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Copper deficiency inhibits Ca^{2+}-induced swelling in rat cardiac mitochondria

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Abstract

Cu deficiency disrupts the architecture of mitochondria, impairs respiration, and inhibits the activity of cytochrome *c* oxidase — the terminal, Cu-dependent respiratory complex (Complex IV) of the electron transport chain. This suggests that perturbations in the respiratory chain may contribute to the changes in mitochondrial structure caused by Cu deficiency. This study investigates the effect of Cu deficiency on Ca^{2+}-induced mitochondrial swelling as it relates to changes in respiratory complex activities in cardiac mitochondria of rats. Male weanling rats were fed diets containing either no added Cu (Cu0), 1.5 mg Cu/kg (Cu1.5), 3 mg Cu/kg (Cu3) or 6 mg Cu/kg (Cu6). The rate of Ca^{2+}-induced mitochondrial swelling in the presence of succinate and oligomycin was reduced, and the time to reach maximal swelling was increased only in the rats consuming Cu0 diet. Cytochrome *c* oxidase activity was reduced 60% and 30% in rats fed Cu0 and Cu1.5, respectively, while NADH:cytochrome *c* reductase (Complex I+ComplexIII) activity was reduced 30% in rats consuming both Cu0 and Cu1.5. Mitochondrial swelling is representative of mitochondrial permeability transition pore (MPTP) formation and the results suggest that Ca^{2+}-induced MPTP formation occurs in cardiac mitochondria of Cu-deficient rats only when cytochrome *c* oxidase activity falls below 30% of normal. Decreased respiratory complex activities caused by severe Cu deficiency may inhibit MPTP formation by increasing matrix ADP concentration or promoting oxidative modifications that reduce the sensitivity of the calcium trigger for MPTP formation.
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Keywords: Copper deficiency; Heart; Mitochondria; Permeability transition pore; Cytochrome *c* oxidase; Rats

1. Introduction

Histological studies have shown that Cu deficiency disrupts the architecture of cardiac mitochondria. The structural alterations caused by Cu deficiency include mitochondrial enlargement, fragmentation and disappearance of the cristae, pronounced vacuolization, and decreased matrical density [1–5]. In addition to structural changes, it has been reported that Cu deficiency also reduces cardiac mitochondrial respiration [6–8]. These findings suggest that impaired respiration contributes to the perturbations in mitochondrial architecture caused by Cu deficiency.

Mitochondrial respiration depends on the flow of electrons through four oligomeric Respiratory Complexes that comprise the electron transport chain. The energy released by electron flow through the respiratory complexes is conserved in an electrochemical potential consisting of a proton gradient and membrane potential ($\Delta\Psi_m$) produced by the coupled translocation of protons through the inner mitochondrial membrane at Complexes I, II and IV. Energy stored in the electrochemical potential is coupled to ATP synthesis by translocation of protons into the mitochondrial matrix through complex V (ATP synthase) [9]. Respiratory Complex IV, also known as cytochrome *c* oxidase, is a cuproenzyme whose activity is reduced in a variety of

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tissues, including the heart, by Cu deficiency [10–12]. Although the role of Cu as an essential cofactor most likely contributes to the inhibitory effect of Cu deficiency on cytochrome *c* oxidase activity, Medeiros and coworkers [13–15] reported that the contents of two nuclear encoded subunits of cytochrome *c* oxidase are reduced in cardiac mitochondria of Cu-deficient rats indicating that structural changes in the holoenzyme may also contribute to the loss of activity. Mederios et al. [13,16] have also reported that Cu deficiency alters ATP synthase by decreasing the content of the δ subunit. These findings indicate that Cu deficiency may exert effects at two loci in the electron transport chain and oxidative phosphorylation system.

It has been reported that inhibition of respiratory Complex I by rotenone [17,18] and cytochrome *c* oxidase by azide [19] leads to reductions in $\Delta\Psi_m$. Thus, a reduction in $\Delta\Psi_m$ may occur as a consequence of the inhibitory effect of Cu deficiency on cytochrome *c* oxidase. Although a definite association between reduced cytochrome *c* oxidase activity and $\Delta\Psi_m$ has not been established, reports showing that $\Delta\Psi_m$ is compromised in cardiac mitochondria of Cu-deficient rats [8] and Cu-deficient HL-60 cells [20] suggest that such an association is possible. A potential mechanism linking reduction in cytochrome *c* oxidase activity caused by Cu deficiency to reduced $\Delta\Psi_m$ may involve compromised permeability of the inner membrane resulting from alterations in the mitochondrial permeability transition pore (MPTP). The MPTP can exist in two conformations, a low conductance state that allows the diffusion of small ions across the inner membrane and a high conductance state that allows the unselective diffusion of large molecules [21]. Oxidative stress is a factor that can cause the transition of the MPTP from a low to a high conductance state [22,23]. Inhibition of cytochrome *c* oxidase can promote oxidative stress by increasing the mitochondrial production of reactive oxygen species (ROS). The redox state of the respiratory complexes is a major determinant of mitochondrial ROS production and is highest when the complexes are highly reduced [24]. Inhibition of cytochrome *c* oxidase may increase the reducing potential of the upstream respiratory complexes causing increased ROS production through single electron transfer to molecular oxygen. This has been demonstrated using flight muscles from houseflies where partial inhibition of cytochrome *c* oxidase increases mitochondrial hydrogen peroxide [25]. Cu deficiency has also been shown to increase hydrogen peroxide generation in hepatic mitochondria [26]. Thus, by inhibiting cytochrome *c* oxidase activity, Cu deficiency may produce sufficient oxidative stress to cause a transition of the MPTP from a low to a high conductance conformation. Because increased inner membrane permeability is associated with the high conductance state of the MPTP, the transition to a higher conductance conformation may also help explain the changes in cardiac mitochondrial structure caused by Cu deficiency. Ca^{2+} is an important determinant of the conductance state of the MPTP. Saturation of internal Ca^{2+} binding sites of the MPTP produces a conformation change that leads to the high conductance state [21]. It is possible that changes in the MPTP caused by Cu deficiency may also alter its sensitivity to the Ca^{2+}-induced switch from the low to the high conductance state. Accordingly, the purpose of this study was to investigate the effect of Cu deficiency on the Ca^{2+}-induced mitochondrial transition in rat heart mitochondria.

2. Materials and methods

2.1. Animals and diets

Male weanling Sprague–Dawley rats (Charles River, Wilmington, MA, USA) were housed in a room maintained at 22±2°C and 50±10% humidity with a 12-h light/dark cycle. The rats were divided into four treatment groups (11 rats/group) and fed AIN-93 G diet [27] formulated with $CuSO_4 \cdot 5H_2O$ to contain <1 mg Cu/kg (Cu0), 1.5 mg Cu/kg (Cu1.5), 3.0 mg Cu/kg, (Cu3) or 6.0 mg Cu/kg (Cu6). The analyzed Cu concentrations in the diets were: Cu0=0.457 mg/kg, Cu1.5=1.61 mg/kg, Cu3=3.22 mg/kg, and Cu6=6.39 mg/kg. Rats had free access to diets and demineralized water for 5 weeks. The study was approved by the Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the NIH guidelines for the care and use of laboratory animals.

2.2. Analyses

After 5 weeks of dietary treatment, the rats were anesthetized with ketamine/xylazine, and livers, hearts and blood were removed for analysis. Liver Cu and iron concentrations were measured by atomic absorption spectrophotometry [28]. Plasma ceruloplasmin was assayed in serum by its amine oxidase activity [29]. An electronic cell counter (Cell-Dyne 3500, Abbott Diagnostics, Abbott Park, IL, USA) was used to measure hemoglobin concentrations and hematocrits.

Individual hearts were homogenized in isolation buffer (1 g heart/ml buffer) containing 0.225 M mannitol, 0.075 M sucrose, 0.02 M HEPES and 0.01 mM EGTA, pH 7.4, to which bovine serum albumin (0.1% final concentration) and trypsin (2 mg/ml final concentration) were added. The homogenate was centrifuged at 9900*g* for 10 min, and the supernatant was discarded in order to limit exposure to trypsin. The pellet was resuspended in isolation buffer containing 0.1% bovine serum albumin and centrifuged at 500*g*. Mitochondria were obtained from the resulting supernatant by centrifuging at 9900*g* for 10 min. The pellet was collected and washed once with isolation buffer containing 0.1% bovine serum albumin, followed by a final wash in isolation buffer. The final mitochondrial pellet was suspended in 1 ml of isolation buffer. All isolation steps were performed at 4°C.

Cytochrome *c* oxidase activity in the isolated mitochondria was assayed by monitoring the oxidation of ferrocytochrome *c* spectrophotometrically at 550 nm [30]. NADH:cytochrome *c* reductase activity was assayed by monitoring the reduction of ferricytochrome *c* spectrophotometrically at 550 nm [31]. Protein concentrations in the mitochondrial preparations were determined with bichinchoninic acid (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA).

Mitochondrial swelling was measured by monitoring the decrease in absorbance at 540 nm [32]. Assay mixtures (3 ml total volume) contained buffer (200 mM sucrose, 10mM Tris base, 10 mM MOPS (3-(N-Morpholine)propane-sulfonic acid)), 5 mM KH_2PO_4, 10 μM EGTA, pH 7.4), 8 mM succinate, 0.5 μg oligomycin, 4 μM rotenone and 0.5 mg mitochondrial protein. In order to determine whether swelling resulted from MPTP formation, cyclosporine A, a specific inhibitor of the MPTP [33,34], was added to the assay mixture (1 μM final concentration). The mixture was preincubated at 25°C for 3 min in a temperature-controlled cuvette before swelling was initiated by the addition of 500 μM $CaCl_2$. The swelling data were modeled separately for each animal by using the 4-parameter equation:

$$\text{Absorbance} = \alpha + \beta\left[1 + \gamma\left(t - \tau_p\right)^2\right]^{-1}$$

where α is the lower asymptote of the observed absorbance curve, $\alpha + \beta$ is the peak absorbance, γ is a rate constant, t is time in seconds and τ_p is the time, in seconds, at which the peak absorbance value occurred (Fig. 1) [35]. The NLIN procedure in SAS V9.1 (SAS Institute, Cary, NC, USA) was used to obtain parameter estimates for α, β, γ and τ_p. An approximate coefficient of determination (R^2) was calculated for model to assess goodness of fit. Time to half-maximum swelling was calculated directly from each estimate of γ as: $t_{½Max}=(1/\gamma)^{0.5}$.

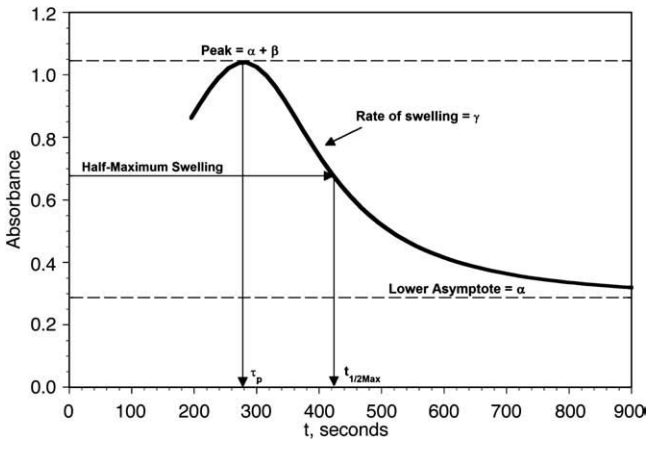

Fig. 1. Plot from the mathematical model for Ca^{2+}-induced swelling of cardiac mitochondria. The model was based on the equation: absorbance=-$\alpha+\beta[1+\gamma(t-\tau_p)^2]^{-1}$. The rate of swelling was determined directly from γ, the downward slope of the curve, and the time to half-maximal swelling was calculated as $(1/\gamma)^{0.5}$.

Table 1
The effect of dietary Cu on indices of Cu status

Diet	Liver Cu (μg/g dry tissue)	Kidney Cu (μg/g dry tissue)	Ceruloplasmin (U/L)	Hemoglobin (g/L)	Hematocrit
Cu0	2.3±0.6[a]	13.0±0.7[a]	0.4±0.7[a]	111±7[a]	0.34±0.02[a]
Cu1.5	10.2±1.7[b]	23.1±7.6[b]	28.9±21.3[b]	131±6[b]	0.39±0.02[ab]
Cu3	10.4±0.9[b]	31.2±4.9[c]	53.6±23.9[c]	128±9[b]	0.38±0.03[ab]
Cu6	11.9±0.8[c]	31.9±4.6[c]	47.5±16.7[cb]	133±4[b]	0.42±0.06[b]

Values are means±S.D. Means having different superscripts are significantly different (P<.05, Tukey's test).

2.3. Statistics

Data were analyzed by analysis of variance (ANOVA) to determine the effect of diet. Tukey's studentized range test was used to detect significant differences between individual means (SAS/STAT Version 9.1, SAS Institute, Cary, NC, USA).

3. Results

Dietary Cu significantly affected (P<.05, ANOVA) liver and kidney Cu concentrations, ceruloplasmin activity, hemoglobin concentration and hematocrit (Table 1). Liver Cu concentration was significantly lower in rats fed Cu0 diet compared to rats fed Cu1.5, Cu3 and Cu6. However, liver Cu concentrations in rats fed Cu1.5 and Cu3 were also significantly lower than the concentration in rats fed Cu6. Kidney Cu concentration was lower in rats fed Cu0 and Cu1.5 diets compared to rats fed Cu3 and Cu6. Although kidney Cu concentration was significantly reduced in rats fed Cu1.5, it was higher than the concentration in rats fed Cu0. Ceruloplasmin activity was lower in rats fed Cu0 compared to the rats in the other diet treatment groups and was lower in rats fed Cu1.5 compared to rats fed Cu3. Significant reductions in hemoglobin concentration and hematocrit occurred only in rats fed Cu0.

Dietary Cu significantly affected (P<.05, ANOVA) cytochrome *c* oxidase and NADH:cytochrome *c* reductase activities in isolated heart mitochondria (Table 2). The activities of both enzymes were reduced in rats fed Cu0 and

Table 2
Effect of dietary Cu on cytochrome *c* oxidase and NADH:cytochrome *c* reductase activities in isolated heart mitochondria

Diet	Cytochrome *c* oxidase [μmol cyt *c* oxidized/(min·mg protein)]	NADH:cytochrome *c* reductase [μmol cyt *c* oxidized/(min·mg protein)]
Cu0	0.42±0.07[a]	0.13±0.02[a]
Cu1.5	0.79±0.12[b]	0.12±0.03[a]
Cu3	1.13±0.18[c]	0.18±0.04[b]
Cu6	1.10±0.80[c]	0.19±0.05[b]

Values are means±S.D. Means having different superscripts are significantly different (P<.05, Tukey's test).

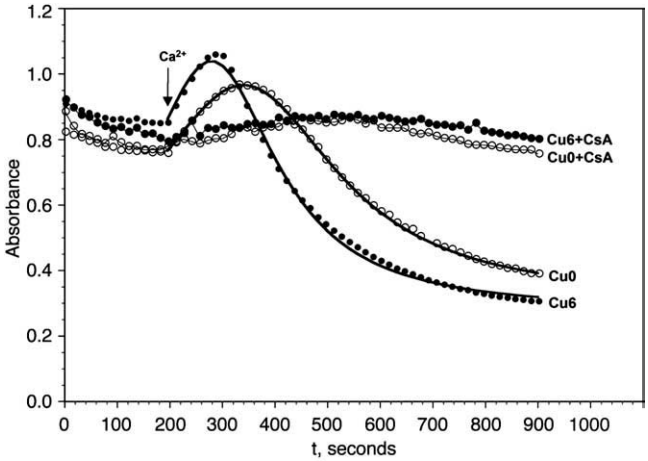

Fig. 2. Representative plots from rats fed Cu0 diet (open circles) and Cu6 diet (black circles) for the swelling of cardiac mitochondria induced with 500 μM Ca^{2+} in the absence and presence of 1 μM cyclosporine A (CsA). The circles represent data points and the solid lines represent the plot generated by the model described in Fig. 1.

Cu1.5 compared to rats fed Cu3 and Cu6. Cytochrome *c* oxidase activity was higher in rats fed Cu1.5 compared to rats fed Cu3.

Upon the addition of Ca^{2+}, the absorbance at 540 nm of cardiac mitochondria suspended in sucrose-based buffer initially increased and then decreased with time (Fig. 2). The decrease in absorbance at 540 nm is attributed to Ca^{2+}-induced mitochondrial swelling. In the examples shown, differences in swelling occurred between the rats fed Cu0 and Cu6. The swelling also was completely inhibited by cyclosporin A. The examples shown also demonstrate that the mathematical model closely fit the swelling data. The range of R^2 values for all animals was 0.94–0.99. Parameters obtained from the mathematical model describing the swelling showed that dietary Cu had a significant effect ($P<.05$, ANOVA) in the rate of swelling and the time to reach half-maximal swelling (Table 3). Significant reductions in the rate of swelling and increases in the time to half-maximal swelling occurred only in the rats fed Cu0.

4. Discussion

The present study examined the influence of dietary Cu on Ca^{2+}-induced swelling of cardiac mitochondria in a sucrose-based media. By mathematical modeling, we were able to determine the rate of swelling and the time to half maximal swelling. Our results showed that the rate of swelling was decreased and the time to half maximal swelling was extended in rats having severe Cu deficiency characterized by reduced liver and kidney Cu, extremely low ceruloplasmin activity and anemia. Swelling was not affected by moderate Cu deficiency characterized by moderately reduced liver and kidney Cu and ceruloplasmin activity in the absence of anemia. Changes in mitochondrial volume measured by monitoring the decrease in spectral absorbance have been shown to parallel the number of mitochondria undergoing permeability transition [36]. Thus, the influence of dietary Cu on mitochondrial swelling reflects an effect on the MPTP and our findings indicate that severe Cu deficiency impairs the permeability transition in cardiac mitochondria. Furthermore, mitochondrial swelling was completely inhibited by cyclosporine A, a specific inhibitor of the MPTP [33,34], indicating that the swelling measured under our experimental conditions resulted from formation of the MPTP.

Severe Cu deficiency produced by feeding Cu0 diet caused significant reductions in cytochrome *c* oxidase and NADH:cytochrome *c* reductase activities. NADH:cytochrome *c* oxidase activity represents the combined electron transport activities of respiratory complexes I and III, and our results indicate that severe Cu deficiency can create partial blockages in the electron transport chain at Complex IV (cytochrome *c* oxidase) and at complex I and/or complex III. It has been shown that electron flux through complex I has a role in regulating MPTP formation and that inhibition of complex I can impair MPTP formation [37,38]. Thus, the inhibition of complex I by Cu deficiency may contribute to the inhibition of Ca^{2+}-induced MPTP in cardiac mitochondria from the severely Cu-deficient rats. However, in the present study, MPTP formation, characterized by mitochondrial swelling, was induced by Ca^{2+} in the presence of rotenone, a complex I inhibitor, and succinate, a complex II substrate. Under these conditions, electron flow through complex I would be prevented because rotenone would block the reverse flow of electrons from complex II. Since electron flow is possible only through complexes downstream from Complex I under these conditions, impaired electron flow through Complex I resulting from inhibition of the complex activity by Cu deficiency is not likely to contribute directly to the observed inhibition of MPTP formation.

Although impaired electron flow through Complex I does not explain the effect of Cu-deficiency on Ca^{2+}-induced MPTP formation in cardiac mitochondria, the general impairment of the mitochondrial electron transport chain resulting from the inhibition of cytochrome *c* oxidase, upstream Cu-independent complexes and ATPase may have indirect effects that contribute to impaired MPTP formation. Previous studies have shown that Cu deficiency causes a structural change in mitochondrial F_0F_1-ATP synthase that

Table 3
The effect of dietary Cu on the rate of mitochondrial swelling (γ) and the time to half-maximal swelling ($t_{½Max}$)

Diet	$\gamma\times10^5$ ΔA_{540}/(sec·mg protein)	$t_{½Max}$ (sec)
Cu0	2.80±0.58[a]	192±21[a]
Cu1.5	5.82±1.81[b]	135±21[b]
Cu3	4.80±1.51[b]	150±27[b]
Cu6	4.61±1.26[b]	152±23[b]

Values are means±SD. Means having different superscripts are significantly different ($P<.05$, Tukey's test).

apparently decreases its sensitivity to oligomycin inhibition [7,8,13,16]. Thus, even though mitochondrial swelling was conducted in the presence of oligomycin in our study, residual ATP synthase activity may have existed in the mitochondria from the severely Cu-deficient rats. ATP synthase is capable of catalyzing both ATP synthesis and hydrolysis depending on the proton gradient between the matrix and inter membrane space with a more acidic matrix favoring ATP hydrolysis [39,40]. The high degree of inhibition of cytochrome *c* oxidase (60%) and NADH: cytochrome *c* reductase (30%) caused by severe Cu deficiency may have produced a relatively acidic matrix by lowering proton translocation from the matrix. This would promote ATP hydrolysis and elevate levels of ADP in the mitochondria from the severely Cu-deficient rats compared to mitochondria from the marginally Cu-deficient and Cu-adequate rats where ATPase was inhibited to a greater degree by oligomycin. The binding of matrix ADP to the adenine nucleotide translocase, an important modulator and component of the MPTP, can inhibit MPTP formation by decreasing the sensitivity of the calcium trigger site to Ca^{2+} [41]. Thus, the combination of reduced respiratory complex activities and higher residual ATP synthase activity in the mitochondria from severely Cu-deficient rats may allow the level of matrix ADP to achieve a level capable of inhibiting Ca^{2+}-induced MPTP formation as observed in our study. Furthermore, the absence of an effect on MPTP formation in the marginally Cu-deficient rats suggest that greater than 30% inhibition of cytochrome *c* oxidase, the degree of inhibition observed in rats fed Cu1.5, must occur before an effect is observed on MPTP formation.

Another potential mechanism for inhibition of Ca^{2+}-induced MPTP formation by severe Cu deficiency may be related to chronic exposure to increased levels of mitochondrially generated ROS. Respiratory Complexes I and III are major sites for mitochondrial ROS production, and the activities of these complexes are determinants of ROS production [42–45]. Mitochondrial ROS production at these sites can also be exacerbated by the partial blockage of electron flow at Complex IV. Thus, cardiac mitochondria from the severely Cu-deficient rats may have experienced increased, chronic exposure to ROS generated by the electron transport chain. Ca^{2+} channels are susceptible to oxidative modifications that can cause their inactivation [46] and oxidative damage to Ca^{2+} channels during Cu deficiency cannot be discounted as a mechanism for the alterations of cellular Ca^{2+} homeostasis in platelets [47] and reduced Ca^{2+} signaling in the endothelium [48] reported in Cu-deficient rats. Saturation of internal Ca^{2+} binding sites leads to the formation of the high-conductance MPTP responsible for mitochondrial swelling [21]. However, in the present study, oxidative modification of these sites by chronic exposure to mitochondrially generated ROS may have impaired their Ca^{2+} binding properties, reducing the sensitivity of mitochondria to Ca^{2+}-induced MPTP formation and causing the inhibition of swelling observed in the cardiac mitochondria from the Cu-deficient rats.

Our findings indicate that the formation of the MPTP in cardiac mitochondria is impaired by Cu deficiency. Although the physiological relevance of the impairment is not clear, it may be a survival response. Formation of the MPTP is a predecessor for apoptosis and necrosis. Inhibition of MPTP may help protect cardiomyocytes from the consequences of sustained oxidative stress resulting from chronically decreased mitochondrial respiratory complex activities during Cu deficiency.

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