp Biochem Physiol 1993;104A:163–8.

[15] Liz MA, Gomes CM, Saraiva MJ, Sousa MM. ApoA-I cleaved by transthyretin has reduced ability to promote cholesterol efflux and increased amyloidogenicity. J Lipid Res 2007;248:2385–95.

[16] Singh N, Medeiros DM. Liver fatty acid profiles and trace element concentrations in heart and liver of copper deficient rats. Biological Trace Element Research 1984;6:423–9.

[17] Chao CJJ, Medeiros DM, Davidson J, Shiry L. Decreased 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.

[18] Mao S, Leone TC, Kelly DP, Medeiros DM. 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: 2143–50.

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

[20] 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;92:829–39.

[21] Finck BN, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. Circulation 2007;115:2540–8.

[22] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mortha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115–24.

[23] Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest 2000;106: 847–56.

[24] Gleyzer N, Vercauteren K, Scarpulla RC. Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. Mol Cell Biol 2005;25:1354–66.

[25] Saari JT, Wold LE, Duan J, Ren J, Carlson HL, Bode AM, et al. Cardiac nitric oxide synthases are elevated in dietary copper deficiency. J Nutr Biochem 2007;18:443–8.

[26] Klaahsen D, Ricklefs K, Medeiros DM. 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. J Nutr Biochem 2007;18:719–26.

[27] Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonella C, Sciorata C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 2003;299:896–9.

[28] Nisoli E, Carruba MO. Nitric oxide and mitochondrial biogenesis. J Cell Sci 2006;119:2855–62.

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