Effects of copper supplementation on copper absorption, tissue distribution, and copper transporter expression in an infant rat model

Kathryn A. Bauerly, Shannon L. Kelleher, and Bo Lönnlerdal

Department of Nutrition, University of California, Davis, California

Submitted 7 May 2004; accepted in final form 7 December 2004

Bauerly, Kathryn A., Shannon L. Kelleher, and Bo Lönnlerdal. Effects of copper supplementation on copper absorption, tissue distribution, and copper transporter expression in an infant rat model. Am J Physiol Gastrointest Liver Physiol 288: G1007–G1014, 2005. First published December 9, 2004; doi:10.1152/ajpgi.00210.2004.—Infants are exposed to variable copper (Cu) intake; Cu in breast milk is low, whereas infant formulas vary in Cu content as well as the water used for their preparation. Little is known about the regulation of Cu absorption during infancy. The objectives of this study were to determine effects of Cu supplementation on Cu absorption and tissue distribution and the expression of Cu transporters in an infant rat model. Suckling rat pups were orally dosed with 0, 10, or 25 μg Cu/day. Intestine and liver were collected at days 10 and 20, and Cu concentration, Cu transporter-1 (Ctr1), Atp7A, Atp7B, and metallothionein (MT) mRNA and protein levels were measured. Sixty Cu absorption was measured at days 10 and 20. Total Cu absorption decreased, and intestinal Cu retention increased with increased Cu intake. At day 10, intestine Cu concentration, MT mRNA, and Ctrl protein levels increased with supplementation, but no changes in Atp7A or Atp7B levels were observed. At day 20, intestine Cu concentration was unaffected by Cu supplementation, but Ctrl protein and Atp7A mRNA and protein levels were higher than in controls. In liver, Cu level reflected Cu intake at days 10 and 20. There was a significant increase in Ctrl, Atp7B, and MT mRNA expression in liver at both ages with Cu supplementation. In conclusion, the ability of suckling rat pups to tolerate varying amounts of dietary Cu may be due to changes in Cu transporters, facilitated by transcriptional and posttranslational mechanisms. Despite these adaptive changes, Cu supplementation resulted in elevated alanine aminotransferase levels, suggesting a risk of Cu toxicity with supplementation during infancy.

COPPER (Cu) is an essential mineral that is required for normal growth and the development of bone, brain, immune system, and red blood cells during infancy (4, 11). However, high Cu intake during infancy can also be toxic and has been associated with impaired growth, hepatic accumulation, jaundice, and necrosis (34). Daily Cu intake of infants varies depending on diet. Breast milk Cu concentration is low containing ~0.2–0.3 mg Cu/l. Term infant formulas contain from 0.4 to 0.8 mg Cu/l, whereas formulas designed for premature infants can contain up to 2 mg Cu/l (21). An additional variable is the Cu content of the water used to reconstitute powdered infant formula. Although the World Health Organization has established a limit of 2 mg Cu/l for the Cu concentration in drinking water (41), some areas of the world that mine Cu or that use Cu plumbing or well water have a water Cu concentration that exceeds this guideline, which has been linked to toxic effects (7). However, data are currently insufficient to set an upper limit for Cu intake and exposure for infants under 1 year of age (5).

Studies in human infants show that even over this range of Cu intake, Cu status remains largely unchanged, as assessed by serum Cu concentration and ceruloplasmin activity (24, 25, 30, 31). This suggests that Cu absorption in infants may be tightly regulated; however, little is known about the regulation of Cu absorption and transport mechanisms. Although it is well established that the intestine and liver play key roles in Cu metabolism, the consequences of high Cu intake on Cu regulatory mechanisms in the intestine and liver of infants have not been well characterized.

Recently, several Cu transporters have been identified that are involved in Cu uptake and transport by cells. Cu transporter-1 (Ctrl) is a Cu import protein that is Cu specific and transports Cu in an energy-independent and saturable manner (17). In addition, Ctrl is expressed in the enterocytes of the small intestine and in enterocyte-like Caco-2 cells in culture (14, 16). These observations suggest that Ctrl may play a role in absorption of dietary Cu. Although mRNA and protein levels did not change with Cu supplementation in Caco-2 cells, Ctrl protein translocated to an intracellular endosomal compartment and represents a novel way to limit Cu uptake by the intestine (3). Atp7A is a Cu efflux protein with a dual role: delivering Cu to Cu-containing enzymes in the trans-Golgi network and cycling to the cellular membrane to participate in Cu efflux during Cu excess in transfected cells (27). Menkes disease is the consequence of a defect in Atp7A resulting in excessive Cu accumulation in the intestine and systemic Cu deficiency (33). Atp7B, or the Wilson’s gene product, has functional similarity to Atp7A and is localized predominately in the liver, with lower expression found in the intestine, kidney, and placenta (10, 15, 20). In liver, Atp7B functions to export Cu into bile for excretion (29). A defect in Atp7B results in Wilson disease, characterized by Cu toxicity and liver damage (35). The role of Atp7B in the small intestine remains largely unknown.

Excess intracellular Cu may be bound to metallothionein (MT). MT functions include intracellular metal metabolism/ storage, metal donation, metal detoxification, and protection against oxidants (6). MT may also play a role in regulating absorption by trapping Cu intracellularly until the enterocyte is sloughed off and excreted by the intestine or secreted into bile by the liver.

We hypothesized that Cu uptake and transport in the infant are regulated by changes in Cu transporter expression levels.

http://www.ajpgi.org

0193-1857/05 $8.00 Copyright © 2005 the American Physiological Society

G1007

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The objectives of this study were to determine the effects of Cu supplementation on tissue Cu distribution, Cu transport, and Cu transporter levels in an infant rat model. Because infants are born with high levels of Cu stored in the liver that decline with age (13), the effect of Cu supplementation in early and late infancy were examined. The study was designed to explore the effects of exposing suckling rat pups to amounts of Cu that artificially fed infants may receive and to understand the molecular events that occur that may protect the infant against Cu toxicity.

**METHODS**

**Experimental design.** This study was approved by Institutional Animal Care and Use Committee at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Pregnant Sprague-Dawley rats (n = 16) were obtained from a commercial source (Charles River, Wilmington, MA). Rats were maintained in polycarbonate cages with wood shavings in a temperature-controlled facility with a 12:12-h light-dark cycle and allowed to consume purified, deionized water and a standard rat diet containing 13 µg/g Cu (Ralston Purina, St. Louis, MO) ad libitum through pregnancy and lactation. Rats were acclimated to their environment and allowed to deliver normally. On postnatal day 1, newborn pups were randomly assigned to Cu treatment groups. Suckling rat pups were given a daily dose of either 0, 10, or 25 µg Cu/day as CuSO4 in 10% sucrose solution by oral gavage during the suckling period and weaned to a standard diet containing 13 µg/g Cu. Pups were weighed daily. On postnatal days 10 and 20, pups were killed by asphyxiation with CO2 and the intestine was isolated and frozen for analysis.

Rat pup small intestine, liver, kidney, brain, and spleen were collected and snap frozen and stored at −80°C for mineral analysis. Samples of the small intestine and liver were dissected and immediately homogenized in TRIzol reagent (Life Technologies, Rockville, MD) for RNA extraction or were snap frozen for assessment of protein expression and stored at −80°C. Total RNA was isolated following the TRIzol protocol. Blood was removed by cardiac puncture into heparinized tubes, and plasma was separated by centrifugation at 2,000 × g for 15 min at 4°C and stored at −20°C for mineral analysis and assay of ceruloplasmin (Cp) activity. Cp oxidase activity in plasma was assayed with o-dianisidine dihydrochloride following the method of Schosinsky et al. (36).

**Cu uptake.** 67Cu absorption was measured in control and Cu supplemented pups at days 10 and 20. The design of this model of Cu absorption in suckling rat pups has previously been described (22). Pups were fasted for 5 h, then intubated with 0.1 µCi 67CuCl2 (specific activity 8.4 mCi/µg; Brookhaven National Laboratory, New York, NY) in 1 ml 3 µM CuSO4 in PBS. After 6 h, animals were killed by asphyxiation with CO2 and the intestine was isolated and perfused with cold PBS. Radioactivity in the perfusate, small intestine, cecum/colon, liver, brain, spleen, kidney, and remaining carcass was counted in a γ-counter (Gamma 8500, Beckman, Irvine, CA). The results were expressed as a percentage of the total radioactivity received. Total percent body absorption was calculated as the total radioactivity given minus the sum of the radioactivity in the small intestine, perfusate, and cecum/colon divided by total radioactivity given times 100. Total percent unabsorbed was calculated as the sum of the radioactivity in the perfusate and cecum/colon divided by the total radioactivity given times 100.

**Cu analysis.** Plasma was digested at room temperature for 5 days with 0.1 mol/l ultrapure nitric acid; small intestine, liver, kidney, brain, and spleen were wet ashed with concentrated nitric acid, and samples were analyzed for Cu by flame atomic absorption spectrophotometry (Smith-Heifjie 4000, Thermo Jarrell Ash, Franklin, MA).

**Liver enzymes.** A complete small animal liver panel was performed on plasma from control and supplemented pups by University of California Davis Department of Veterinary Medicine, Teaching Hospital Clinical Pathology Laboratory. Liver panel was analyzed in 200 µl plasma on a Hitachi chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Included in the analyses were albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), bilirubin, and glutamate dehydrogenase (GD).

**Real-time quantitative PCR.** Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) as previously described (12) and diluted to 1 µg/ml in RNase-free water. RNA integrity and genomic DNA contamination were evaluated following electrophoresis through 2% agarose and staining with ethidium bromide (Sigma). cDNA was generated from 1 µg RNA using reverse transcription kit (PerkinElmer Applied Biosystems, Foster City, CA) following the manufacturer’s instructions, and the reaction was performed at 48°C for 30 min followed by 95°C for 5 min. Gene-specific primers to rat Ct1, Atp7A, Atp7B, MT, and GAPDH (control gene) were chosen using Primer Designer software (Perkin-Elmer Applied Biosystems) and purchased from Qiagen (Valencia, CA). For Ct1, the primers used were 5′-GGA AAT GCT GGA GCT TTT-3′ and 5′-CCG GCT ATC TTG AGT CCT TCA-3′. For Atp7A, the primers used were 5′-AAG CCA CAT TAC GAT AAT TAT GAG TTG-3′ and 5′-TGC GTA CTC TGA GAT TTC-3′. For Atp7B, the primers used were 5′-TTG GAA ACC GAA GCT GGA-3′ and 5′-CTG TCG CTG TAC TGA-3′. For MT, the primers used were 5′-GCG GAC CAA CTC AGA CTC TTG-3′ and 5′-TTC ACA TGC TCG GTA GAA AAC G-3′. For GAPDH, the primers used were 5′-TGC CAA GTA TGA TGA CATCAA GAA G-3′ and 5′-AGC CCA GGA TGC CCT TTA GAT-3′. Real-time PCR was performed on 4 µl of the cDNA reaction mixture using the ABI 7900HT real-time thermocycler (Perkin-Elmer Applied Biosystems) coupled with SYBR Green technology (Perkin Elmer Applied Biosystems) and the following cycling parameters: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 40 cycles of 95°C for 15 s; 60°C for 1 min; and stage 4, 95°C for 15 s; 60°C for 15 s; 95°C for 15 s.

The linearity of the dissociation curve was analyzed using the ABI 7900HT software, and the mean cycle time of the linear part of the curve was designated Ct. Each sample was analyzed in duplicate and normalized to GAPDH using the following equation: ΔCtGENE = CtGENE − CtGAPDH. The fold change relative to control animals was calculated using the following equation: 2ΔCtGENE, where ΔCtGENE = mean CtGENE of the control animals − ΔCtGENE of each supplemental animal. Values represent mean fold change ± SD.

**Western blot analysis.** For membrane protein isolation, intestine samples were homogenized in 20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose, with 1% protease inhibitor cocktail containing 200 µg/ml soybean trypsin inhibitor, 14 µg/ml benzamidine, 14 µg/ml phenylmethylsulfonyl fluoride, and 0.3 µM aprotinin. Samples were first centrifuged at 1,500 g for 15 min, 4°C, and supernatant was collected, and then were ultracentrifuged at 100,000 g for 30 min (Beckman Ti75.1, H650 rotor, DuPont, Newton, CT). The pellet containing the crude membrane fraction was suspended in 1 ml homogenization buffer. Protein concentration was determined by the Bradford method (Bio-Rad). Membrane proteins (50 µg) were electrophoresed through polyacrylamide gels (Ct1, 10%; Atp7A and Atp7B, 8%), transferred onto nitrocellulose membrane at 350 mA for 60 min, blocked overnight in PBS/0.1% Tween-20 (PBST) with 5% nonfat milk at 4°C. Blots were incubated with Ct1, Atp7A, or Atp7B antisera (1:3,000 in PBST) washed three times with PBST. Donkey anti-rabbit Ig hors eradish peroxidase-linked antibody (Amersham) was used as the secondary antibody (1:20,000 in 5% milk). Bands were detected using Super Signal Femto chemiluminescent reagent (Pierce). Processed blots were exposed to X-ray film for the optimum exposure time. Specific bands were quantified by densitometry using the Chemi-doc gel quantification system (Bio-Rad).
RESULTS

Tissue Cu. There was no significant effect of Cu supplementation on body weight, serum Cu, or ceruloplasmin activity (Table 1). There was a significant effect of age on serum Cu, ceruloplasmin activity, and body weight; however, there was no interaction between Cu supplementation and age observed for these variables.

There was a significant effect of Cu supplementation and age on intestine Cu concentration and a significant interaction between supplementation and age (Table 2). Further analysis by one-way ANOVA determined that at day 10, intestine Cu concentration was significantly higher in pups supplemented with 25 µg Cu/day (P < 0.0001), whereas no significant effect of Cu supplementation was observed at day 20 (P = 0.6).

There was a significant effect of Cu supplementation and age on liver, kidney, and brain Cu concentration; however, no interaction was observed (Table 2). There was a significant effect of age on spleen Cu concentration; however, no significant effect of Cu supplementation was observed (Table 2).

Liver enzymes. There was a significant effect of Cu supplementation on ALT levels in plasma, with no effect of age or interaction between Cu supplementation and age (Table 3). There was no significant effect of Cu supplementation or interaction with age on total protein, albumin, AST, AP, bilirubin, or GD activity.

67Cu uptake. Table 4 represents 67Cu retention in the intestine and liver as well as total Cu absorbed. There was a significant decrease in total 67Cu absorption in Cu-supplemented pups with no significant effect of age and no significant interaction between Cu supplementation and age. There was a significant increase in intestine 67Cu retention in Cu-supplemented pups with no significant effect of age or an interaction between Cu supplementation and age. There was a significant interaction between Cu supplementation and age in the percent of unabsorbed 67Cu. Further analysis by one-way ANOVA determined there was no significant effect of Cu supplementation in day 10 pups; however, at day 20, pups supplemented with Cu had a significantly lower percentage of unabsorbed 67Cu (P < 0.01) compared with control pups. There was a significant decrease in liver 67Cu retention in Cu-supplemented pups with no significant effect of age or an interaction between Cu supplementation and age.

There was a significant decrease in kidney 67Cu in Cu-supplemented pups, with no significant effect of age or an interaction between Cu supplementation and age. There was no
significant effect of Cu supplementation, age, or their interaction on \(^{67}\)Cu uptake into spleen or brain. There was a significant effect of age on \(^{67}\)Cu uptake in the remaining carcass but no effect of Cu supplementation or interaction between Cu and age (data not shown).

**mRNA expression.** In the small intestine, there was no effect of Cu supplementation, age, or interaction between Cu supplementation and age on Ctrl1 mRNA level (Fig. 1). There was a significant effect of Cu supplementation on MT mRNA expression \((P = 0.006)\) but no interaction between Cu supplementation and age. There was a significant interaction between Cu supplementation and age on intestine Atp7A mRNA expression \((P = 0.005)\). Atp7A was not significantly affected by Cu intake at day 10 \((P = 0.5)\); however, Cu supplementation significantly increased Atp7A mRNA expression in the small intestine of day 20 pups \((P = 0.005)\); Fig. 1). There was no effect of Cu supplementation, age, or interaction between Cu supplementation and age on Atp7B mRNA levels in the small intestine. GAPDH mRNA levels did not change with Cu supplementation and served as an appropriate control gene (data not shown).

In liver, Cu supplementation significantly increased Ctrl1 mRNA expression levels \((P < 0.001);\) Fig. 2) with no significant effect of age or an interaction between Cu supplementation and age. There was a significant interaction between Cu supplementation and age on MT mRNA expression \((P < 0.001).\) Further analysis by one-way ANOVA determined that Cu supplementation increased MT mRNA levels at days 10 \((P = 0.01)\) and 20 \((P < 0.001).\) There was a significant effect of Cu supplementation on Atp7B mRNA expression \((P < 0.001),\) with no effect on age or an interaction between Cu supplementation and age. There was no significant effect of Cu supplementation, age, or interaction between supplementation and age on liver Atp7A mRNA levels. GAPDH mRNA levels did not change with Cu supplementation and served as an appropriate control gene (data not shown).

**Protein expression.** There was a significant effect of Cu supplementation on intestine Ctrl1 protein expression \((P = 0.01);\) however, no effect of age or an interaction between Cu and age was observed (Fig. 3). There was a significant interaction between Cu supplementation and age on Atp7A protein expression in the small intestine \((P = 0.03).\) Further analysis by one-way ANOVA determined that Cu supplementation significantly increased intestine Atp7A protein expression at day 20 \((P = 0.02)\) but not at day 10 (Fig. 3). Atp7B protein levels did not change with Cu supplementation in either day 10 or day 20 small intestine (data not shown).

There was a significant interaction between Cu supplementation and age on liver Ctrl1 protein expression \((P = 0.01);\) Fig. 4). Further analysis by one-way ANOVA determined that Cu supplementation increased Ctrl1 protein expression at day 10 \((P = 0.04),\) with no significant effect observed at day 20. There

### Table 3. Plasma analysis of liver enzymes from pups supplemented with Cu

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 µg/day</th>
<th>25 µg/day</th>
<th>Copper</th>
<th>Age</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>day 10</td>
<td>2.2±0.4</td>
<td>2.3±0.1</td>
<td>2.7±0.04</td>
<td>0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>3.5±0.3</td>
<td>3.7±0.1</td>
<td>3.9±0.3</td>
<td>0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin</td>
<td>day 10</td>
<td>1.2±0.2</td>
<td>0.9±0.2</td>
<td>1.5±0.03</td>
<td>0.04</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>1.9±0.3</td>
<td>2.2±0.05</td>
<td>2.4±0.2</td>
<td>0.06</td>
<td>0.6</td>
</tr>
<tr>
<td>ALT</td>
<td>day 10</td>
<td>0.8±0.5</td>
<td>0.6±0.2</td>
<td>1.2±0.2</td>
<td>0.06</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>1.3±0.3</td>
<td>0.4±0.2</td>
<td>1.4±0.5</td>
<td>0.06</td>
<td>0.6</td>
</tr>
<tr>
<td>AST</td>
<td>day 10</td>
<td>86.0±27.8</td>
<td>73.0±28.0</td>
<td>64.3±3.2</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>62.5±7.9</td>
<td>74.0±12.0</td>
<td>66.6±8.2</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>AP</td>
<td>day 10</td>
<td>110.5±21.4</td>
<td>126.5±7.0</td>
<td>135.5±7.7</td>
<td>0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>219.0±15.4</td>
<td>214.0±15.3</td>
<td>213.6±15.0</td>
<td>0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>day 10</td>
<td>0.35±0.2</td>
<td>0.5±0.1</td>
<td>0.2±0.04</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>0.3±0.1</td>
<td>0.1±0.02</td>
<td>0.2±0.02</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td>GD</td>
<td>day 10</td>
<td>3.0±0.7</td>
<td>2.0±1.0</td>
<td>3.3±0.3</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>3.3±1.0</td>
<td>4.0±0.8</td>
<td>2.8±0.6</td>
<td>0.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Values are means ± SD \((n = 8 \text{ per group})\). Two-way ANOVA determined significance of Cu supplementation, age, and their interaction. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase; GD, glutamate dehydrogenase.

### Table 4. Percent \(^{67}\)Cu uptake in tissues of pups supplemented with varying levels of Cu

<table>
<thead>
<tr>
<th></th>
<th>% (^{67})Cu</th>
<th>Two-way ANOVA ((P \text{ values}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total absorbed</td>
<td>day 10</td>
<td>46.7±12.9</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>36.1±6.7</td>
</tr>
<tr>
<td>Intestinal retention</td>
<td>day 10</td>
<td>41.9±4.1</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>35.5±7.8</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>day 10</td>
<td>11.5±5.3a</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>26.6±8.7b</td>
</tr>
<tr>
<td>Liver</td>
<td>day 10</td>
<td>8.4±3.8</td>
</tr>
<tr>
<td></td>
<td>day 20</td>
<td>16.2±2.1</td>
</tr>
</tbody>
</table>

Values are means ± SD \((n = 8 \text{ per group})\). Two-way ANOVA determined significance of Cu supplementation, age, and their interaction. One-way ANOVA determined significance if an interaction was detected. Values with different letters in a row are significantly different \((P < 0.05)\).
was no significant effect of Cu supplementation or age or an interaction between Cu and age on Atp7B protein levels in liver of pups at day 10 or day 20 (data not shown).

**DISCUSSION**

Liver Cu stores are high at birth, thus infants may be at risk for the physiological consequences of excess Cu if Cu intake is high. On the other hand, infants may be protected against Cu toxicity because the liver may accommodate substantial Cu levels during infancy. To address concerns regarding the safety of high Cu intake during infancy, we investigated effects of Cu supplementation during infancy in a rat pup model. The Cu levels used in this study were chosen to simulate the Cu intake
of artificially fed infants. The supplementation level of 10 μg Cu/day is, on a body weight basis, similar to the Cu intake of an infant fed infant formula. This level of Cu intake did not affect small intestine and liver Cu concentration, suggesting that this moderate level of Cu intake is safe for suckling rat pups. However, pups supplemented with 25 μg Cu/day, similar to the Cu intake of infants fed formula made with Cu-contaminated water, retained Cu in their liver and small intestine, suggesting that they may be at risk for Cu toxicity.

There was a striking effect of age on tissue Cu level as a result of Cu supplementation. At day 10, Cu-supplemented pups retained 149.9 μg Cu/g in the intestine (compared with 34.6 μg Cu/g in controls), whereas at day 20, intestine Cu concentration was no longer different from controls. We speculated that this is explained by the development of Cu-transport regulation between these days and that the machinery to handle Cu is immature in young pups (day 10) and matures to adult levels by weaning (day 20). Developmental regulation of intestine Cu transport may occur as infants may not rely on dietary Cu until weaning, resulting in intestinal Cu “trapping” when Cu intake is high (2, 3, 8, 9, 42). Therefore, intestine Cu accumulation may be a protective mechanism to prevent the transfer of excess Cu into the body.

As an intracellular Cu-binding protein, MT sequesters excess Cu, thus protecting the cell from redox toxicity. Similar to reports from others (23), MT gene expression was upregulated in response to Cu supplementation in the small intestine at both ages. Whereas there are Cu-responsive transcription factors such as Ace1, Mac1, and Amt-1 (38), Cu may also indirectly induce MT gene expression by displacing Zn, allowing Zn to bind to the metal responsive element in the MT promoter region and induce MT expression. Interestingly, MT expression was higher in Cu-supplemented pups independent of intestine Cu concentration perhaps as a response to oxidative stress (1, 32).

Despite increased tissue Cu concentration in day 10 pups, serum Cu level and ceruloplasmin activity remained unchanged, similar to results from human infant studies (24, 25, 31, 43). This is possibly due to increased intestine Cu retention because Cu-supplemented pups had higher 67Cu retention following an oral dose. Although Cu supplementation (80 μg Cu·kg⁻¹·day⁻¹ for 15 days) did not affect 65Cu absorption in 1- to 3-mo-old infants, there was an inverse correlation between fecal Cu excretion and the percentage of 65Cu absorbed, indicating that Cu absorption is regulated at the absorptive level in young infants (24); however, the mechanisms behind this regulation during infancy are poorly understood.

Ct1 is believed to mediate Cu uptake into the small intestine. Similar to observations by Lee et al. (18), Cu supplementation did not affect intestine Ct1 mRNA level. Instead, posttranscriptional events altering Ct1 protein level or cellular localization may primarily regulate Ct1 function as we (3) and others (28) have shown that Ct1 is endocytosed in response to Cu in cultured cells. Although our suckling pup model was not sensitive enough to assess intracellular trafficking, we speculate that increased Ct1 protein levels in combination with potential Ct1 endocytosis may be responsible for the higher
amount of unabsorbed $^{67}$Cu in young rat pups, thereby limiting intestine Cu uptake.

Atp7A is presumed to mediate Cu efflux across the plasma membrane (26). In contrast to Ctrl1, Atp7A expression at day 10 was not affected by Cu supplementation, which may help to explain the lower total body $^{67}$Cu absorption observed in Cu-supplemented young pups. Atp7A is also regulated post-translationally by Cu exposure and translates to intracellular vesicles or to the plasma membrane to sequester Cu or increase Cu efflux, respectively, in transfected Chinese hamster ovary cells (27). As a polarized cell, the enterocyte may regulate Cu absorption to the body by relocating Atp7A to vesicles or either the apical or serosal membrane, depending on cellular/whole body needs and thus may functionally trap Cu in the small intestine in young infants. In contrast, by weaning (day 20), although intestine Cu concentration was no longer different from controls, the amount of $^{67}$Cu retained by the small intestine remained higher, whereas the amount of unabsorbed Cu in the gastrointestinal tract was lower, indicating that Cu is more effectively transported through the small intestine most likely as a result of increased Ctrl1 and Atp7A expression.

The liver is considered the main site of Cu homeostasis in the body (19). In this study, Cu supplementation increased liver Cu concentration, which has been shown in other animal (9) and cell culture models (37). Similar to what we observed in the small intestine, Cu supplementation increased Ctrl1 mRNA and protein levels perhaps to increase liver Cu uptake, quickly clearing free or loosely bound Cu from circulation to maintain serum Cu concentration. Wilson disease results from Cu toxicity due to liver Cu accumulation as a result of impaired biliary Cu excretion, indicating that Atp7B plays a major role in Cu excretion. Cu supplementation did not affect Atp7B protein level, which may have resulted in liver Cu accumulation. Because bile is the main route for Cu excretion (19), it represents a site for homeostatic regulation of Cu metabolism (39, 40). However, biliary excretion, and thus Cu excretion, is immature at birth, and we have shown that Cu excretion by the liver develops at approximately days 14–16 postnatally in rats (22). Therefore, whereas already burdened with normally high hepatic Cu levels, infants exposed to excess Cu accumulate additional amounts of Cu in liver during a time when they lack an effective excretion mechanism, putting them at risk for Cu toxicity. However, the effect of Cu supplementation on the rate of biliary excretion during the postnatal period was not addressed in this study and remains unknown.

Human infants exposed to modestly elevated Cu intake do not have altered liver enzymes, suggesting healthy liver function. There was no significant difference in total bilirubin or liver enzymes in infants supplemented with 2 mg/l Cu in drinking water compared with unsupplemented infants at 6, 9, or 12 mo (25). In addition, no signs of adverse health effects, including elevated liver enzymes or serum Cu, were found in children from households with elevated Cu in tap water (43). However, we found elevated ALT levels in rat pups supplemented with the higher level of Cu. In addition, we have preliminary results (M. Araya, S. Kelleher, M. Arredondo, W. Sierraalta, M. Vial, R. Uauy, B. Lönnerdal, unpublished data) from controlled studies in infant rhesus monkeys that indicate that whereas there was no significant effect of Cu treatment on liver enzyme activities at 3 mo of age (early infancy), infants supplemented with ~3 mg Cu/day from birth until weaning (6 mo) had higher alkaline phosphatase and γ-glutamyl transferase activity than control infants, suggesting that liver function may be compromised at higher levels of Cu exposure.

In summary, although infants are believed to be at risk for Cu toxicity in early life, our data suggest that young infants are capable of regulating Cu absorption to some extent. With the use of a suckling rat pup model, we have shown that the level of Cu present in infant formula was well tolerated. However, pups fed Cu at levels similar to those present in formula made with Cu-contaminated water are presented with more of a challenge. At young age (day 10), protein expression of intestinal Ctrl1, but not Atp7A, increased, resulting in an increased intestinal Cu content and limited the amount of Cu transferred to the body. In older pups (day 20), both Ctrl1 and Atp7A were upregulated, resulting in no Cu accumulation in the intestine and enhanced transport into circulation. MT expression was also upregulated as a protective mechanism in response to the additional Cu. However, despite these adaptive changes, Cu accumulates in the liver of these animals, possibly due to immature biliary excretion of Cu. Finally, liver enzymes were elevated, indicating that liver Cu accumulation had adverse effects and raising concern over potential Cu toxicity when high amounts of Cu are consumed during infancy.

REFERENCES


