Intestinal regulation of copper homeostasis: a developmental perspective

Bo Lönnerdal

ABSTRACT

Stable-isotope studies in human infants and adults have shown that copper homeostasis occurs, but the contribution of the small intestine to this regulation is still not well understood. Copper first needs to be reduced to the cuprous form, most likely by Steap proteins on the apical membrane. Copper is subsequently absorbed by Ctr1 and then transferred in the enterocyte by the chaperone Atox1 to reach ATP7A for export from the cell. The role of ATP7B, shown to be present in the small intestine, is still poorly understood. In situations of high copper exposure, Ctr1 is endocytosed, metallothionein is induced, and ATP7A moves to a more basolateral localization. However, the ontogeny of regulation of copper homeostasis has received little attention. In rat pups, tissue copper and total-body 67Cu retention decrease throughout postnatal development, whereas liver 67Cu retention, serum copper, and ceruloplasmin activity increase. Total 67Cu absorption decreases and intestinal 67Cu retention increases with increased copper intake. During early infancy (day 10), copper supplementation increases intestinal copper and metallothionein gene expression, and Ctr1 protein levels increase, whereas Atp7A and Atp7B are unaffected. However, during late infancy (day 20), intestinal copper concentrations are unaffected by supplementation, but Ctr1, ATP7A, and Atp7B protein levels are higher than in controls. Thus, maturation of small intestine copper transport occurs through increased abundance and altered localization of Ctr1, Atp7A, and Atp7B. The mechanisms behind this maturation, including both transcriptional and posttranscriptional regulation, require further studies.

INTRODUCTION

A fundamental issue when considering the risk of copper deficiency and excess in human populations is the extent to which copper homeostasis occurs. It was shown by stable-isotope studies in adults that copper absorption is 36% when a copper-adequate diet (1.68 mg/d) is fed, 56% when a low-copper diet (0.78 mg/d) is fed, and 12% when a high-copper diet (7.5 mg/d) is fed (1). Subsequent studies showed that both copper absorption (uptake) and copper excretion respond to copper intake (2).

Whether homeostatic regulation of copper absorption in infants occurs is uncertain. Infants can be exposed to highly variable copper intakes; breast milk contains 0.15–0.20 mg Cu/L, infant formula 0.4–0.6 mg/L, and formula for preterm infants even higher concentrations of 0.6–1.0 mg/L (3). The risk of excess copper exposure in infants fed formula prepared with water containing high concentrations of copper is a concern of pediatricians in areas where dinking water may contain 1–2 mg Cu/L. We studied the absorption of copper in infants exposed to 80 μg Cu · kg⁻¹ · d⁻¹ for 15 d by using the stable isotope 65Cu (4). Copper absorption was similarly high in both groups at 1 and 3 mo of age, which suggests either that young infants cannot down-regulate copper absorption as found in adults (1) or that the dose or duration (or both) of the load used in our study was insufficient to affect copper homeostasis.

To explore this further, we used a nonhuman primate animal model, infant rhesus monkeys, and exposed them to a substantially higher intake of copper during early life (5). Copper retention was 19% and 11% at 1 and 5 mo of age, respectively, whereas it was ≈75% in control monkeys at 2 mo of age, which suggests that copper absorption was down-regulated. Liver copper content was markedly increased at 1, 5, and 8 mo of age, and although no clinical evidence of copper toxicity was observed, histology revealed ultrastructural changes that may signal early cellular damage. The exposure level in this study was 6000 μg Cu · kg⁻¹ · d⁻¹ or ≈75 times that in the study on human infants, which suggests that a trigger point for homeostatic regulation may have been reached at this considerably higher exposure. The extent to which the small intestine will respond to increased copper exposure is not yet known, particularly at young ages.

Studies in adult rodents also show regulation of copper homeostasis. However, mechanisms that control the intestinal absorption of copper (discussed below) are immature during the newborn period in rats (6), which potentially puts younger animals at greater risk of adverse consequences of high copper exposure. Copper supplementation of suckling rat pups resulted in considerably higher copper concentration in the small intestine; however, plasma copper was not affected (7, 8). Liver copper concentrations were also high, either because less copper was mobilized from the liver or some copper was transferred from the small intestine to the liver. We have studied the effects of moderately increased copper exposure on copper uptake and retention in the human intestinal Caco-2 cell line and the ontogeny of intestinal regulation of copper homeostasis in a suckling rat pup model, with special emphasis on copper transporters.

1 From the Department of Nutrition, University of California, Davis.
2 Supported by intramural grants.
3 Presented at the symposium “Molecular Biomarkers of Copper Homeostasis,” held in Viña del Mar, Chile, September 26–29, 2007.
4 Reprints not available. Address correspondence to B Lönnerdal, Department of Nutrition, University of California, One Shields Avenue, Davis, CA 95616. E-mail: blonnerdal@ucdavis.edu.
INTESTINAL UPTAKE AND TRANSPORT OF COPPER

Dietary copper is likely to be in the Cu\(^{2+}\) form. Because copper is absorbed as Cu\(^{2+}\), it needs to be reduced before uptake at the apical membrane of the enterocyte. The presence of a reductase localized in the brush border membrane was suggested (9), and recently a family of metalloreductases, called Steap proteins, were shown to be expressed in the duodenum (10). Steap proteins can reduce both ferric iron and cupric ions when transfected into HEK293 cells (10), but their functional significance in vivo has not yet been shown. The major transporter of copper across the apical membrane of the intestinal cell is Ctrl (copper transporter 1), which is a homotrimer that facilitates the transport of Cu\(^{2+}\) (11). Studies in various cell lines (12, 13), as well as in vivo (14), have shown that Ctrl1 is localized at the apical membrane and intracellular vesicular compartments. Transfection studies with epitope-tagged Ctrl1 have shown that Ctrl1 undergoes constitutive recycling, and that high copper exposure results in rapid endocytosis of Ctrl1 (15). Thus, it is likely that Ctrl1 cycles from and to the apical membrane in intestinal cells (14). The critical importance of Ctrl1 for intestinal uptake of copper was shown by the conditional knockout of Ctrl1 in mouse small intestine by using the cre-lox method (15). Homozygous mouse pups without Ctrl1 became severely copper deficient and had poor growth and viability, but intraperitoneal injection of copper could at least partially restore copper status. However, some copper was still accumulated, which suggests alternative mechanisms for copper uptake.

The major transporter of iron across the apical membrane of the enterocyte, DMT1 (divalent metal transporter 1), has been suggested to also transport copper (16). By knocking down DMT1 in Caco-2 cells, copper uptake was reduced (17), and induction of DMT1 was shown to increase copper uptake (18). The quantitative significance of DMT1 for copper uptake under physiologic ranges of copper concentrations is still not known. Other mechanisms, such as pinocytosis, may also play a role, particularly during early life (19).

Somewhat surprisingly, copper accumulation by the intestine was considerably higher than normal in the small intestine, tissue-specific Ctrl1 knockout mice (14), which suggests that copper was taken up but was not biologically available. The authors suggested that Ctrl1 is also essential for the transport of copper from the lumen of an unidentified intracellular compartment, or that Ctrl1 transports copper by a 2-step process including binding of Cu\(^{2+}\) at the enterocyte surface followed by mobilization of copper from endosomal compartments that accumulate copper in these mice.

After uptake by the cell, copper is most likely delivered to Atox1, a copper chaperone that can deliver copper to ATP7A for uptake by the trans-Golgi network (19). Mice with a mutation in Atox1 accumulate high concentrations of intracellular copper, showing that delivery of copper for export out of the cell does not occur (20). Some copper will also most likely be delivered by the chaperone COX17 to cytchrome oxidase in mitochondria and to superoxide dismutase (SOD1) by copper chaperone SOD (CCS) (21). The export of copper from the epithelial cell is mediated by ATP7A, and a recent study in Caco-2 cells showed that the vast majority of ATP7A is intracellular under all copper conditions (22). The fate of copper in the trans-Golgi network was found to be determined by cellular copper status. In copper-depleted cells, virtually all ATP7A is localized to a post-trans-Golgi network compartment with <3% located at the basolateral membrane. When exposed to high copper concentrations, however, ATP7A dispersed to novel vesicles in the cell periphery, but only 8–10% co-localized with a marker for the basolateral membrane. The authors suggested that the copper-loaded vesicles release their contents into the basolateral medium by exocytosis, thereby fusing with the basolateral membrane and increasing ATP7A levels there. A role for Ctrl1 in the basolateral uptake of copper was recently suggested (23). The authors suggested that this pathway would ensure an adequate copper supply to the enterocyte from serum, but this observation is somewhat difficult to reconcile with the findings of Ctrl1 being at the apical membrane. In addition, absorption of dietary copper is likely to be adequate for the cellular needs of the small intestine in most situations. Further research on Ctrl1, its localization, and physiologic significance is clearly needed.

COPPER EXPOSURE OF HUMAN INTESTINAL CACO-2 CELLS

The human intestinal Caco-2 cell line is a well-established enterocyte model that has been used extensively for studies on nutrient uptake and transport. When Caco-2 cells grown in monolayers on Transwell filters (Corning Life Sciences, Wilkes Barre, PA) were exposed to copper at concentrations ranging from that of breast milk (3 \(\mu\)mol/L) to that of formula made up with copper-contaminated water (94 \(\mu\)mol/L) for 7 d (7), intracellular copper concentrations increased with copper supplementation. At higher copper concentrations, however, cellular copper uptake as a percentage decreased compared with that in untreated cells. Arredondo et al (24) also exposed Caco-2 cells to copper concentrations of up to 20 \(\mu\)mol/L and found increasing copper accumulation. They showed that copper transport across the cell was saturable, reaching a plateau at 4–6 \(\mu\)mol/L, suggesting a carrier-mediated mechanism. These observations support that copper transport is affected by intracellular copper concentrations, which may be an adaptive response that results in increased intestinal storage of copper, preventing acute changes in plasma copper.

Interestingly, copper uptake increased 10-fold when the intracellular copper concentration was increased. This resulted in only a small fraction (≈4%) of fresh copper introduced to the cells being transferred to the basal chamber, whereas ≈64% was transferred by cells incubated with low-copper medium (24). Studies in copper-deficient cells show homeostatic up-regulation of copper uptake and transport (25), with less copper retained by the cell, supporting higher flux through the cell. Specific mechanisms for this were not explored, but it is likely that copper transporters play a role.

Copper treatment was not found to affect the gene expression of Ctrl1 after 1 wk, but metallothionein mRNA levels increased at the higher copper exposures (7). Metallothionein has been suggested to regulate copper absorption (24), but there is no evidence to this effect. Rats fed high amounts of copper were found to have high metallothionein concentrations in villous epithelial cells as well as in Paneth cells (26), which suggests that metallothionein may play a protective role by limiting copper transport across the basolateral membrane. Metallothionein knockout models have shown that metallothionein protects against copper toxicity, supporting a “trapping” role of metallothionein.

No effect of copper was found on the protein expression of Ctrl1 in the isolated membrane fraction (7). Confocal microscopy
showed that Ctr1 was localized at the apical membrane as well as intracellularly, and copper exposure at 94 μmol/L resulted in less Ctr1 being located on the apical membrane. The intracellular Ctr1 stained diffusely and co-localized with transferrin. This is similar to results from transfected cells (15).

No effect of copper treatment on Atp7A gene expression was found, which is similar to results from copper-exposed mice (27), or on protein expression. Atp7A was predominantly found intracellularly, although some was found on the cell membrane. Alterations in the localization of Atp7A in response to copper to change copper efflux have been observed in other cell types (28), but no effect of copper treatment was found at these levels on the localization of Atp7A. It is possible, however, that the enterocyte has another role in copper metabolism by limiting the amount of copper passed into the circulation (7). The lack of effect of copper on Atp7A expression and localization may explain the lack of an effect on 67Cu export across the basolateral membrane despite increased intracellular copper. A proposed model of enterocyte copper trafficking is shown in Figure 1.

COPPER SUPPLEMENTATION OF SUCKLING RAT PUPS

A suckling rat pup model has been used to study developmental changes in copper absorption (29). At a young age (<10 d), a large percentage (>50–60%) of 64Cu was absorbed and retained by the small intestine, whereas little (<10%) was taken up by the liver (29). In older pups (>14 d), however, much less 64Cu was absorbed and retained by the small intestine (~25%) and more by the liver (~25%). To study the ontogeny of copper homeostasis in vivo, rat pups were exposed daily from birth with 10 or 25 μg Cu by oral gavage, and serum and tissue copper concentrations, copper absorption, and expression of copper transporters at postnatal days 10 and 20 were analyzed (8). These levels of copper supplementation, which are similar per kilogram of body weight to what breastfed and formula-fed infants, respectively, are exposed to, did not result in any adverse effects on growth, and serum copper concentrations were not affected, which suggests that these are mild to moderate levels of exposure.

A significant interaction between age and copper supplementation was observed. At day 10, the intestinal copper concentration was 5-fold higher in pups supplemented with 25 μg Cu/d, whereas no significant effect was found at day 20. Because there was no significant effect on the intestinal copper concentration at 10 μg Cu/d, this suggests that the intestine has some, but limited, capacity to homeostatically adapt at modest copper exposures. At 20 d of age, there was no effect of copper supplementation on the intestinal copper concentration; however, at this age, the intestinal copper concentration was very low, which suggests that little copper was retained in this tissue. These observations are possibly explained by the development of copper-transport regulation between these ages (8) and that the machinery to handle copper is immature in young pups (day 10) and matures to adult levels by weaning (day 20). Developmental regulation of intestinal copper transport may occur because infants may not depend on dietary copper until weaning because of liver copper stores, resulting in intestinal “trapping” of copper when copper intake is high.

A significant decrease in total 67Cu absorption was found in copper-supplemented pups, measured 6 h after giving 67Cu by oral intubation, with no significant effect of age (8). There was also a significant increase in intestinal 67Cu in copper-supplemented pups. Further analysis by ANOVA showed that there was no significant effect of copper supplementation at day 10, but at day 20, copper-supplemented pups had a significantly lower percentage of unabsorbed 67Cu than did control pups. Although tissue copper concentrations increased with copper supplementation in 10-d-old pups, serum copper and ceruloplasmin activity were unchanged (8), which is similar to observations in human infants (4, 30). This may be the result of increased intestinal copper retention, because copper-supplemented pups had higher 67Cu retention after being given an oral dose. Although copper supplementation of human infants at 1 and 3 mo
of age did not affect $^{65}$Cu absorption, there was an inverse correlation between fecal copper excretion and the percentage of $^{65}$Cu absorbed, which suggests that copper absorption is regulated at the absorptive level in young infants (4); however, the mechanisms behind this regulation during infancy are poorly understood.

Ctrl1 is believed to mediate copper uptake into the enterocyte. Ctrl1 mRNA levels in the small intestine increase during early life, but were not affected by copper supplementation (31). However, Ctrl1 protein expression increased significantly with copper supplementation at a young age (10 d old), but decreased in older rat pups (8). It is likely that posttranscriptional events alter Ctrl1 protein abundance or cellular localization and thereby regulate Ctrl1 function, because Ctrl1 is endocytosed in response to copper in cultured cells (7, 15). In mice, Ctrl1 is predominantly intracellular during early life (32), possibly as the result of high copper concentrations in early milk. Ctrl1 endocytosis may therefore be responsible for the higher amount of unabsorbed $^{67}$Cu in young rat pups, thereby limiting intestinal copper uptake. In mice fed a low-copper diet, however, Ctrl1 had a more apical localization.

Metallothionein mRNA levels were significantly affected by copper supplementation, with no effect of age. Metallothionein is an intracellular copper-binding protein that sequesters copper at higher intakes and thereby protects the cell against redox toxicity. Interestingly, metallothionein gene expression was up-regulated in both younger and older copper-supplemented pups independent of intestinal copper concentration, possibly as a response to oxidative stress (33).

Atp7A mRNA expression and Atp7A protein abundance were not affected by copper supplementation at day 10, but increased significantly at day 20. Atp7A is presumed to mediate copper efflux from the plasma membrane (34, 35). In contrast with Ctrl1, Atp7A was not affected by copper supplementation at day 10, which may explain the lower total-body $^{67}$Cu absorption observed in copper-supplemented young pups. Atp7A is also regulated posttranslationally by copper exposure and translocates to intracellular vesicles or to the plasma membrane to sequester copper or increase copper efflux, respectively, in transfected cells (28, 36). Polarized enterocytes may regulate copper uptake into the body by translocating Atp7A to vesicles or the apical or serosal membrane (37) depending on cellular or body requirements and may thus trap copper in the small intestine of young infants. By weaning (day 20), although intestinal copper concentration was no longer different from controls, $^{67}$Cu retained by the small intestine remained higher, whereas the amount of unabsorbed $^{67}$Cu in the gastrointestinal tract was lower (8). These findings suggest that copper is more effectively transported through the enterocyte, most likely as a result of increased Ctrl1 and Atp7A expression.

No significant effect of copper supplementation or age was found on Atp7B mRNA expression (8). Atp7B has been found to be expressed in the intestine of mice (38) and sheep (39), but its role in intestinal copper metabolism is still not known. It is possible that Atp7B serves to deliver copper to hephaestin (40) bound to the serosal membrane.

CONCLUSIONS

At birth, liver copper stores are high, and infants may therefore be at risk of the physiologic consequences of excess copper if copper intake is high. However, it is also possible that infants may be protected against copper toxicity because the liver may accommodate substantial levels of copper during infancy. Rat pups supplemented with a modest amount of copper, 10 $\mu$g/d, which on a body weight basis is similar to the copper intake of formula-fed infants, did not have increased small intestinal or liver copper concentrations, thus suggesting that this level of copper is safe. However, pups supplemented with 25 $\mu$g Cu/d retained more copper in their small intestine and liver, suggesting that they may be at risk of copper toxicity. It is possible that down-regulation of absorption occurs at higher intakes, which would protect against excess copper. However, if absorption remains high, toxicity may be possible even at moderately high exposures. Further knowledge about copper homeostasis and its regulation during higher copper intakes is needed, both at the cellular level and on a whole animal and human level.

I am grateful to Kathryn Bauerly and Shannon L. Kelleher for stimulating collaboration and discussions and to Ming-yu Jou for help with the illustrations. The author had no conflicts of interest.

REFERENCES


