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COPPER DEFICIENCY: A POTENTIAL MODEL FOR DETERMINING THE ROLE OF MITOCHONDRIA IN CARDIAC AGING

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ABSTRACT

Heart mitochondria experience age-related declines in cytochrome c oxidase (CCO) activity and increases in the generation of reactive oxygen species (ROS) that may contribute to loss of cardiac function and the development of disease that occur with advancing age. In a manner similar to aging, copper deficiency also suppresses heart CCO activity and has cardiovascular consequences related to increased peroxidation. Food restriction is often used as a tool to study oxidative mechanisms of aging and the present study examines the potential of copper deficiency to model the role of mitochondria in cardiac aging by determining if the effect of food restriction on CCO activity and oxidative stress in heart mitochondria parallels its effect on cardiac mitochondria during aging. Overall, copper deficiency severely inhibited CCO activity and increased both Mn superoxide dismutase (MnSOD) and glutathione peroxidase (GPX) in isolated heart mitochondria. However, a 20% reduction in food intake by copper-deficient rats increased CCO activity by 65% and decreased MnSOD activity by 25% but had no effect in rats fed adequate copper. Copper deficiency also reduced the carbonyl content of 80-100 kDa mitochondrial proteins, but the reduction in carbonyl content was unaffected by food restriction. Food restriction did, however, completely prevent the enlargement of cardiac mitochondria in copper-deficient rats. Together, these findings indicate that copper deficiency induces mitochondrial antioxidant enzyme activity and hypertrophy in cardiac tissue in response to reduced CCO activity and that food restriction may counteract these changes by reducing oxidative stress. Because the action of food restriction on CCO activity and mitochondrially generated oxidative stress are similar in copper deficiency and aging, copper deficiency may serve as a short-term model for studying the potential roles of mitochondria in cardiac aging.

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INTRODUCTION

Aging in mammals involves the senescence-related loss of function of postmitotic tissues. A predominant hypothesis for aging is the free radical theory which attributes the loss of function to the accumulation of molecular oxidative damage (1). The free radical theory is supported by several studies showing that oxidative damage to lipids, proteins, and DNA accumulates with advancing age (2). Molecular damage caused by reactive oxygen species (ROS) also may contribute to a variety of diseases including cancer, cardiovascular disease, Parkinson's disease, ischemia and chronic inflamation (3, 4). Reports showing that copper deficiency in animals increases lipid hydroperoxide content in hepatic mitochondria and microsomes (5), the susceptibility of tissues to lipid peroxidation (6, 7), and the production of breath ethane (8) indicate that low copper intake is a dietary condition that causes molecular oxidative damage. In both aging and copper deficiency, the suppression of antioxidant enzyme activities may increase oxidative stress and facilitate damage by ROS (9,10). These parallels between aging and copper deficiency suggest that mechanisms contributing to the biological consequences of increased oxidative stress caused by copper deficiency may simulate similar mechanisms related to aging.

Mitochondria are a major source of ROS that contribute to the increase in oxidative stress associated with aging (11). A mechanism proposed for the role of mitochondria in aging involves the age-dependent decline in the activities of mitochondrial respiratory complexes. As these activities decline with age, there is an increasing tendency for the respiratory complexes to become more autoxidizable and donate single electrons to molecular oxygen to form superoxide and related ROS which damage mitochondrial components (12). The accumulation of oxidative damage to mitochondria eventually impairs their function to the extent that optimal organ performance cannot be maintained. Cytochrome c oxidase (CCO) is among the respiratory complexes whose activities have been reported to decline with age (13-16). Furthermore, the decline in CCO activity may be an important determinant for increasing mitochondrial ROS production. It has been shown that partial inhibition of CCO activity in mitochondria from flight muscle of houseflies increases hydrogen peroxide production (12). This finding indi-

cates that declining CCO activity may contribute to the increase in mitochondrial ROS production that occurs with advancing age. Suppression of CCO activity in a variety of tissues is also one of the most prominent biochemical changes that occurs during copper deficiency (17-19). However, it is not known whether the inhibition of CCO activity resulting from copper deficiency is similar to the age-dependent decline in CCO activity in its ability to stimulate mitochondrial ROS production.

Oxidative stress is an important factor in causing subcellular changes that lead to cardiomyopathy and heart failure (20). Previous studies have shown that the activity of cardiac CCO declines with advancing age in rats and humans (15, 16). This suggests that intensified mitochondrial ROS production resulting from declining CCO activity is a potential mechanism for the cardiac oxidative damage leading to the decline in cardiac function and the increase in some cardiac diseases that occur with advancing age. Dietary copper deficiency produces a variety of cardiovascular consequences including cardiac hypertrophy and mitochondrial enlargement that are related, at least in part, to increased peroxidation (21). Thus, cardiac oxidative damage is an attribute of both aging and copper deficiency. However, it is not known whether CCO inhibition contributes to cardiac oxidative damage by increasing oxidative stress during copper deficiency. If CCO inhibition leads to increased cardiac oxidative stress, then copper deficiency may serve as a model in which to investigate, over a short period of time, the role of mitochondria in cardiac aging. It also has been reported that long-term food restriction decreases mitochondrial ROS production in rat heart (22). This finding suggests that food restriction may ameliorate the decline in cardiac function that occurs with advancing age by attenuating oxidative stress caused by increased mitochondrial ROS production resulting from the loss of CCO activity. For copper deficiency to be a valid model for investigating the role of CCO and oxidative stress on cardiac aging, it must be capable of lowering oxidative stress resulting from increased mitochondrial ROS production in hearts of copper-deficient rats. Accordingly, we compared the effects of mild (20%) food restriction on CCO, manganese superoxide dismutase (MnSOD), and glutathione peroxidase (GPX) activities in heart mitochondria of rats fed adequate and inadequate copper. The influence of food restriction on oxidative damage to proteins and mitochondrial size also was evaluated.

MATERIAL AND METHODS

Animals and diets

Forty, male, weanling Sprague-Dawley rats (Sasco, Omaha, NE) were divided into 4 groups of 10 rats each. All rats were housed individually in stainless steel cages in a room maintained at 22±2°C and 50±10% humidity with a 12-h light dark cycle. Two groups of rats were fed copper-deficient diet (0.3 μg Cu/g diet) and two groups were fed copper-adequate diet (5.0 μg Cu/g diet) for 5 weeks. One group in each of these diet treatments was allowed to feed ad libitum and the other group in each diet treatment received food at 80% of the ad libitum consumption in a 2 x 2 factorial design. The groups are designated as CuA and CuD, respectively, for those consuming copper-adequate and copper-deficient diets ad libitum, and CuA80 and CuD80, respectively, for those consuming copper-adequate and copper-deficient diets at 80% of the ad libitum consumption. Diets were composed of 940.0 g of a casein-based copper- and iron-free basal diet (#TD84469, Teklad Test Diets, Madison, WI†), 50.0 g of safflower oil and 10 g of Cu-Fe mineral mix per kg of diet to give the desired copper and iron contents. The specific composition of this diet has been described previously (23).

The study was approved by the Institutional Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center and the animals were maintained in accordance with the National Research Council guidelines for the care and use of laboratory animals.

Mitochondrial preparation and analyses

Individual hearts were homogenized in 5 mL of cold (4°C) buffer containing 0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, and 10 mg trypsin, pH 7.4. Mitochondria were obtained from the homogenates by differential centrifugation as previously described (24). Protein concentrations in suspensions of heart mitochondria were determined with bichinchoninic acid (25).

Mitochondria were stored at -70°C until assayed for CCO (EC 1.9.3.1), GPX (EC 1.15.1.1) and MnSOD (EC 1.11.1.9) activities. CCO activity was determined by monitoring the loss of ferrocytochrome c at 550 nm (17, 26). A unit of CCO activity was that amount which catalyzed the oxidation of 1 μmol ferrocytochrome c / min at 30°C. The activity of GPX was assayed with a glutathione reductase-coupled assay in which the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was measured at 340 nm with t-butyl hydroperoxide as substrate (27, 28). A unit of GPX was that amount which catalyzed the oxidation of 1 nmol NADPH / min at 37°C. MnSOD activity was assayed by monitoring the inhibition of pyrogallol autoxidation spectrophotometrically at 320 nm (17, 29) following the inactivation of copper, zinc-superoxide dismutase with 1 mM KCN. The autoxidation rate for pyrogallol was determined in the presence of 1mM KCN to correct for cyanide inhibition of pyrogallol autoxidation. A unit of MnSOD was defined as that amount which caused 50% inhibition in the rate of pyrogallol autoxidation at 30°C.

Determination of oxidatively modified proteins

Carbonyl groups of oxidatively modified mitochondrial proteins were detected by Western blot immunoassay (30) using a commercially available kit (Oxyblot Oxidized Protein Detection Kit, Intergen Company, Purchase, NY). Briefly, the carbonyl groups of mitochondrial proteins were derivatized to 2,4-dinitrophenylhydrozone by treating the mitochondria with 2,4-dinitrophenylhydrazine. Untreated mitochon-

dria served as negative controls. The derivatized and nonderivatized mitochondrial proteins (6 μg / lane) were separated by SDS polyacrylamide gel electrophoresis on 10% acrylamide gels and then transferred to polyvinylidene fluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA) by semidry electroblotting. One lane on each gel contained molecular weight standards (Kaleidoscope Prestained Standards, Bio-Rad Laboratories, Inc., Hercules, CA) that were simultaneously transferred with the samples to estimate molecular weights of the blotted proteins. Following transfer of the proteins, the membrane was processed by a method that takes advantage of the hydrophobicity of polyvinylidene fluoride to eliminate the blocking step and reduce the number of wash steps (Millipore Technical Note RP562). Dried blots were incubated at room temperature for 1h with the anti-dinitrophenyl antibody supplied in the Oxyblot Oxidized Protein Detection Kit. The blots were then incubated for 0.5h with horseradish peroxidase-coupled anti-sheep IgG (Amersham Life Science, Inc., Arlington Heights, IL) and visualized by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western blotting detection reagents and Hyperfilm-ECL, Amersham Life Sciences, Inc.).

Electron microscopy and morphometry

Heart tissue was taken as an oblique slice from the external lateral aspect of the left ventricle and diced to small (1.0-1.5 mm) blocks in 2% glutaraldehyde, 0.05 M cacodylic acid, 1 mM calcium chloride, pH7.4. Following storage in fixative at 4°C for several days, the tissue was washed with 0.05 mM cacodylic acid, 7.5% sucrose, 1 mM calcium chloride, pH 7.4. The tissue was then treated for 1h at 4°C with 1% osmium tetroxide in buffer with sucrose adjusted to maintain osmolarity. After washing , the tissue was dehydrated in a graded series of ethanol concentrations and flat embedded in an epoxy resin mixture (EMbed-812, Electron Microscopy Sciences, Fort Washington, PA). Tissue pieces were sawn from the flat embedments and mounted without orientation onto resin cylinders. Sections were cut at a thickness of 70-90 nm (LKB Ultratome V, LKB Produkter AB, Bromma, Sweden) equipped with a diamond knife (RMC Inc., Tucson, AZ). The sections were mounted on 200 mesh copper grids (Ted Pella, Inc., Redding, CA) and stained for 7-10 min in an alcoholic uranyl acetate solution prepared from equal volumes of absolute ethanol and a saturated solution of uranyl acetate in 50% ethanol, followed by lead citrate for 3-5 min (31).

Tissue sites for morphometry micrographs were selected by orienting the electron microscope camera to the grid bars at the corners of the grid opening. Sites were examined beginning with the left most corner of the grid square and proceeding clockwise around the square, then moving to an adjacent square. A micrograph was recorded when the area examined was free of staining and sectioning artifacts and was estimated to be comprised of a minimum of 50% in area of cardiac myofibers, thus avoiding large blood vessels, connective tissue, and intercellular spaces. Four micrographs per animal were recorded. Micrographs of heart tissue were recorded at a magnification of 5,240 on the negative. The micrograph negatives were overlayed with a grid whose squares approximated the average narrow dimension of the mitochondrial profiles. The composite image was printed with an enlargement factor of 2.5. This resulted in working images of heart tissue at a magnification of 13,100 with approximately 9 mm grid squares superimposed on the images. Grid line intercepts overlaying mitochondria, nuclei, and myocardium were counted in the heart micrographs. The points over these cellular components were used to estimate the relative volume of mitochondria (32). Mitochondrial volumes were represented per volume of myocardium minus nuclear volume.

Statistics

Effects of dietary copper and food restriction were tested by two-way analysis of variance (ANOVA). Feeding pairs, ad libitum and 80% ad libitum, were used as blocking factors. For variables showing a significant interaction, Tukey's studentized range test was used for post hoc comparisons (SAS/STAT 6.12, SAS Institute, Inc., Cary, NC).

RESULTS

As shown in table 1, body weights after five weeks of dietary treatment were lower ($P<0.05$, Tukey's test) in animals subjected to food restriction than in their counterparts consuming diet ad libitum regardless of the copper content of the diet. Also, body weights of animals consuming CuD and CuD80 were significantly lower ($P<0.05$, Tukey's test) than the body weights of their counterparts fed CuA and CuA80. Liver copper concentrations were lower and liver iron concentrations were higher in rats fed CuD80 and CuD compared to rats fed CuA80 and CuA. Food restriction did not significantly affect hepatic copper concentration in rats fed either copper-deficient or copper-adequate diet but did increase iron concentration ($P<0.05$ by ANOVA for the main effect of food restriction). However, differences in either liver copper or iron concentrations between the restricted animals and those fed ad libitum in either the copper-deficient (CuD80 vs CuD) or copper-adequate (CuA80 vs CuA) treatment groups were not significant.

Figure 1 shows the activities of CCO, MnSOD, and GPX activities measured in heart mitochondria. For CCO activity there was a significant interaction between dietary copper and food restriction ($P<0.05$, ANOVA). Although dietary copper deficiency inhibited CCO activity, the activity was 65% higher in mitochondria from rats fed CuD80 compared to those fed CuD ($P<0.05$, Tukey's test). However, CCO activity in mitochondria from rats fed CuA80 was not significantly different from the activity in mitochondria from rats fed CuA.

There also was a significant interaction between dietary copper and food restriction for MnSOD in heart mitochondria ($P<0.05$, ANOVA). Although dietary copper deficiency elevated MnSOD activity in heart mitochondria, the activity in rats fed CuD80 was 25% lower

than the activity in rats fed CuD ($P<0.05$, Tukey's test). MnSOD activities in heart mitochondria from rats fed CuA80 and CuA were not significantly different.

The activity of GPX in heart mitochondria was 44.4 U/mg protein over all rats fed copper deficient diet (CuD80 and CuD) and 31.9 U/mg protein over all rats fed copper-adequate diet (CuA80 and CuA). The effect of dietary copper was significant ($P<0.05$, ANOVA) indicating that consumption of copper-deficient diet raised GPX activity in heart mitochondria. However, food restriction did not significantly affect GPX activity in rats fed either copper-deficient or copper adequate diets.

Figure 2 shows typical Western blots of proteins from heart mitochondria that were immunoreactive to antidinitrophenyl antibody following treatment of the mitochondria with 2,4-dinitrophenylhydrazine. Although no immunoreactive proteins were observed in untreated mitochondria (blots not shown), numerous immunoreactive proteins were present in mitochondria treated with 2,4-dinitrophenylhydrazine. Qualitatively, some differences between rats fed copper-deficient and copper-adequate diets were observed in the profiles of the immunoreactive proteins. Immunoblots of heart mitochondrial proteins typically showed that proteins of approximately 80-100 kDa appeared less immunoreactive to antidinitrophenyl antibody in rats fed copper-deficient diet (CuD or CuD80) than in rats fed copper-adequate diet (CuA or CuA80). Food restriction had no discernable effect on the profiles of immunoreactive proteins in heart mitochondria from rats fed copper-deficient or copper-adequate diet.

Typical electron micrographs of heart mitochondria from rats that consumed copper-deficient and copper-adequate diets ad libitum are shown in figure 3. Dietary copper did not affect the gross structure of the mitochondria. The inner and outer membranes as well as the cristae were intact and matrix opacity was normal in mitochondria of rats fed copper-deficient diet. Matrical density, cristae, and membrane structure of mitochondria from rats whose consumption of copper deficient and copper-adequate diets was restricted were not visibly different from their counterparts consuming diet ad libitum (micrographs not shown). Morphological measurements shown in Figure 4 indicate that mitochondrial size was significantly affected by an interaction between dietary copper and food restriction ($P<0.05$, ANOVA). Mitochondrial size was 32% greater in hearts of rats fed CuD compared to rats fed CuD80 ($P<0.05$, Tukey's test), but the difference in mitochondrial size between rats fed CuA and CuA80 was not significant. Furthermore, mitochondrial size in rats fed CuD80 was equivalent to mitochondrial size found in rats fed CuA and CuA80.

DISCUSSION

Rats consuming copper deficient diet showed retarded growth, reduced hepatic copper concentrations, elevated hepatic iron concentrations, and reduced CCO activity in heart mitochondria, all of which are typical signs of copper deficiency and confirm the efficacy of the dietary treatment in lowering copper status. Restricting food intake by 20% in rats consuming copper-deficient diet did not significantly increase hepatic copper concentrations or lower hepatic iron concentrations. This indicates that food restriction had little effect on copper status.

Our results indicate that copper deficiency has some effects on heart mitochondria that parallel the effects of aging on mitochondria. The decline in respiratory complexes, including CCO, is a characteristic of the aging heart (15, 16,). It has been reported that CCO activity in rat heart is 35% lower in 26 month old rats compared to 5 month old rats (16). In the present study, copper deficiency caused a 74% decrease in CCO activity in the absence of food restriction. A key feature of mitochondria that places them in a role of critical importance for aging is the increase in mitochondrial ROS production that accompanies the age-dependent decline in respiratory complex activities (11). MnSOD is a mitochondrial matrix enzyme whose expression is increased by H_2O_2 and other oxidants (33, 34). The close proximity of MnSOD to the electron transport chain, a major source of ROS, indicates that mitochondrial generation of oxidants is an important factor in regulating MnSOD expression. Our observation that copper deficiency significantly elevated MnSOD activity in heart mitochondria is an indirect indication that ROS production by these mitochondria was increased. Thus, both aging and copper deficiency cause a decrease heart mitochondrial CCO activity that is accompanied by an increase in ROS production.

Dietary restriction, because it extends life and delays many biochemical, physiological, and behavioral changes that occur with advancing age, is often used as an experimental approach to investigate the basic mechanisms of aging (2). It has been shown in mice that dietary restriction significantly increases life span and reduces age-related increases in ROS production by heart, brain, and kidney mitochondria (35). It has also been shown that dietary restriction strongly opposes declines in CCO activity that occur in mouse gastrocnemius muscle with advancing age (14). These findings suggest that dietary restriction retards aging in part by moderating the loss of mitochondrial respiratory complex activities and lowering mitochondrial ROS production. In the present study, 20% reduction in food intake reduced the loss of CCO activity and lowered MnSOD activity in heart mitochondria of copper-deficient rats. The reduction in MnSOD activity likely reflects a reduction in mitochondrial ROS production caused by reduced food intake. Thus, the effect of food restriction on mitochondrial changes that occur during both aging and copper deficiency has a common basis, namely, the reduction in loss of CCO activity and the lowering of mitochondrial ROS production. The parallel effects of copper deficiency and aging on CCO activity and mitochondrial ROS production and the ability of food restriction to oppose these effects suggest that copper deficiency may be a useful model for investigating the biochemical mechanisms through which mitochondria contribute to aging.

It has been shown that mitochondrial GPX can be induced in cultured cells by increased hydrogen peroxide production caused by deficiencies in respiratory

complex activities (36) and in rat liver by the ROS-generating drug, bleomycin (37). Thus, our observation that GPX is elevated in heart mitochondria of copper deficient rats is further evidence that copper deficiency increases mitochondrial ROS production in the heart. However, food restriction had no effect on the increase in GPX activity caused by copper deficiency. If the decrease in heart mitochondrial MnSOD activity caused by food restriction is related to reduced mitochondrial ROS production in copper-deficient rats, it is not clear why food restriction failed to lower GPX activity. This discrepancy may reflect a greater susceptibility of GPX to induction by mitochondrially generated hydrogen peroxide. Even though MnSOD activity was lower in heart mitochondria of copper-deficient rats subjected to food restriction, its activity was still higher than normal indicating that mitochondrial ROS production was above normal. It is possible that even with 20% food restriction, MnSOD activity remained high enough to produce hydrogen peroxide through dismutation of mitochondrially generated superoxide at an optimal level for the induction of GPX, but at an insufficient level for maximal induction of MnSOD.

Induction of MnSOD and GPX activity in heart mitochondria indicates that mitochondrial antioxidant defenses were increased during copper deficiency. The increase in the activities of these antioxidant enzymes caused by copper deficiency apparently protected some mitochondrial proteins against oxidative damage. Oxidation of proteins converts some of their amino acid side chains to carbonyl derivatives and the presence of carbonyl groups is used as an indicator of protein oxidation by ROS (30, 38). In the present study, carbonyl groups were present in proteins over a broad range of molecular weights in heart mitochondria. This finding is consistent with previous findings showing that oxidative stress, a common consequence of aerobic metabolism, causes extensive oxidative damage to the DNA, lipids, and proteins of mitochondria (11, 39). In heart mitochondria, copper deficiency led to the disappearance of carbonyl groups in proteins with molecular weights of 80-100 kDa without observable changes in the carbonyl content in lower molecular weight proteins. This finding suggests that increased activity of antioxidant enzymes in heart mitochondria selectively protects proteins within a narrow range of molecular weights (80-100 kDa) from oxidative modifications during copper deficiency. Although copper deficiency altered the carbonyl content of some proteins in heart mitochondria, restricting food intake by 20% had no qualitatively observable effect on the immunoblot profiles of carbonyl-bearing proteins. Food restriction also had no effect on the carbonyl content of proteins in heart mitochondria of rats fed adequate dietary copper. Thus, over a short period of five weeks, food restriction did not enhance protection of heart mitochondrial proteins from oxidative damage resulting from normal aerobic metabolism or from increased oxidative damage caused by copper deficiency.

The moderating effect of food restriction on the loss of CCO activity during copper deficiency and aging is a key feature that relates copper deficiency to proposed roles for mitochondria in the process of aging. However, it is not clear how food restriction increases the activity of CCO in copper-deficient rats. Although CCO is a copper-dependent enzyme, our study indicates that the reduction in CCO activity of heart mitochondria in copper-deficient rats caused by food restriction occurs without a significant improvement in copper status. Also, a previous study showed that a 20% reduction in food intake by copper-deficient rats has no effect on heart copper content (40). These findings suggest that the effect of food restriction on CCO activity in heart mitochondria of copper-deficient rats most likely results from an action that is unrelated to the copper dependency of this enzyme.

Several explanations are possible that may account for the moderating effect of food restriction on the loss of CCO caused by copper deficiency. Previous studies have shown that copper deficiency decreases nuclear-encoded subunits IV and V of CCO in heart mitochondria through a mechanism that may involve impaired importation of these subunits into the mitochondria (41, 42). It is possible that food restriction counteracts the inhibitory effects of copper deficiency on CCO by promoting the synthesis and importation of nuclear encoded subunits into the mitochondria.

The effect of food restriction on CCO activity in heart mitochondria of copper-deficient rats may also be related to an antioxidant action. Food restriction has been shown to decrease the rate of hydrogen peroxide and superoxide production by heart mitochondria from mice at 9, 17, and 23 months of age (35). Another study showed that the basal rate of ROS production by heart mitochondria was significantly reduced in 2-year-old rats who had been subjected to caloric restriction for 1 year (22). Both of these studies demonstrate that ROS production by heart mitochondria is decreased by long-term food restriction. Although the copper-deficient rats were subjected to short-term food restriction, if the loss of CCO activity during copper deficiency is in part caused by oxidative damage to the protein resulting from increased mitochondrial ROS production, then food restriction, by decreasing production of these ROS, may improve CCO activity by lowering the severity of inactivation caused by oxidative damage.

Another potential explanation for the effect of food restriction on heart mitochondrial CCO activity during copper deficiency may involve changes in the lipid composition of the mitochondrial membranes. A previous study examining the effects of aging on heart mitochondrial membrane fluidity and fatty acid composition found that dietary restriction attenuated the age-dependent loss in membrane fluidity and prevented increases in 22:4 fatty acid content and decreases in 18:2 fatty acid content that result from aging (43). The preservation of membrane fluidity by dietary restriction during aging most likely reflects a decrease in lipid peroxidation resulting from the combination of lower content of highly unsaturated fatty acids and lower ROS production. These findings suggest that dietary restriction may preserve physical properties, such as fluidity, of heart mitochondrial membranes during aging. Although the effect of copper deficiency on the lipid

composition of heart mitochondria is not known, increased 16:1, 22:5 and 22:6 fatty acid content and decreased 18:2 and 20:4 fatty acid content have been reported in liver mitochondria of copper-deficient rats (44, 45), suggesting that copper deficiency increases the degree of unsaturation in the fatty acids of mitochondrial membranes. This could result in increased susceptibility of mitochondrial membranes to peroxidative damage during copper deficiency because mitochondrial ROS production may be increased. Thus, changes in the physical properties of heart mitochondrial membranes caused by peroxidative damage during copper deficiency may be similar to those caused by aging and also may be ameliorated by food restriction through reduction in mitochondrial ROS generation. By preventing changes in the physical properties of the inner mitochondrial membrane related to altered fatty acid composition and peroxidative damage, food restriction may stabilize lipid-protein interactions that help maintain membrane-bound CCO in a more active configuration during copper deficiency.

Mitochondrial hypertrophy is a common outcome of copper deficiency (46-49). In the present study, copper deficiency increased the size of heart mitochondria but the hypertrophy was completely prevented by a 20% restriction in food intake. The preventative effect of food restriction on mitochondrial hypertrophy may be related to its protective effect on CCO activity during copper deficiency. Hypertrophy of heart mitochondria during copper deficiency in our study occurred without producing evident abnormalities in the cristae or matrical density. Mitochondrial hypertrophy of this nature is found in a number of tissues where pathological or experimental conditions place an increased functional demand on the tissue (50). In the case of copper deficiency, mitochondrial hypertrophy is considered compensatory to reduced oxidative phosphorylation caused by inhibition of CCO activity (48). Because food restriction reduced the severity of CCO inhibition during copper deficiency, the impact of copper deficiency on oxidative phosphorylation may have been insufficient to require a compensatory increase in mitochondrial size. As indicated by the ability of food restriction to decrease MnSOD activity in heart mitochondria of copper-deficient rats, the reduction in CCO inhibition by food restriction was also accompanied by a reduction in mitochondrial ROS generation. Thus, food restriction likely prevented hypertrophy of heart mitochondria during copper deficiency by moderating the loss of CCO activity and maintaining CCO activity at a level that provided adequate oxidative phosphorylation and also prevented electron transport chain blockage that could cause oxidative damage to mitochondria through a major increase in ROS generation.

It has been shown previously that, in contrast to long-term dietary restriction beginning when rats are 1 year old, 6 weeks of dietary restriction beginning when rats are 8 weeks old has little effect on ROS production in heart mitochondria (22). The contrasting effects of short-term dietary restriction in young rats versus long-term diet restriction in old rats suggests that dietary restriction may not have life extending effects related to its antioxidant action until the age-associated decline in respiratory complex activities is sufficient to significantly increase mitochondrial ROS production. Our study shows that copper deficiency causes a rapid decline in CCO activity in heart mitochondria that is apparently accompanied by increased ROS production. Furthermore, short-term food restriction attenuated both the reduction in CCO activity and the increase in ROS production. Although the acute inhibitory effect of copper deficiency on CCO activity depends largely on the absence of copper from the active site of the enzyme and the decline in CCO activity with age depends more on the long-term accumulation of oxidative damage to CCO and to the genes encoding the protein subunits of CCO, food restriction apparently is able to moderate the decline in CCO and associated increase in ROS generation by heart mitochondria in both copper deficiency and aging. This parallel effect of food restriction on mitochondria during copper deficiency and aging suggests that copper deficiency may simulate, over a short time, some of the biological consequences of declining respiratory chain activity, ROS generation, and mitochondrial function as they relate to cardiac aging. The parallels between aging and copper deficiency on CCO activity and mitochondrial ROS generation also suggest that adequate dietary copper intake by the elderly is important for moderating the decline in cardiac function that occurs with advancing age.

Table 1. Effect of food restriction and dietary copper on growth and liver Cu and Fe concentrations.

Diet	Initial Weight	Final Weight	Cu	Fe
	g	g	*nmol/g dry liver*	*•mol/g dry liver*
CuA80	78±6	230±18[a]	242±24	5.1±1.1
CuA	76±4	272±18[b]	228±77	3.9±0.5
CuD80	77±4	204±17[c]	27±11	7.6±1.6
CuD	78±5	242±13[a]	19±3	6.2±1.8
ANOVA			p values	
Cu		0.0002	0.0001	0.0001
Food Restriction		0.0001	0.4	0.006
Cu x FR		0.7	0.8	0.7

Values are means ± SD. Means with different superscripts are significantly different (P<0.05, Tukey's test)

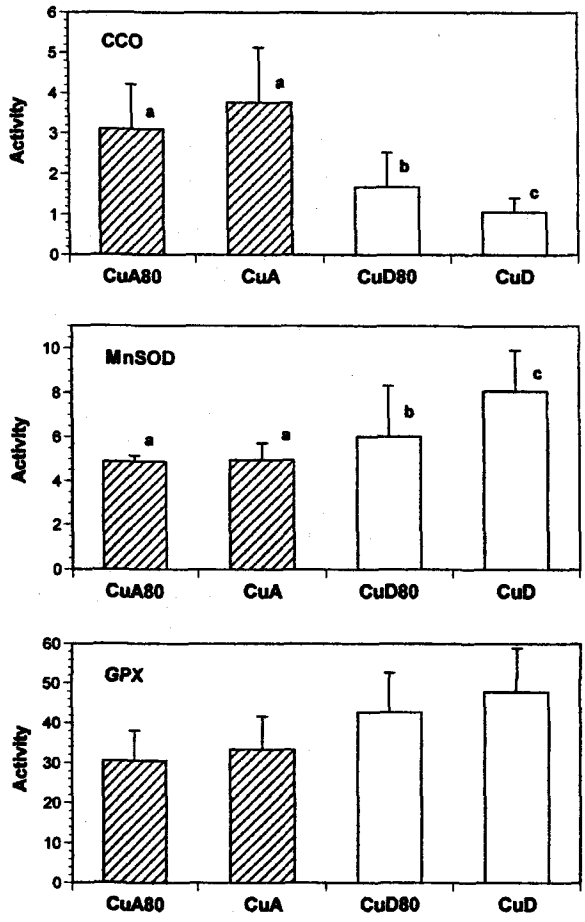

Figure 1: Activities of cytochrome c oxidase (CCO), manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPX) in mitochondria isolated from the hearts of rats fed copper-adequate diet either ad libitum (CuA) or at 80% of the ad libitum intake (CuA80) and copper-deficient diet either ad libitum (CuD) or at 80% of the ad libitum intake (CuD80). Enzyme activities are expressed as Units/mg protein where a unit of CCO is the amount that catalyzes the oxidation of 1µmol ferrocytochrome c / min, a unit of MnSOD is the amount that causes 50% inhibition in the rate of pyrogallol autoxidation, and a unit of GPX is the amount that catalyzes the oxidation of 1 nmol NADPH / min. Values shown are means±SD. Activities for CCO and MnSOD were ln transformed to reduce heterogeneity of variance as required for ANOVA. Results from the 2-way ANOVA were: for CCO activity, P=0.0001 for the effect of dietary Cu, P=0.067 for the effect of food restriction, and P=0.0002 for the effect of dietary Cu x food restriction interaction; for MnSOD activity, P=0.0003 for the effect of dietary Cu, P=0.006 for the effect of food restriction, and P=0.01 for the effect of dietary Cu x food restriction interaction; for GPX activity, P=0.0001 for the effect of dietary Cu, P=0.14 for the effect of food restriction, and P=0.52 for the effect of dietary Cu x food restriction interaction. Means labeled with different letters are significantly different (P<0.05, Tukey's test).

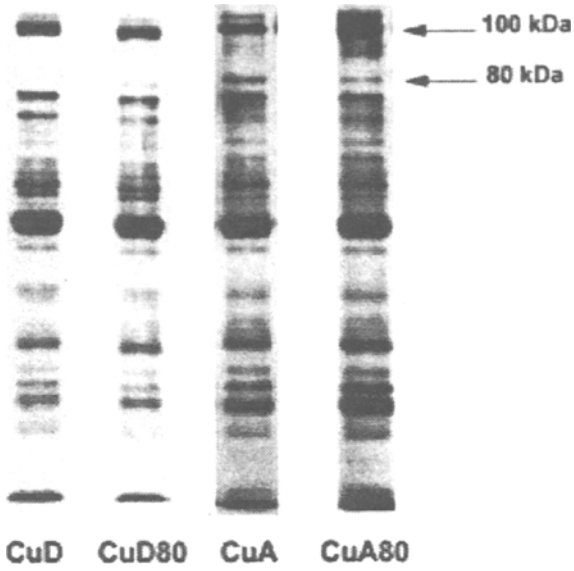

FIGURE 2: Western blots showing immunoreactive dinitrophenyl derivatives of carbonyl groups in proteins of mitochondria isolated from hearts of rats fed copper-adequate diet either ad libitum (CuA) or at 80% of the ad libitum intake (CuA80) and copper-deficient diet either ad libitum (CuD) or at 80% of the ad libitum intake (CuD80). Each lane contained 6 µg of mitochondrial protein.

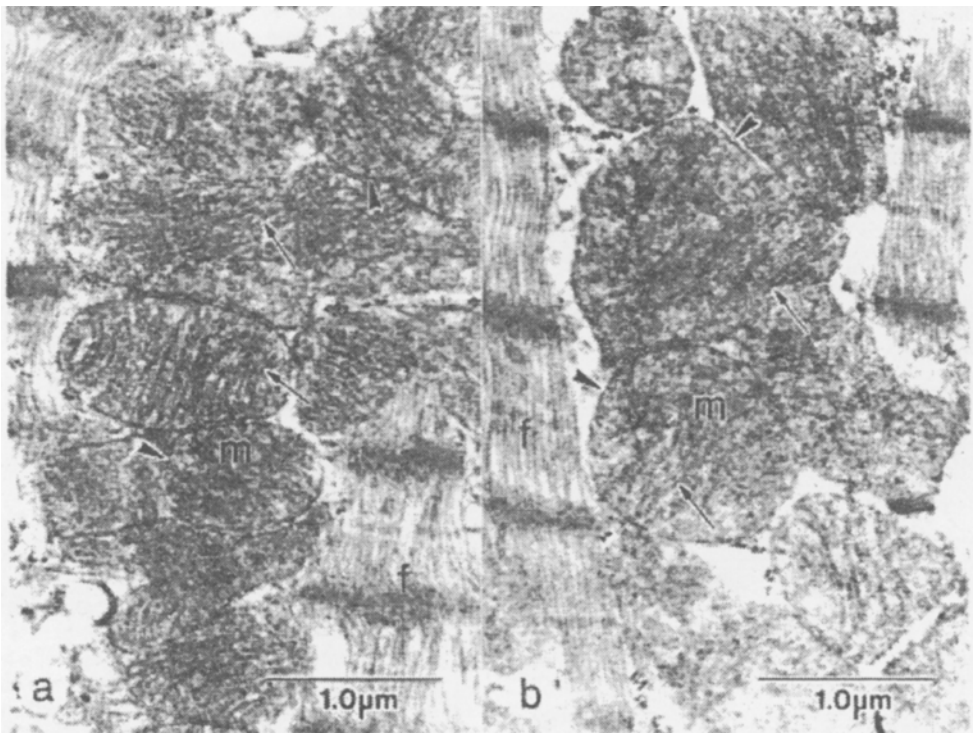

FIGURE 3: Electron micrographs of mitochondria (m) from hearts of rats fed copper-adequate (panel a) or copper-deficient (panel b) diets ad libitum. The myofibrillar component is indicated (f). The general morphology of the mitochondria, the outer membranes (arrowheads) and the cristae (arrows) demonstrate no detrimental effects associated with the copper-deficient diet.

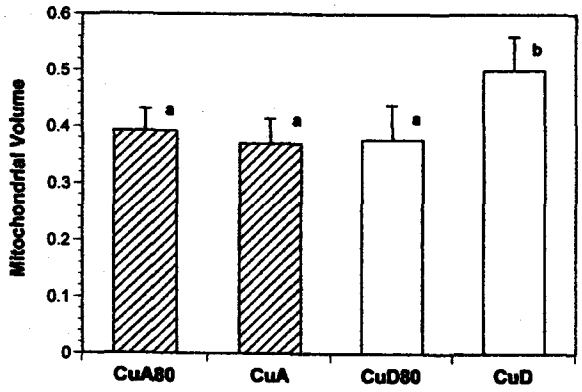

FIGURE 4: Mitochondrial size in hearts from rats fed copper-adequate diet either ad libitum (CuA) or at 80% of the ad libitum intake (CuA80) and copper-deficient diet either ad libitum (CuD) or at 80% of the ad libitum intake (CuD80). Mitochondrial volumes are relative to myocardium minus nuclear volume. Results from the 2-way ANOVA were: P=0.008 for the effect of dietary Cu, P=0.002 for the effect of food restriction, and P=0.0001 for the effect of dietary Cu x food restriction interaction. Means labeled with different letters are significantly different (P<0.05, Tukey's test).

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