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Cardiac levels of fibronectin, laminin, isomyosins, and cytochrome c oxidase of weanling rats are more vulnerable to copper deficiency than those of postweanling rats

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The relative quantities of cardiac laminin, fibronectin, cytochrome c oxidase (CCO), and isomyosin types were studied by gel electrophoresis in male rats fed copper-deficient diets beginning either from the time of weaning for 5 weeks or from 5 weeks postweaning for 6 weeks with one group of copper-repleted rats. Increased levels of fibronectin and V_3 isomyosin but decreased levels of CCO subunit IV and laminin were found in weanling copper-depleted rats. In contrast, postweanling copper-depleted rats exhibited only increased levels of fibronectin and decreased levels of cardiac CCO subunit IV. Repletion of copper-deficient rats for 6 weeks was not sufficient to restore CCO subunit IV to the same level as controls. These results confirm that biochemical lesions in the basal laminae are a result of copper restriction. The decreased nuclear encoded subunits of CCO may help explain some of the mitochondrial pathology observed in dietary copper restriction. Increased V_3 isomyosin levels with low ATPase activity may help to conserve to a limited extent the ATP levels in copper-deficient cardiac tissue. These protein changes are consistent with the known morphological alterations of hearts from copper-restricted rats. (J. Nutr. Biochem. 6:385–391, 1995.)

Keywords: copper; cytochrome c oxidase; fibronectin; laminin; isomyosins

Introduction

Cardiovascular abnormalities in rats fed copper-deficient diets have been well documented. Cardiac hypertrophy with increased mortality,[1–3] hemothorax and aneurysms,[2–4] and abnormal electrocardiograms[3,5–7] have been reported in rats fed copper-deficient diets from weaning for a period of several weeks. A recent study by Davidson et al.[8] compared relative pathologies of hearts from rats fed copper-deficient diets instituted either from weaning or 5 weeks postweaning, as well as repletion of copper-deficient rats. Copper-deficient rats of both ages exhibited significant cardiac pathology and abnormal electrocardiograms, and the postweanling copper-restricted rats demonstrated these abnormalities in the absence of hypertrophy and anemia. Specific alterations in younger and older copper-restricted rats included increased mitochondrial volume density, disarranged cristae, and fragmented basal laminae at capillary-myocyte interfaces. Copper-repleted rats exhibited some but not total reversal of these abnormalities.

With respect to metabolic consequences, ATP levels do not appear to be altered in copper-deficient rat hearts.[9,10] However, cytochrome c oxidase (CCO) activity is depressed and the nuclear encoded subunits of CCO are decreased in copper-deficient rat hearts but not the copper-containing mitochondrial subunits.[11] A possible explanation of why ATP levels do not decline in copper deficiency

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could be due to several factors: (1) CCO is not rate limiting in the production of ATP in well nourished animals and consequently it may not be in copper-deficient animals, (2) ATP levels could be maintained by increased glycolytic activity, or (3) a decreased utilization of ATP may occur. Studies with other models of heart failure in rats has given some credence to the latter possibility with a shift toward a greater level of the slower contracting but less active ATPase V_3 isomyosin type with concomitant decline in V_1 isomyosin types.[12–14]

The major goal of the present study was to examine basal laminae proteins (laminin and fibronectin), CCO subunits, and isomyosin types in rats fed diets deficient in copper from weaning or instituted postweaning as well as to replete copper-deficient rats to determine reversibility of potential changes. The study used the same design reported by our lab previously[8] except that biochemical changes were investigated to give potential insight into some of the ultrastructural changes reported previously.

Methods and materials

Animals and diets

Protocol for this study was approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University. Thirty-five male weanling Long–Evans rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were purchased and weighed upon arrival. The rats were randomly assigned to either copper-adequate (Cu^+) (three groups, n = 7/group) or copper-deficient (Cu^-) diets (two groups, n = 7/group). At the end of 5 weeks, one group (n = 7) from each of the dietary treatments (Cu^+ and Cu^-) was sacrificed. Beginning from week 5, one group (n = 7) fed the Cu^+ diet from weaning remained on the Cu^+ diet to serve as a postweanling control (referred to as the Cu^+ postweanling control group). The remaining Cu^+ group (n = 7) was crossed to the Cu^- diet (referred to as the Cu^+,Cu^- group) and the remaining Cu^- group of rats (n = 7) was switched to the Cu^+ diet (referred to as the Cu^-,Cu^+ repleted group) to evaluate copper repletion. The latter three groups were maintained on these diets for 6 additional weeks. Body weights were measured weekly.

Rats were fed a basal diet (U.S. Biochemical Cleveland, OH, USA), following the recommendation of the American Institute of Nutrition.[15] The diet contained energy yielding macronutrients as follows (g/kg): sucrose, 500; cornstarch, 150; casein, 200; corn oil, 50; with the vitamin mix commonly used in the AIN-76 diets. The Cu^+ diet had 6 mg of Cu/kg of feed added in the form of cupric carbonate, whereas copper was omitted from the Cu^- diet. Dietary Cu was determined by flame atomic absorption spectrophotometry, and the Cu^- diet contained 0.4 mg of Cu/kg of feed and the Cu^+ diet contained 6.4 mg of Cu/kg of feed. Rats were singly housed in stainless steel cages in a room with a 12 hr light–dark cycle and a mean temperature of 21.7°C. The rats had free access to both deionized distilled water and food. At the end of each treatment period, rats were anesthetized with CO_2 inhalation. Blood was obtained by cardiac puncture. Liver and hearts were removed as described later.

Hematocrit and liver superoxide dismutase and plasma ceruloplasmin activities

Blood drawn into heparinized tubes by cardiac puncture had hematocrit values determined using a microhematocrit centrifuge. Liver cytoplasmic Cu-Zn superoxide dismutase (SOD) activity was determined spectrophotometrically as described by Marklund and Marklund[16] and modified by Prohaska[17] to assess relative Cu status of rats in the different treatment groups. The oxidase activity of ceruloplasmin was measured according to the method described by Schosinsky et al.[18]

Gel electrophoresis and Western blotting

Hearts were rinsed in phosphate-buffered saline (PBS), pH 7.4, frozen in liquid nitrogen and stored at −80°C until analysis. All proteins were separated by gel electrophoresis as described later. Western blotting was conducted for laminin and fibronectin in order to identify which bands of the respective gels was the protein of interest. Western blotting of CCO was conducted to identify and quantify the various subunits.

Gel electrophoresis and Western blotting for fibronectin, laminin, and CCO were conducted after these proteins had been extracted. Purified rat plasma fibronectin (Sigma Chemical Co., St. Louis, MO, USA) and laminin from the Engelbreth–Holm–Swarm (EHS) mouse tumor (Sigma Chemical Co.) and purified bovine CCO from Dr. Prochaska (Wright State University, Dayton, OH, USA) were used as standards. Molecular markers used in the fibronectin and laminin gel electrophoresis contained myosin from rabbit muscle, β-galactosase from *E. coli*, phosphorylase B from rabbit muscle, and albumin from bovine plasma, and had molecular weights of 205, 116, 97.4, and 66 kD, respectively (Sigma Chemical Co.).

Fibronectin

Heart tissues were homogenized with a Polytron apparatus using cold PBS, pH 7.4, containing aprotinin (5 trypsin IU/ml), leupeptin (10 mmol/L), pepstain A (1 mmol/L), and phenylmethyl sulfonylfluoride (PMSF, 1 mmol/L), at a 12:1 ratio of buffer volume to tissue wet weight.[19] The homogenate was centrifuged at 11,000g for 2 min at 4°C. The pellet was resuspended in 0.139 mol/L of sodium dodecyl sulfate (SDS) and heated at 100°C for 4 min. The resuspension was centrifuged at 11,000g at room temperature for 2 min. The supernatant contained denatured fibronectin and was stored at −80°C until electrophoresis. The protein concentration was measured by the Lowry method.[20] Electrophoresis of samples in SDS and β-mercaptoethanol (10 μg of protein/well) was conducted with a 7.5% SDS polyacrylamide gel (SDS–PAGE) for 1.5 hr as described by Laemmli.[21] A Bio-Rad Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Richmond, CA, USA) at constant current (10 mA/gel) and room temperature with an ISCO Electrophoresis Power Supply (Model 494, Lincoln, NE, USA) was used. One gel was stained with Commassie brilliant blue R250 for 30 min and destained overnight with acetic acid until the background was clear. Protein in a non-stained gel was then transferred to nitrocellulose membrane by a Trans Blot apparatus (Bio-Rad Laboratories) for 2 hr and stained with Ponceau S and destained with acetic acid. The immunoblot was performed by incubating the membrane with a goat antihuman fibronectin (Sigma Chemical Co.) as the primary antibody overnight. After washing with a buffer the membrane was incubated with a rabbit-biotinylated antisheep IgG (Vector Laboratories, Burlingame, CA, USA) as the secondary antibody for 1 to 2 hr. Streptavidin horseradish peroxidase (HRP) (Arlington Heights, IL, USA) was added to the immunoblot buffer and incubated for 3 to 4 hr. Color was developed by incubating the membrane in 2.80 mol/L of 4-chloro-1-naphthol, 4.68 mol/L of methanol, and 4.41 mmol/L of H_2O_2 dissolved in PBS.

Laminin

Sample preparation, gel electrophoresis, and Western blot for laminin were similar to the fibronectin method described earlier, but

with two major differences. First, laminin was extracted with a slightly modified method of Timpl et al.[22] using 0.5 M NaCl, 0.05 M Tris-HCl (pH 7.4) buffer in the presence of protease inhibitors: 8.0 mmol/L of ethylenediamine-tetraacetic acid (EDTA), 1 mmol/L of PMSF, 1 mmol/L of pepstatin A and aprotinin (5 trypsin IU/ml). The supernatant was used for electrophoresis immediately after extraction. Second, the blotted protein in the nitrocellulose membrane was incubated with rabbit antirat laminin antibody (TELIOS Pharmaceutical, Inc., San Diego, CA, USA) as the primary antibody and followed by a biotinylated goat antirabbit antibody and an avidin/biotinylated HRP complex from Vectastain ABC kit (Vector Laboratories)[23] according to the instructions from the supplier. Finally, the amount of protein loaded onto each well for the gel electrophoresis was 100 μg in order to visualize the 220 kD subunit of laminin in the Western blots.

Cytochrome c oxidase

For the studies of CCO subunits, cardiac tissues were separated into myofibrillar and nonmyofibrillar fractions as previously described[24] and the nonmyofibrillar fraction was electrophoresed using 8 mol/L of urea and 16% acrylamide gels described by Estey et al.[25] Proteins loaded onto each well were 20 μg for gel electrophoresis, and double the amount of protein was loaded for Western blots. Preliminary studies indicated that these levels were on the linear range for detection by densitometry. Proteins from gels were transferred to nitrocellulose membranes as described earlier. The membranes were incubated with a primary antibody (polyclonal CCO to bovine) followed by an antirabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co.) as the secondary antibody. Color development was as previous described by Medeiros et al.[11]

Myosin electrophoresis

Myosin was extracted by homogenization[26] in the following buffer: 0.6 mol/L of NaCl, 20 mmol/L of $MgSO_4$, 0.1 mmol/L of dithiothreitol, 0.1 mmol/L of EGTA, 10 mmol/L of ATP-Na (added when used) and 5 mmol/L of NaH_2PO_4. Crude homogenized samples were centrifuged for 30 sec at 11,000*g* (Microfuge E, Beckman Instruments, Inc., Palo Alto, CA, USA). The supernatant was used for native gel electrophoresis analysis where the running gel contained 3.5% acrylamide. The protein concentration was determinated by the Lowry method.[20] Crude myosin extracts were loaded onto the gel (5 μg of protein/well) and electrophoreses for 1 hr at 100 V and 20 mA, 1 hr at 200 V and 40 mA, and 2 hr at 300 V and 60 mA with a pulse rate of 180 pulses/sec and 1 discharge capacity in a Model 4100 Pulsed Constant Power Supply (EG&G ORTEC Company, Oak, TN, USA). The upper and the lower chamber buffers were recirculated between the two chambers in order to keep a constant pH. After electrophoresis, the gels were stained with Commassie brilliant blue R250 for 30 min and destained overnight with 1.56 mol/L of acetic acid until the background was clear.[27]

Densitometry

Commassie stained gels that contained fibronectin and laminin had the relative levels of protein (identified from Western blots) measured using an ULTROSCANXL Enhanced Laser Densitometer (Broma, Sweden). Relative levels of CCO subunits from the Western blots were determined by densitometry. The relative amounts of isomyosins were determined from the Commassie stained gels using densitometry. Data analyzed were the percent area under the densitometry tracings for the protein or subunit of interest.

Statistics

The dependent variables of body weight, heart weight, heart to body weight ratio, hematocrit, liver and plasma SOD, plasma ceruloplasmin, and data from densitometry of gel electrophoresis for laminin, fibronectin, and isomyosins, and Western blot for CCO were analyzed by one way ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA). The General Linear Models procedure was used to determine significant differences, and when significant F values existed, the least significant difference procedure was used to determine which of group means differed from each other.

Results

There were no differences ($P > 0.05$) in heart and final body weights between Cu^+ rats and Cu^- rats after 5 weeks of copper treatment beginning from weaning and between the three postweanling treatment groups (*Table 1*). However, the ratio of heart to body weight was higher ($P < 0.05$) in Cu^- rats than in Cu^+ rats after 5 weeks of copper treatment, but hematocrit levels were lower ($P < 0.05$) in Cu^- rats compared with Cu^+ rats. These differences were not apparent in the postweanling rats (*Table 1*). Plasma and liver SOD in the Cu^- and Cu^+,Cu^- depleted rats were lower ($P < 0.05$) than in the Cu^+,Cu^+ postweanling and Cu^+,Cu^- depleted rats (*Table 1*). There were no differences ($P > 0.05$) in liver and plasma SOD between Cu^-,Cu^+ repleted and Cu^+ postweanling rats (*Table 1*). Significantly ($P < 0.05$) reduced plasma ceruloplasmin activity was observed in Cu^- rats as compared with Cu^+ rats but there were no significant differences among postweanling rats (*Table 1*).

Cu^- rats had lower ($P < 0.05$) levels of CCO subunit IV and laminin, but higher ($P < 0.05$) levels of fibronectin, isomyosin V_3, and the ratio of V_3 to V_1 (*Table 2*, *Figures 1*, *2*, *3*, and *4*). The Cu^+,Cu^- depleted and Cu^-,Cu^+ repleted groups did not have altered laminin and myosin V_3 levels, but the fibronectin levels were still higher ($P < 0.05$) in Cu^+,Cu^- depleted rats as compared with the Cu^+ postweanling and Cu^-,Cu^+ repleted rats (*Table 2*). Both the Cu^+,Cu^- depleted and Cu^-,Cu^+ repleted rats still had lower ($P < 0.05$) levels of CCO subunit IV than the control Cu^+ postweanling rats (*Table 2*, and *Figure 1*), with no apparent differences between the Cu^+,Cu^- depleted and the Cu^-,Cu^+ repleted groups.

Subunit V is composed of two subunits (a and b) that often comigrated on the gels used. Subunit VI is composed of three subunits (a, b, and c). For subunits Va,b and VIa,b,c in Cu^- rats, the percent area from the densitometer traces were 27.7% and 31.1%, respectively. Inspection of *Figure 1b* reveals that subunit Va,b should be much less than subunit VIa,b,c. However, the dramatic decrease in subunit IV inflated the percentages for these subunits. To correct for this, subunits IV, Va,b, and VIa,b,c were also expressed as a ratio to subunit II, since there were no differences by treatment apparent for this subunit. Similar results were obtained in that subunit IV expressed relative to subunit II was decreased ($P \leq 0.01$) in Cu^- rats compared with all other treatments with no apparent differences in any of the other subunits. There was greater variation in the

Table 1 Indices of copper deficiency among treatments (mean ± SEM)*

Parameter	Weanling		Postweanling		
	Cu^+ (n = 7)	Cu^- (n = 7)	Cu^+ (n = 7) control	Cu^+, Cu^- (n = 7) depleted	Cu^-, Cu^+ (n = 7) repleted
Final body weight (g)	275 ± 10[a]	249 ± 8[a]	404 ± 10[b]	438 ± 6[b]	410 ± 16[b]
Heart weight (g)	1.27 ± 0.05[a]	1.39 ± 0.07[a]	1.61 ± 0.06[b]	1.75 ± 0.09[b]	1.64 ± 0.08[b]
Heart:body weight ($\times 10^{-3}$)	4.6 ± 0.1[a]	5.6 ± 0.04[b]	3.9 ± 0.1[c]	3.9 ± 0.02[c]	3.9 ± 0.1[c]
Hematocrit	0.48 ± 0.02[a]	0.28 ± 0.03[b]	0.45 ± 0.01[a]	0.45 ± 0.03[a]	0.47 ± 0.01[a]
Plasma SOD (U/ml)	59 ± 7[a]	17 ± 3[b]	62 ± 5[a]	27 ± 6[b]	67 ± 8[a]
Liver SOD (U/g wet wt)	13,399 ± 1,468[a]	3944 ± 262[b]	22354 ± 3461[c]	14727 ± 1794[d]	22024 ± 2524[c]
Ceruloplasmin (U/L)	30.4 ± 4.7[a]	5.6 ± 2.1[b]	32.6 ± 2.3[a]	28.9 ± 2.9[a]	29.0 ± 2.6[a]

*Means followed by different superscripts are significantly different at $P < 0.05$ as determined by the least significant difference method.

levels of subunits Va,b and VIa,b,c in the Cu^- group compared with other treatments (*Table 2*).

Discussion

Weanling and postweanling rats fed copper-deficient diets did exhibit some deficiency signs, such as lower enzyme activities of Cu-Zn SOD in liver and plasma and for weanling rats, decreased plasma ceruloplasmin activity (*Table 1*) as compared with copper-adequate rats. The low hematocrit and increased heart to body weight ratio in Cu^- rats are consistent with the copper-deficient states. The enzyme activities of both liver and plasma Cu-Zn SOD returned to the same values as the control group after the 6-week copper repletion period (*Table 1*). Anemia and lower tissue SOD activity induced by copper deficiency may increase the stress upon the heart and result in cardiac injuries.

Previous work showed that cardiac isomyosin shifts from the V_1 isoform to V_3 under conditions of pressure overload in the heart in both humans and animals.[14,28] V_3 isomyosin, which has lower Ca^{2+} and actin-activated ATPase activities, accounts for most of the total myosin in rat fetuses, whereas the V_1 is found mainly in adult rats.[27] In this study, we did find that the V_3 isomyosin increased only in rats fed the Cu^- diet from weaning (*Table 2* and *Figure 4*). This change was less as compared with those hypertrophic hearts induced by aortic constriction.[12] This may be related to the age of the rats and the extent of cardiac hypertrophy because β-heavy myosin chain (HMC) almost disappears at 2 months of age in rats.[28] Cu^- weanling rats had about double the amount of V_3 as compared with the control rats (3.2% versus 5.8%; *Table 2*). The lack of a significant difference for V_1 isomyosins among five groups of rats in this study (*Table 2*) could be due to the high initial content of this isomyosin.

A shift from the V_1 isoform to V_3 isomyosin could be an energy-saving process to reduce myosin ATPase activity. This would decrease the velocity of cardiac contraction due

Table 2 Mean percent (± SEM) of cardiac proteins among treatments as determined by densitometry*

Densitometry	Weanling		Postweanling		
	Cu^+ (n = 7) (%)	Cu^- (n = 7) (%)	Cu^+ (n = 7) control (%)	Cu^+,Cu^- (n = 7) depleted (%)	Cu^-,Cu^+ (n = 7) repleted (%)
Fibronectin†	25.3 ± 1.5[a]	42.3 ± 2.2[b]	19.1 ± 1.2[c]	24.2 ± 1.1[a]	19.9 ± 1.0[c]
Laminin†	30.4 ± 1.2[a]	23.6 ± 1.6[b]	20.0 ± 2.4[c]	17.8 ± 0.8[c]	19.1 ± 1.3[c]
Myosin V_3†	3.2 ± 0.3[a]	5.8 ± 0.9[b]	3.8 ± 0.8[a]	4.5 ± 1.0[a]	5.2 ± 0.9[a]
Myosin V_2†	18.5 ± 2.0	19.5 ± 1.8	9.5 ± 2.1	18.9 ± 1.8	18.9 ± 3.1
Myosin V_1†	78.3 ± 2.1	74.6 ± 2.3	76.7 ± 2.0	76.6 ± 1.4	75.9 ± 3.5
Myosin V_3/V_1 ratio	4.1 ± 0.5[a]	8.0 ± 1.3[b]	5.0 ± 0.1[a]	5.8 ± 1.3[a]	7.1 ± 1.4[a]
CCO II‡	11.6 ± 0.5	12.4 ± 1.2	12.2 ± 0.8	13.3 ± 1.9	11.5 ± 1.0
CCO IV‡	4.2 ± 0.5[ac]	1.1 ± 0.4[b]	4.9 ± 0.4[c]	3.5 ± 0.1[a]	3.9 ± 0.4[a]
CCO V‡	27.3 ± 2.1	27.7 ± 3.1	22.8 ± 1.3	22.8 ± 1.4	23.8 ± 1.7
CCO VI‡	25.7 ± 1.6	31.1 ± 4.2	27.3 ± 3.3	25.1 ± 1.4	31.9 ± 5.3

*Values for each protein or peptide were obtained as percent of total protein areas from densitometry tracings described in the materials and methods section. Means followed by different superscripts are significantly different at $P < 0.05$ as determined by the least significant difference method.
†Values are from Commassie stained gels.
‡CCO II, IV, V, and VI represent cytochrome c oxidase subunits II, IV, V, and VI, respectively, from Western blots.

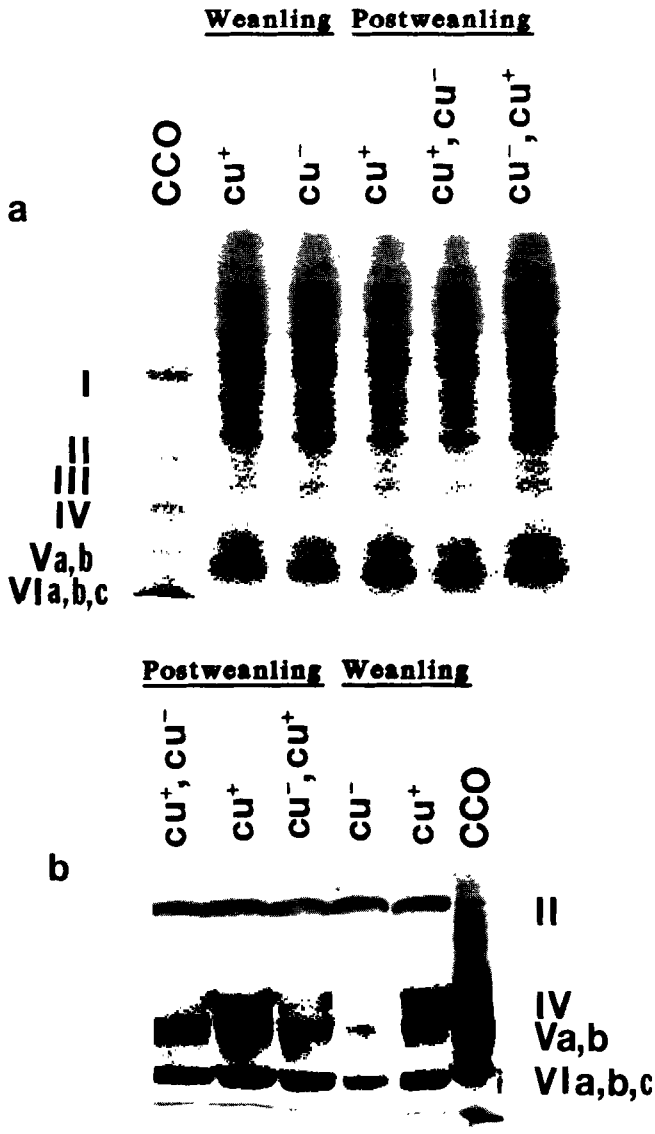

Figure 1 SDS-urea PAGE (a) and Western blot for CCO (b) from copper-deficient and control rats. (a) 16% acrylamide, 8 mol/L of urea gel stained with Commassie blue. (b) Western blot of gel in (a) developed with anti-CCO antibody. Decreased CCO subunit IV was shown in Cu^- rats and its level was lower in Cu^+,Cu^- depleted and Cu^-,Cu^+ repleted than in the Cu^+ postweanling rats.

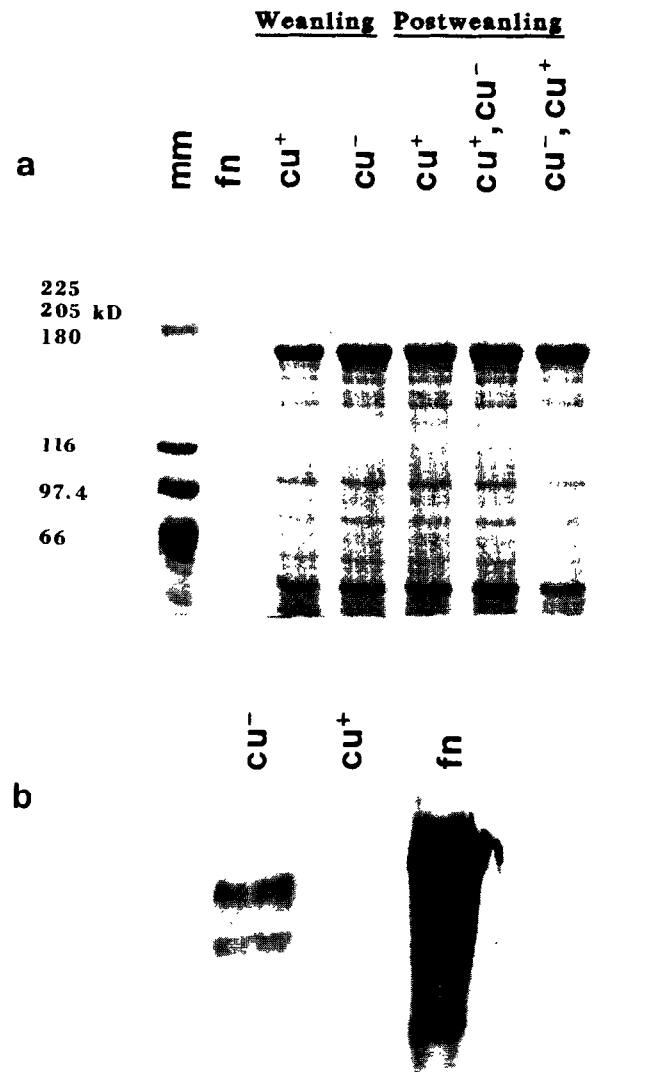

Figure 2 (a) SDS–PAGE of fibronectin levels from hearts of copper restricted, repleted, and control rats stained with Commassie blue. Lane mm is for the molecular weight markers, and lane fn is the fibronectin standard from rat plasma. The fibronectin band among the different treatments is indicated by an arrow. (b) The fibronectin band was identified using a Western blot. All electrophoresis and blotting were as described in the Methods and Materials section.

to decreased cross-bridge recycling[29] and contractility.[13] On the other hand, an isozyme shift of isomyosin may improve the cardiac pumping and the mechanical efficiency of the heart due to lower ATP utilization with increased systole tension.[30] This may partially explain why cardiac ATP levels were not altered after 5 weeks of copper restriction despite decreased cardiac CCO activity.[10] This is also consistent with the decreased cardiac contractility in the copper-deficient spontaneously hypertensive–heart failure rats (SHHF/Mcc-fa^{cp}) reported in another study.[7] However, the small level of V_3 initially present leads one to question whether such an increase from such a low level could dramatically influence ATP levels.

Rat CCO subunit II encoded by mitochondrial DNA contains an oxidation-center (Cu_A) and has catalytic properties, whereas the nuclear encoded subunit IV of CCO may have some regulatory function.[31,32] Copper is located on subunits I and II.[33,34] A decreased protein level of CCO subunit IV, but not subunit II, was reported by Medeiros et al.[11] The data presented here show that copper is not required for protein synthesis of CCO subunit II but is required for the nuclear encoded CCO subunit IV. Other studies have shown that CCO activity in copper-deficient animals is markedly depressed in several tissues including the heart.[35,36] Weisenberg et al.[37] reported that the liver of copper-deficient rats had decreased ATP content in rats fed a copper-deficient diet from weanling until the age of 4 to 6 weeks. Kopp et al.[5] reported that there were significant reductions in rat hepatic, cardiac, and renal ATP levels in copper-deficient rats. This could lead to impaired energy

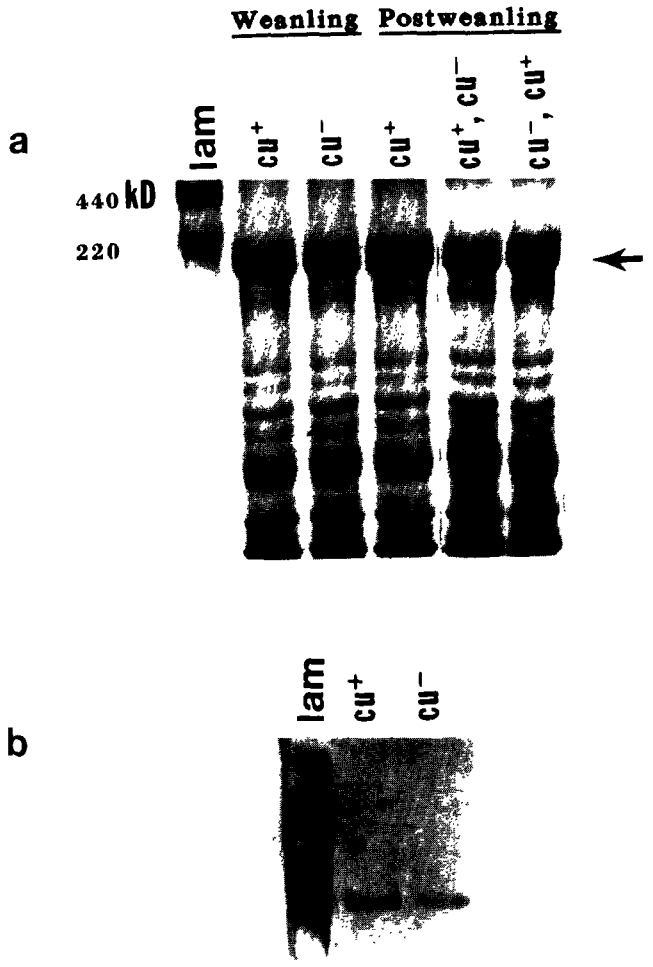

Figure 3 (a) SDS–PAGE of laminin levels from hearts of copper restricted, repleted, and control rats stained with Commassie blue. Lane LAM is the laminin standard protein isolated from mouse EHM tumor. (b) Laminin was identified using a Western blot. The laminin band among the different treatments is indicated by an arrow in (a). All electrophoresis and blotting were as described in the Methods and Materials section.

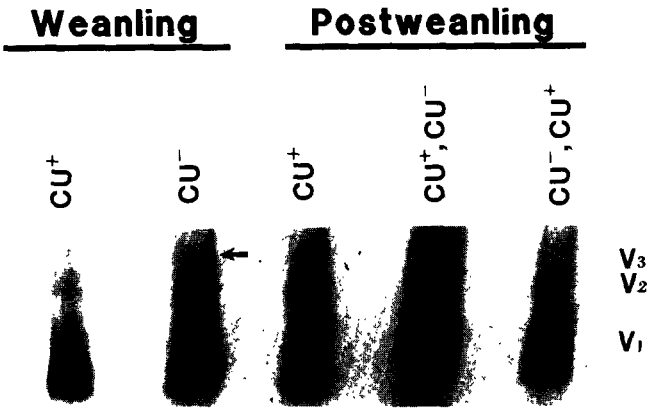

Figure 4 Native gel electrophoresis of myosin extracts from hearts of copper restricted, repleted, and control rats stained with Commassie blue. The V_3 isomyosin was increased in Cu^- rats as indicated by the arrow. The small amount of V_3 isomyosin in weanling Cu^+ rats (3.2% of total myosin) increased to a mean of 5.8% in the weanling Cu^- group as determined by laser densitometry. The electrophoresis procedure is described in the Methods and Materials section.

metabolism and may be one factor in explaining the pathophysiology of copper deficiency. In other work, however, adenine nucleotides did not differ after copper restriction, but CCO activity significantly decreased in the liver, brain, heart, kidney, and spleen of copper-deficient mice[9] and in rat hearts.[10] The results of these studies suggest that there may be an increase in the activity of other ATP production pathways, such as anaerobic glycolysis or creatinine phosphokinase. Also, these results may suggest that CCO activity may not be rate limiting for ATP production.

Basement membrane as a supporting structure for cells can regulate cell attachment, growth, and differentiation.[22] Fibronectin and laminin from basement membrane are associated with these functions.[38] Fragmentation and thickening of the basement membranes was revealed from Davidson's study[8] by transmission electron microscopy in hearts of copper-deficient rats fed a Cu^- diet either from weanling or from postweanling. The morphological changes of basement membrane in the hearts of copper-depleted rats could be related to the changes of fibronectin and laminin as shown by this study. Increased fibronectin of hearts from both weanling and postweanling rats fed copper-deficient diets in this study may indicate that cardiac tissues undergo wound healing because fibronectin can act as a substrate for cell adhesion and wound healing.[39]

Copper-repleted rats had the same level of cardiac fibronectin and laminin as control rats. This is in contrast to a study by Davidson et al.[8] in which rat hearts of copper-repleted rats still had the thickened and fragmented basement membrane. This may indicate that the morphological changes of cardiac basement membranes in copper-depleted rats are irreversible even though some of the biochemical indices such as SOD, ceruloplasmin, hematocrit, fibronectin, laminin, and isomyosin return to control levels upon copper repletion. This apparent contradiction between the morphological results of Davidson et al.[8] and the biochemical results presented here for copper-repleted rats could be due to a longer period of time needed for the heart to regain normal morphology after the proteins have normalized to control levels. Additional work could confirm this by examining further repletion times and conducting simultaneous morphological and biochemical observations.

Laminin can work as a stimulator for cell growth and tissue differentiation, a mediator for cell communication, and a promotor for neurite growth.[40] The decreased laminin levels from male weanling Cu^- rats may indicate cardiac cell detachment because of the involvement of laminin in cell attachment and cell signaling.[41,42] Disrupted basal laminae at the capillary–myocyte interface[8] may be related to lower laminin levels. Copper depletion of postweanling rats had no significant effect upon laminin levels as analyzed by gel electrophoresis (*Table 2* and *Figure 3*). Therefore, copper had greater effects on hearts from male rats fed the Cu^- diet from weanling than on those from male postweanling in maintaining structural and functional integrity of the rat hearts.

In summary, cardiac levels of fibronectin, laminin, V_3 isomyosins, and subunits of cytochrome c oxidase were

altered by copper deficiency in weanling rats and these proteins in postweanling rats were less affected by copper restriction. However, postweanling rats either fed Cu^- diets or repleted were of marginal copper status as compared with younger copper-deficient rats. The changes in CCO subunit IV and in fibronectin suggest that marginal copper status has effects upon these proteins.

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