Marginal Copper-Restricted Diets Produce Altered Cardiac Ultrastructure in the Rat

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Abstract. **To determine if chronic ingestion of a diet containing a marginally low level of Cu could cause deleterious alterations in cardiac ultrastructure, male offspring were nursed by dams fed a diet containing either 6.7 or 2.8 mg Cu/kg from midgestation through lactation before weaning to the same diet. Conventional measures of Cu status, including growth, relative heart weight, tissue concentrations of Cu, ceruloplasmin activity, and tissue activity of Cu,Zn-superoxide dismutase (SOD) were similar in both dietary treatment groups at 5.5 months of age. However, significant increases in the number and volume of lipid droplets and an increased incidence of pathological abnormalities in mitochondria and basal laminae were observed in sections of hearts from rats chronically fed the diet containing 2.8 mg/kg Cu. Reduction of the dietary level of Cu from 2.8 to 1.3 mg/kg from 4 to 5.5 months of age caused significant reductions in the concentration of Cu in serum and liver, but Cu content, Cu,Zn-SOD activity, pathological scores, and morphometric parameters in hearts were not modified by the greater restriction of dietary Cu in adult rats. This study suggests that abnormalities in cardiac ultrastructure occurred in rats chronically fed diets marginally low in Cu, despite minimal changes in conventional biochemical indicators of Cu status.** [P.S.E.B.M. 1995, Vol 210]

Reports of cellular and extracellular ultrastructure changes in hearts of Cu-deficient animals are well established. Intracellular alterations associated with severe Cu-deficiency include augmentation of mitochondrial volume density (1–5), marked increase in the number of lipid droplets (1–5), sarcomere degeneration (3), and mitochondrial hypertrophy with disruption of cristae (1–5). Extracellular alterations include collagen fibrosis (1, 4, 5) and thickening and disorganization of the basal laminae (1, 4, 5). Generally, such studies have utilized young rodents weaned to diets containing very low levels of Cu (<1 mg/kg). The severity of the Cu restriction on young animals can cause death due to ventricular rupture or aneurysms after 6–10 weeks (1).

Daily Cu consumption in the United States has been estimated at 1.2 and 0.9 mg for males and females, respectively (6). This is well below the Estimated Safe and Adequate Daily Dietary Intake of 1.5–3.0 mg Cu/day for adults (7). Thus, although Cu consumption in the Unites States can be described as "marginally low," consequences of severe Cu deficiency in rapidly growing rodent models may not be relevant to the human situation. The purpose of this study was to examine whether chronic consumption of a diet marginally restricted in Cu perturbed cardiac ultrastructure of adult male rats. Since the requirement of growing laboratory rats for Cu is 5 mg/kg of diet (7), we provided male rats with diets containing either an adequate level or 50%–60% of the requirement throughout the periods of development and rapid growth. A subset of the latter group had the Cu content of the diet further reduced to about 25% of the requirement beginning at 4 months of age to determine

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if greater restriction exacerbated the impact of chronic intake of suboptimal level of Cu on cardiac structure.

Materials and Methods

Animals. Hearts were taken from adult male Sprague-Dawley rats that were part of an investigation also evaluating the effects of chronic marginal Cu-restriction on in vitro activities of immune cells. Data related to immunocompetence has been reported separately (Hopkins RG, Failla ML, submitted). The feeding trial was initiated by purchasing pregnant dams from Harlan Laboratories (Indianapolis, IN). Animals arrived at 11 days gestation and were randomly assigned to shoebox cages with corn cob bedding and fed a semipurified diet containing either 6.7 (adequate) or 2.8 (marginally low) mg Cu/kg, respectively, by analysis. The basal diet containing casein (200 g/kg), sucrose (500 g/kg), cornstarch (150 g/kg), corn oil (50 g/kg), cellulose (50 g/kg), choline bitartrate (2 g/kg), DL-methionine (3 g/kg), AIN-76A vitamin mix (10 g/kg) (8), and AIN-76 mineral mix (35 g/kg) (8) with the indicated levels of Cu. Throughout the study, all animals had free access to the diet and deionized water. Following parturition, litters were culled to 10 pups per dam as necessary. Lactating dams continued to be fed the diet that had been assigned during pregnancy. At weaning, pups were transferred to individual hanging stainless steel cages and fed the same diet as their mothers. At 4 months of age, one half of the animals in the group fed the diet containing 2.8 mg Cu/kg were randomly selected and subsequently fed a diet containing 1.3 mg Cu/kg diet (by analysis) for the remainder of the study (moderately low). Animals were sacrificed at 5.5 months of age following an overnight fast as described below. Six rats from each of the three dietary groups, viz. 6.7, 2.8, and 2.8 → 1.3 mg/kg Cu diet, were used in this study.

Assays. Cu and zinc content of hearts and diets were determined by atomic absorption spectrophotometry after combined dry and wet ashing procedure of Hill *et al.* (9). Because hearts were perfused with glutaraldehyde (see below), a sample of right ventricle was dried at 105°C overnight to determine dry weight prior to initiating the ashing procedure. Metal levels in hearts are reported per gram dry weight. Analysis of samples of standard reference materials, viz., bovine liver 1577A and rice flour 1568, from the National Institute of Science and Technology were prepared similarly and demonstrated that recovery of Cu and zinc was 98%–101%. Serum was diluted 1:4 with deionized water and directly aspirated into a flame atomic absorption spectrophotometer for analysis. Ceruloplasmin activity was determined by measuring serum-mediated oxidation of O-dianisidine, as reported previously (10). Cu,Zn-superoxide dismutase (SOD) activity was measured in a sample removed from the right ventricle of the heart. The inhibition of the rate of autoxidation of pyrogallol using a modification of the method of Prohaska *et al.* (11), as described elsewhere (Hopkins RG, Failla ML, submitted). The modified procedure was standardized using known activities of purified bovine erythrocyte Cu,Zn-SOD (Sigma Chemical Co., St. Louis, MO). Serum total cholesterol was measured by a coupled enzyme assay (Sigma kit #352).

Transmission Electron Microscopy. Rats were anesthetized with ketamine and xylazine (85 and 15 mg/kg body wt, respectively) injected ip. Once fully anesthetized, thoracic cavities were opened by midline incision, and a blood sample was removed by right ventricular cardiac puncture. Hearts were then perfused with KCl (1.0 *M*) and removed by severing the great arteries. Hearts were quickly perfused with 2% glutaraldehyde in 0.1 *M* Sorenson's phosphate-buffered saline (PBS) in 0.1 *M* sucrose. Hearts were weighed and processed for electron microscopy as described previously (1).

All electron microscopy processing, examination, micrography, and scoring were completed blindly to minimize bias. Electron micrographs were printed at ×4000 to allow a broader perspective of cardiomyocyte composition, ×17,500 to allow accurate resolution between cellular components for volume density determination, and ×24,000 to allow for scrutiny of the perivascular space for basal laminae organization and presence of collagen fibrosis. Volume density measurements followed a point system previously described by Weibel (12) and Steer (13). A 20 × 25 cm transparent grid was utilized which allowed approximately 1000 points of reference per micrograph.

Mitochondrial appearance was evaluated on a 1–4 scale, with 0.5 increments, with 4.0 being the highest score of normalcy. Briefly, mitochondria were scored on shape and degree of swelling, organization of membranes, cristae density and appearance, presence of vacuoles, and mitochondrial intrusion upon other cellular compartments. Scoring of the basal laminae was performed using a 1–4 scale, with 0.5 increments, as described previously (2).

Statistical Applications. For mitochondria and basement pathology scores, the number of rats with scores of 1–2, 2–3, and 3–4 were determined for each level of dietary Cu treatment. A chi-square analysis with Fisher's exact test was used to test for independence of association. For all other data, a one-way analysis of variance (ANOVA) was applied to determine if significant differences existed in the variations of sample means using the Statistical Analysis Software system (SAS, Cary, NC). When significant differences were determined the least significant differences (LSD) post-ANOVA test was utilized to determine which means differed. Linear regression was

applied for trend analysis. The α level was set *a priori* at 0.05.

Results

Body weight, heart weight and the ratio of heart: body weights were similar for the three dietary treatment groups (Table I). Chronic ingestion of marginally low Cu diet (i.e., 2.8 mg/kg) was not associated with significant changes in growth or conventional measures of Cu status, including the levels of the micronutrient in serum, liver, or heart. Similarly, the activities of serum Cp and cardiac Cu,Zn-SOD were not altered by feeding a marginally low Cu diet. Further reduction of the level of dietary Cu to 1.3 mg/kg for a subgroup of adult rats that had been previously fed 2.8 mg/kg resulted in significant decreases in the concentrations of serum and hepatic Cu and the activity of serum ceruloplasmin. However, cardiac Cu and Cu,Zn-SOD activity were not altered by further reduction of dietary Cu intake in adult male rats. Cardiac Zn was independent of dietary Cu status. Hearts from rats fed diets with adequate, marginally and moderately low Cu contained 111 ± 4, 109 ± 11, and 116 ± 7 mg Zn/kg dry weight, respectively. Serum cholesterol levels did not differ among the three diet groups.

Pathology scores of the mitochondria revealed greater pathology in animals chronically fed marginally and moderately low Cu diets (Table II). For example, mitochondria from three of the six hearts from rats fed a moderately low Cu diet as adults appeared swollen and the fine cristae structure appeared fragmented in areas, with vacuolization apparent (Fig. 1). Focal regions of collagen fibrosis and leukocyte infiltration (Fig. 2) were observed. For the basal lamina, greater pathology was observed in rats fed marginally and moderately low Cu diets ($P = 0.102$) (Table II). Nonuniform and thickened basement membranes as well as disorganization along both capillaries and myocytes were observed in sections for some rats fed marginally (Fig. 3) and moderately low (data not shown) Cu diets (Fig. 3). Generally, there was less variation in pathology scores in the Cu-adequate groups compared with the Cu-restricted groups (Table II). For the mitochondria pathology scores, three rats from each of the marginally and moderately low Cu groups were below 3.0. Likewise, analysis of sections from four rats in each of the Cu-restricted groups had pathology scores for the basement membrane below 3.0, whereas only one of the rats fed the Cu-adequate diet had such a score.

Lipid inclusion volume density was about 2.5-fold ($P \leq 0.05$) higher in rats chronically fed either the marginally or moderately low Cu diet compared with the Cu-adequate diet. A representative electron micro-

Table I. Body and Heart Weights, Tissue Cu Status, and Serum Cholesterol for Rats Fed Control (Cu-Adequate) and Cu-Restricted Diets[a]

Variable	Treatment		
	Cu-adequate (6.7 mg Cu/kg) (n = 6)	Marginally low (2.8 mg Cu/kg) (n = 6)	Moderately low Cu (2.8→1.3 mg Cu/kg) (n = 6)
Body weight (g)	580.8 ± 46.6	584.0 ± 69.8	589.3 ± 86.4
Heart weight (g)	1.62 ± 0.3	1.54 ± 0.2	1.47 ± 0.1
Heart:body weight (g/100 g)	0.28 ± 0.05	0.27 ± 0.03	0.25 ± 0.02
Serum Cu (μmol/l)	18.7 ± 3.5[a]	19.8 ± 1.7[a]	10.9 ± 6.5[b]
Serum Cp (U/l)	90.6 ± 16.8[a]	92.9 ± 8.0[a]	39.0 ± 41.4[b]
Liver Cu (μmol/kg wet wt)	86.5 ± 8.8[a]	80.8 ± 14.6[a,b]	65.6 ± 16.7[b]
Heart Cu (μmol/kg dry wt)	418 ± 14	398 ± 76	395 ± 41
Heart Cu,Zn-SOD (U/g tissue)	1389 ± 124	1451 ± 98	1521 ± 86
Serum cholesterol (mM)	2.56 ± 0.66	2.82 ± 0.57	2.72 ± 0.93

[a] *Note.* Values are expressed as mean ± SD. Measures followed by different superscripts are significantly different ($P \leq 0.05$).

Table II. Pathology Scores from Adult Rats Chronically Fed Cu-Restricted or Control Diets[a]

Variable	Treatment												
	Cu-adequate (6.7 mg Cu/kg) (n = 6)				Marginally low (2.8 mg Cu/kg) (n = 6)				Moderately low Cu (2.8→1.3 mg Cu/kg) (n = 6)				
	Score distribution			Mean	Score distribution			Mean	Score distribution			Mean	P[b]
	1–2	2–3	3–4		1–2	2–3	3–4		1–2	2–3	3–4		
Mitochondria[a]	—	—	6	3.3	—	3	3	3.0	2	1	3	2.7	0.048
Basement membrane[a]	—	1	5	3.2	3	1	2	2.1	1	3	1	2.3	0.102

[a] Values are mean scores using a 1–4 scale with 4 indicating normal morphology. See text for details.
[b] Determined using chi-square with Fisher's exact test.

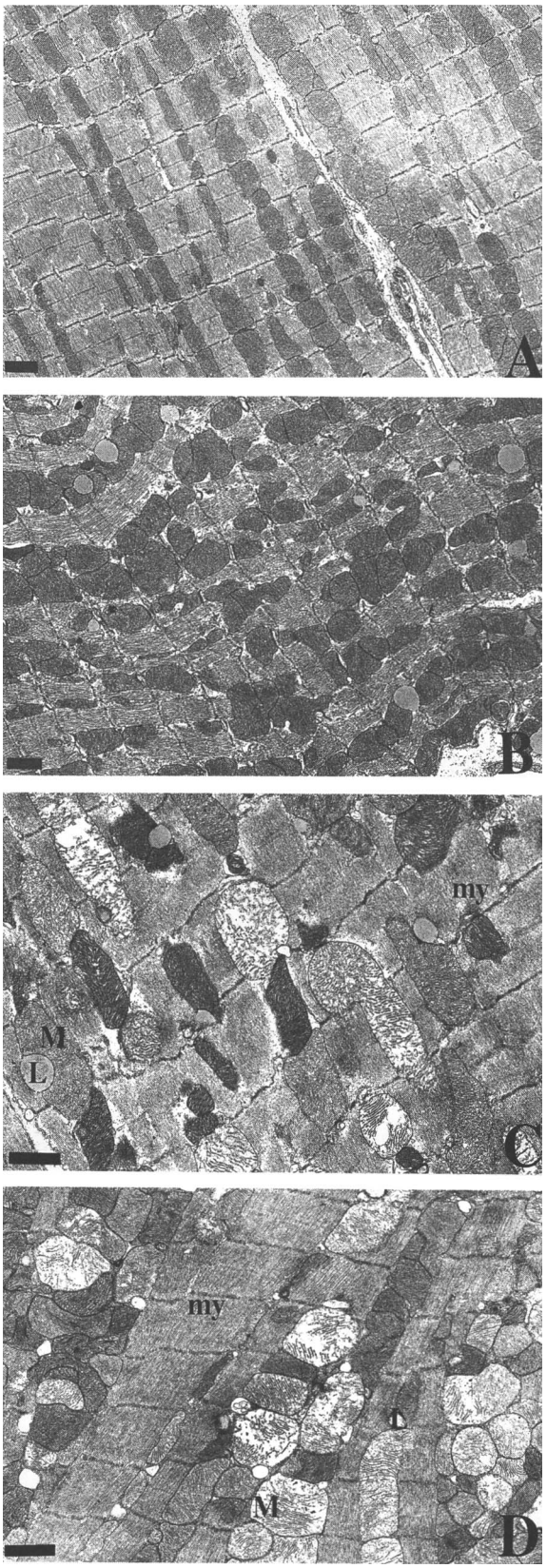

Figure 1. Cardiomyocytes presenting volume density of cellular components: 6.7 mg Cu/kg (A), 2.8 mg Cu/kg (B), and micrographs from two different rat hearts from the group fed the moderately low Cu diet (C and D). M, mitochondria; my, myofibril; L, lipid. Bar = 1 μm.

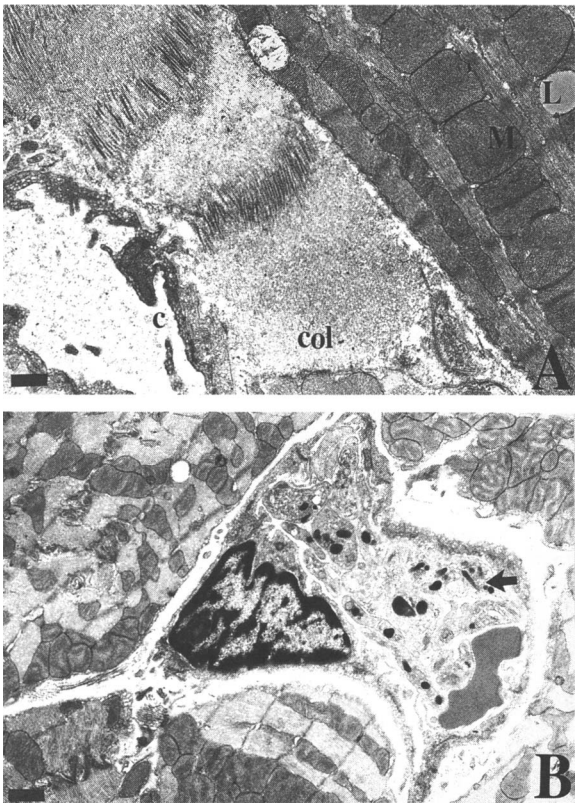

Figure 2. Interstitial space of a heart from a rat fed either the moderately low Cu diet showing increased collagen deposition (Panel A), or the marginally low Cu diet showing presence of increased capillary leukocytes (arrow, Panel B). L, lipid; M, mitochondria; col, collagen; c, capillary. Bar = 1 μm.

graph in Figure 4 displays an increased lipid compartment of many of the marginally low Cu-restricted cardiomyocytes. Mitochondrial and myofibrillar densities were not altered by dietary Cu treatment (Table III). However, increased mitochondrial volume density and concomitant increase in mitochondria:myofibrillar volume density ratio were apparent in some micrographs (Fig. 1). Rats that had low pathology scores for both mitochondria and basal lamina also tended to have elevated mitochondria volume density and mitochondria:myofibril.

As an alternative approach to evaluate the impact of marginal Cu restriction on cardiac ultrastructure, data from the three groups were pooled and linear regression and correlation coefficients of Cu status measures with pathology scores and volume density measures were determined (Table IV). Several indicators of Cu status were related to heart morphology. As serum ceruloplasmin and liver Cu levels increased to normal, the basal lamina scores increased toward normal ($P \leq 0.05$). Similarly, as liver Cu levels increased there was a trend toward higher mitochondria scores ($P = 0.06$). As expected, when liver Cu levels increased, volume density occupied by lipid droplets decreased ($P \leq 0.05$). These relationships indicate that some of the rats fed the Cu-restricted diets responded

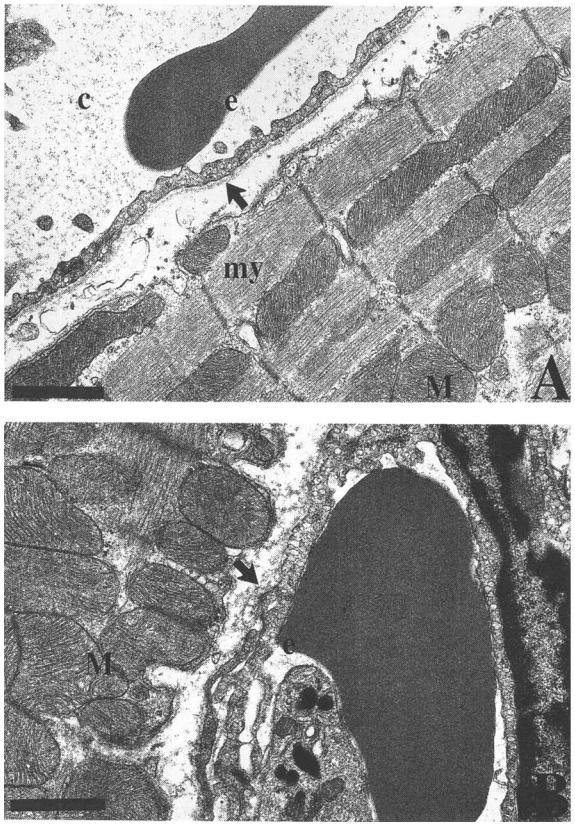

Figure 3. Interstitial space in sections from hearts of rats fed adequate (6.7 mg Cu/kg; Panel A) or restricted in Cu (2.8 → 1.3 mg Cu/kg; Panel B). Focal regions of disorganization of basal laminae (arrow) were observed in the hearts of the rat fed Cu-restricted diet. M, mitochondria; my, myofibril; c, capillary; e, erythrocyte. Bar = 1 μm.

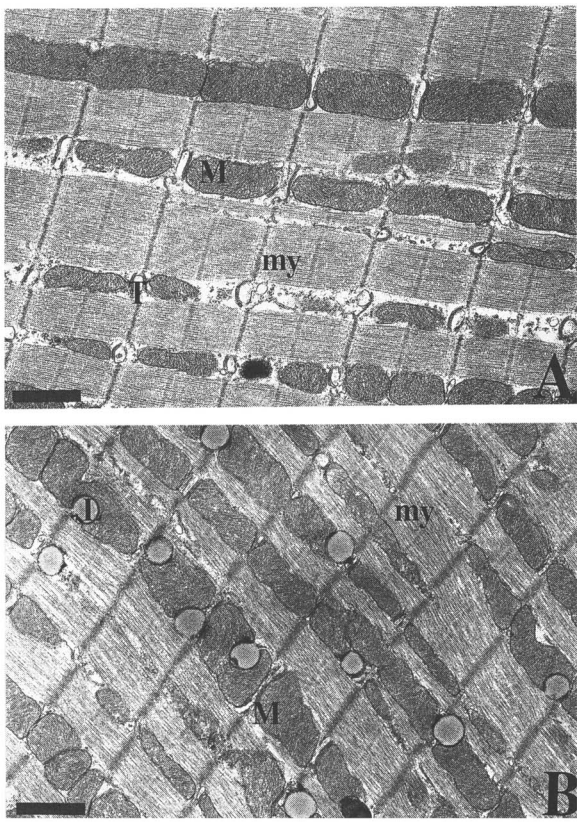

Figure 4. Cardiomyocytes of hearts from 6.7 mg Cu/kg (A) and 2.8 mg Cu/kg (B). Note the presence of more lipid droplets and their larger diameter in the section from the rat chronically fed the marginally low Cu diet. M, mitochondria; my, myofibril; L, Lipid. Bar = 1 μm.

to inadequate intake of the micronutrient more than others.

Discussion

Dietary restriction of the essential trace element Cu has been shown to have a detrimental effect upon the heart. Alterations are observed in electrocardiography (1, 2, 4, 5), gross morphometry including concentric hypertrophy (1, 5), and ultrastructure (1–5). Recently, weaned male rats fed a severely Cu-deficient (<1 mg Cu/kg diet) diet exhibit a decelerated body weight gain (1, 2, 5) and often die after 6–10 weeks as a result of ventricular rupture or aneurysm (1). Liver, cardiac, and serum concentrations of Cu as well as activities of cardiac and liver Cu,Zn-SOD, markedly decrease and are used to assess the degree of Cu depletion in the animal (1–5).

In the present study, traditional indicators of Cu status, including relative heart weight, serum and hepatic concentrations of the trace metal, and the activity of serum ceruloplasmin, were not significantly decreased by supplying a diet with about 60% of the NRC requirement for Cu from midgestation to adulthood. When the level of dietary Cu was further reduced to 1.3 mg/kg from 4 to 5.5 months of age (i.e., moderately low Cu group), the levels of Cu in serum and liver decreased significantly. However, relative cardiac mass and the level of Cu and the activity of Cu,Zn-SOD in the heart of these adult male rats was not altered by the greater restriction of dietary Cu.

Despite the relative stability of tissue Cu in the marginally and moderately low dietary groups, alterations in cardiac structure were apparent in this study. Indices of mitochondria and basal laminae integrity exhibited trends towards greater pathology in the marginally Cu-restricted diet groups and decreases in liver Cu and serum ceruloplasmin levels were associated with greater basement membrane pathology. These findings are consistent with previous reports of greater mitochondrial and basal laminae pathology in Cu-restricted rodents and pigs (1–5). The preliminary signs of cardiomyopathy can be detected before changes in conventional indicators of Cu status (e.g., serum and tissue Cu and Cu,Zn-SOD activity) are evident. Results from this study further revealed that, as diet Cu content becomes limited, lipid volume density increased in the cardiomyocytes of adult animals. Although the basis for the increase in lipid droplets or possible consequences of such accumulation are unclear, greater numbers of lipid inclusions also have

Table III. Volume Density Measures from Adult Rats Chronically Fed Cu-Restricted or Control Diets[a]

Variable	Treatment		
	Cu-adequate (6.7 mg Cu/kg) (n = 6)	Marginally low (2.8 mg Cu/kg) (n = 6)	Moderately low Cu (2.8→1.3 mg Cu/kg) (n = 6)
Lipid ($\mu m^3/\mu m^3$)	0.0046 ± 0.004[a]	0.014 ± 0.007[b]	0.011 ± 0.006[a,b]
Mitochondria ($\mu m^3/\mu m^3$)	0.40 ± 0.033	0.42 ± 0.045	0.44 ± 0.026
Myofibril ($\mu m^3/\mu m^3$)	0.50 ± 0.04	0.48 ± 0.05	0.48 ± 0.03
Mitochondria:myofibril	0.81 ± 0.13	0.88 ± 0.17	0.92 ± 0.10

[a] *Note.* Values are expressed as mean ± SD for n = 6 per group, measures with different superscript are significantly different ($P \leq 0.05$).

Table IV. Linear Regression Coefficients for Selected Indicators of Cu Status with Cardiac Morphology Measures

Dependent variable	y-intercept	Slope (b)	r	Independent variable
Basal lamina morphology	1.724	+0.0118[a]	0.48[a]	Ceruloplasmin
Basal lamina morphology	0.381	+0.6453[a]	0.40[a]	Liver Cu
Mitochondria morphology	1.668	+0.2789[b]	0.38	Liver Cu
Lipid droplet volume density	0.1054	−0.0175[a]	−0.31[c]	Liver Cu

[a] $P \leq 0.05$.
[b] $P = 0.06$.
[c] $P = 0.10$.

been reported in severely Cu-deficient young rats (1, 14). The present results suggest that the appearance of more and larger lipid droplets in the heart was the most evident abnormality in reference to marginal Cu status.

Previous reports on severely Cu-deficient rats have demonstrated elevations in serum cholesterol levels (15, 16). This study, however, suggests that marginally and moderately low levels of dietary Cu do not influence serum cholesterol levels. The presence of cardiac pathology in Cu-restricted groups would therefore appear to be unrelated to serum cholesterol levels.

The adverse effect of chronic ingestion of the marginally low Cu diet upon cardiac ultrastructure of the adult male rat is similar to its impact on immunocompetence. Spleens isolated from the same rats that provided the hearts analyzed and discussed above were used to prepare splenic mononuclear cell cultures (Hopkins RG, Failla ML, submitted). Relative weight of spleen, splenic Cu,Zn-SOD activity, and the yield of mononuclear cells (MNC) were similar for rats fed diets containing adequate and marginally low levels of Cu. However, splenic MNC from male rats fed the diet with marginally low Cu exhibited a markedly decreased response to T-cell mitogens and generated significant liver IL-2 bioactivity. These activities were restored to normal by dietary Cu repletion for two weeks. Also, neutrophils elicited by thioglycollate administration to an additional group of rats fed marginally low Cu diet produced only 60% as much superoxide anion when stimulated with phorbol myristic acetate as cells isolated from control animals (Hopkins RG, Failla ML, submitted). These data demonstrate that abnormalities are present in both the heart and immune system of adult males chronically fed the marginally low Cu diet, despite the absence of changes in the traditional indices of Cu deficiency.

The observed defects in both the heart and immune system of adult males chronically fed the marginally low Cu diet in the absence of reduced levels of tissue Cu and cuproenzyme activities raise the possibility that failure to provide adequate Cu at one or more critical times during development may have long-term consequences on tissue function. This is supported by the finding that perinatal Cu deficiency causes irreversible changes in Cu, cuproenzymes and catecholamines in specific regions of the mouse brain (17). In this and the companion study (Hopkins RG, Failla ML, submitted), our examination of the impact of chronic feeding of a marginally low Cu diet on cardiac ultrastructure and immunocompetence was limited to animals at six months of age. However, the relative size of the heart, the levels of Cu in heart, spleen and liver, and hepatic Cu,Zn-SOD activity at weaning (21 days of age) were similar ($P > 0.05$) for animals delivered and nursed by dams fed the adequate and marginally low Cu diets. The need for further examination of the effect of suboptimal Cu intake by the pregnant and lactating dam on cardiac structure and function of the neonatal and weaned rat is clearly merited.

In conclusion, compromised cardiomyocyte ultrastructure was observed in adult male rats chronically fed marginally and moderately Cu-restricted diets in the absence of significant changes in traditional indi-

cators of Cu status in the groups fed the marginally low Cu diet. Noted changes are for the same ultrastructural parameters reported for young, severely Cu-deficient rats and pigs. Regarding human Cu nutriture in the United States where heart disease remains a great concern, our results are the basis for several questions. Is it possible for a marginally Cu-restricted diet to lead to cardiac damage despite only mild, if any, decreases in traditional biochemical indicators of Cu status? Also, since a marginally Cu-restricted diet may not evoke cardiac enlargement or a decelerated body weight gain, what are overt indicators of a marginal Cu status? Finally, do the observed alterations in the heart, while not necessarily severe by themselves, increase risk of damage when the individual is subjected to additional stresses, such as aerobic exercise, ischemia/reperfusion, and cardiotoxic drugs? Further investigation of these possibilities is merited.

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