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Impaired Cardiac Mitochondrial Membrane Potential and Respiration in Copper-Deficient Rats

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Cardiac mitochondrial respiration, ATP synthase activity, and membrane potential and intactness were evaluated in copper-deficient rats. In the presence of NADH, both copper-deficient and copper-adequate mitochondria had very low oxygen consumption rates, indicating membrane intactness. However copper-deficient mitochondria had significantly lower oxygen consumption rates with NADH than did copper-adequate mitochondria. Copper-deficient mitochondria had significantly lower membrane potential than did copper-adequate mitochondria using fluorescent dyes. Copper-deficient mitochondria had significantly lower state 3 oxygen consumption rates and were less sensitive to inhibition by oligomycin, an ATP synthase inhibitor. Copper-deficient and copper-adequate mitochondria responded similiarly to CCCP. No difference was observed in mitochondrial ATPase activity between copper-deficient and copper-adequate rats using submitochondrial particles. We conclude that cardiac mitochondrial respiration is compromised in copper-deficient rats, and may be related to an altered ATP synthase complex and/or a decreased mitochondrial membrane potential.

KEY WORDS: Copper deficiency; mitochondria; ATP synthase; oligomycin; membrane potential; heart.

INTRODUCTION

Dietary copper depletion results in cardiac hypertrophy and ultrastructual pathology. Copper-deficient rats have enlarged cardiac myocytes, which are characterized by increased mitochondria volume density and mitochondria–myofibril ratio with mitochondria exhibiting vacuoles and disrupted cristae (Medeiros *et al.*, 1991a,b). It has been well established that copper deficiency results in decreased activity of cytochrome *c* oxidase (CCO), a cuproenzyme, which is a key enzyme in the mitochondrial electron transport chain (Chao *et al.*, 1993). Medeiros *et al.* (1997) reported significant decreases in the protein levels of nuclear encoded subunits of CCO in copper deficient rats, but no detectable changes in the mitochondrially encoded subunits. However the mRNA levels of the nuclear encoded subunits were not altered in copper deficient rats. One explanation for this may be that the nuclear encoded subunits are unable to be transported across the mitochondrial membrane and thus degraded in the cytosol. Protein translocation across the mitochondrial membrane requires an electrochemical gradient and the gross morphological changes in mitochondria caused by the copper deficiency may negatively affect this potential.

In addition to CCO, Chao *et al.* (1994) also reported changes in ATP synthase, the enzyme adjacent to CCO that catalyzes the conversion of ADP to ATP, in copper deficient rats. In particular they noted that there was a decreased amount of δ subunit in copper deficient rats. A subsequent study (Mao *et al.*, 2000) revealed decreased level of the β subunit mRNA in copper deficient animals. Matz *et al.* (1995) did not find any changes in the rate of ATP hydrolysis in copper deficient rats but they did report that oligomycin, an ATP synthase inhibitor, had less inhibitory effect upon ATP hydrolysis in copper deficient animals.

According to Mitchell's chemiosmotic theory, the electron transport chain pumps protons across the mitochondrial inner membrane and this establishes a proton gradient. This gradient is essential for ATP formation.

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Protons reenter the matrix through F_1F_0-ATP synthase. The electron transport chain must be tightly coupled to ATP synthase in order for ATP to be synthesized. The mitochondria respiratory control ratio (RCR), which is expressed as the ratio of state 3 (oxygen consumption in the presence of ADP) and state 4 (oxygen consumption when ADP is limiting), can indicate how tight the coupling is. Both Chao *et al.* (1993) and Matz *et al.* (1995) reported decreased states 3 and 4 respiration rates, but Chao *et al.* also reported decreased RCR. One unresolved question is whether these changes in mitochondrial respiration are due to impairments in CCO and/or ATP synthase function. Alternatively, since the mitochondria are structurally compromised (Medeiros *et al.*, 1991a,b), the question arises as to whether these previously reported observations are due to compromised membrane intactness. Mitochondrial respiration in the presence of NADH was used to evaluate membrane intactness. In addition, we used membrane potential-sensitive dyes to determine if there are changes in the mitochondrial membrane potential associated with copper deficiency. As to whether CCO and/or ATP synthase are impaired, it is necessary to separate the functions of these enzymes as they relate to copper deficiency. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) can conduct protons across the mitochondria membrane by bypassing F_1F_0-ATP synthase. This leads to collapse of the electrochemical gradient, and uncoupling of oxygen consumption by CCO from ATP formation by F_1F_0-ATP synthase. The result is a measure of CCO function. On the other hand, oligomycin binds to ATP synthase and blocks the formation of ATP. To determine where potential alteration in oxidative phosphorylation exists in copper deficiency, mitochondrial respiration studies using oligomycin and CCCP were conducted. To further test the effect of copper deficiency on ATP synthase, we examined ATP hydrolysis in the presence and absence of oligomycin.

METHODS AND MATERIALS

General Procedure

The protocol used was approved by the Kansas State University Institutional Animal Care and Use Committee. Male weanling Long-Evans rats were purchased from Charles River Laboratory. Upon arrival, the rats were housed singly in stainless steel cages with 12-h L:D cycle at 22°C. In the first study, the function of mitochondria respiration and ATP synthase were evaluated. Fifteen rats were randomly assigned to two dietary treatment groups (copper-adequate, $n = 7$; and copper deficient, $n = 8$) and fed the diets for 5 weeks. In the second study, mitochondrial membrane potential was tested in six rats randomly assigned to two treatment groups (copper-adequate and copper deficient, $N = 3$ in each group) for 5 weeks. Dietary copper levels were verified by flame atomic absorption spectrophotometry. The copper-adequate diet contained 7.19 mg Cu/kg (0.11 mmol/kg) diet, and the copper deficient diet contained 0.78 mg Cu/kg (0.01 mmol/kg) diet. All rats were given free access to food and deionized–distilled water and weighed weekly. At the completion of the 5-week period, rats were anesthetized by injecting with 65 mg/kg pentobarbital intraperitoneally. The thoracic cavity was opened and a small volume of blood was obtained from the heart by cardiac puncture and used for hematocrit determination. Hearts were quickly removed and kept in isolation buffer at 0°C. Livers were removed and frozen at −20°C and later assayed for Zn–Cu superoxide dismutase (SOD) activity.

Isolation of Intact Mitochondria From Heart

Mitochondria were isolated according to the protocol from Mela and Seitz (1979) with some modifications. The mitochondrial isolation buffer consisted of 0.225 M mannitol, 0.075 M sucrose, 20 mM HEPES, 1 mM EGTA, and 0.5 mg/mL BSA, pH 7.4. After weighing, the hearts were chopped in isolation buffer, and the chopped tissue was suspended in 10 mL of isolation buffer followed by addition of 10 mg Nargase. The finely minced tissue was homogenized with three strokes in loose-fitting glass Teflon homogenizer at 150 rpm and then immediately diluted to 30 mL with isolation buffer. The homogenate was centrifuged at 1000*g* for 5 min. The resulting supernatant was filtered through two layers of cheesecloth and centrifuged at 7700*g* for 10 min. A light fluffy layer, consisting of membrane fragments, surrounding the pellet, was carefully discarded using a brush. The dark brown pellet was saved and gently resuspended in 30 mL isolation buffer and centrifuged at 7700*g* for 10 min. The final mitochondria pellet was suspended in 400 μL isolation buffer. Protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratory, Hercules, CA), and protein concentrations were adjusted to 6–8 μg protein/μL.

Submitochondrial Particle Preparation

Submitochondrial particles were prepared according to the protocol of Catterall and Pederson (1971). An aliquot of 200 μL of mitochondria suspension obtained above was diluted in 4 mL of isolation buffer and subjected to sonic oscillation at 50% maximal intensity for a

total of 2 min in 10-s intervals at 0°C. After sonication, the volume of the submitochondrial solution was adjusted to 10ml with isolation buffer and centrifuged at speed of 150,000*g* for 45 min at 0°C. The submitochondrial pellet was resuspended in 200 μL of isolation buffer and protein concentration adjusted to 6–8 μg protein/mL and used for an ATP hydrolysis assay.

Mitochondrial Respiration and Membrane Intactness Assay

Air-saturated respiration buffer (225 mM sucrose, 10 mM KH_2PO_4, 20 mM KCl, and 5 mM $MgCl_2$, pH 7.4) was added to a sample oxygen monitor chamber (YSI Model 5300 biological Oxygen Monitor, Yellow Springs Instrument Co., Yellow Springs, OH) in the presence of 10 mM glutamate and 10 mM malate as substrate and equilibrated to 30°C with a magnetic stirrer. The final volume was 3.0 mL. After reaching constant temperature, the monitor was set to 100%. Mitochondria were added to the sample chamber and reached a final concentration of 66.7 μg/mL. A slow rate of oxygen uptake was detected by an oxygen probe (YSI 5338 "O" Ring Applicator) in the presence of substrate and absence of ADP. State 3 respiration was recorded following addition of ADP to a final concentration of 0.067 mM. State 4 respiration appeared when ADP became limiting. The ratio of state 3 and state 4 oxygen consumption rates was expressed as RCR. Mitochondrial respiration in the presence of oligomycin and CCCP was also studied. After ADP induction of state 3 respiration, oligomycin was added to a final concentration of 0.07 mg/mL and oxygen consumption rate was recorded. The effect of CCCP upon mitochondrial respiration was tested by adding CCCP to a final concentration of 0.08 μM into the respiration buffer containing substrate and mitochondria.

Mitochondrial membrane intactness was assessed by measuring respiration in the presence of NADH. NADH was added to a final concentration of 90 mM into the mitochondria respiration buffer in the presence of mitochondria and oxygen consumption rate was recorded.

ATP Hydrolysis Measurement

Hydrolysis of ATP was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions (Pedersen *et al.*, 1981). The submitochondrial suspension (150 μg protein) was added to 1 mL of reaction mixture (4 mM ATP, 65 mM Tris-Cl, 4.8 mM $MgCl_2$, 2.5 mM KPi, 0.4 mM NADH, 0.6 mM phosphoenolpyruvic acid, 5 mM KCN, 1 unit of lactic dehydrogenase, 1 unit of pyruvate kinase). The decreasing rate of absorbance at 340 nm was recorded, which represents the rate of oxidation of NADH to NAD^+. The effect of oligomycin was studied by adding 2.5 μg/mL oligomycin to the reaction system. ATPase specific activity was calculated by the formula: specific activity = (ΔA340 nm/min)/(6.2 $\times$ mg protein/mL reaction mixture).

Fluorometric Analysis of Membrane Potential

Mitochondria were prepared as described earlier and suspended in the isolation buffer at 8 μg protein/μL. Each mitochondria sample was used for both fluorescence measurement and microscope analysis. RCR was determined for each sample using methods described earlier.

Mitochondria were incubated in 200 μL succinate–rotenone buffer (120 mM KCl, 10 mM Hepes, 5 mM succinate, 1 mM potassium phosphate, and 1 μM rotenone; pH 7.2.) at 0.3 mg protein/mL at room temperature. JC-1 dye was added to a final concentration of 0.3 μM. JC-1 dye was excited at 490 nm, and fluorescent emission was monitored at 530 nm and 590 nm using a fluorescence spectrophotometer (Turner Quantech^{TM} Digital Filter Fluorometer, Model FM 109535, Barnstead Thermolyne, Dubuque, IA). The maximum emission light intensity was set by using 1.2 μM JC-1 dye in DMSO at 530 nm as the standard and then subsequent emission was expressed as relative fluorescence intensity.

Mitochondrial membrane potential was also measured using rhodamine 123, another membrane potential probe. Rhodamine123 was added to the succinate-rotenone buffer to a final concentration of 1 mM. Dye was excited at 490 nm and emission monitored at 530 nm. Mitochondria were added to a final concentration of 0.3 mg protein/mL.

For microscopy, a Zeiss LSM 410 microscope with a 40 $\times$ 11.3 objective lense was used. Filters used included a KP600 line filter, BP 515-540 (Green filter) and LP590 (Red filter). Images were recorded and stored as digital files.

Liver SOD Activity Assay

Liver SOD activity was measured by monitoring inhibition of the oxidation of pyrogallol as described by Marklund and Marklund (1974) and modified by Prohaska (1983). One unit of SOD activity was the amount of sample needed to inhibit the reaction of pyrogallol oxidation by 50%.

Statistical Analysis

Data were analyzed by a one-way ANOVA using the General Linear Models procedure of Statistical Analysis System (SAS Institute, Cary, NC). The 0.05 level of significance was set as the critical value for analysis of appropriate data. All values were expressed as mean ± SEM.

RESULTS

Copper Status

Indices of copper status in both copper-adequate and copper deficient rats are summarized in Table I for rats used in the mitochondrial respiration, membrane intactness, and the ATP hydrolysis studies, and for the mitochondrial membrane potential studies. For both sets of studies, the copper deficient animals had significantly lower body weights than did copper-adequate animals ($p < 0.05$). The heart weights of copper deficient rats were significantly higher than those of copper-adequate rats ($p < 0.05$). The ratio of heart-to-body weight was also significantly increased in the copper deficient group ($p < 0.05$). Liver superoxide dismutase (Zn, Cu–SOD) activities from copper-adequate rats were much higher than those from copper deficient rats ($p < 0.05$). These findings indicate that rats fed a copper-deficient diet for 5 weeks were deficient in copper.

Membrane Intactness Assay

NADH does not directly penetrate the intact mitochondrial membrane, but it can be transferred into mitochondria by substrate shuttles at very low levels. A low rate of mitochondrial respiration in the presence of NADH is often used as an indicator of the intactness of the mitochondrial membrane (Richwood *et al.*, 1987). In this study, in the presence of NADH, mitochondria had a very low oxygen consumption rate in both copper deficient animals (81.8 nmol O/min/mg) and copper-adequate animals (143.7 nmo O/min/mg) (Fig. 1). This indicates that the inner membrane of the mitochondria from both groups were intact. Interestingly baseline oxygen consumption rates and oxygen consumption in the presence of NADH in the copper-adequate rats were significantly higher than those in the copper deficient rats (Fig. 1).

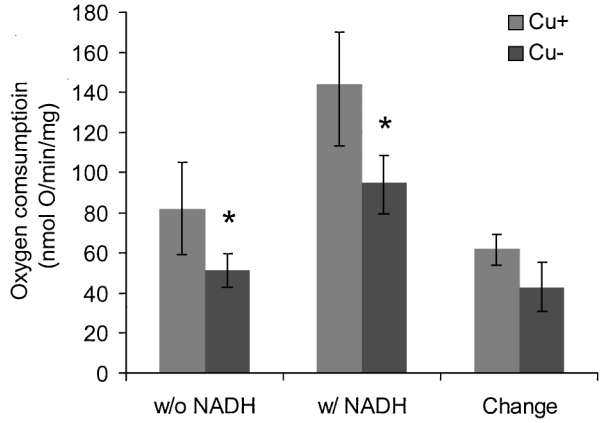

Fig. 1. Mitochondrial respiration in the presence of NADH in Cu^+ ($n = 7$) and Cu^- ($n = 8$).Mitochondrial respiration rate was recorded before (without NADH) and after adding NADH (with NADH). There were very low oxygen consumption rates in both Cu^+ and Cu^- in the presence of NADH (90 mM), which indicated good membrane integrity in both Cu^+ and Cu^- cardiac mitochondria preparations.The copper-adequate group had a significantly higher oxygen consumption rate in the presence of NADH (means ± SE, $*p < 0.05$).

Table I. Indices of Copper Deficiency in Copper-Adequate (Cu^+) and Copper-Deficient (Cu^-) Rats

Variables	Cu^+ ($n = 7$)	Cu^- ($n = 8$)
Mitochondrial respiration, membrane intactness, and ATP hydrolysis studies		
Final body weight (g)	291 ± 10.7	277 ± 11.1[a]
Heart weight (g)	0.98 ± 0.04	1.38 ± 0.09[a]
Heart/body weight ($\times 10^{-3}$)	3.38 ± 0.05	4.96 ± 0.22[a]
Liver SOD activity (units/g liver)	12060 ± 677 ($n = 3$)	3051 ± 69[a] ($n = 3$)
Mitochondrial membrane potential study		
Final body weight (g)	302 ± 21.2	227 ± 9.0[a]
Heart weight (g)	1.15 ± 0.07	1.68 ± 0.46[a]
Heart/body weight ($\times 10^{-3}$)	3.79 ± 0.03	7.39 ± 1.08[a]
Liver SOD activity (units/g liver)	12519 ± 775	3982 ± 953[a]

*Note.*Values are mean ± SEM.
[a]Significant difference ($p < 0.05$) between Cu^+ and Cu^-.

Mitochondrial Respiration

Evaluation of mitochondrial respiration is illustrated in Fig. 2 by representative traces. The addition of ADP (0.067 mM) to the mitochondria and substrate mixture induced rapid oxygen consumption, state 3 respiration. The state 3 respiration rate was 20% lower in the copper deficient animals than in controls. When ADP became limiting, state 4 respiration,the mitochondrial respiration rate decreased. There was no significant difference ($p > 0.05$) with regard to state 4 respiration rate by treatment. The respiratory control ratio (state 3 respiration rate divided by state 4 respiration rate) decreased slightly in the copper deficient rats, but was not significantly different ($p > 0.05$). The addition of oligomycin inhibited mitochondrial respiration in both sets of rats but the inhibitory effect in the copper deficient rats was significantly lower than that in copper-adequate rats (Fig. (2b) and (3a)). When mitochondria were treated with CCCP, oxygen consumption rate increased dramatically in both groups, but there was no significant difference ($p > 0.05$) between copper-adequate and copper deficient rats (Fig. 3b).

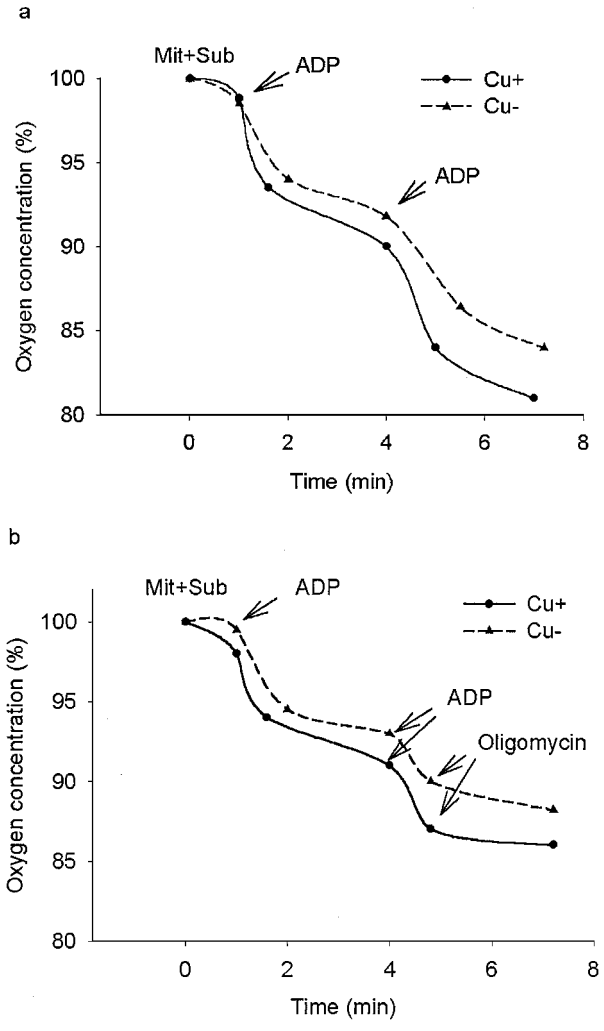

Fig. 2. Cardiac mitochondrial respiration in Cu^- ($n = 7$) and Cu^+ rats ($n = 8$) (representative traces) in the absence (a) and presence (b) of oligomycin. In panel a, state 3 was induced by adding ADP to a final concentration of 0.067 mM. It was followed by state 4 when ADP became limited. Cu^- rats had depressed state 3 respiration. In panel b, in the process of state 3, the addition of oligomycin (0.07 mg/mL) decreased the oxygen consumption rate in both Cu^+ and Cu^- rats.The inhibitory effect of oligomycin was less in Cu^- than Cu^+ in mitochondria. (Mit, mitochondria; Sub, substrate).

Hydrolysis of ATP

ATP hydrolysis in submitochondrial particles from copper deficient hearts was not significantly different ($p > 0.05$) from that of copper-adequate hearts (Fig. 4). The addition of oligomycin produced significant inhibition ($p < 0.05$) in the rates of ATP hydrolysis, but there was no significant difference ($p > 0.05$) observed for oligomycin sensitivity between copper deficient and copper-adequate rats.

Fluorometric Analysis of Mitochondrial Inner Membrane Potential

JC-1 is a membrane potential-sensitive probe that accumulates in energized mitochondria and subsequently forms J-aggregates from monomers. The depolarization of the mitochondrial membrane is associated with an increase in monomer fluorescence at 535 nm, as well as a decrease in the fluorescence of J-aggregates at 590 nm (Lisa *et al.*, 1995 and Reers *et al.*, 1991). The ratio between 530 nm and 590 nm emission can be used as an indicator of the mitochondrial inner membrane potential (Nuydens *et al.*, 1999). Mitochondria from copper deficient rats had significantly higher 530:590 ratios than those of the copper-adequate rats (Fig. (5a)) ($p < 0.05$), which indicated decreased membrane potential in copper deficient animals.

Rhodamine 123, another membrane potential-sensitive probe (Johnson *et al.*, 1980), was utilized as a secondary measure of membrane potential changes. The percentage of quenching of the fluorescence at 530 nm was used as measure of mitochondrial membrane potential. In copper deficient rats, the quenching rate (24%) was significantly lower than that of copper-adequate rats (34%) (Fig. (5b)) ($p < 0.05$).

The same mitochondrial suspensions, stained with JC-1 and rhodamine 123, were visualized by microscopy

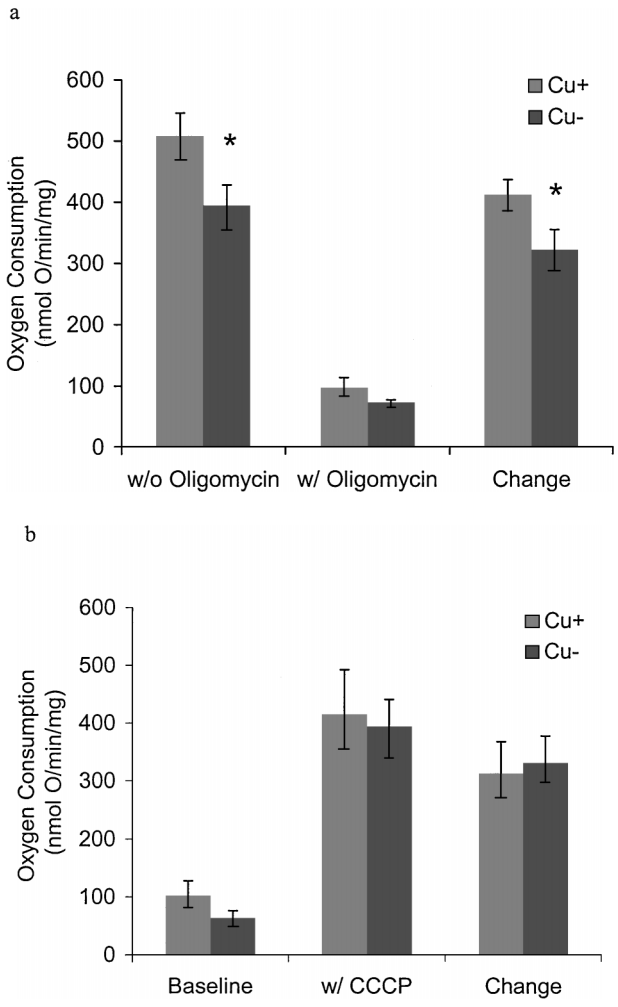

Fig. 3. The effect of oligomycin (a) and CCCP (b) on mitochondrial respiration in Cu^+ ($n = 7$) and Cu^- ($n = 8$) rats. In panel a, mitochondrial state 3 respiration rate was recorded before (without oligomycin) and after adding oligomycin (with oligomycin) in Cu^+ and Cu^- rats.The change was calculated by subtracting the mitochondrial oxygen consumption rate in the presence of oligomycin from that in the absence of oligomycin. The Cu^- rats had decreased state 3 respiration and were less sensitive to oligomycin inhibition (mean $\pm$ SE, $*p < 0.05$). In panel b, baseline respiration in the absence of ADP was recorded.The addition of CCCP (0.08 μM) induced increased mitochondrial respiration (with CCCP).The change was calculated by subtracting the baseline respiration rate from the respiration rate in the presence of CCCP.The effect of CCCP was not significantly different between Cu^+ and Cu^- rats. (Mean $\pm$ SE, $p > 0.05$).

(Fig. 6). In the presence of JC-1 dye, green fluorescence indicated the presence of JC-1 monomers and red fluorescence indicated the presence of JC-1 aggregates in mitochondria. The orange region was where both green and red fluorescence coexisted. Copper adequate mitochondria samples exhibited more red fluorescence than did copper deficient mitochondria samples (Fig. 6(c) and 6(d)). Correspondingly copper deficient mitochondria had more green fluorescence. This indicates a decrease in the mitochondrial membrane potential of copper deficient mitchondria.

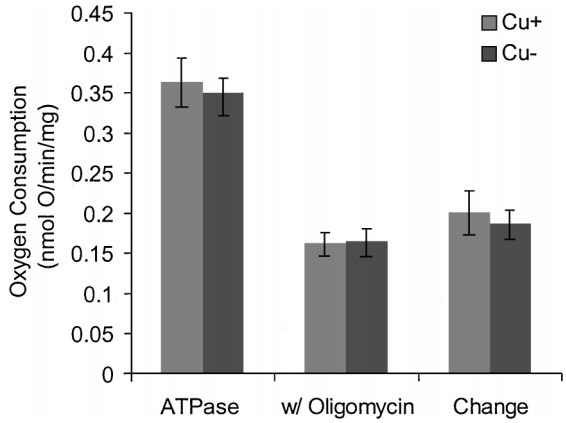

Fig. 4. ATP hydrolysis and the effect of the oligomycin on Cu^+ and Cu^- rats. Mitochondrial ATPase activity was determined in the absence and presence of oligomycin.Change was calculated by subtracting the ATPase activity in the presence of oligomycin from that in the absence of oligomycin.There was no difference between Cu^+ and Cu^- with regard to ATPase activity in the presence or in the absence of oligomycin, nor was there any indication of altered oligomycin sensitivity (means $\pm$ SE, $p > 0.05$).

Mitochondria with "healthy" membrane potentials take up and sequester rhodamine123 ("quench" it). This results in bright red mitochondria when visualized by microscopy. If the mitochondria lack or have poor membrane potentials, they do not take up rhodamine 123, and the whole slide is a dull red. In this study, in the presence of rhodamine 123, copper-adequate mitochondria samples exhibited more red fluorescence (Fig. 6(e)) than did copper deficient mitochondria samples (Fig. 6(f)). Again this indicates that copper deficient mitochondria have lower membrane potentials than do copper-adequate mitochondria.

To confirm the effectiveness of the JC-1 dye and rhodamine123 as mitochondrial membrane potential probes, control mitochondria were de-energized by CCCP after being stained by JC-1 and rhodamine123. In the presence of CCCP, mitochondria stained with JC-1 dye exhibited little-to-no red fluorescence (Fig. 6(a)) compared to mitochondria that were not treated with CCCP (Fig. 6(b)). De-energized mitochondria also exhibited less rhodamine123 quenching when compared to untreated controls (data not shown).

DISCUSSION

Previous studies have demonstrated that cardiac mitochondria from rats fed a copper-deficient diet have

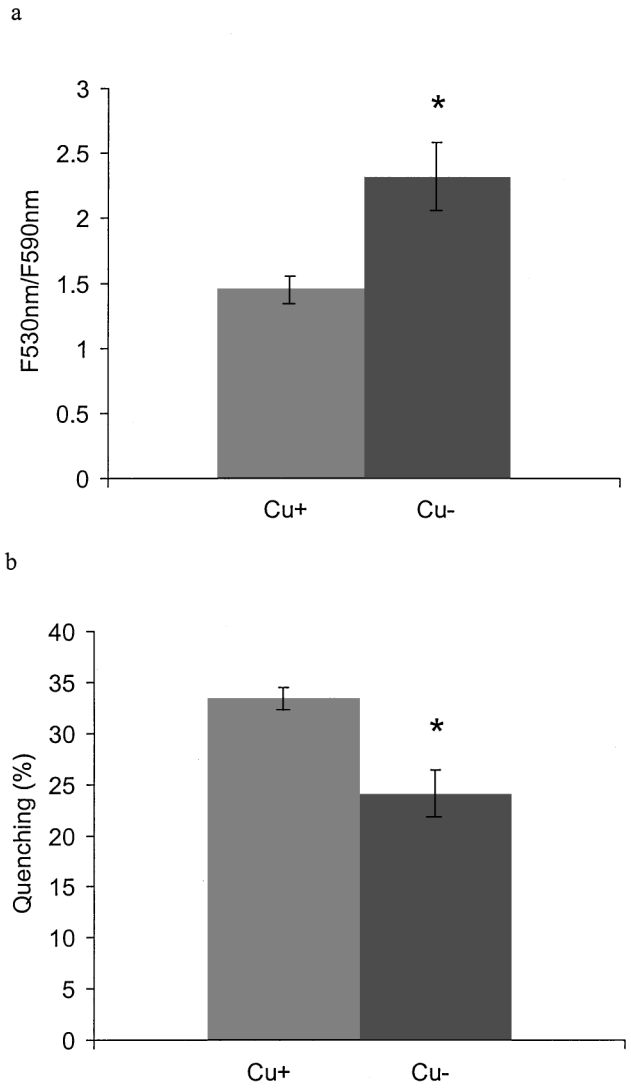

Fig. 5. Evaluation of mitochondrial membrane potential using JC-1 dye (a) and rhodamine 123 (b) in Cu^+ ($n = 3$) and Cu^- ($n = 3$) rats. JC-1 dye was excited at 490 nm and fluorescence at 530 nm and 590 nm were recorded. The ratio of the fluorescence at 530 nm to the fluorescence at 590 nm(F530 nm/F590 nm) was used as an indicator of the membrane potential. Cu^- mitochondria had significantly higher ratios ($*p < 0.05$), indicating lower membrane potentials compared with Cu^+ mitochondria. In panel b, rhodamine 123 was excited at 490 nm, and fluorescence at 530 nm was recorded. After adding mitochondria (0.3 mg/mL), rhodamine 123 fluorescence dropped because of the uptake of rhodamine 123 by mitochondria. The drop in fluorescence value at 530 nm was divided by the value before adding mitochondria and was referred to as quenching.The mitochondria from Cu^+ rats had significantly higher quenching than did the mitochondria from Cu^- rats, again indicating a decrease in mitochondrial membrane potential in Cu^- rats.

impaired mitochondrial respiration (Chao *et al.*, 1993; Matz *et al.*, 1995) and altered membrane pathology (Medeiros *et al.*, 1991a,b). Concern with overall mitochondria membrane intactness in hearts of copper-deficient rats prompted us to utilize NADH to evaluate mitochondria inner membrane intactness. Both copper-adequate and copper deficient mitochondria had very low oxygen consumption rates in the presence of NADH. This indicated good membrane intactness in both groups. Surprisingly, the copper-adequate mitochondria had a significantly higher oxygen consumption rate than did copper deficient mitochondria. One explanation for this unexpected result may be that the function of the substrate shuttle for NADH is impaired in copper deficient mitochondria, due to a change either in the shuttle itself or in membrane potential.

Mitochondrial membrane potential is a vital component of respiring mitochondria and is linked to a host of mitochondrial functions including import of nuclear encoded mitochondrial proteins and translocation of some metabolites and drugs. Utilizing membrane potential-sensitive dyes, we have shown in this study that copper deficient mitochondria had significantly lower membrane potential compared to control mitochondria. This decrease is mitochondrial membrane potential may impair the tanslocation of proteins and other substances into mitochondria, providing an explanation as to why the protein level of the nuclear encoded subunits of CCO are decreased in copper deficient rats, but the mRNA levels of the subunits do not differ (Medeiros *et al.*, 1997). It may be that in copper deficient rats, the nuclear encoded subunits of CCO are synthesized in the cytosol but cannot be translocated into the mitochondria, resulting in their subsequent degradation in the cytosol as suggested by the findings of Medeiros *et al.* (1997).

In this study, state 4 and state 3 respiration rates decreased, with state 3 respiration rate significantly lower in copper deficient rats than that in copper-adequate rats. RCR, a measure of the degree of coupling of phosphorylation to oxidation, did not differ by group, which is in agreement with results reported by Matz *et al.* (1995), but in contrast to that reported by Chao *et al.* (1993) in which significantly lower RCR values were observed in copper deficient rats. These inconsistent data might be due to different mitochondria preparation conditions used between these studies. First, Chao *et al.* (1993) reported much lower RCR values in both controls (3.6) and copper-deficient (2.6) rats than did the present study, where they were around 5. Second, we used mannitol in the current study in preparation of isolated mitochondria, whereas Chao *et al.* (1993) did not. On the basis of our present study, the coupling of phosphorylation to oxidation in copper deficient rats appeared intact.

To identify the possible mechanisms within the oxidative-phosphorylation pathway for the observed changes in cardiac mitochondrial respiration, respiration

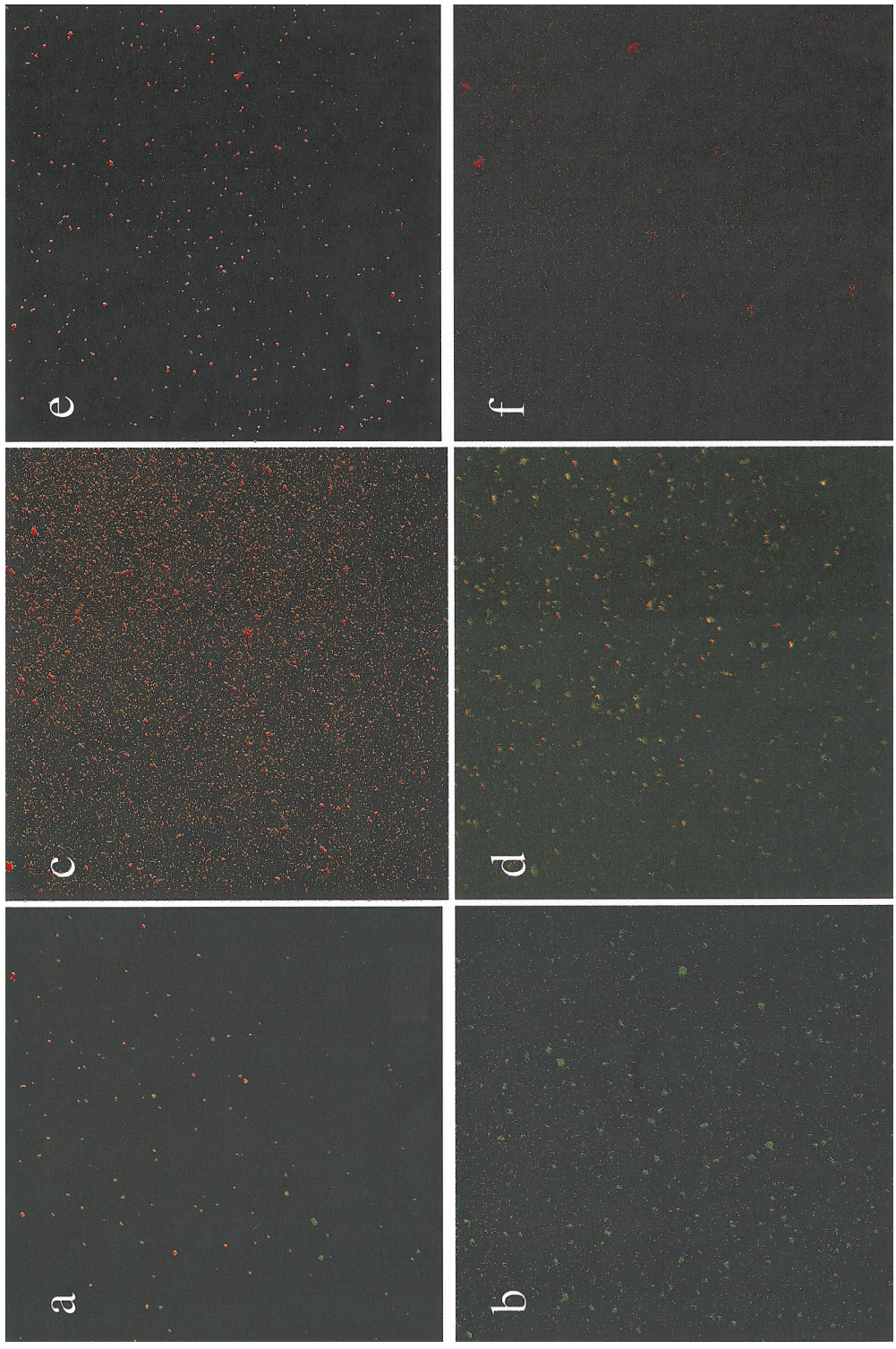

Fig. 6. Fluorescence microscope analysis of mitochondrial membrane potential using JC-1 dye (a–d) and rhodamine 123 (e,f) in Cu^+ ($n = 3$) and Cu^- ($n = 3$) rats. Panel a: control mitochondria (0.3 mg protein/mL) were stained with JC-1 dye (1 μM) in the absence of CCCP. Panel b: mitochondria from control rats were stained with JC-1 dye (1 μM) in the presence of CCCP (2 μM). Red fluorescence represents mitochondria with high membrane potential. Green fluorecence represents mitochondria with low membrane potential. The addition of CCCP deenergized the mitochondria and red fluorescence disappeared almost completely. In the presence of JC-1 dye, mitochondria from Cu^+ rats (panel c) exhibited more red fluorescence than did mitochondria from Cu^- rats (panel d). In the presence of rhodamine123 (1 mM), red dots represents the mitochondria with sequestered rhodamine123.Mitochondria from Cu^+ rats (panel e) exhibited more red fluorescence than did mitochondria from Cu^- rats (panel f).These findings indicate lower mitochondrial membrane potential in Cu^- rats.

was studied using two drugs, CCCP and oligomycin. As mentioned previously, CCCP allows protons to cross the mitochondrial inner membrane, thereby disrupting the proton gradient and dissociating oxidation in the respiratory chain from phosphorylation, thus allowing the function of the electron transport chain to be evaluated separately from potential changes in ATP synthase. Mitochondrial respiration did not differ between copper deficient and copper-adequate groups in the presence of CCCP, suggesting that the potential mitochondrial respiration defect in copper deficiency might not lie within the electron transport chain. The classical explanation for the impaired mitochondrial function in copper deficiency has been the reduced activity of cuproenzyme CCO (Chao *et al.*, 1994),the terminal enzyme complex of the electron transport chain. While CCO activity was not measured in this study, it has been reported to be decreased in copper deficiency (Chao *et al.*, 1993). On the basis of this study, a CCO defect did not appear to significantly compromise the electron transport chain function which suggests that other factors may be involved.

Chao *et al.* (1994) showed that there is a decrease in ATP synthase δ subunit protein levels in copper deficient rats. A subsequent study (Mao *et al.*, 2000) revealed that the mRNA level of ATP synthase β subunit also decreased in copper deficient animals. These data indicate another potential mechanism by which mitochondrial respiration may be impaired in copper deficient animals. To investigate the role of ATP synthase in the alteration of mitochondrial function, oligomycin was used as a tool. Oligomycin binds to ATP synthase, blocking the translocation of protons into the mitochondria through the enzyme and eventually inhibiting the mitochondrial respiration rate. In agreement with results reported by Matz *et al.* (1995), we observed that oligomycin had less inhibitory effect on mitochondrial respiration in copper deficient rats than that in the copper-adequate rats. Collectively, these data suggest that copper deficiency directly affects ATP synthase function rather than overall mitochondrial respiration.

In addition to mitochondrial respiration assays, mitochondrial ATP hydrolysis was also measured. ATP hydrolysis and ATP synthesis occur by a common reversible catalytic process in mitochondria, thus measurement of ATP hydrolysis is often used as a measure of ATP synthase activity. In contrast to the data from the respiration assay, there was no difference observed between copper deficient and copper-adequate rats in ATP hydrolysis (or ATPase) activity when submitochondrial particles were used. Matz *et al.* (1995) also saw no changes in ATPase activity in copper deficiency, however they did report less of an inhibitory effect of oligomycin on ATP hydrolysis in the copper deficient rats. In the present study there was no significant difference in the inhibitory effect of oligomycin between copper deficient and copper-adequate rats. This inconsistency might be due to the fact that we used submitochondrial particles whereas Matz *et al.* (1995) used intact mitochondria. By using submitochondrial particles, we eliminated any effect that changes in the inner membrane due to copper deficiency may have had.

There are at least two possible reasons why oligomycin may have had less of an effect upon ATP synthase in copper deficiency. First, there may be a defect in the ATP synthase enzyme, possibly in one of the oligomycin sensitivity conferring proteins. Oligomycin binds to the F_0 subunit of ATP synthase and blocks the proton translocation through F_0. Both the oligomycin sensitivity conferring protein (OSCP) and F_6 play important roles in the inhibition of ATP synthase by oligomycin (Golden *et al.*, 1998). Another possible explanation for the decreased sensitivity is that oligomycin is unable to penetrate the mitochondrial membrane, perhaps because of decreased membrane potential, and thus is unable to bind to the F_0 unit.

In summary, our data indicates that (1) mitochondria from hearts of copper deficient rats appear intact as indicated by the low NADH-dependent oxygen consumption, (2) the oxygen consumption in the presence of NADH was lower than that of controls, (3) mitochondria from copper-deficient rats had a lower membrane potential, (4) there was no effect of CCCP on oxygen consumption as a function of copper, and (5) copper-deficient mitochondria were less sensitive to oligomycin in intact mitochondria. It is important to note that although the function of CCO has been found to be impaired in copper deficiency, it does not appear to be rate limiting. This study demonstrates a potential defect in mitochondrial ATP synthase activity.

Further studies should address changes in various ATP synthase proteins including F_6 and OSCP which are involved in sensitivity to oligomycin (Golden *et al.*, 1998). Additionally there is a strong possibility that the differences we observed, even the changes in protein levels, may be entirely due to membrane changes. Cell culture studies will enable us to examine how rapidly the membrane changes occur and whether they are a primary or secondary effect of copper deficiency.

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