Cardiac Cytochrome c Oxidase Activity and Contents of Subunits 1 and 4 Are Altered in Offspring by Low Prenatal Copper Intake by Rat Dams¹,²

W. Thomas Johnson³* and Cindy M. Anderson⁴

³USDA, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034 and
⁴Family and Community Nursing Department, College of Nursing, University of North Dakota, Grand Forks, ND 58202-9025

Abstract

It has been reported previously that the offspring of rat dams consuming low dietary copper (Cu) during pregnancy and lactation experience a deficiency in cardiac cytochrome c oxidase (CCO) characterized by reduced catalytic activity and mitochondrial and nuclear subunit content after postnatal d 10. The present study was undertaken to determine whether the cardiac CCO deficiency was caused directly by low postnatal Cu intake or whether it was a prenatal effect of low Cu intake by the dams that became manifest postnatally. Dams were fed either a Cu-adequate diet (6 mg Cu/kg) or Cu-deficient diet (1 mg Cu/kg) beginning 3 wk before conception and throughout gestation and lactation. One day following parturition, several litters from Cu-adequate dams were cross fostered to Cu-deficient dams and several litters from Cu-deficient dams were cross fostered to Cu-adequate dams. Litters that remained with their birth dams served as controls. CCO activity, the content of the mitochondrial-encoded CCO subunit 1 (COX1), and the content of the nuclear-encoded subunit COX4 in cardiac mitochondria were reduced in the 21-d-old offspring of Cu-deficient dams. COX1 content was normal in the 21-d-old cross-fostered offspring of Cu-deficient dams, but CCO activity and COX4 were reduced. Cross fostering the offspring of Cu-adequate dams to Cu-deficient dams did not significantly affect CCO activity, COX1 content, or COX4 content in cardiac mitochondria of 21-d-old offspring. These data indicate that low prenatal Cu intake by dams was the determinant of CCO activity in cardiac mitochondria of the 21-d-old offspring and may have led to the assembly of a less-than-fully active holoenzyme. J. Nutr. 138: 1269–1273, 2008.

Introduction

Past studies have provided ample evidence demonstrating that dietary manipulation of the periconceptual, embryonic, fetal, or neonatal environment can influence cardiovascular and metabolic function. The responses to dietary manipulation can become permanent if they operate during critical time windows of development (1). Copper (Cu) is essential for development (2,3) and several studies suggest that the outcomes of Cu deficiency during pregnancy on the phenotype of the offspring depend on the timing of the deficiency during development. For instance, presenting mice with a severely Cu-deficient diet on embryonic d 13 causes death in the offspring before postnatal d 21 but does not affect survivability if the diet is presented on embryonic d 19 (4). Aspects of heart development are also affected by pre- and postnatal Cu intake. Consumption of a marginally Cu-deficient diet by rat dams from mid-gestation through lactation produces abnormalities in the heart and mitochondria of their offspring that consume marginally Cu-deficient diets after weaning (5). Other studies have shown that Cu deficiency during pregnancy causes a reduction in cardiac cytochrome c oxidase (CCO) activity in offspring that is resistant to repair by adequate dietary Cu intake after weaning (6,7). The influence of maternal Cu deficiency on cardiac mitochondrial function in the first generation can persist into adulthood as demonstrated by increased hydrogen peroxide generation by cardiac mitochondria in adult offspring of Cu-deficient rat dams (8). Together, these studies show that Cu is important for development and that pregnancy and lactation are critical time windows during which low Cu intake can produce...
persistent alterations in cardiac CCO activity and mitochondrial function in the first generation.

A recent study showing that a significant decline in cardiac CCO in the offspring of Cu-deficient rats does not occur until after postnatal d 10 (7) suggests that the decline in CCO activity is a direct consequence of low postnatal intake. However, this does not rule out the possibility that maternal Cu deficiency produces an intracellular influence that alters the developmental trajectory of cardiac CCO in such a manner that reduced enzymatic activity does not become manifest until postnatal maturation of the heart occurs. The aim of this study was to determine the relative importance of the gestational and postnatal periods in the reduction of cardiac CCO activity in offspring of dams consuming a low-Cu diet. To accomplish this, pups from dams consuming a low-Cu diet were cross fostered to dams consuming an adequate Cu diet and vice versa. Cu status, CCO activity, and subunit composition in heart and liver mitochondria in the cross-fostered pups were compared with the Cu status of pups that remained with their birth dams throughout gestation and lactation.

Materials and Methods

**Animals and diets.** Adult (145–150 g) female Sprague-Dawley rats (Charles River) were housed in a room maintained at 22 ± 2°C and 50 ± 10% humidity with a 12-h-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 NRC guidelines for the care and use of laboratory rats. The rats were divided into 2 groups and fed an AIN-93 G diet (9) formulated with CuSO$_4$·5H$_2$O to contain either 1 mg Cu/kg (Cu-deficient diet) or 6 mg Cu/kg (Cu-adequate diet). The analyzed Cu contents of the diets were 0.82 mg Cu/kg and 5.95 mg Cu/kg in the Cu-deficient and Cu-adequate diets, respectively. After 3 wk of dietary treatment, the rats were mated with male Sprague-Dawley rats that had been maintained on a non-Cu diet (0.43 mg Cu/liter diet) to develop anemia as indicated by hematocrits (145 ± 6.4% in dams fed the Cu-deficient diet). The pregnant dams were maintained on their respective diets throughout gestation and lactation. On the day following birth, the litters were adjusted to 8 pups (4 male, 4 female) and 5 litters from dams fed Cu-deficient diet were cross fostered to dams fed Cu-adequate diet and 4 litters from dams fed the Cu-adequate diet were cross fostered to dams fed the Cu-deficient diet. The 4 groups of offspring were designated as: CuA, offspring of dams fed the Cu-adequate diet suckled by the same dams; CuACuD, offspring of dams fed the Cu-adequate diet suckled by dams fed the Cu-deficient diet; CuD, offspring of dams fed the Cu-deficient diet suckled by the same dams; and CuDCuA, offspring of dams fed the Cu-deficient diet suckled by dams fed the Cu-adequate diet. Livers and blood were collected from the dams on postnatal d 21 and hearts and livers from pups in each litter also were harvested at this time. Four hearts obtained from male pups in each litter were combined, as were 4 hearts obtained from female pups in each litter, to provide single samples for analysis. Livers from the pups in each litter were combined in a similar manner.

**Analytical methods.** We measured hepatic Cu and Fe concentrations by atomic absorption spectrophotometry (10). Heart Cu was measured by atomic absorption spectrophotometry using acid digests of fresh homogenates containing 1 kg tissue/L (see below). Ceruloplasmin activity was assayed in plasma by its amine oxidase activity (11). Hemoglobin concentrations and hematocrits were measured with an automated electronic cell counter (Cell-Dyne 3500, Abbott Diagnostics). We isolated mitochondria from heart and liver as described previously (12) with modifications in the homogenization buffers. In brief, heart and liver samples were weighed and homogenized in 10 volumes of either heart homogenizing buffer (0.225 mol/L mannitol, 75 mmol/L sucrose, 20 mmol/L HEPES, 1 mmol/L EGTA) or liver homogenizing buffer (0.25 mol/L sucrose, 10 mmol/L HEPES, 0.1 mmol/L EGTA, pH 7.4). The homogenates were centrifuged at 600 × g for 10 min and the resulting pellets were discarded. Supernatant fractions were centrifuged at 7700 × g for 10 min and the resulting mitochondrial pellets were washed once and resuspended in either heart homogenizing buffer or liver homogenizing buffer (1 kg tissue/L buffer).

CCO activity in isolated mitochondria was assayed by monitoring the oxidation of ferrocytochrome c at 550 nm (13). Protein concentrations in the mitochondrial preparations were determined with bichinonic acid (BCA Protein Assay Reagent kit, Pierce) using bovine serum albumin as the standard.

Heart and liver mitochondrial proteins were separated by SDS-PAGE using 10% acrylamide gels and MOPS running buffer (NuPAGE Novex Bis Tris gel, Invitrogen Life Technologies). Mitochondrial samples were prepared for electrophoresis according to the manufacturer's directions (NuPAGE Technical Guide, Invitrogen Life Technologies). Each lane of the gel was loaded with 20 μg of mitochondrial protein. Following electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane. The blots were probed with monoclonal antibodies (MitoSciences) specific for subunit 1 (COX1) and subunit 4 (COX4) of CCO. The COX1 and COX4 subunits were detected by chemiluminescence (ECL Western Blotting Substrate, Pierce Biotechnology) and quantified by imaging densitometry (EpiChem Imaging system, UVP).

**Statistics.** Significance of the effects of dietary Cu treatment on the Cu status of the dams was determined by Student’s t test for unequal variance. Values in the text related to the Cu status of the dams are means ± SD. Data from the pups were analyzed by 3-way ANOVA to determine the significance of the effects for Cu status of the birth dam, Cu status of the postnatal dam, the sex of the pups, and their interactions (14). None of the variables measured in the pups were significantly affected by sex or by interactions between sex and the Cu status of the birth mother or postnatal mother. Therefore, all values for the pups reported in the text, tables, and figures are pooled means ± SEM for male and female pups obtained from the 3-way ANOVA. OD for CCO subunits was obtained from multiple Western blots, each of which contained samples from each treatment group. The ANOVA for optical densities treated each blot as a blocking factor to account for between-blot variability. Differences between means were tested for significance with Tukey’s multiple comparison test when interactions were significant (14). Differences were considered significant at P ≤ 0.05.

Results

Dams consuming the Cu-deficient diet beginning 3 wk before conception showed signs of decreased Cu status 21 d after parturition. The liver Cu concentration in dams fed the Cu-deficient diet was 80.1 ± 28.3 nmol/g dry liver compared with 171.1 ± 23.6 nmol/g dry liver in dams fed the Cu-adequate diet (P < 0.05). Plasma ceruloplasmin activities were 14 ± 23 U/L and 109 ± 35 U/L (P < 0.05) in dams fed the Cu-deficient and Cu-adequate diets, respectively. However, dams fed the Cu-deficient diet did not develop anemia as indicated by hemocrits (0.43 ± 0.02) and hemoglobin concentrations (145 ± 6 g/L) that did not differ from those of dams fed the Cu-adequate diet (0.43 ± 0.02, 149 ± 7 g/L). Their hepatic Fe concentrations (14.3 ± 6.4 μmol/g dry liver) were not elevated compared with those in dams fed the Cu-adequate diet (11.8 ± 6.8 μmol/g dry liver). Hepatic Cu concentrations depended only on the Cu status of the birth mother and were lower in the CuD and CuDCuA offspring than in the CuA and CuACuD offspring (Table 1). The Cu status of the birth mother and of the postnatal mother interacted to affect hepatic Fe concentration; it was higher in CuD offspring (P < 0.05) than in all other groups, which did not differ from one another. The Cu status of the birth mother and of the postnatal mother interacted to affect heart Cu concentration in the offspring.
activity in either CuA or CuACuD offspring, which did not differ from one another. COX4 content in heart mitochondria (Fig. 2B) depended only on the Cu status of the birth mother and was lower in the offspring of Cu-deficient dams in CuA and CuACuD offspring. Heart weight and the heart:body weight ratio depended only on the Cu status of the birth mother. Heart weights were 0.34 ± 0.01 g for combined CuA and CuACuD offspring and 0.36 ± 0.01 g for combined CuD and CuDCuA (P = 0.02 for the effect of birth mother). Heart weights relative to body weight were 0.52 ± 0.01% for combined CuA and CuACuD offspring and 0.57 ± 0.01% for combined CuD and CuDCuA offspring (P = 0.0009 for the effects of birth mother).

The Cu status of the birth mother and the postnatal mother interacted to affect CCO activity in heart and liver mitochondria (Table 2), whereby activity in heart mitochondria was higher in CuDCuA offspring than in CuD offspring but lower than the activity in either CuA or CuACuD offspring, which did not differ from one another. CCO activity in liver mitochondria was lower in CuD offspring than in any other group of offspring, which did not differ from one another.

The contents of CCO subunits COX1 and COX4 were determined in 20 μg of heart or liver mitochondrial protein by Western blotting followed by densitometry measurements of the bands representing the subunits. A significant interaction between the Cu status of the birth mother and the postnatal mother affected the content of COX1 in heart mitochondria (Fig. 2A), whereby the content was lower in CuD offspring than in any other group of offspring, which did not differ from one another. COX4 content in heart mitochondria (Fig. 2B) depended only on the Cu status of the birth mother and was lower in the offspring of dams fed the Cu-deficient diet. Optical densities of the bands representing COX4 in heart mitochondria were 9.2 ± 2.7 in combined CuA and CuACuD offspring and 6.5 ± 2.7 in combined CuD and CuDCuA offspring.

COX1 and COX4 contents in liver mitochondria depended only on the Cu status of the birth mother. COX1 (Fig. 3A) and COX4 (Fig. 3B) were both higher in the liver mitochondria of the offspring of dams fed the Cu-adequate diet than in the offspring of dams fed the Cu-deficient diet. Optical densities representing COX1 in liver mitochondria were 15.5 ± 2.6 in the combined Cu and CuACuD offspring and 8.6 ± 2.6 in the combined CuD and CuDCuA offspring. OD representing COX4 in liver mitochondria were 4.2 ± 1.0 in the combined Cu and CuACuD offspring and 3.5 ± 1.0 in the combined CuD and CuDCuA offspring.

**Discussion**

A previous study showed that cardiac CCO activity in the offspring of Cu-deficient dams is normal on postnatal d 1–10 and then declines to produce significant reductions in activity and in the contents of the COX1 and COX4 subunits on postnatal d 21 (7). The present study was conducted by cross fostering the offspring from the dams in one dietary treatment group to dams in the opposite treatment group to determine whether the late postnatal loss of cardiac CCO activity and altered subunit content represent a postnatal effect of low maternal Cu intake or a prenatal effect that becomes manifest postnatally. If the influence of maternal Cu intake on cardiac CCO were operative only during postnatal development, then cross fostering would tend to normalize CCO in the offspring of Cu-deficient dams and lower CCO in the offspring of Cu-adequate dams. Our results showed that cross fostering the

**TABLE 1** Hepatic Cu and Fe concentrations in the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Cu</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuA</td>
<td>28</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>CuACuD</td>
<td>10</td>
<td>1.18 ± 0.10</td>
</tr>
<tr>
<td>CuDCuA</td>
<td>10</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td>CuD</td>
<td>20</td>
<td>0.07 ± 0.07</td>
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</table>

Effect | P-value |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth mother (BM)</td>
<td>&lt;0.0001 0.001</td>
</tr>
<tr>
<td>Postnatal mother (PM)</td>
<td>0.68 0.01</td>
</tr>
<tr>
<td>BM × PM</td>
<td>0.09 0.001</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column with superscripts without a common letter differ, P < 0.05.

**TABLE 2** CCO activity in heart and liver mitochondria of the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation

<table>
<thead>
<tr>
<th>Offspring</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuA</td>
<td>26</td>
<td>3.25 ± 0.06</td>
</tr>
<tr>
<td>CuACuD</td>
<td>8</td>
<td>3.29 ± 0.11</td>
</tr>
<tr>
<td>CuDCuA</td>
<td>10</td>
<td>2.52 ± 0.10</td>
</tr>
<tr>
<td>CuD</td>
<td>18</td>
<td>1.81 ± 0.07</td>
</tr>
</tbody>
</table>

Effect | P-value |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth mother (BM)</td>
<td>&lt;0.0001 0.0002</td>
</tr>
<tr>
<td>Postnatal mother (PM)</td>
<td>0.0005 0.019</td>
</tr>
<tr>
<td>BM × PM</td>
<td>&lt;0.0001 0.006</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column with superscripts without a common letter differ P < 0.05.
2 A unit of CCO activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of ferrocytochrome c per minute at 30°C.

![FIGURE 1](https://academic.oup.com/jn/article-abstract/138/7/1269/4670197/138712946701707) Heart Cu concentrations in the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation. Data from male and female offspring were combined. Values are means ± SEM, n = 26 (CuA), 8 (CuACuD), 10 (CuDCuA), or 18 (CuD). Means without a common letter differ, P < 0.05 (Tukey’s test).
offspring of Cu-deficient dams to Cu-adequate dams immediately following parturition did not lead to normal cardiac CCO activity on postnatal d 21. The results also showed that cross fostering the offspring of Cu-adequate dams to Cu-deficient dams did not affect cardiac CCO activity. These findings suggest that low prenatal Cu intake was a major determinant of CCO activity in the 21-d-old offspring.

Our results showing that cross fostering did not lead to normal liver and heart Cu concentrations in the offspring of Cu-deficient dams suggests that Cu in the milk of Cu-adequate dams was insufficient to completely restore the low Cu status established prenatally in these offspring. Cu deficiency produces cardiomyopathy (15) and the slight elevation in heart weight and heart:body weight ratio in the offspring of Cu-deficient rats regardless of whether or not they were cross fostered may also reflect low Cu status in these offspring. The low Cu status in the cross-fostered offspring of the Cu-deficient dams likely reflects the fact that milk Cu concentrations are relatively low and tend to decline late in the postnatal period in rats (16). However, even though liver Cu concentration was low, hepatic CCO activity in the cross-fostered offspring of the Cu-deficient dams was normal. This suggests that other factors in addition to low Cu status influenced CCO activity in the cross-fostered offspring of Cu-deficient dams.

A factor that may have influenced the effect of cross fostering on CCO activity is the difference in turnover between cardiac and hepatic mitochondria. The rate of recovery for CCO activity after Cu repletion of Cu-deficient rats is determined, at least in part, by mitochondrial biogenesis and is slower in the heart than in the liver (17). Cardiac mitochondria in differentiated cardiomyocytes have a half-life of ~18 d compared with 9 d for hepatic mitochondria (18). CCO activity, representing the activity in preexisting and newly synthesized mitochondria, was measured on postnatal d 21, well after terminal differentiation of cardiomyocytes, which occurs during the first 14 d of postnatal life (19,20). Even though Cu in the milk of Cu-adequate dams may have promoted normal CCO activity in newly synthesized mitochondria in the cross-fostered offspring of Cu-deficient dams, slow turnover of cardiac mitochondria whose CCO activity was reduced by low prenatal Cu intake may have limited the normalization of CCO activity in the cardiac mitochondrial population once the cardiomyocytes became terminally differentiated. Cross fostering may have produced normal hepatic CCO activity, because the relatively fast turnover of hepatic mitochondria may have permitted rapid replacement of mitochondria affected by low prenatal Cu intake with newly synthesized mitochondria having normal CCO activity.

**Figure 2** The contents of COX1 (A) and COX4 (B) subunits in heart mitochondria isolated from the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation. Representative Western blots showing COX1 and COX4 contents in 20 μg mitochondrial protein are placed above the graphs. Values are means ± SEM, n = 26 (CuA), 8 (CuAxCuD), 10 (CuDCuA), or 18 (CuD). Results of the ANOVA for the effects of the birth mother (BM), postnatal mother (PN), and birth mother × postnatal mother interaction (BM × PN) are shown in each panel. Means without a common letter differ, P < 0.05 (Tukey’s test).

**Figure 3** The contents of COX1 (A) and COX4 (B) subunits in liver mitochondria isolated from the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation. Designations for the offspring are given in the legend to Figure 1. Representative Western blots showing COX1 and COX4 contents in 20 μg mitochondrial protein are placed above the graphs. Values are means ± SEM, n = 26 (CuA), 8 (CuAxCuD), 10 (CuDCuA), or 18 (CuD). Results of the ANOVA for the effects of the birth mother (BM), postnatal mother (PN), and birth mother × postnatal mother interaction (BM × PN) are shown in each panel.
Low Cu concentrations in the heart of the cross-fostered offspring of the Cu-deficient dams may have influenced the subunit composition of cardiac CCO. CCO is composed of 13 subunits, 3 of which (COX1, COX2, and COX3) are encoded by the mitochondria DNA. COX1 and COX2 contain Cu and heme in their active sites and COX3 modulates the proton pumping activity of COX1 and COX2. Although the mitochondrial-encoded subunits comprise the catalytic core of CCO, the nuclear-encoded subunits may influence CCO activity by modulating catalysis, stabilizing the catalytic subunits, or providing stability during the assembly of the holoenzyme (21). Our current findings are consistent with a previous report showing that COX1 and COX4 contents are reduced in cardiac mitochondria from 21-d-old offspring of Cu-deficient dams (7). The present study also showed that cross fostering produced relatively normal COX1 content but did not increase COX4 content in cardiac mitochondria. This suggests that low maternal Cu intake may operate prenatally to limit COX4 but not COX1 content in the heart. Mechanistically, the limitation placed on COX4 content may be related to the low heart Cu concentrations in the cross-fostered offspring of the Cu-deficient dams. It is well established that Cu deficiency lowers the content of nuclear-encoded subunits in cardiac mitochondria through mechanisms that may involve increased degradation or reduced mitochondrial importation of the subunits (22–24). Furthermore, it has been shown that cardiac COX4 in rats is particularly resistant to repair by Cu supplementation once its content is reduced by Cu deficiency (23). Thus, the reduced content of nuclear-encoded COX4 content in cardiac mitochondria of the cross-fostered offspring of Cu-deficient dams may be a consequence of the resistance of COX4 to repair coupled with the low heart Cu concentration in these offspring. However, further research is required to determine whether cardiac COX4 can be completely normalized in the offspring of Cu-deficient rats by long-term postweaning Cu supplementation.

Low COX4 content may have contributed to the low cardiac CCO activity found in the cross-fostered offspring of the Cu-deficient dams. It has been reported that the kinetic properties of cardiac CCO depends on the contents of the nuclear-encoded subunits COX4 and COX5b in the holoenzyme (25). Thus, normal COX1 content together with low COX4 content in cardiac mitochondria may have altered subunit stoichiometry in a manner that limited the activity of the fully assembled holoenzyme.

In summary, our findings indicate that the reductions in cardiac CCO activity and subunit content that occur late in the postnatal period in the offspring of dams whose dietary Cu intake was low during pregnancy and lactation result primarily from a prenatal effect, possibly on heart Cu concentration. The prenatal effect produced a decline in heart Cu concentration that was not readily reversed by normal Cu intake from milk during the suckling period. The low heart concentration was likely a determinant of the suppressed CCO activity and COX4 content in the offspring of the dams that consumed the Cu-deficient diet.

Acknowledgments

We thank Steve Dufault, Lana Demars, Terry Schuler, and Kim Michelsen for technical assistance, Denice Schafer for animal care, and LuAnn Johnson for statistical analysis.

Literature Cited