Cardiac Cytochrome c Oxidase Activity and Contents of Subunits 1 and 4 Are Altered in Offspring by Low Prenatal Copper Intake by Rat Dams\textsuperscript{1,2}

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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)\textsuperscript{5} 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

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\textsuperscript{5} Abbreviations used: CCO, cytochrome c oxidase; COX1, cytochrome c oxidase subunit 1; CuA, offspring of dams fed Cu-adequate diet; CuACuD, offspring of dams fed Cu-adequate diet that were cross fostered to dams fed Cu-deficient diet; CuD, offspring of dams fed Cu-deficient diet; CuDCuA, offspring of dams fed Cu-deficient diet that were cross fostered to dams fed Cu-adequate diet.
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 CuSO4 6H2O 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 nonpurified diet (no. 5012 rat diet, PMI Nutrition International) commercial rat nonpurified diet. Mating produced litters in 14 of the 20 dams fed the Cu-deficient diet and 17 of the 20 dams fed the Cu-adequate 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; CuD, offspring of dams fed the Cu-deficient diet; CuACuA, offspring of dams fed the Cu-deficient diet that were cross fostered to dams fed the Cu-adequate diet; and CuDCuA, offspring of dams fed the Cu-deficient diet that were cross fostered to dams fed the Cu-deficient diet. Livers and blood were collected from the dams on postnatal d 21 and hearts and livers from pups in each litter 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 heart homogenate samples containing 1 kg tissue/L (see below). Ceruloplasmin activity was assayed in plasma by its amine oxidase activity (11). 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-SDS 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 COC subunits were 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 hematocrits (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.
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 lactation1

<table>
<thead>
<tr>
<th>Offspring</th>
<th>n</th>
<th>Cu  µmol/g dry liver</th>
<th>Fe µmol/g dry liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuA</td>
<td>28</td>
<td>1.00 ± 0.06</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>CuACuD</td>
<td>10</td>
<td>1.18 ± 0.10</td>
<td>1.25 ± 0.13</td>
</tr>
<tr>
<td>CuDCuA</td>
<td>10</td>
<td>0.19 ± 0.10</td>
<td>0.14 ± 0.19</td>
</tr>
<tr>
<td>CuD</td>
<td>20</td>
<td>0.07 ± 0.07</td>
<td>1.98 ± 0.09</td>
</tr>
</tbody>
</table>

Effect | P-value
Birth mother (BM) | <0.0001 | 0.001
Postnatal mother (PM) | 0.68 | 0.01
BM × PM | 0.09 | 0.001

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

FIGURE 1  Heart Cu concentrations in the offspring and cross-fostered offspring of dams fed Cu-adequate or Cu-deficient diets throughout pregnancy and lactation.

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 lactation1

<table>
<thead>
<tr>
<th>Offspring</th>
<th>n</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuA</td>
<td>26</td>
<td>3.25 ± 0.06</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>CuACuD</td>
<td>8</td>
<td>3.29 ± 0.11</td>
<td>1.11 ± 0.05</td>
</tr>
<tr>
<td>CuDCuA</td>
<td>10</td>
<td>2.52 ± 0.16</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>CuD</td>
<td>18</td>
<td>1.81 ± 0.07</td>
<td>0.83 ± 0.03</td>
</tr>
</tbody>
</table>

Effect | P-value
Birth mother (BM) | <0.0001 | 0.0002
Postnatal mother (PM) | 0.005 | 0.019
BM × PM | <0.0001 | 0.006

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

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 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
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 differen-
tiation 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 turn-
over of hepatic mitochondria may have permitted rapid re-
placement 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 off-
spring and cross-fostered offspring
of dams fed Cu-adequate or Cu-
deficient diets throughout preg-
nancy and lactation. Representative
Western blots showing COX1 and
COX4 contents in 20 μg mitochon-
drial protein are placed above the
graphs. Values are means ± SEM,
\( n = 26 \) (CuA), 8 (CuACuD), 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 with-ou- t 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 off-
spring and cross-fostered offspring
of dams fed Cu-adequate or Cu-
deficient diets throughout preg-
nancy 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 mitochon-
drial protein are placed above the
graphs. Values are means ± SEM,
\( n = 26 \) (CuA), 8 (CuACuD), 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.

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Literature Cited