Cardiac Cytochrome-*c* Oxidase Deficiency Occurs During Late Postnatal Development in Progeny of Copper-Deficient Rats

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Although cytochrome-*c* oxidase (CCO) is a copper-dependent enzyme, the effect of maternal copper deficiency on the expression of CCO activity during postnatal development of the neonatal rat heart has not been investigated extensively. Here, we show that CCO activity in heart mitochondria isolated from neonates of copper-deficient dams did not exhibit significant reductions until postnatal days (PND) 15 and 21. In addition, immunoblot analysis indicated that the CCO subunit (Cox-1) was reduced on postnatal Days 10 and 21, and that Cox-4 was reduced on PND 21 in heart mitochondria of the neonates from copper-deficient dams. These findings indicate that the impairment of CCO activity in neonatal heart by maternal copper deficiency occurs late in the postnatal heart development. Furthermore, the concurrent reductions in Cox-1 and Cox-4 suggest that the impaired CCO activity reflects a CCO deficiency in heart mitochondria. CCO activity and Cox-1 in heart mitochondria were not fully restored by 6 weeks of postweaning copper repletion in the pups of copper-deficient dams. This indicates that prolonged maternal intake of moderately low dietary copper produces CCO deficiency in cardiac mitochondria of neonates during late postnatal heart development, after terminal differentiation of cardiomyocytes occurs. The resistance of CCO deficiency to repair by dietary copper supplementation may be related to the relatively slow turnover of the affected mitochondria in the terminally differentiated heart. Exp Biol Med 231:172–180, 2006

Key words: copper deficiency; pregnancy; rats; neonatal heart; cytochrome-*c* oxidase

Cytochrome-*c* oxidase (CCO) is the terminal respiratory complex of the mitochondrial respiratory chain and is responsible for catalyzing the reduction of molecular oxygen. CCO is a heteromultimer composed of 13 subunits, three of which (subunits I, II, and III) are encoded by mitochondrial DNA. The three mitochondrially encoded subunits serve as the catalytic core of CCO. Subunits I and II contain copper and heme in their active sites and subunit III apparently modulates the proton-pumping activity of subunits I and II. The nuclear-encoded subunits may modulate catalysis, stabilize the catalytic subunits, or provide for stable assembly of the holoenzyme (1). The activity of CCO is sensitive to copper status and is reduced by copper deficiency in a variety of organs (2–4). Although the reduction of CCO activity caused by copper deficiency likely reflects the role of copper as an essential cofactor, additional mechanisms may also contribute to the loss of CCO activity in the hearts of copper-deficient animals. Medeiros and coworkers (5–7) reported that the contents of nuclear-encoded subunits IV and V are reduced in cardiac mitochondria of copper-deficient rats. Rossi *et al.* (8) also reported that copper deficiency reduced CCO protein and heme content associated with cytochrome aa3 in cardiac mitochondria of rats. These findings indicate that impaired CCO assembly or holoenzyme stability contribute to the loss of CCO activity in cardiac mitochondria of copper-deficient rats.

A 5-fold increase in heart weight occurs in rats during the first 11 days after birth (9), illustrating that considerable growth occurs in mammalian heart during early neonatal development. Functionally, contractile aspects of rat cardiac muscle increase between 10 and 16 days after birth, and by postnatal day (PND) 16, the heart is structurally and functionally equivalent to the adult heart (10). The rapid postnatal growth and development of the heart is accompanied by increased CCO activity in rats and humans (11, 12) and coordinate increases in mitochondrial and nuclear-encoded CCO mRNA in mice (13). Although it is known that copper deficiency can reduce CCO activity and contents of nuclear-encoded CCO subunits in the heart, it is not clear

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whether copper deficiency in neonates adversely affects the postnatal developmental expression of cardiac CCO activity. A report by Prohaska *et al.* (14) shows that heart CCO activity is severely reduced in 1-month-old offspring of copper-deficient rat dams. In this study, dams began consuming a copper-deficient diet on the seventh day of pregnancy and their offspring were fed the same copper-deficient diet for 9 days after weaning before CCO activity was measured. Although this study demonstrates that severe copper deficiency initiated during pregnancy and maintained throughout lactation leads to reduced CCO activity in the hearts of weaned offspring, the effect of copper deficiency on temporal changes in activity or subunit content of heart CCO in the neonates during postnatal development was not examined.

Whether low copper intake during pregnancy and lactation impairs the developmental expression of cardiac CCO activity in newborns is a relevant human nutrition question. Estimated average requirements (EAR) and recommended dietary allowances (RDA) have been recently established for copper during pregnancy and lactation. For 19- to 50-year-old women, the EAR and RDA are 800 and 1000 μg Cu/day, respectively, during pregnancy and 1000 and 1300 μg Cu/day, respectively, during lactation (15). Data for usual copper intakes from food compiled in the National Health and Nutrition Survey II, the Continuing Survey of Food Intakes by Individuals II, and the Total Diet Studies provide estimates for copper intakes for women of reproductive age and pregnant and lactating women. These data indicate that a substantial number of pregnant and lactating women and 19- to 50-year-old women do not meet either the ERA or RDA for copper (see Tables C-15, D-2, and E-3 in Ref. 15). Although the results from these surveys provide widely varying estimates of copper intake from food, they indicate that copper intakes below the ERA and RDA are not uncommon for pregnant and lactating women and for women of reproductive age in general. Whether the normal postnatal pattern for the expression of CCO activity in the developing neonatal heart is affected by low maternal copper intake during pregnancy and lactation is not known. A rat model that simulates human intake by maintaining a low, but not severely deficient, copper intake throughout pregnancy and lactation may be useful for determining the potential effects of prolonged, moderate maternal copper deficiency on the expression of CCO activity in neonates during postnatal heart development. Accordingly, the present study examined the temporal expression of CCO activity and CCO subunits Cox-1 and Cox-4 in heart mitochondria of neonates whose dams consumed a moderately deficient diet before and during pregnancy and lactation.

Materials and Methods

Animals and Diets. Adult (145–150 g), female, Sprague-Dawley rats (Charles River, Wilmington, MA) were housed in a room maintained at 22 ± 2°C and 50 ± 10% humidity with a 12:12-hr light:dark cycle. The study was approved by the Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the National Research Council Guidelines for the care and use of laboratory rats. The rats were placed into two groups (25 rats/group) and fed AIN-93 G diet (16) formulated with $CuSO_4 \cdot H_2O$ to contain either 1 mg Cu/kg (CuD diet) or 6 mg Cu/kg (CuA diet). The analyzed copper content of the diets were CuD = 1.13 mg Cu/kg; and CuA = 5.65 mg Cu/kg. After 3 weeks of dietary treatment, the rats were mated with male Sprague-Dawley rats that had been maintained on commercial rat chow. Immediately after successfully mating (determined by the presence of copulation plugs), the rats fed CuA diet were switched to CuA diet containing 8 mg Cu/kg as recommended for pregnant and lactating dams (17). The analyzed copper content of this CuA diet was 7.40 mg Cu/kg. The pregnant dams were maintained on CuD diet and CuA diet throughout pregnancy and lactation. All litters were adjusted to eight pups and cross-fostered to dams within the same dietary treatment group when necessary to have four female and four male pups in each litter. Hearts and livers were harvested from all female and male pups in individual litters from five dams in each diet treatment group on PND 1, 5, 10, and 15. The four hearts from the male pups in each litter were combined, as were the four hearts from the female pups, to provide single samples for analysis. Livers were similarly combined. On PND 21, hearts and livers were harvested from three male and three female pups from five dams in each dietary treatment group and combined, as described, for analysis. A male and a female pup from each litter was placed on CuA diet containing 6 μg Cu/kg for 6 weeks before individual hearts and livers were harvested for analysis.

Liver copper concentrations were measured by atomic absorption spectrophotometry (18). Plasma ceruloplasmin was assayed in serum by its amine oxidase activity (19). An electronic cell counter (Cell-Dyne 3500; Abbott Diagnostics, Abbott Park, IL) was used to measure hemoglobin concentrations and hematocrits.

Assay of Mitochondrial Enzyme Activities. Liver and heart mitochondria were prepared as described previously (20). In brief, liver and heart samples were weighed and homogenized in 10 volumes of either liver homogenizing buffer (0.25 *M* sucrose, 10 m*M* HEPES, and 0.1 m*M* ethyleneglycoltetraacetic acid (EGTA), pH 7.4) or heart homogenizing buffer (0.225 *M* mannitol, 0.075 *M* sucrose, 20 m*M* HEPES, and 1 m*M* EGTA). The homogenates were centrifuged at 600 *g* for 10 mins and the resulting pellets were discarded. The supernatants were centrifuged at 7700 *g* for 10 mins and the resulting mitochondrial pellets were washed once and resuspended in either liver homogenizing buffer or heart homogenizing buffer (1 ml/g tissue).

The activities of NADH:cytochrome-*c* oxidoreductase (NADHCR) and succinate:cytochrome-*c* oxidoreductase

(SucCR) were assayed in isolated heart and liver mitochondria by monitoring the reduction of ferricytochrome-*c* spectrophotometrically at 550 nm (21). CCO activity in the isolated mitochondria was assayed by monitoring the oxidation of ferrocytochrome-*c* at 550 nm (22). Protein concentrations in the mitochondrial preparations were determined with bichinchoninic acid (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL) using bovine serum albumin as the standard.

Measurement of Mitochondrial Respiratory Complex Subunits. Heart mitochondrial proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of urea, as described by Capaldi *et al.* (23) for the analysis of Cox-1–Cox-4. Previously developed immunoblotting procedures (24, 25) were used to detect the protein levels of Cox-1 and Cox-4 and the 39-kDa subunit of complex I (CMPLX1–39) in heart mitochondria. Briefly, heart mitochondrial proteins (5 μg of protein/lane) were separated by SDS-PAGE 12% Tris gels (BioRad Laboratories, Hercules, CA) and transferred to polyvinylidene fluoride membrane. The blots were probed with anti-Cox-1 (clone 1D6), anti-Cox-4 (clone 20E8), and anti-CMPLX1–39 (clone 20C11) monoclonal antibodies (Molecular Probes, Eugene, OR), which are highly specific to the subunits for which they are prepared (23). Cox-1, Cox-4, and CMPLX1–39 were detected by chemiluminescence (Immun-Star HRP Chemiluminescence Kit; Bio-Rad Laboratories, Hercules, CA) and quantified by imaging densitometry (EpiChemi^3 Imaging System; UVP, Inc., Upland, CA). Optical densities of the digital images of the bands representing the subunits were calculated by using a calibration curve to relate pixel intensity to optical density (O.D.) in arbitrary units for each blot. The optical density was then calculated from O.D. = (total O.D. of all pixels in band − O.D. of background pixels in band) × band area. Molecular weights of the subunits were determined by comparing their position on the blots to the positions of protein markers with known molecular weights (ProSieve Color Protein Markers; Cambrex Bio Science Rockland, Inc., Rockland, ME). Protein concentrations of the mitochondrial preparations used for immunoblotting were determined using the Bradford assay (26).

Statistics. Values are either means ± SD or means ± pooled SEM for data combined from males and females. Data were analyzed by either 3-way ANOVA to determine the effects of maternal copper status, sex, age, and their interaction; or by 2-way ANOVA to determine the effects of maternal copper status, sex, and their interaction; followed by Tukey's multiple comparison test when the interactions were significant. Data regarding the copper status of the dams were evaluated by 2-way ANOVA to determine the effects of dietary copper, PND, and their interaction followed by Tukey's multiple comparison test when the interaction was significant (27). Differences were considered significant at $P < 0.05$.

Results

Dams in this study consumed either CuD diet or CuA diet beginning 3 weeks before conception and ending 3 weeks after parturition. Copper status of the dams was assessed on the same PND their litters were sampled (Table 1). The effects of maternal diet significantly influenced liver copper concentrations, liver iron concentrations, and ceruloplasmin, independently of PND ($P > 0.05$ for the effect of interaction of diet and PND; ANOVA). Liver copper concentrations were lower, liver iron concentrations were higher, and ceruloplasmin activities were lower on each PND in dams fed CuD compared with dams fed CuA ($P < 0.05$ for the effect of diet, ANOVA). Liver copper concentrations (pooled means ± SEM) in dams consuming CuD diet and CuA diet were 78 ± 3 nmol/g dry liver and 153 ± 4 nmol/g dry liver, respectively. Liver iron concentrations (pooled means ± SEM) in dams consuming CuD diet and CuA diet were 12.5 ± 1.1 μmole/g dry liver and 8.4 ± 1.2 μmole/g dry liver, respectively. Plasma ceruloplasmin activities (pooled means ± SEM) in dams consuming CuD diet and CuA diet were 5 ± 5 U/liter and 88 ± 5 U/liter, respectively. The reductions in hepatic copper content and ceruloplasmin activity and elevation in hepatic iron content are signs of copper deficiency in the dams fed CuD. However, anemia was absent in these dams. Maternal diet did not significantly affect hematocrits or hemoglobin concentrations ($P > 0.05$ for effect of diet, ANOVA). Hematocrits (pooled means ± SEM) were 0.41 ± 0.004 and 0.41 ± 0.004, and hemoglobin concentrations (pooled means ± SEM) were 134 ± 1 g/liter and 136 ± 1 g/liter in dams fed CuD diet and CuA diet, respectively.

Dams fed CuA diet and CuD diet had litters of normal size (6–16 pups/litter) and the litter size was not influenced by the copper status of the dams. Also, the neonates of the copper-deficient dams did not exhibit any visible, gross abnormalities. However, pups of the copper-deficient dams experienced growth retardation. On PND 21, body weights of pups from copper-deficient and copper-adequate dams were 66.1 ± 1.8 g and 74.0 ± 2.0 g, respectively ($P < 0.05$ for the effect of maternal copper status). Heart weight was not significantly affected by the copper status of the dams. Heart weights were 0.43 ± 0.01 g and 0.41 ± 0.01 g in pups from copper-deficient and copper-adequate dams, respectively, on PND 21.

Hepatic copper concentrations in the pups (Fig. 1) were affected by a significant interaction between maternal copper status and age ($P < 0.05$). In the pups of copper-adequate dams, hepatic copper concentrations increased between PND 1 and 10 before declining. However, hepatic copper concentrations in the pups of copper-deficient dams did not significantly change between PND 1 and 21 and were significantly lower compared with the pups of copper-adequate dams on PND 1 thru PND 21. Sex did not significantly affect hepatic copper concentrations in pups of either copper-deficient or copper-adequate dams.

Table 1. The Effects of CuA and CuD Diets on the Copper Status of Dams on PND 1–21[a]

PND	Liver Cu (nmol/g dry liver)		Liver Fe (μmol/g dry liver)		Ceruloplasmin (U/liter)		Hematocrit		Hemoglobin g/liter	
	CuA	CuD	CuA	CuD	CuA	CuD	CuA	CuD	CuA	CuD
1	161 ± 16	106 ± 9	3.09 ± 0.85	3.57 ± 0.77	89 ± 34	2.8 ± 3.4	0.32 ± 0.01	0.34 ± 0.05	106 ± 9	110 ± 4
5	133 ± 20	55 ± 10	6.34 ± 5.13	8.53 ± 4.67	60 ± 31	0.1 ± 0.2	0.38 ± 0.02	0.39 ± 0.02	127 ± 4	126 ± 7
10	139 ± 26	54 ± 18	5.52 ± 2.34	14.42 ± 3.92	86 ± 37	0.2 ± 0.3	0.42 ± 0.03	0.42 ± 0.02	139 ± 8	136 ± 7
15	169 ± 10	74 ± 18	7.98 ± 0.89	18.7 ± 6.64	95 ± 20	7.3 ± 12.0	0.44 ± 0.08	0.43 ± 0.02	146 ± 4	142 ± 5
21	162 ± 14	104 ± 16	18.9 ± 3.52	17.4 ± 12.63	109 ± 24	15.5 ± 34.6	0.48 ± 0.02	0.47 ± 0.01	158 ± 9	156 ± 6
ANOVA										
Diet	$P < 0.05$		$P < 0.05$		$P < 0.05$		NS		NS	
PND	$P < 0.05$		$P < 0.05$		NS		$P < 0.05$		$P < 0.05$	
Diet × PND	NS		NS		NS		NS		NS	

[a] Values are means ± SD. NS, not significant, $P > 0.05$.

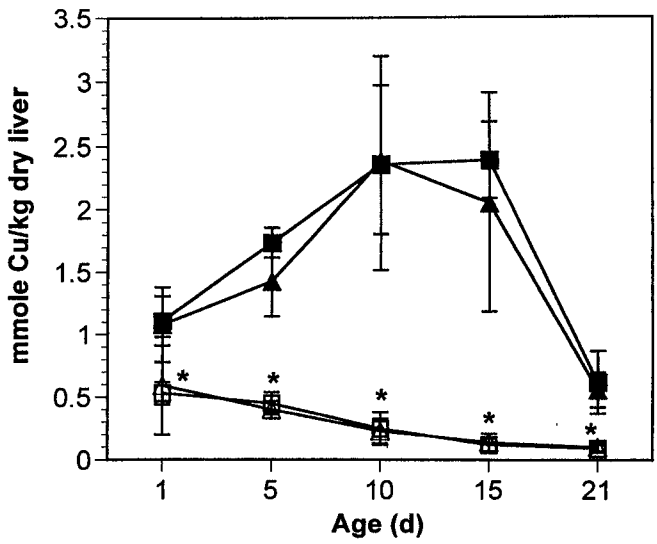

Figure 1. The variation in liver copper concentration with age in male (squares) and female (triangles) pups of copper-deficient dams (open symbols) and copper-adequate dams (filled symbols). *P* values from the 3-way ANOVA were $P < 0.05$ for the effects of maternal copper status, age, and maternal copper status × age interaction; $P > 0.05$ for the effects of sex, age × sex, maternal copper status × sex interaction, and maternal copper status × sex × age interaction. Asterisks indicate significant differences between the offspring of copper-deficient and copper-adequate dams ($P < 0.05$; Tukey's multiple comparison test).

NADHCR activity in heart mitochondria of pups of copper-deficient and copper-adequate dams was significantly affected only by age ($P < 0.05$). NADHCR showed a general rise in activity between PND 1 and PND 21 that was independent of either the sex of the pups or maternal copper status (Fig. 2A). SucCR activity in heart mitochondria (Fig. 2B) was affected by a significant interaction between maternal copper status and age ($P < 0.05$). SucCR activity was higher on PND 15 in pups of copper-deficient dams than in pups of copper-adequate dams. SucCR activity was also significantly higher on PND 5 than on PND 1 in all neonates, regardless of the copper status of their dams. SucCR activity was not affected by sex ($P > 0.05$). CCO activity in heart mitochondria (Fig. 2C) was affected by a significant interaction between maternal copper status and age ($P < 0.05$). CCO activity was lower on PND 15 and PND 21 in pups of copper-deficient dams than in pups of copper-adequate dams. CCO activity was also significantly higher on PND 5 than on PND 1 in all pups, regardless of maternal copper status. CCO activity was slightly higher in heart mitochondria from males compared with females. Pooled means ± SEM for CCO activities were 3.60 ± 0.08 U/mg protein in males and 3.37 ± 0.08 U/mg protein for females ($P < 0.05$ for the effect of sex, ANOVA).

The effects of maternal copper status on the content of mitochondrially encoded Cox-1 and nuclear-encoded Cox-4 and the CMPLX1–39 in isolated heart mitochondria were investigated by immunoblotting. The monoclonal antibodies used for analysis of CCO subunits are highly specific for the

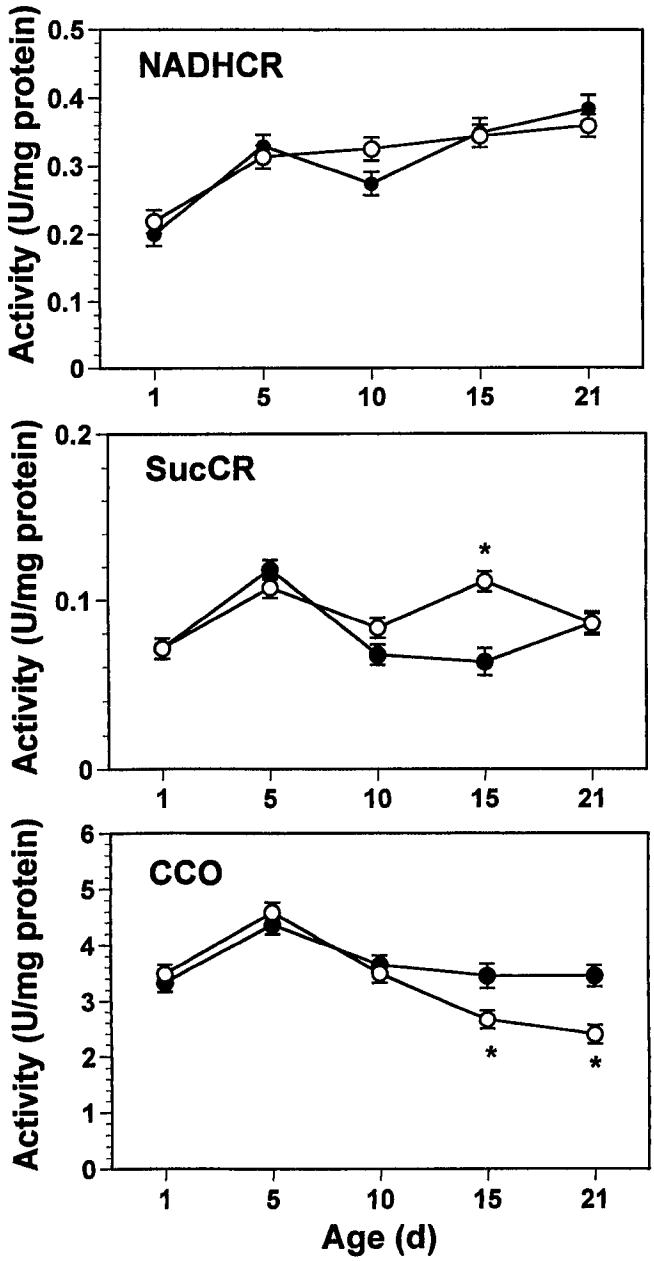

Figure 2. The variation in activities of NADHCR, SucCR, and CCO with age in heart mitochondria isolated from pups of copper-deficient dams (open circles) and copper-adequate dams (closed circles). A unit of activity for NADHCR or SucCR is defined as the amount of enzyme that catalyzes the reduction of 1 μmole of ferricytochrome-*c*/min at 30°C. A unit of activity for CCO is defined as the amount of enzyme that catalyzes the oxidation of 1 μmole of ferrocytochrome-*c*/min at 30°C. Significant effects of maternal copper status, age, sex, and their interactions were $P < 0.05$ for the effect of age on NADHCR activity; $P < 0.05$ for the effects of maternal copper status, age, and maternal copper status × age interaction on SucCR activity; and $P < 0.05$ for the effects of maternal copper status, age, and maternal copper status × age interaction on CCO activity. The effects of sex, age × sex interaction, maternal copper status × sex interaction, and maternal copper status × age × sex interaction were not significant ($P > 0.05$) for any of the enzyme activities. The values shown in each panel are means ± pooled SEM for combined male and female pups. Symbols labeled with an asterisk indicate a significant difference between pups from copper-deficient and copper-adequate dams ($P < 0.05$; Tukey's multiple comparison test).

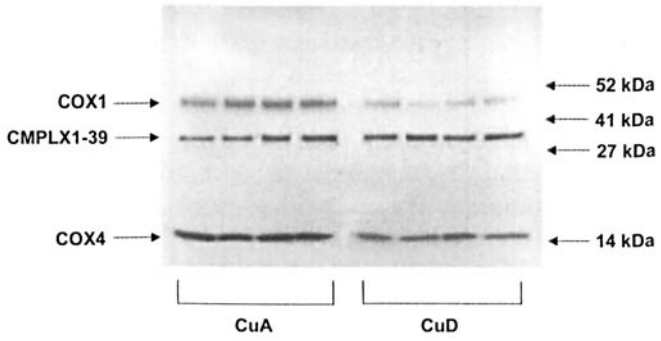

Figure 3. Representative Western blot of Cox-1, Cox-4, and CMPLX1–39 in heart mitochondria of neonates from copper-adequate (CuA) and copper-deficient (CuD) dams on PND 21. The mitochondrial proteins were separated by SDS-PAGE and immunoblotted with monoclonal antibodies to Cox-1 (clone 1D6), Cox-4 (clone 20E8), and CMPLX1–39 (clone 20C11). Migration of molecular weight markers on the SDS-PAGE gel are shown on the right.

subunit to which they are prepared (23), and detected proteins with molecular weights of approximately 45 kDa for Cox-1 and 15 kDa for Cox-4 (Fig. 3). These molecular weights for Cox-1 and Cox-4 are in agreement with those reported for the subunits in human cell extracts separated by SDS-PAGE without urea (23). The immunoblot shown for heart mitochondria sampled on PND 21 (Fig. 3) qualitatively shows that Cox-1 and Cox-4 contents were lower in the pups of copper-deficient dams. Analysis by imaging densitometry of the immunoblots for heart mitochondria showed that Cox-1 content on PND 10 and PND 21 and Cox-4 content on PND 21 were significantly reduced in the pups of copper-deficient dams (Fig. 4). CMPLX1–39 was not affected by maternal copper status on PND 10 or PND 21 but was slightly, though significantly higher, on PND 1 in the heart mitochondria of the pups of copper-deficient dams.

Groups of male and female offspring of copper-deficient and copper-adequate dams were fed CuA diet for 6 weeks after PND 21. Consumption of CuA diet restored the liver copper concentration and the CCO activity in hepatic mitochondria in the offspring of the copper-deficient dams. Liver copper concentrations in the offsprings of copper-deficient dams were 171 ± 19 mmol/kg dry liver and 221 ± 18 mmol/kg dry liver in males and females, respectively, compared with 187 ± 14 mmol/kg dry liver and 214 ± 14 mmole Cu/kg dry liver in male and female offspring, respectively, of copper-adequate dams ($P > 0.05$ for the effect of maternal copper status; ANOVA). CCO activities in hepatic mitochondria from offspring of copper-deficient dams were 1.32 ± 0.16 U/mg protein and 1.48 ± 0.12 U/mg protein in males and females, respectively, compared with 1.42 ± 0.20 U/mg protein and 1.54 ± 0.21 U/mg protein in males and females, respectively, of copper-deficient dams ($P > 0.05$ for the effect of maternal copper status; ANOVA). However, CCO activity in heart mitochondria (Fig. 5) was significantly lower in the offspring of

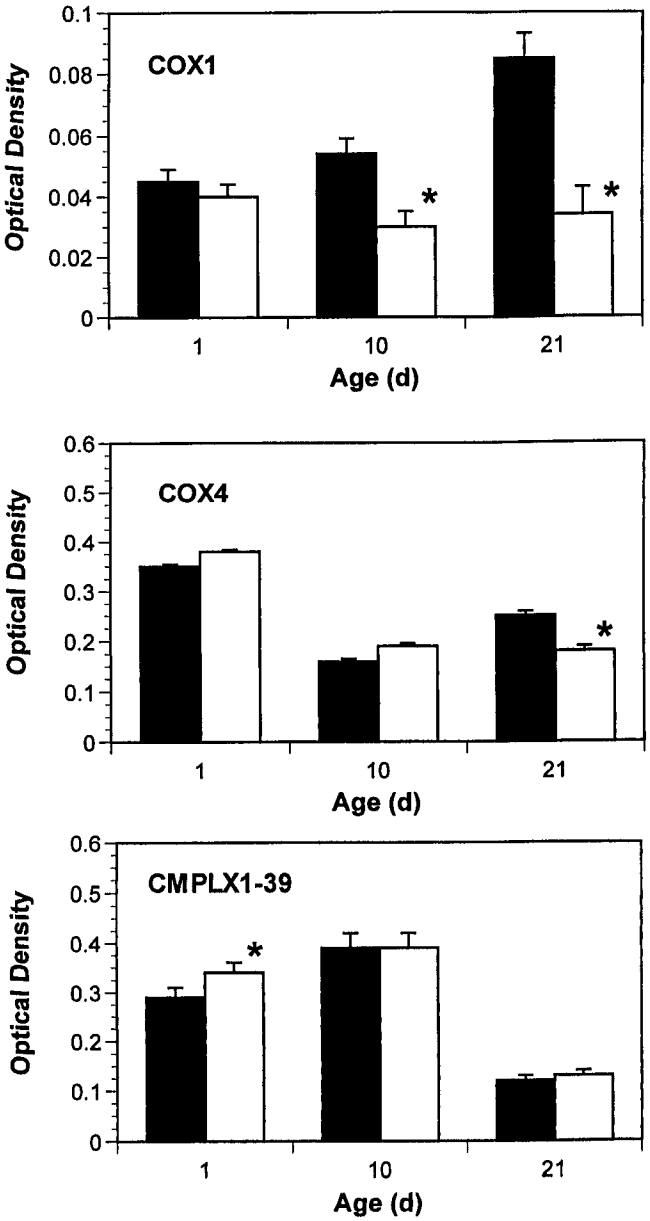

Figure 4. The content of Cox-1, Cox-4, and CMPLX1–39 of respiratory complex I in heart mitochondria of neonates from copper-adequate dams (black bars) and copper-deficient dams (white bars) on PND 1, 10, and 21. The optical densities (arbitrary units) of the subunits were calculated from the intensities of the pixels in the digital images of the immunoreactive protein bands, as described in Materials and Methods. The values shown are the mean optical densities ± pooled SEM for combined male and female neonates. Data were analyzed by 2-way ANOVA for the effects of maternal copper status and sex. Asterisks signify a significant difference between neonates from copper-adequate and copper-deficient dams ($P < 0.05$ for the effect of maternal copper status). The effects of sex and maternal copper status × sex interaction were not significant ($P > 0.05$) for any subunit on PND 1, 10, and 21.

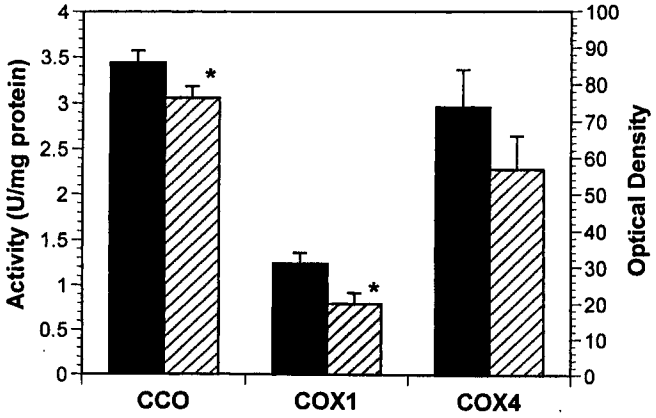

Figure 5. CCO activity and the contents of Cox-1 and Cox-4 in heart mitochondria from the pups of copper-adequate dams (black bar) and copper-deficient dams (white bar) after 6 weeks of copper supplementation. Values shown are mean CCO activities ± pooled SEM and mean optical densities ± pooled SEM for pooled male and female pups. Units of CCO activity are defined in Figure 2. Data were analyzed by 2-way ANOVA for the effects of maternal copper status and sex. Asterisks signify a significant difference between pups from copper-adequate and copper-deficient dams ($P < 0.05$ for the effect of maternal copper status). The effects of sex and maternal copper status × sex interaction were not significant ($P > 0.05$) for either enzyme activities or subunit contents.

copper-deficient dams compared with the offspring of copper-adequate dams ($P < 0.05$ for the effect of maternal copper status) after both groups consumed adequate dietary copper for 6 weeks after PND 21. The reduced activity of CCO in heart mitochondria of the offspring copper-deficient dams was as also accompanied by a significant reduction in Cox-1 ($P < 0.05$ for the effect of maternal copper status).

Discussion

Hepatic copper concentrations in neonatal rats normally increase after birth, reach a maximum on PND 12, and decline to adult levels (28). In our study, hepatic copper concentrations failed to rise and remained abnormally low from PND 1 through PND 21 in the pups of dams fed a moderately copper-deficient diet. The abnormal pattern of postnatal hepatic copper accrual in these pups and their slightly lower body weights at weaning indicate that copper deficiency was induced in the pups because of moderately low copper intake by their dams during pregnancy and lactation.

The activity of CCO in neonatal rat heart normally increases rapidly after birth and peaks approximately 2–3 days after birth (11, 12) as mitochondria adjust to meet the expanding postnatal energy requirements as the heart transitions from a hypoxic fetal environment and rapidly matures in the oxygen-rich neonatal environment. Our data showing that CCO activity in heart mitochondria peaked at PND 5 are in close agreement with these reports. Furthermore, neonatal copper deficiency did not affect the magnitude of the postparturient rise in cardiac CCO activity in our study. It is somewhat surprising, given that copper is an essential cofactor for CCO activity, that neonatal copper deficiency did not adversely affect the normal rise in CCO activity in heart mitochondria during early postnatal cardiac development. However, the failure of copper-deficient neonates to accumulate hepatic copper after birth suggests that rather than entering hepatic copper stores, what little

copper is acquired from the copper-deficient dams or available from internal neonatal stores is diverted directly to critical biochemical processes connected to rapid postnatal growth and development of tissues and organs.

During postnatal cardiac development, cardiomyocytes become terminally differentiated, and the continued increase in heart mass occurs by enlargement of preexisting cardiomyocytes. A report showing that the levels of Cyclin A mRNA and protein in rat cardiomyocytes decrease 37% by PND 2 and become undetectable by PND 14 (29) indicates that terminal differentiation of cardiomyocytes begins shortly after birth and is completed within the first 14 days of postnatal life. The transition from cardiomyocyte hyperplastic growth to hypertrophic growth that marks terminal differentiation may occur as early as PND 3 or 4 (30). In our study, reductions in CCO activity in heart mitochondria from copper-deficient pups occurred on PND 15 and PND 21. Thus, the late postnatal reduction of CCO activity we observed likely occurred after or at least during the late stages of terminal cardiomyocyte differentiation. In the control pups, hepatic copper stores rapidly declined during the late postnatal period. This suggests that stored copper was mobilized to meet increasing late postnatal requirements for growth and development in the control pups. However, the copper-deficient pups were not able to accrue hepatic copper. Thus, copper stores in the copper-deficient pups may not have been sufficient to meet the various competing demands for copper during late postnatal development, and the supply of copper from internal stores may have been inadequate for maintaining CCO activity as mitochondria proliferated in growing cardiomyocytes after terminal differentiation occurred.

Heart mitochondrial NADHCR activity, which represents the combined activities of complexes I and III, increased continually from PND 1 and PND 21 in our study. These data are consistent with a previous report showing that the activity of respiratory complex I in rat heart mitochondria increases during the transition from newborn to adult (31). Neither the rise in activity nor the magnitude of the activity was altered by copper deficiency in the pups. Copper deficiency in the neonates also did not suppress heart mitochondrial SucCR activity, which represents the combined activities of complexes II and III, at any point during the postnatal period. These results are not surprising, because NADHCR and SucCR are not cuproenzymes, but they suggest that our results regarding the effects of neonatal copper deficiency on CCO reflect a specific adverse effect on CCO activity during late postnatal cardiac development.

Although the late postnatal reduction in heart mitochondrial CCO activity in the copper-deficient pups may result from the inability of the limited internal copper stores to supply sufficient copper to the catalytic core of CCO, other factors may also be involved. Our results are consistent with the presence of late postnatal CCO deficiency in the heart mitochondria of the copper-deficient pups because Cox-1, which is encoded by mitochondrial DNA, and Cox-4, which is encoded by nuclear DNA, were both reduced by PND 21. However, our findings are not in complete agreement with previous reports by Medeiros and coworkers showing that copper deficiency in weanling rats decreases Cox-4 and Cox-5 in hearts but has no effect on mitochondrially encoded Cox-2 (5–7). The discrepancy between our finding showing a suppressive effect of copper deficiency on mitochondrially encoded Cox-1 in hearts and the previous reports may reflect the stage of heart maturation when copper deficiency was initiated. In our study, moderate copper deficiency was maintained throughout pregnancy and lactation and the neonates were copper-deficient throughout the entire period of postnatal heart development. The data in the previous reports were obtained from weanling rats whose copper-deficiency was initiated after heart tissue was terminally differentiated.

Our finding that mitochondrial- and nuclear-encoded CCO subunits are both reduced in heart mitochondria in copper-deficient neonates by PND 21 suggests that lower content of either the total holoenzyme or subunits required for activity and stability of the holoenzyme contributed to the loss of CCO activity during the late postnatal period. It has been suggested that copper deficiency may impair the importation of the nuclear-encoded Cox-4 and Cox-5 subunits into heart mitochondria (5, 7). Whether the loss of CCO activity and concomitant loss of Cox-1 and Cox-4 content we observed at the later stage of postnatal heart maturation results from impaired mitochondrial importation of nuclear-encoded subunits and repressed expression of mitochondrial-encoded subunits, a combination of impaired importation and repressed expression of nuclear- and mitochondrial-encoded subunits, or impaired assembly of the subunits into the active oligomeric enzyme remains to be determined.

In our study, 6 weeks of postweaning copper repletion restored hepatic copper concentration and CCO activity in the offspring of the copper-deficient dams. However, copper repletion did not fully restore CCO activity and Cox-1 content in heart mitochondria isolated from these offspring. Copper repletion also may not have fully restored Cox-4 content in heart mitochondria, although the difference in Cox-4 content between the repleted and control offspring did not achieve statistical significance. These results are consistent with a previous report (14) showing that 1 month of postweaning dietary copper repletion did not fully restore heart CCO activity in the offspring of dams that began consuming a severely copper-deficient diet on the seventh day of pregnancy and also with a previous report (6) showing that 6 weeks of copper repletion did not restore Cox-4 content in the hearts of rats that had been copper-deficient for 5 weeks after weaning. In our study, the resistance of the low CCO activity and Cox subunit contents in heart mitochondria to restoration by copper repletion may be related to the late postnatal stage of heart development during which the initial reductions in activity and subunit content occurred. A previous report by Dallman (32)

indicated that the recovery of CCO activity after copper repletion of copper-deficient rats is determined, in part, by the rate of mitochondrial biogenesis. The half-life of mitochondria in the adult, fully differentiated heart is approximately 18 days compared with approximately 9 days for hepatic mitochondria (33). In our study, CCO deficiency in heart mitochondria of the copper-deficient neonates occurred during late postnatal development when cardiomyocytes are terminally differentiated. Thus, once CCO deficiency was established after the heart was fully differentiated in the copper-deficient neonates, the slow turnover rate of the affected mitochondria may have contributed to the resistance of CCO activity to restoration by postweaning copper repletion.

Adequate dietary copper intake for 6 weeks after weaning failed to fully restore cardiac CCO activity and content in the offspring of copper-deficient dams, but the activity was only approximately 10% lower, and the content, based on Cox-1, was approximately 35% lower than the activity in the control offspring. However, this modest reduction has potential biological consequences. Approximately 1%–2% of the oxygen used by the electron transport chain is converted to reactive oxygen species (ROS) (34, 35). Furthermore, mitochondrial ROS production is largely determined by the redox state of the respiratory complexes and is increased as the complexes become more reduced (34). Blockage of the electron transport chain near its terminus increases ROS production by increasing the reducing potential of the upstream complexes. Although mitochondrial ROS production was not measured in the present study, it has been shown that partial inhibition of CCO can increase mitochondrial generation of hydrogen peroxide (36). Thus, the prolonged deficiency of cardiac CCO that we observed in the offspring of copper-deficient dams has the potential for exposing the heart to elevated levels of mitochondrially generated ROS. The heart is particularly susceptible to oxidative stress because of its relatively low activities of superoxide dismutase, catalase, and glutathione peroxidase (37). Thus, increased oxidative stress resulting from CCO deficiency and overproduction of mitochondrial ROS may promote damage to intercellular proteins, lipids, and DNA in cardiomyocytes.

In conclusion, our study indicates that prolonged intake of moderately low dietary copper by rats starting before pregnancy and continuing throughout lactation leads to CCO deficiency in neonates during late postnatal heart development after cardiomyocytes have become terminally differentiated. Once CCO deficiency is established at this stage of postnatal cardiac development, it may be resistant to repair by adequate dietary copper because mitochondrial turnover is relatively slow in the terminally differentiated heart. Further research is required to determine whether cardiac CCO deficiency in the offspring of copper-deficient dams represents an alteration in the developmental trajectory of the heart resulting from changes in developmental regulation during the fetal or early neonatal phase that do not become manifest until the heart becomes terminally differentiated.

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