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Functional aspects of oxidative phosphorylation and electron transport in cardiac mitochondria of copper-deficient rats

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Although dietary copper deficiency causes physiological, morphological, and biochemical abnormalities in cardiac mitochondria, the relationship observed between abnormalities of mitochondrial structure and function have been inconsistent in previous studies. The purpose of the present study was to re-evaluate the respiration rates of cardiac mitochondria from copper-deficient rats and to use several drugs that uncouple and inhibit mitochondrial respiration in order to clarify the mechanisms of mitochondrial dysfunction found in several laboratories. Copper deficiency reduced state 4 and state 3 cardiac mitochondrial respiration rates with all substrates tested. However, neither the ratio of ADP/oxygen consumed nor the acceptor control index was affected by copper deficiency. Cardiac mitochondria of copper-deficient rats showed a resistance to respiratory blockade by oligomycin and an increased ability to hydrolyze ATP in the presence of oligomycin compared with mitochondria of copper-adequate rats. This suggests that copper deficiency affects the function of the cardiac mitochondrial ATP synthase. (J. Nutr. Biochem. 6:644–652, 1995.)

Keywords: oxidative phosphorylation; ATP synthase; electron transport; mitochondrial respiration

Introduction

The cardiovascular system is a focus for damage caused by dietary copper deficiency.[1] In particular, the copper-deficient (CuD) heart exhibits both morphological defects including cardiac enlargement[2,3] and the loss of connective tissue integrity[4] and functional impairment represented by abnormal electrocardiograms[5] and reduced contractile function.[6]

Histological studies have shown that CuD hearts have enlarged and fragmented mitochondria[7] showing pronounced vacuolization throughout the organelle.[8,9] The markedly enlarged mitochondria apparently displace and distort the myofibrils.[10] Copper-deficient rats have a significantly increased mitochondrial/myofibrillar ratio.[10] Although cardiac mitochondrial morphology is severely altered by copper deficiency, parallel alterations in mitochondrial function have not been completely defined. Copper deficiency significantly reduces the activity of the cuproenzyme cytochrome c oxidase (EC 1.9.3.1) which is the terminal link in the electron transport chain.[3,11] Since cytochrome c oxidase activity is reduced in copper deficiency,[3,11] one might expect that a decrease in respiration and/or ATP production would be evident. We[12] and others[13] have shown that cardiac mitochondrial respiration rates are reduced by copper deficiency. Whether the observed reduction is caused by decreased cytochrome c oxidase activity or whether the decrease has additional mechanistic bases is unknown.

The generation of ATP by mitochondria is commonly described by the chemiosmotic theory.[14] This theory postulates that the respiratory chain on the inner membrane translocates protons at complexes I, III, and IV into the intermembrane space.[14] The inner membrane is highly im-

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permeable to protons thereby creating an electrochemical potential difference from the asymmetric distribution of the protons.[14] The electrochemical potential difference drives the ATP synthase or F_1F_oATPase to phosphorylate ADP to ATP.[14] The mechanism by which proton translocation from the respiratory chain is coupled to ATP synthesis by ATP synthase is not known in molecular detail.[14] The ATP synthase is reversible and is only constrained to run in the direction of net ATP synthesis by the continual regeneration of the proton gradient by the electron transport chain and the use of ATP by the cell.[14] If the respiratory chain is inhibited and/or ATP is supplied to the mitochondria, the ATP synthase functions as an ATPase generating a proton gradient comparable to that produced by the respiratory chain.[14] However, the essence of the chemiosmotic theory is that the respiratory chain generates such a high gradient of protons that the enzyme normally produces ATP.[14]

The purpose of the present study was to examine the susceptibility of mitochondria from CuD hearts to several agents that affect oxidative phosphorylation and electron transport in order to identify possible mechanisms for observed changes in cardiac mitochondrial respiration and to identify possible consequences or implications of a decreased respiration rate. Oxidative phosphorylation is commonly studied by the use of agents that either uncouple oxidation from phosphorylation or inhibit ATP formation.[15] We used 2,4 dinitrophenol (2,4 DNP), a proton translocator, and the K^+ ionophore, valinomycin, to uncouple oxidation from phosphorylation by reducing the electrochemical gradient for protons which drives phosphorylation. Uncoupling oxidation from phosphorylation is useful for examining electron transport (respiratory) chain function independent of the phosphorylating ability of the mitochondria. Oligomycin, which inhibits ATP synthase activity by binding to an oligomycin-sensitive subunit in the ATP synthase complex and inhibiting proton translocation by the enzyme was used to examine the effect of copper deficiency on phosphorylation. Observed differences in mitochondrial oxygen consumption in the presence of some of these agents in copper deficiency led to the investigation of the activity of the ATP synthase in the presence of the same agents.

Methods and materials

Animals

Male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI USA) were housed in the Animal Resource Facilities at the USDA, ARS Grand Forks Human Nutrition Research Center. The animals were maintained at 22 to 24°C with a 12-hr light-dark cycle and one rat was housed per standard wire rack cage. They consumed copper-adequate (CuA) diet or CuD diet and deionized water ad libitum for 4 weeks.

Diets

CuA and CuD diets were composed of 940.0 g of basal diet (catalog #TD84469, Teklad Test Diets, Madison, WI USA), 50.0 g of safflower oil (Hollywood Foods, Los Angeles, CA USA), and 10.0 g of a mineral mix. The mineral mix contained cornstarch (Best Foods, Englewood Cliffs, NJ USA) and iron with or without copper and was designed to provide 0.22 g of ferric citrate (16% Fe) (JT Baker Chemical Co., Phillipsburg, NJ USA) and either 0.02 g or no $CuSO_4 \cdot 5H_2O$ (JT Baker Chemical Co.) per kg of diet. The final diet contained 200 g/kg of casein, 386 g/kg of sucrose, 304 g/kg of cornstarch, 50 g/kg of safflower oil, and all other known essential vitamins and minerals[16] for vitamin and mineral composition. Diet analysis by atomic absorption spectrophotometry indicated that the CuA diet contained 5.3 to 6.0 mg of copper per kg of diet and the CuD diet contained 0.4 to 0.6 mg of copper per kg of diet. The National Institute of Standards and Technology (NIST, Gaithersburg, MD USA) reference samples (citrus leaves, #1572) yielded values within the specified range.

Serum and organ assays

Rats were anesthetized with ether. Blood was drawn from the inferior vena cava in an open thorax for hematocrit determination and to obtain serum samples. The liver and kidneys were removed for mineral assays. Assay for copper concentration in serum was initiated by precipitating serum proteins from 0.5 mL of serum with 0.5 mL of 0.785 mol/L of nitric acid. The mixture was allowed to sit at room temperature for 24 hr and then centrifuged at 3,000*g* for 10 min. The supernatant fraction was analyzed for copper by atomic absorption spectroscopy according to standard procedures. Organs not used for mitochondrial isolation were digested with nitric acid-hydrogen peroxide[17] and assayed for copper by inductively coupled argon plasma emission spectrophotometry.[18] NIST reference samples (bovine liver, #1577a) yielded values within the specified range. Serum was assayed for ceruloplasmin by the method of Sundermann and Nomoto[19] and for cholesterol by the method of Allain et al.[20]

Mitochondrial isolation

Mitochondria were isolated at 0 to 4°C according to Mela and Seitz[21] with some modifications. Briefly, hearts were removed and homogenized in isolation buffer consisting of 0.225 M mannitol, 0.075 M sucrose, 20 mM HEPES, and 1 mM EGTA. A protease (trypsin) was included with the hearts to ensure a high yield of mitochondria with optimal respiratory control (acceptor control index) and P/O ratios. Either two CuA or CuD hearts were placed in a homogenizer with 5 mL of isolation buffer to which had been added 10 mg of trypsin. The hearts were homogenized immediately on ice with three strokes and then quickly diluted with 15 mL of isolation buffer to limit the exposure of the heart to concentrated trypsin. The homogenate was then centrifuged at 480*g* for 5 min, and the pellet was discarded. The supernatent was collected and filtered through two layers of cheesecloth into two clean centrifuge tubes and centrifuged at 7,700*g* for 10 min. The resulting pellet contained the mitochondria with some contaminants such as lysosomes, peroxisomes, and membrane fragments. The mitochondrial pellet was further purified by repeated washing with isolation buffer. Specifically a light, fluffy layer consisting of membrane fragments was removed, and the resulting pellet containing the mitochondria was carefully resuspended in isolation buffer and centrifuged at 7,700*g* for 5 min. The mitochondrial pellet was resuspended in the same volume (100 μL of isolation buffer per heart) regardless of whether the hearts were from CuA or CuD rats. The entire isolation procedure was completed within 1 hr.

Citrate synthase activity

Citrate synthase (EC 4.1.3.7) activity was assayed in supernatant fractions of cardiac mitochondria in the presence and absence of the detergent CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate) to verify the intactness of the mitochondrial membrane.[22] Because citrate synthase is a marker enzyme for mitochondrial matrix,[15,23] supernatant fractions from intact mito-

chondria exhibited no citrate synthase activity in the absence of detergent but showed a substantial increase in activity upon disruption with detergent.[24] The activity was assayed by the method of Srere[22] with modification as described previously.[12] The enzyme activity was calculated from the change in the absorbency over a linear period of reaction and expressed as micromoles per minute. The mitochondrial membrane was assumed to be intact if mitochondria exhibited little or no citrate synthase activity in the absence of detergent but showed a substantial increase in activity upon disruption with detergent. In addition, protein concentration in the supernate was considered to be an estimate of protein released from disrupted mitochondria.

Mitochondrial measurements

Mitochondrial respiratory measurement values were determined at 30°C polarographically by the method of Estabrook.[25] The reaction medium was saturated with air and consisted of isolation buffer, 0.25 M sucrose, 200 mmol/L of $MgCl_2$, and 200 mmol/L of sodium phosphate buffer (pH 7.4). Substrates are linked to the mitochondrial respiratory chain at different sites; therefore, a variety of substrates were used in order to give an indication of problems at different points in the respiratory chain. Substrates (final concentrations) used were succinate (10 mM) plus rotenone (0.004 mM), pyruvate plus malate (10 mM each), or glutamate plus malate (10 mM each).

Mitochondria were isolated from two CuD and two CuA rat hearts per day. Each mitochondrial pellet was subjected to the same treatment. State 3 respiration states were determined following the addition of ADP to a final concentration of either 0.08 mmol/L for the measurement of the P/O ratio or 0.8 mmol/L for the measurement of the acceptor control index (ACI). The state 4 respiration rate was measured in the presence of a substrate but in the absence of ADP as the phosphate acceptor. Respiration was expressed as nanograms of oxygen atoms consumed/min · mg of protein. The P/O ratio or ADP/O ratio was defined as the amount of ADP phosphorylated per unit of oxygen consumed. The ACI was obtained by dividing the oxygen consumption (nanomoles of oxygen consumed per milligram mitochondria protein) in state 3 by the oxygen consumption in state 4. A 3.5 to 4 fold increase in oxygen consumption with the addition of ADP was accepted as one indication of the mitochondrial membrane integrity. The remaining CuA and CuD mitochondria were subjected to three experiments involving uncouplers and/or inhibitors. In the first and second experiments, a state 4 respiration rate was recorded and then 2,4 DNP or valinomycin plus KCl was added to a final concentration of 0.5 mM 2,4 DNP or 0.05 μM valinomycin plus 15 mM KCl and a respiration rate was recorded. The third experiment examined the inhibitory effects of oligomycin by the addition of oligomycin to a reaction medium containing mitochondria stimulated by ADP. After state 4 respiration was recorded, 10 mM ADP was added. Before the ADP supply was exhausted or all was converted into ATP, an oligomycin solution was added to a final concentration of 0.05 mg/mL, and the respiration rate recorded. To investigate whether respiration rates could be stimulated differentially in the presence of oligomycin, 2,4 DNP was then added as above to preparations of mitochondria from CuA and CuD rat hearts, and the final respiration rates recorded.

Measurement of mitochondrial protein concentration

Mitochondrial protein measurements were made by utilizing the bicinchoninic acid (BCA) reagent kit from Pierce (Rockford, IL USA) according to instructions included with the kit.

Hydrolysis of ATP

Hydrolysis of ATP was assayed as described by Lardy[26] with modifications. For every mitochondrial pellet, four hydrolysis experiments were performed. The amount of ATP hydrolysis was assayed: in the absence of inhibitors or uncouplers; in the presence of 2,4 DNP (0.01 mM); in the presence of oligomycin (2.5 μg/mL); and in the presence of both oligomycin and 2,4 DNP.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed for unequal sample size with copper status serving as the independent variable. The dependent variables were the P/O ratio, the ACI, state 3 respiration, state 4 respiration, the amount of stimulation from 2,4 DNP and valinomycin, the inhibitory effects of oligomycin, and the amount of hydrolysis of ATP in the absence of inhibitors or uncouplers, in the presence of 2,4 DNP, in the presence of oligomycin, and in the presence of both oligomycin and 2,4 DNP. The accepted level of significance for F_{obs} was $P < 0.05$.

Results

Copper status

Evidence of copper deficiency in rats fed the CuD diet is provided in *Table 1*, which lists several direct and indirect indices of copper status. Copper concentrations in serum, liver, and kidney were all significantly lower ($P < 0.05$) in CuD rats than in CuA rats. Activity of serum ceruloplasmin, a copper-dependent protein, was also lower ($P < 0.05$) in CuD rats. Enlarged hearts, anemia, and hypercholesterolemia, characteristics of animals severely deficient in copper, were also evident in CuD rats ($P < 0.05$). The amount of protein in mitochondria isolated from hearts in CuD rats was significantly greater ($P < 0.05$) than in mitochondria from CuA rats. The body weights of CuD rats were significantly lower ($P < 0.05$) than those of CuA rats, and the heart-to-body weight ratio was significantly greater ($P < 0.05$) in CuD rats than CuA rats. All direct and indirect indices indicate that the 4-week diet of the CuD rats

Table 1 Characteristics of rats fed copper-adequate (CuA) or copper-deficient (CuD) diets for 4 weeks

Characteristics	CuA		CuD	
Body weight (g)	221.5 ± 2.9	(66)	192.5 ± 2.6	(66)*
Heart weight (g)	1.2 ± 0.1	(66)	1.8 ± 0.1	(66)*
Heart/body weight ratio (g/kg)	5.42 ± 0.01	(66)	9.35 ± 0.03	(66)*
Hematocrit (%)	40.5 ± 0.2	(64)	20.9 ± 0.5	(66)*
Serum Cu (μg/mL)	0.58 ± 0.01	(66)	ND	(66)*
Liver Cu (μg/g of dry weight)	12.8 ± 0.5	(51)	1.6 ± 0.3	(53)*
Kidney Cu (μg/g of dry weight)	28.2 ± 0.8	(66)	11.97 ± 0.09	(66)*
Serum ceruloplasmin (mg/dL)	28.4 ± 0.8	(66)	4.5 ± 0.1	(66)*
Serum cholesterol (mg/dL)	77 ± 2	(66)	92 ± 2	(66)*
Mitochondrial protein yield (mg of protein/mL)	34 ± 2	(33)	56 ± 3	(33)*

Values are means ± SEM. Numbers in parenthesis indicate the number of separate experiments. ND means variable is not detectable.
*Significant difference ($P < 0.05$) from the corresponding CuA value.

was deficient in copper and did in fact produce copper deficiency.

Citrate synthase activity

The effectiveness of CHAPS in liberating citrate synthase activity was determined by a titration with increasing concentrations of CHAPS. The maximal activity observed in mitochondria from CuD and CuA hearts was similar in both at 0.05% CHAPS whereas higher concentrations of CHAPS resulted in an inhibition of activity (data not shown). Supernatant fractions of intact and detergent-disrupted mitochondria were assayed for crude protein content and citrate synthase activity. These assays indicated no differences between CuA and CuD rats in the amount of protein or citrate synthase activity released from untreated mitochondria (*Figures 1A and 1B*). Treatment of mitochondria with 0.05% CHAPS increased significantly the amount of protein and citrate synthase activity released from mitochondria in both CuA and CuD preparations (*Figures 1A and 1B*). In the presence of CHAPS, significantly ($P < 0.05$) more protein and citrate synthase activity were found in the mitochondrial supernatant fraction from CuD rats as compared with CuA rats (*Figures 1A and 1B*).

Mitochondrial respiration and ATP production

State 4, state 3, P/O ratio, and ACI in copper deficiency. Changes in variables related to cardiac mitochondrial respiratory function are shown in *Table 2*. Mitochondria from hearts of CuD rats showed significantly depressed state 4 and state 3 respiration rates relative to the mitochondria from hearts of CuA rats when succinate plus rotenone, pyruvate plus malate, or glutamate plus malate were used as substrates ($P < 0.05$). Neither the ACI nor the ratio of ATP produced to oxygen consumed (P/O ratio) were affected by copper deficiency in the presence of any of the substrates.

In each of the following experiments (A, B, C below), oxygen consumption of mitochondria was measured in the absence of ATP (state 4) prior to the addition of any other agents such as ADP, 2,4 dinitrophenol, valinomycin, and/or oligomycin. Because the state 4 respiration rate was significantly lower in the mitochondria of CuD rats compared with the mitochondria of CuA rats initially, the amount of stimulation of respiration by ADP, 2,4 DNP, or valinomycin was calculated by subtracting the state 4 respiration rate from the respiration rate in the presence of one of the above substrates. The data are presented in tables as the mean ± standard error (SE). The number in parentheses indicates the number of separate samples or experiments.

A. Effect of 2,4 dinitrophenol. Mitochondrial respiration rates in the presence of 2,4 DNP are summarized in *Table 3*. State 4 was significantly depressed ($P < 0.05$) by copper deficiency with all three substrates as before (at least a 20 ng of atoms of O_2/min · mg decrease in CuD rats compared with CuA rats) and the addition of 2,4 DNP increased respiration 2 to 3 fold as expected in mitochondria from both CuA and CuD rat hearts. Again because the state 4 respiration rate was significantly lower in the mitochondria of CuD rats compared with the mitochondria of CuA rats, the amount of stimulation of respiration by 2,4 DNP was calculated by subtracting the state 4 respiration rate from the respiration rate in the presence of 2,4 DNP. There was no significant difference between CuA and CuD mitochondria in the amount of stimulation with 2,4 DNP with any of the three substrates.

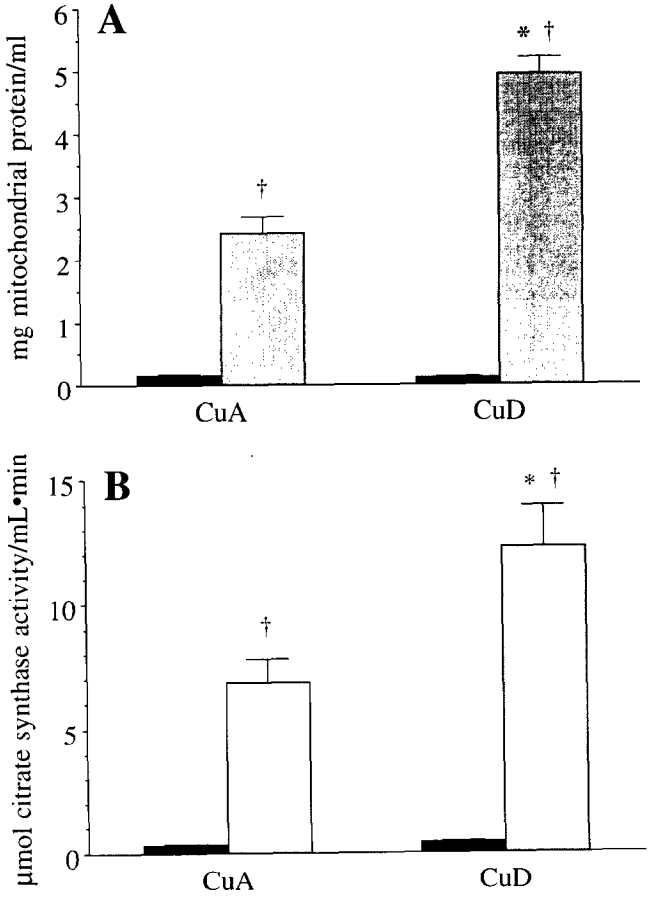

Figure 1 Mitochondrial intactness in CuA and CuD cardiac muscle. (A) Represents the protein concentration in supernates prepared as described previously.[12] No significant difference was observed in the amount of protein present in the supernate with 0% CHAPS ■ in mitochondria from hearts of CuA ($n = 15$) and CuD ($n = 15$) rats. However, almost twice as much protein (a significant increase $P < 0.05$) was released from the mitochondria of CuD rats when disrupted with 0.05% CHAPS ▨ as compared with the mitochondria from CuA rats disrupted with 0.05% CHAPS ▨ as indicated by the asterisk (*). The cross (†) indicates a significant increase ($P < 0.05$) in protein released between mitochondria treated with 0% CHAPS ■ and mitochondria treated with 0.05% CHAPS ▨. (B) Represents total citrate synthase activity as the change in µmol/min in supernates prepared as described in Methods and materials section. No significant difference was observed between CuD ($n = 15$) and CuA ($n = 15$) total citrate synthase activity before addition of detergent ■. Total citrate synthase activity of both groups was stimulated approximately 20 to 30 fold with the addition of detergent ▨. Total citrate synthase activity was significantly greater ($P < 0.05$) in mitochondria from hearts of CuD rats as compared with mitochondria from hearts of CuA rats after disruption by 0.05% CHAPS as indicated by the asterisk (*). After disruption, both hearts of CuA and CuD rats had significantly greater ($P < 0.05$) activities of citrate synthase (indicated by the cross (†)) than before the addition of the detergent indicating that both were equally intact.

B. Effect of valinomycin. Mitochondrial respiration rates in the presence of valinomycin plus K^+ are shown in *Table*

Table 2 A comparison of state 4, state 3, the ACI, and the P/O ratio in cardiac mitochondria from copper-adequate (CuA) and copper-deficient (CuD) rats

Characteristic	CuA		CuD	
State 4 (ng of atoms of O_2/min · mg)				
succinate + rotenone	176 ± 11	(33)	113 ± 9	(33)*
pyruvate/malate	79 ± 7	(33)	60 ± 6	(32)*
glutamate/malate	86 ± 8	(14)	63 ± 7	(13)*
State 3 (ng of atoms of O_2/min · mg)				
succinate + rotenone	620 ± 40	(33)	400 ± 30	(33)*
pyruvate/malate	560 ± 40	(33)	360 ± 30	(33)*
glutamate/malate	580 ± 60	(13)	420 ± 50	(13)*
ACI or RCR (St. 3/St. 4 respiration)				
succinate + rotenone	3.6 ± 0.1	(33)	3.8 ± 0.2	(33)
pyruvate/malate	7.7 ± 0.4	(33)	6.5 ± 0.4	(32)
glutamate/malate	6.6 ± 0.3	(14)	7.2 ± 0.7	(14)
P/O ratio				
succinate + rotenone	1.85 ± 0.03	(33)	1.89 ± 0.04	(33)
pyruvate/malate	2.84 ± 0.05	(33)	2.83 ± 0.05	(33)
glutamate/malate	2.69 ± 0.11	(14)	2.76 ± 0.09	(14)

Values are means ± SEM. Numbers in parenthesis indicate number of separate experiments.
*Significant difference ($P < 0.05$) from the corresponding CuA value.

4. State 4 was significantly depressed ($P < 0.05$) by copper deficiency with all three substrates as before, and when valinomycin was added respiration was stimulated 2 to 3 fold in mitochondria from both CuA and CuD rats. The amount of stimulation or increase in respiration was determined by subtracting the state 4 respiration rate from the valinomycin-stimulated respiration rate. Copper deficiency significantly reduced the stimulation in mitochondria treated with valinomycin in the presence of succinate plus rotenone ($P < 0.05$) and a tendency toward a decreased stimulation in mitochondria treated with pyruvate plus malate or glutamate plus malate was observed.

C. Effect of oligomycin. The mitochondrial respiration rates in the presence of oligomycin are shown in *Table 5*. State 4 was significantly depressed ($P < 0.05$) by copper deficiency with all three substrates as before. Addition of 10 mM ADP caused significantly less stimulation ($P < 0.05$) of respiration in mitochondria from CuD rat hearts than in mitochondria from CuA rat hearts regardless of substrate. The amount of inhibition by oligomycin was calculated by subtracting the respiration rate in the presence of oligomycin from the rate in the presence of 10 mM ADP. Calculated this way, results show that the amount of inhibition with oligomycin was significantly lower ($P < 0.05$) in mitochondria from CuD rat hearts for all three substrates. 2,4 DNP was then added to stimulate respiration in the presence of oligomycin by uncoupling oxidation from phosphorylation. The amount of stimulation in respiration was calculated by subtracting the respiration rate in the presence of 2,4 DNP plus oligomycin from the respiration rate in the presence of oligomycin alone. Release of inhibition with the addition of 2,4 DNP was significantly greater ($P < 0.05$) in mitochondria from hearts of CuD rats than in those from CuA rats when succinate plus rotenone or glutamate plus malate were used as substrate. Copper deficiency did not affect this release of inhibition in the presence of pyruvate plus malate.

Hydrolysis of ATP

The results of the hydrolysis of ATP by mitochondria are shown in *Table 6*. Basal (control) hydrolysis of ATP in mitochondria from CuA rat hearts was not significantly different from that of CuD rat hearts. The addition of 2,4 DNP produced significant increases in the rates of ATP hydrolysis which were not different between CuA and CuD rat hearts. ATP hydrolysis in the presence of oligomycin was significantly greater ($P < 0.05$) in mitochondria from CuD rat hearts than in mitochondria from CuA rats. Oligomycin is known to inhibit ATPase activity even in the presence of 2,4 DNP, and mitochondria from CuD rat hearts exhibited a significantly greater rate of hydrolysis ($P < 0.05$) than mitochondria from CuA hearts when both oligomycin and 2,4 DNP were present.

Discussion

Functional changes in cardiac mitochondria from CuD rats as measured by respiration and coupling of oxidative phosphorylation have not been correlated consistently to structural changes observed in cardiac mitochondria from CuD rats. In the present study, changes in the rate of oxygen consumption suggest that cardiac mitochondrial function is altered by copper deficiency. In addition, the specific effects of the antibiotic oligomycin suggest that the functional changes observed in copper deficiency are at least partially related to a change in the F_1F_o ATP synthase.

Table 3 The addition of 2,4 DNP to cardiac mitochondria of copper adequate (CuA) and copper deficient (CuD) rats

Characteristic	CuA	CuD
State 4 (ng of atoms of O_2/min · mg)		
succinate + rotenone	211 ± 27 (13)	119 ± 13 (13)*
pyruvate/malate	86 ± 13 (15)	56 ± 6 (17)*
glutamate/malate	94 ± 13 (13)	62 ± 9 (13)*
2,4 DNP respiration rate (ng of atoms of O_2/min · mg)		
succinate + rotenone	430 ± 60 (13)	320 ± 40 (13)
pyruvate/malate	190 ± 30 (15)	160 ± 20 (17)
glutamate/malate	220 ± 30 (13)	170 ± 20 (13)
Amount of stimulation by 2,4 DNP (ng of atoms of O_2/min · mg)		
succinate + rotenone	220 ± 30 (13)	200 ± 30 (13)
pyruvate/malate	100 ± 20 (15)	110 ± 20 (17)
glutamate/malate	120 ± 20 (13)	110 ± 20 (13)

Values are means ± SEM. Numbers in parenthesis indicate number of separate experiments.
*Significant difference ($P < 0.05$) from the corresponding CuA value.
Amount of stimulation by 2,4 DNP = 2,4 DNP respiration rate − state 4.

Table 4 The addition of valinomycin to cardiac mitochondria of copper-adequate (CuA) and copper-deficient (CuD) rats

Characteristic	CuA	CuD
State 4 (ng of atoms of O_2/min · mg)		
succinate + rotenone	183 ± 22 (9)	113 ± 11 (9)*
pyruvate/malate	85 ± 9 (9)	55 ± 7 (9)*
glutamate/malate	121 ± 15 (11)	81 ± 11 (11)*
Valinomycin respiration rate (ng of atoms of O_2/min · mg)		
succinate + rotenone	460 ± 60 (9)	260 ± 30 (9)*
pyruvate/malate	270 ± 40 (9)	190 ± 30 (9)
glutamate/malate	240 ± 30 (11)	160 ± 20 (11)*
Amount of stimulation by valinomycin (ng of atoms of O_2/min · mg)		
succinate + rotenone	280 ± 50 (9)	150 ± 30 (9)*
pyruvate/malate	180 ± 30 (9)	140 ± 20 (9)
glutamate/malate	120 ± 20 (11)	80 ± 20 (11)

Values are means ± SEM. Numbers in parenthesis indicate number of separate experiments.
*Significant difference ($P < 0.05$) from the corresponding CuA value.
Amount of stimulation by valinomycin = valinomycin respiration rate − state 4.

Membrane integrity

Because cardiac mitochondria from CuD rats are enlarged and morphologically altered, functional changes could result from a greater disruption of the mitochondrial membrane during isolation. Therefore, evaluation of membrane intactness is important. Membrane intactness was assessed by measuring the mitochondrial protein concentration and total citrate synthase activity in supernatant fractions. No significant difference was observed in either the amount of protein released or in the total citrate synthase activity in the supernates of cardiac mitochondria of CuD and CuA rats, thereby suggesting that the mitochondrial membranes were intact (*Figure 1*). However, disrupting the cardiac mitochondria with 0.05% CHAPS resulted in a significant increase in the protein released and the total citrate synthase activity in stimulation mitochondrial supernatant fractions of CuD rats compared with cardiac mitochondrial supernatant fractions of CuA rats which could be a consequence of enlarged mitochondria[7] or increased mitochondrial volume density.[8,9]

Mitochondrial oxygen consumption

The ability of cardiac mitochondria from CuD rats to consume oxygen was reduced both in the presence and absence of ADP with all substrates tested, as indicated by decreased state 3 and state 4 respiration rates (*Table 2*). The finding of decreased state 3 and state 4 respiration rates in cardiac mitochondria of CuD rats is consistent with previous observations[12] and with the decreased state 3 respiration rates observed by Chao et al.[13] The reduced stimulation of respiration by ADP in CuD rats may be a consequence of altered levels of ATP present in cardiac mitochondria of CuD rats. Kopp et al.[9] detected decreased levels of ATP in hearts of CuD rats as compared with hearts of CuA rats, whereas Rusinko and Prohaska[27] and Chao et al.[13] indicate that there is no differences in ATP levels despite decreased cytochrome c oxidase activity in CuD rats. Inconsistencies in the ATP levels appear to stem from the severity of copper deficiency and the method of analysis. Even though the ATP levels in the hearts of CuD animals have not been determined conclusively, decreased respiration rates in cardiac mitochondria of CuD rats correlate with the reduced activity of cytochrome oxidase observed in copper deficiency.[3,11] In addition, the depression of respiration with copper deficiency was similar regardless of the substrate used. Because these substrates enter the respiratory chain at different sites but have a common step at complex IV (cytochrome oxidase), an association between decreased respiration rates and cytochrome oxidase activity in copper deficiency is further supported.

Because heart size[2,3] and mitochondrial yield per heart[10] are increased with copper deficiency, one might ask how respiratory capacity of the whole heart changes. When mitochondrial respiration (state 3 or state 4) is expressed per whole heart, CuA and CuD rats do not appear to differ. The Law of Laplace states that at a constant internal pressure, wall tension increases with the radius of a spherical surface. This suggests that more energy is required to support the increase in wall tension of an enlarged heart. The similar whole-heart mitochondrial respiration rates of copper-adequate and copper-deficient rats reveal that an increase in

Table 5 The inhibition of oligomycin and reversal by 2,4 DNP in mitochondria of copper-adequate (CuA) and copper-deficient (CuD) rats

Characteristic	CuA	CuD
State 4 (ng of atoms of O_2/min · mg)		
succinate + rotenone	229 ± 30 (13)	112 ± 13 (13)*
pyruvate/malate	82 ± 11 (16)	59 ± 7 (16)*
glutamate/malate	85 ± 8 (13)	62 ± 7 (13)*
ADP respiration rate (ng of atoms of O_2/min · mg)		
succinate + rotenone	610 ± 80 (13)	270 ± 30 (13)*
pyruvate/malate	270 ± 30 (17)	190 ± 20 (16)*
glutamate/malate	350 ± 30 (13)	230 ± 40 (12)*
Amount of inhibition by oligomycin (ng of atoms of O_2/min · mg)		
succinate + rotenone	270 ± 40 (13)	80 ± 10 (13)*
pyruvate/malate	170 ± 20 (17)	120 ± 20 (17)*
glutamate/malate	240 ± 20 (13)	110 ± 30 (10)*
Amount of recovery by 2,4 DNP (ng of atoms of O_2/min · mg)		
succinate + rotenone	60 ± 13 (9)	140 ± 30 (13)*
pyruvate/malate	48 ± 10 (13)	49 ± 10 (13)
glutamate/malate	30 ± 5 (9)	69 ± 5 (11)*

Values are means ± SEM. Numbers in parenthesis indicate number of separate experiments.
*Significant difference ($P < 0.05$) from the corresponding CuA value.
Amount of inhibition by oligomycin = ADP respiration rate − oligomycin respiration rate (not shown).
Amount of recovery by 2,4 DNP = 2,4 DNP respiration rate (not shown) − oligomycin respiration rate (not shown).

Table 6 Hydrolysis of ATP by cardiac mitochondria in copper-adequate (CuA) and copper-deficient (CuD) rats

Characteristic (nmol of Pi/min · mg)	CuA	CuD
Control	121 ± 12 (21)	150 ± 14 (21)
2,4 DNP	355 ± 18 (21)	336 ± 19 (19)
Oligomycin	26 ± 4 (20)	41 ± 6 (19)*
Both 2,4-DNP and oligomycin	84 ± 5 (21)	100 ± 6 (19)*

Values are means ± SEM. Numbers in parenthesis indicate number of separate experiments.
*Significant difference ($P < 0.05$) from the corresponding CuA value.

respiratory capacity does not accompany the increase in heart size and that copper-deficient hearts will be compromised in their ability to provide energy for force development. This may explain the reduced force development of copper-deficient hearts.[6,28]

Coupling of oxidation and phosphorylation as indicated by the ratio of ADP to oxygen consumed

The P/O ratio was not affected by copper deficiency (*Table 2*) which is consistent with the findings of Chao et al.[13] Because protons produced from the electron transport chain in mitochondria from CuD rats result in the same relative amount of ATP being synthesized as in mitochondria from CuA hearts, mitochondria from CuA and CuD hearts may be considered to be equally coupled.

Acceptor control index

Because both state 3 and state 4 respiration rates were decreased relatively equally by copper deficiency, no changes were observed in the ratio of state 3 to state 4 or in the ACI (*Table 2*). This is consistent with prior findings from this laboratory[12] but different from those of Chao et al.[13] who reported a decreased ACI in cardiac mitochondria of CuD rats. A low ACI would indicate an energy leakage and a relative uncoupling of oxidation from respiration. Because of the similarity of ACI values observed in the present study, we conclude that the mitochondria from CuD rats have an energy-conserving capacity at least equal to that observed in the mitochondria from CuA rats.

Effects of 2,4 dinitrophenol

The consequence of the addition of 2,4 DNP is to uncouple oxidation from phosphorylation with the direct effect of flooding the matrix with protons, thus eliminating the driving force for ATP synthase. The increase in respiration that results is a measure of the ability of the electron transport chain to extrude the excess protons. The amount of stimulation of respiration by 2,4 DNP from state 4 was not significantly different between mitochondria from CuA and CuD rats (*Table 3*). Although no difference was found in the stimulation of oxygen consumption with 2,4 DNP between CuA and CuD rats, the impairment of proton translocation by a specific proton pump has not been eliminated. Isolation and study of complexes I, III, or IV from the mitochondria of CuD rats may determine specifically which, if any, of the complexes is functionally altered.

Effects of valinomycin

Valinomycin is a mobile carrier ionophore that complexes with potassium and also uncouples oxidation from phosphorylation by translocating K^+ into the matrix, lowering the membrane potential, and hence reducing the proton motive force (i.e., the proton electrochemical gradient). The respiratory chain responds to the lowering of the proton motive force by net extrusion of protons into the intermembrane space.[29] The addition of valinomycin resulted in significantly less stimulation of respiration in the presence of succinate; although apparent decreases in stimulation were observed with other substrates, these decreases were not statistically significant. Further studies are needed before generalized conclusions may be made regarding the effect of this ionophore.

Effects of oligomycin

The enzyme complex that synthesizes ATP is called the F_1F_o ATPase (also called the mitochondrial ATP synthase) and is embedded in the mitochondrial inner membrane. The F_1F_o ATPase drives ATP synthesis in the mitochondrial matrix using the energy generated from the electrochemical proton gradient across the mitochondrial inner membrane.[30] Fourteen different subunits in bovine heart mitochondrial ATP synthase have been determined, and all but two subunits are encoded by the nuclear genes.[31] The F_1 portion of the complex is a water-soluble sector and the F_o is a hydrophobic protein complex. Oligomycin is a specific ATPase inhibitor that binds to the F_o portion of the ATP synthase and inhibits oxidative phosphorylation.[26,29] When oligomycin is added to mitochondria stimulated by ADP (state 3), oxygen consumption was less inhibited in mitochondria from CuD rats than those from CuA rats (*Table 5*). This indicates that the oligomycin-sensitive subunits in the F_o fraction of the ATP synthase were altered. Because matrix protons drive respiration, we conclude that incomplete blockage of the ATP synthase with oligomycin allowed protons to continue to move through the inner membrane into the mitochondrial matrix of CuD rats. The relative inability of oligomycin to retard proton movement was also apparent in 2,4 DNP-treated mitochondria from CuD rat hearts (*Table 5*). The observation of altered ATP synthase in cardiac mitochondria of CuD rats is supported by Medeiros[32] who observed decreased amounts of δ subunit in the F_1 fraction of the ATP synthase in cardiac mitochondria of CuD rats. The δ subunit in the F_1 fraction of the ATP synthase is essential for proper binding of the F_1 to F_o portion. Decreased subunits and an incomplete inhibition of respiration by oligomycin on the ATP synthase may be the reason for the inconsistencies reported in the level of ATP in hearts of CuD rats.[9,13]

Hydrolysis of ATP

The finding of Medeiros et al.[32] of a diminished δ subunit of the F_1 fraction of the ATP synthase and our finding of incomplete inhibition of respiration by oligomycin in cop-

per deficiency suggest that subunits of the ATP synthase are altered and may affect enzyme activity. The activity of the mitochondrial ATP synthase was studied by inducing it to act as an ATPase. This was done by providing ATP for hydrolysis and withholding substrate to inhibit the respiratory chain. Normally protons enter the matrix through the ATP synthase and are used in the synthesis of ATP. As an ATPase, the ATP synthase acts as a pump that moves protons from the matrix into the intermembrane space when hydrolyzing ATP. The hydrolysis of ATP was determined in the absence of inhibitors or uncouplers or in the presence of 2,4 DNP, oligomycin, or both oligomycin and 2,4 DNP. 2,4 DNP is known to accelerate ATP hydrolysis by maximizing proton translocation into the matrix,[14,33] and oligomycin inhibits hydrolysis even in the presence of 2,4 DNP by binding to the oligomycin-sensitive subunits and preventing proton translocation through the ATP synthase complex in either direction.[34–36] In the absence of inhibitors and uncouplers, no significant difference between CuD and CuA rat hearts was observed in the hydrolysis of ATP by the F_1F_o ATPase (*Table 6*). These data indicate that the ability to hydrolyze ATP is similar for mitochondria from CuA and CuD rats. In the presence of 2,4 DNP, no difference because of copper status was observed in the amount of ATP hydrolyzed, which further supports the finding that proton translocation was not affected by copper deficiency. However, when oligomycin was present, with or without 2,4 DNP, significantly more hydrolysis of ATP occurred in mitochondria from CuD rats (*Table 6*) which suggests an incomplete inhibition of the ATP synthase with oligomycin. This agrees with observations made in the respiratory studies. The increase in hydrolysis of ATP in mitochondria from CuD hearts again indicates that oligomycin was not completely blocking the translocation of protons from the matrix into the inner membrane space. The results from the respiration and hydrolysis studies strongly suggest that the function of the oligomycin-sensitive subunits in the F_1F_o ATP synthase are altered in mitochondria from CuD rats. This indicates that molecular components of the ATP synthase in the CuD rat should be investigated further.

We have shown that HSP60 mRNA is reduced in the atria of hearts of CuD rats compared with the atria of CuA rats.[37] Both biochemical and genetic studies have shown HSP60 to be a critical component for protein maturation within the mitochondria.[38] HSP60 facilitates the orderly association of monomeric proteins into their final oligomeric structures.[39] The suggestion has been made that HSP60 is involved in the assembly of the ATP synthase complex.[40] In fact, one of the ATP synthase subunits has been shown to be a member of the HSP60 family.[41] Because HSP60 may be essential for the folding[39] and assembly[38] of imported mitochondrial proteins, the alteration of HSP60 in hearts of CuD rats suggests that these mitochondria may have difficulty in assembling proteins into macromolecules including those that make up the ATP synthase. This could explain the altered subunits to which oligomycin binds.

Another point of consideration is the severity of copper deficiency. One factor that contributes to the inconsistencies in the copper deficiency literature arises from differences in the severity of copper deficiency induced by various diets and the duration of the diets. The rats in this study were severely copper-deficiency which means the hearts had several cardiac abnormalities including pathological, physiological, and biochemical defects. Therefore it is possible that some of the findings may be secondary to the damage. However, Chao et al.[42] suggest that the ATP synthase is altered in CuD rats when a strong antioxidant is given to minimize the secondary damage, which suggests that the effect on ATP synthase is due to copper deficiency and not to secondary damage.

In summary, this study examines two aspects of the mitochondrial respiration process: uncoupling oxidation from phosphorylation and inhibiting ATP synthase activity. The use of uncouplers suggests that respiration in the absence of phosphorylation indicates that the overall ability of mitochondria to translocate matrix protons is not impaired by copper deficiency, although potential defects in individual primary proton pumps were not addressed. This study has, however, brought attention to a potential functional defect in cardiac mitochondrial ATP synthase in dietary copper deficiency. Because of the importance of the ATP synthase in cardiac energy metabolism and hence in the overall function of the heart, further studies of the enzyme are warranted.

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