Mitochondrial respiration in heart, liver, and kidney of copper-deficient rats

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Morphological observations in some tissues indicate that dietary copper deficiency results in structural damage to mitochondria. The purpose of this study was to determine whether mitochondrial function is impaired as well. Male, weanling Sprague-Dawley rats were fed diets deficient or sufficient in copper for 4 weeks. Mitochondria were isolated from heart, liver, kidney cortex, and kidney medulla. P/O ratio, state 3 and state 4 respiration rates (oxygen consumed in the presence and absence of ADP, respectively), and acceptor control index (ratio of state 3:state 4) were determined using succinate or pyruvate/malate as substrate. State 3 respiration rate in mitochondria from copper-deficient hearts and livers was lower than in mitochondria from copper-sufficient hearts. Copper deficiency reduced the state 4 respiration rate only in cardiac mitochondria. Neither respiration rate was affected by copper deficiency in mitochondria from kidney medulla or cortex. P/O ratio was not significantly affected by copper deficiency in any tissue examined. Acceptor control index was reduced only in liver mitochondria. The observed decreases in respiration rates are consistent with decreased cytochrome c oxidase activity, shown by others to occur in mitochondria isolated from hearts and livers of copper-deficient rats.

Keywords: copper deficiency; cardiac mitochondria; mitochondrial respiration

Introduction

Studies with rats have shown mitochondria to be a focus of damage in dietary copper deficiency. Findings include markedly enlarged and structurally disrupted mitochondria in heart and liver,[1-5] depressed cytochrome $a+a_3$ (cytochrome oxidase) activity in hearts and livers, and alteration of activity of several other mitochondrial enzymes in livers of copper-deficient as compared with normal rats.[1,6-7]

In hearts, though mitochondrial morphology is severely altered by copper deficiency, parallel alterations in respiration or phosphorylation rates (except for cytochrome oxidase activity) have not been found in previous studies.[1] In contrast to heart mitochondria, some investigators have found that liver mitochondria of copper-deficient rats, in addition to showing morphological changes,[3] exhibit changes in respiratory rate (in particular, state 3 respiration) with a variety of substrates.[6,7] In addition, others have shown that kidney mitochondria, while exhibiting the characteristic (though relatively small) reduction in cytochrome oxidase activity with copper deficiency,[6] exhibit no apparent morphological changes.[8]

The objectives of the present study were to: (1) study the function of copper-deficient heart mitochondria, because the lack of respiratory changes found by others seems inconsistent with the observed structural damage; (2) confirm the findings of others regarding respiration of liver mitochondria; and (3) initiate studies on kidney mitochondria, in which, to our knowledge, functional characteristics, including respiration, have not been measured in copper deficiency.

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Materials and methods

Diets

Copper-sufficient (CuS) and copper-deficient (CuD) diets were composed of 940.0 g of copper-free, iron-free 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 copper-iron mineral mix. The basal diet was a casein- (20%), sucrose- (39%), cornstarch- (29%) based diet containing all known essential minerals and vitamins except copper and iron.[9] 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) (J.T. Baker Chemical Co., Phillipsburg, NJ USA) and either 0.02 g or no $CuSO_4$-$5H_2O$ (J.T. Baker Chemical Co.) per kg of diet. Diet analysis by atomic absorption spectrophotometry indicated that the CuS diet contained 5.2 g copper per kg diet, and the CuD diet contained 0.4 g copper per kg diet. National Institute of Standards and Technology (NIST) reference samples (citrus leaves, #1572) yielded values within the specified range.

Animals

Male, weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI USA) were housed in quarters maintained at 22–24° C with a 12-hr light/dark cycle. They were fed the CuS diet ($n=16$) or the CuD diet ($n=16$) and deionized water ad libitum for 4 weeks.

Assays

Rats were anesthetized with ether. Blood was drawn from the inferior vena cava for hematocrit determination and to obtain serum samples. Hearts, livers, kidneys, and one hind limb muscle (quadriceps group) were removed for mineral assays or mitochondrial isolation. After dilution with nitric acid, serum was assayed for copper by atomic absorption spectrophotometry. Organs not used for mitochondrial isolation were digested with nitric acid-hydrogen peroxide[10] and assayed for copper by inductively coupled argon plasma emission spectrophotometry. NIST reference samples (bovine liver, #1577a) yielded values within the specified range. Serum was assayed for ceruloplasmin by the method of Sundermann and Nomoto[11] and for cholesterol by the method of Allain et al.[12]

Citrate synthase activity was assayed in cardiac mitochondria to verify the intactness of the mitochondria membrane in the presence and absence of detergent.[13] Briefly, activity was assayed by resuspending an aliquot of the mitochondrial suspension (1 mg protein) in either 0.05% CHAPS or an equal volume of buffer, centrifuging at 7700*g*, and assaying the supernatant for citrate synthase activity. Intact mitochondria would exhibit no citrate synthase activity in the absence of detergent, but would show a substantial increase in activity upon disruption with detergent.

Mitochondrial measurements

Mitochondria were isolated at 0–4° C according to Mela and Seitz[14] with some modification. Briefly, hearts were removed and homogenized in isolation buffer consisting of 0.225 M mannitol, 0.075 M sucrose, 1 mmol/L EGTA and washed with buffer consisting of 0.225 M mannitol, 0.075 M sucrose. A protease (trypsin) was initially included with the hearts to insure a high yield of mitochondria with optimal respiratory control (acceptor control index) and P:O ratios[15]; each heart was placed in a homogenizer with 5 mL isolation buffer to which had been added 10 mg of trypsin. The heart was homogenized immediately on ice with three strokes and then quickly diluted with 15 mL more 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 discarded. The supernatant was collected and filtered through two layers of cheesecloth into two clean centrifuge tubes and centrifuged at 7700*g* for 10 min. The resulting pellet contained the mitochondria, which were carefully resuspended in isolation buffer without EGTA and centrifuged at 7700*g* for 5 min. The entire isolation procedure was complete within 1 hour, and mitochondria were resuspended in appropriate isolation buffer without EGTA. Mitochondrial protein measurements were made utilizing the BCA reagent kit from Pierce (Rockford, IL USA) using bovine serum albumin as a standard.

Isolation of kidney mitochondria was similar to that for heart mitochondria except that kidney cortex and medulla were separately treated, and no trypsin was used in the isolation buffer.

Isolation of liver mitochondria differed from that for heart in that no trypsin was used and the isolation buffer consisted of 0.25 M sucrose, 10 mmol/L Hepes, and 0.1 M EDTA.

Mitochondrial respiratory measurement values were determined at 30° C, polarographically, by the method of Estabrook.[16] The reaction medium was saturated with air and consisted of appropriate isolation buffer, 0.25 M sucrose, 200 mmol/L $MgCl_2$ and 200 mmol/L sodium phosphate buffer (pH 7.4). Substrates used were either succinate (10 mmol/L) or pyruvate/malate (10 mmol/L). Respiration states were defined according to Chance and Williams.[17,18] State 3 respiration was determined following addition of ADP to a final concentration of either 0.08 mmol/L for measurement of P/O ratio or 0.8 mmol/L for measurement of acceptor control index (ACI). State 4 respiration rate was measured in the presence of succinate or pyruvate/malate, but in the absence of ADP as the phosphate acceptor. Respiration was expressed as ng atoms oxygen consumed/min·mg protein. P:O ratio or ADP:O ratio is defined as the amount of ADP phosphorylated per unit of oxygen consumed. ACI was obtained by dividing the oxygen consumption (nmol of oxygen consumed per mg mitochondrial 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 mitochondrial membrane integrity.[18,19]

Statistics

An overall analysis of variance (ANOVA) was performed for unequal ns, with copper status serving as the independent variable. P/O, ACI, state 3 respiration, or state 4 respiration were the dependent variables. If an overall significant F value was obtained, univariate ANOVAs were performed to compare the copper-sufficient and the copper-deficient groups. 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*, in which several direct and indirect indices of copper status are listed. Copper concentration in serum, skeletal muscle, liver, and kidney were all significantly lower in CuD rats than in

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

	Diet	
	CuS	CuD
Body weight (g)	249 ± 4 (15)	225 ± 4 (15)†
Hematocrit	38 ± 1 (15)	24 ± 1 (15)†
Serum Cu (μg/mL)	0.53 ± 0.03 (15)	ND (15)†
Liver Cu (μg/g dry wt)	11.2 ± 0.5 (11)	1.3 ± 0.2 (11)†
Kidney Cu (μg/g dry wt)	21.6 ± 1.9 (4)	0.5 ± 0.2 (4)†
Skeletal muscle Cu (μg/g dry wt)	4.4 ± 0.2 (7)	0.8 ± 0.2 (6)†
Serum ceruloplasmin (mg/dL)	29 ± 2 (15)	5.3 ± 0.1 (15)†
Serum cholesterol (mg/dL)	105 ± 5 (15)	131 ± 5 (15)†
Total organ weight (g)		
Heart	1.2 ± 0.6 (8)	1.7 ± 0.1 (9)†
Liver	12.0 ± 0.6 (4)	13.3 ± 0.5 (4)
Kidney cortex	1.0 ± 0.2 (5)	1.0 ± 0.1 (5)
Kidney medulla	1.1 ± 0.1 (5)	1.4 ± 0.2 (5)
Mitochondrial yield (mg protein/g wet wt)		
Heart	20.0 ± 1.7 (8)	16.0 ± 1.8 (9)
Liver	2.5 ± 0.1 (4)	2.4 ± 0.2 (4)
Kidney cortex	29.7 ± 12.6 (5)	25.6 ± 3.1 (5)
Kidney medulla	31.6 ± 5.2 (5)	24.8 ± 3.7 (5)

Values are means ± SEM (number of samples).
ND Indicates variable is not detectable
†Indicates a significant difference ($P < 0.05$) from the corresponding CuS value.
‡Note that organ weights are given only for animals for which mitochondrial yield and respiratory measurements *(Table 2)* were made. Numbers of samples for these values are less than the total number of animals because (with a few exceptions) mitochondria from different organs were obtained from different rats. Also, organs used for mitochondrial respiration were not available for mineral analysis.

Table 2 Comparison of respiration rates, P/O ratio, and acceptor control index (ACI) in heart, liver, and kidney mitochondria from copper adequate (CuS) and copper deficient (CuD) rats

	Diet			
	CuS		CuD	
	Substrate			
	pyruvate/malate	succinate	pyruvate/malate	succinate
State 3 respiration (ng·atoms/min·mg)				
Heart	212 ± 13	259 ± 33	157 ± 11†	170 ± 12†
Liver	—	49 ± 1	—	33 ± 5†
Kidney cortex	50 ± 8	150 ± 21	83 ± 12	146 ± 15
Kidney medulla	59 ± 8	115 ± 9	61 ± 4	110 ± 11
State 4 respiration (ng·atoms/min·mg)				
Heart	49 ± 6	92 ± 15	39 ± 6	50 ± 7†
Liver	—	24 ± 3	—	24 ± 2
Kidney cortex	24 ± 1	76 ± 9	27 ± 3	61 ± 4
Kidney medulla	19 ± 2	52 ± 6	19 ± 1	48 ± 1
ACI (State 3/State 4 respiration)				
Heart	4.8 ± 0.6	3.0 ± 0.2	4.6 ± 0.6	3.6 ± 0.1
Liver	—	2.1 ± 0.2	—	1.4 ± 0.1†
Kidney cortex	2.1 ± 0.3	2.0 ± 0.2	2.9 ± 0.1	2.5 ± 0.4
Kidney medulla	3.2 ± 0.3	2.2 ± 0.2	3.2 ± 0.4	2.3 ± 0.2
P/O Ratio				
Heart	2.1 ± 0.2	1.6 ± 0.1	2.0 ± 0.2	1.6 ± 0.2
Liver	—	1.5 ± 0.2	—	1.3 ± 0.1
Kidney cortex	2.1 ± 0.4	1.5 ± 0.1	1.4 ± 0.6	1.4 ± 0.1
Kidney medulla	2.5 ± 0.2	1.5 ± 0.3	2.3 ± 0.2	1.4 ± 0.1

Values are means ± SEM (number of samples). Numbers of samples are the same as those listed for mitochondrial yields in *Table 1.*
Mitochondria were incubated with designated substrate as described in Materials and methods.
†Indicates a significant difference ($P < 0.05$) from the corresponding CuS tissue using the same substrate.

CuS rats. Activity of serum ceruloplasmin, a copper-dependent enzyme, was also lower in CuD rats. Enlarged hearts, anemia, and hypercholesterolemia, characteristics of animals severely deficient in copper, were also evident in CuD rats.

Mitochondrial respiration

Variables related to mitochondrial respiratory function in hearts, livers, and kidneys are listed in *Table 2*. Mitochondria from hearts of CuD rats showed significantly depressed state 3 respiration rates relative to hearts of CuS rats when either succinate or pyruvate/malate was used as substrate. State 4 respiration rate was also significantly depressed when succinate was used as substrate; a nonsignificant tendency toward depression of state 4 respiration with pyruvate/malate was observed. Neither ACI nor P/O ratio was affected with either substrate.

Mitochondria from livers of CuD rats showed significantly depressed state 3 respiration rates relative to hearts of CuS rats with succinate as substrate. Pyruvate/malate was not used. State 4 respiration and P/O ratio were not affected by copper status. Because there was a significant decrease in state 3 but not in state 4, ACI was significantly decreased in copper-deficient livers.

Copper status appeared to have no effect on mitochondrial function in kidney medulla or cortex.

Cardiac mitochondria appear to be the most structurally affected by copper deficiency, and therefore the intactness of the membrane is a consideration when studying function. If the mitochondria from copper-deficient hearts are more fragile and therefore more subject to disruption from the isolation procedure, this could account for any functional differences that might be observed. Citrate synthase is considered to be the best marker enzyme for mitochondria matrix. If mitochondria are completely intact, little or no citrate synthase activity can be detected in the supernatant fraction.[20] Mitochondria were therefore assayed for citrate synthase activity in intact and detergent-disrupted fractions as described in materials and methods. Results of these studies indicate no difference in citrate synthase activity in the nondetergent-treated cardiac mitochondrial supernatant from either CuD or CuS rats *(Figure 1A)*. Citrate synthase activity was increased approximately five-fold in both CuS and CuD in the presence of 0.05% CHAPS. In addition, the amount of protein released into the supernatant of the same mitochondrial fractions from CuS and CuD was not different *(Figure 1B)* in the absence of detergent.

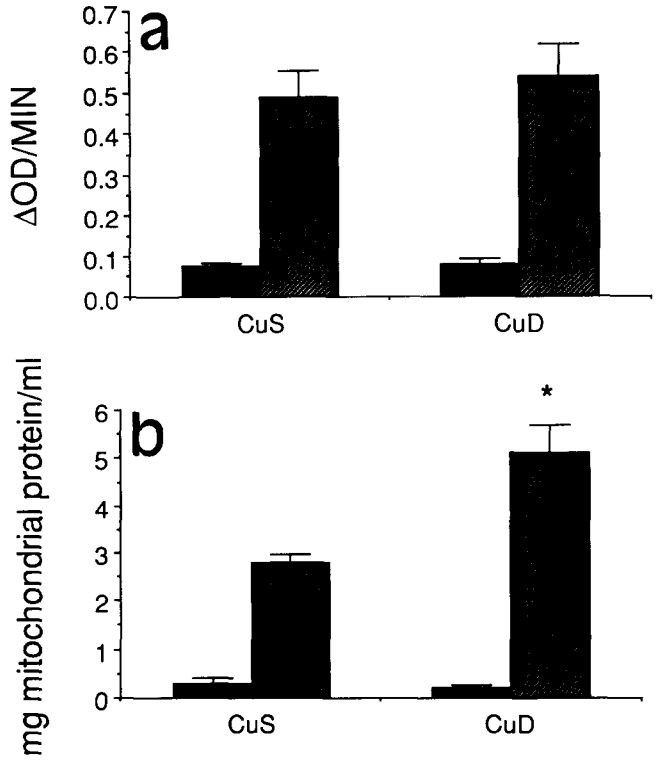

Figure 1 (a) Represents total citrate synthase activity as the change in OD/min in supernates prepared as described in Materials and methods. No significant difference was observed between copper deficient and copper adequate total citrate synthase activity ($P > 0.05$). Total citrate synthase activity of both groups was simulated approximately fivefold with the addition of detergent. (b) Represents protein concentration in supernates prepared as described in Materials and methods. No significant difference was observed in the amount of protein present in the supernate with 0% CHAPS. However, almost twice as much protein was released from copper-deficient mitochondria when disrupted with 0.05% CHAPS ($^*P < 0.05$).

Discussion

Previous research showing severe structural changes in cardiac mitochondria from copper-deficient rats has not shown a corresponding change in mitochondrial respiration or phosphorylation ability.[1] However, cytochrome oxidase activity and concentration have been shown to decrease in hearts from copper-deficient rats.[1,2] In addition, one group observed decreased succinate dehydrogenase activity in mitochondria from copper-depleted rat hearts.[4] The present results suggest that cardiac mitochondrial function, as determined by state 3 and state 4 respiration rates, is altered in copper deficiency, and the decreased respiration rate is related to a defect in respiratory control brought about by copper deficiency. No change in ACI was observed because of the decreases in both respiration states. Phosphorylation ability appears normal, which is in agreement with past studies.[1] Because mitochondrial yield appeared similar in both groups, the question may arise as to why there are more disrupted mitochondria present in the copper-deficient group. However, the citrate synthase data *(Figure 1A, 1B)* strongly indicate that both populations of mitochondria are similar in intactness. In addition, phosphorylation ability (P/O ratio) is unimpaired by copper deficiency, indicating that oxidation is still coupled to phosphorylation.

In liver mitochondria, a consistent effect of copper deficiency on function has not been observed.[7,21] Some

investigators[21] concluded that copper deficiency had no effect on liver mitochondrial respiration rates in the presence of succinate, glutamate, or ascorbate plus tetramethylphenylenediamine. More recently,[7] a marked decrease in state 3 respiration was observed with succinate, glutamate/malate, and β-hydroxybutyrate as substrates. State 4 respiration appeared relatively unaffected by copper deficiency, the result of which was a decreased ACI. This group also observed a decrease in succinate cytochrome c reductase and NADH cytochrome c reductase, as well as cytochrome c oxidase after 8 weeks of copper deficiency. The present study agrees most closely with the work of Davies, et al.[6] in that a depression of state 3 respiration was observed with succinate as substrate, but no difference was observed in state 4 respiration.

At least one study has investigated the effect of copper deficiency on kidney structure.[8] This group observed no abnormalities in mitochondrial structure, and suggested that cytochrome oxidase activity in the rat kidney is conserved in copper deficiency although others have observed somewhat decreased activity.[6] However, they observed lesions in the convoluted tubules and loop of Henle. Data from the present studies appear to support the work of Fell, et al.[8] in that no differences in mitochondrial respiratory function or phosphorylation ability were observed with copper deficiency.

The mechanisms of alterations in mitochondrial respiratory function in copper deficiency need to be investigated further. Direct effects on copper-dependent cytochrome c oxidase may play a role in altered respiration. In addition, indirect effects may be the result of damage to other respiratory enzymes by oxygen-derived reactive species,[22] which is supported by observed reduced activity of copper-dependent antioxidant enzymes such as serum ceruloplasmin and tissue Cu, Zn superoxide dismutase.[23] Evidence for this suggestion is the observation that dimethyl sulfoxide, a hydroxyl radical scavenger, can ameliorate structural damage to mitochondria caused by copper deficiency.[24] That mitochondria from various tissue appear to be affected differently by copper deficiency, both in terms of structural and functional changes, is of interest. More work is needed to determine why these differences occur.

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