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Functional and molecular responses of suckling rat pups and human intestinal Caco-2 cells to copper treatment

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Abstract

Ctr1 and Atp7A are copper (Cu) transporters that may play a role in the regulation of intestinal Cu absorption; however, intestinal regulation of these transporters by Cu *in vivo* has not been well defined. In this study, we hypothesized that Cu supplementation would alter the expression of intestine Ctr1 and Atp7A *in vivo* and further documented effects of Cu exposure on Cu transport, Ctr1 and Atp7A levels and localization in enterocyte-like Caco-2 cells. Suckling rat pups were supplemented with Cu (0 and 25 μg Cu/day) for 10 days and small intestine Cu concentration, Ctr1, Atp7A and metallothionein (MT) gene expression were measured by Northern blot analysis. Caco-2 cells were treated with basal medium, or medium supplemented with 3 and 94 μM $CuSO_4$ and ^{67}Cu transport, Ctr1 and Atp7A levels and localization were determined. In rat pups, Cu supplementation increased intestinal Cu, Ctr1 and MT gene expression; however, Atp7A gene expression was not significantly affected. Caco-2 cells treated with 94 μM Cu had lower cellular Cu uptake and export compared to untreated cells. While Ctr1 and Atp7A gene and protein levels were unaffected, confocal microscopy indicated that Ctr1 was endocytosed and co-localized with transferrin in Cu treated cells. This study demonstrates the functional response of intestinal cells to Cu treatment and suggests that both Ctr1 and Atp7A may regulate Cu absorption.

Keywords: Cu, Cu transport; Intestine; Caco-2 cells; Ctrl; Atp7A

1. Introduction

Copper (Cu) is a nutrient that is required for a number of enzymes involved in oxidation/reduction reactions; however, due to its oxidative potential, excess copper can also be toxic [1]. Therefore, tight regulation of Cu homeostasis is important and while homeostasis is believed to be predominantly regulated through biliary excretion, regulation of intestinal uptake may also play a role.

Although little is known about the mechanisms regulating Cu absorption at the intestinal level it appears to occur in the small intestine by 3 distinct processes; uptake of Cu into the enterocyte across the brush border membrane, translocation of Cu across the enterocyte, and the export of Cu from the enterocyte into circulation. Recently, several Cu transporters and binding proteins have been identified that may play a role in regulating Cu absorption at the intestinal level. Copper transporter-1 (Ctr1) is believed to play a role in Cu uptake into cells [2–5]. Ctr1 is a Cu-specific transport protein first discovered in a yeast model defective in iron metabolism and was subsequently found to be associated with the plasma membrane, transporting Cu in a saturable manner, dependent upon time, concentration and pH [3]. In addition, Ctr1 has been shown to be expressed in the villi of the small intestine and in Caco-2 cells in culture [2,6], an established model of intestinal enterocytes [7], suggesting that Ctr1 may play a role in absorption of dietary Cu. While Lee et al. [5] determined that intestine Ctr1 mRNA levels are not affected by dietary Cu status in growing male rats, the effect of Cu deficiency on Ctr1 protein levels has not been addressed. Cu must later be exported across the basolateral membrane of the enterocyte to supply the body with Cu. Atp7A is a Cu transport protein which delivers Cu to Cu-containing enzymes in the *trans* Golgi network and translocates to the plasma membrane of transfected Chinese Hamster Ovary (CHO) cells in response to Cu exposure to facilitate Cu efflux [8]. The role of intestinal Atp7A in Cu homeostasis is supported by the occurrence of Menkes disease, as multiple mutations in the Atp7A gene result in the inability of Atp7A to translocate to the plasma membrane

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facilitating systemic Cu deficiency and overload of Cu in the intestine [9,10]. Any excess Cu that is not exported from the enterocyte may be bound to metallothionein (MT), which can function as an intracellular metal binding protein that acts to detoxify the cell of metals when in excess [11]. Cu may influence the expression of MT in multiple ways. While zinc (Zn) is a well-known inducer of MT, Cu may indirectly induce MT gene expression by displacing Zn, due to its higher binding affinity for MT. Zn may then bind to the metal responsive element (MRE) in the MT promoter region, inducing MT expression. MT gene expression has also been shown to be regulated by the oxidative status of the cell, which may be altered by Cu status, through an antioxidant responsive element (ARE) [12,13]. Finally, the Cu responsive transcription factors Ace1, Mac1, and Amt-1 have been identified in yeast, and have been shown to regulate MT gene expression [14].

Copper (Cu) is an essential mineral during infancy. It is required for normal growth and the development of bone, brain, immune system, and red blood cell maturation [15]. Yet, Cu can also be toxic to the infant at high levels which has been associated with impaired growth, hepatic accumulation, jaundice, and necrosis, as seen in patients with Wilson's disease or Cu related Indian childhood cirrhosis [16]. In adults, dietary Cu intake is quite variable, ranging from 0.6 to 1.6 mg/day [1] and balance studies suggest that Cu absorption is inversely related to Cu intake [17,18]. The Cu intake of infants is also variable. Breast milk contains approximately 0.2 to 0.3 mg Cu/L (3 μM). Regular infant formulas are supplemented with $CuSO_4$ at levels ranging from 0.4 to 0.8 mg Cu/L (6 to 9 μM), while formulas for premature infants can contain up to 2 mg Cu/L (30 μM). An additional variable is the Cu concentration of the water used to mix infant formulas. While the World Health Organization (WHO) has established a limit of 2 mg Cu/L for the Cu concentration in drinking water, water in some areas of the world that mine Cu or use Cu plumbing exceeds this guideline (up to 6 mg/L, 94 μM) [19]. Despite the wide range in dietary Cu that infants are exposed to, little is currently known regarding the regulation of Cu homeostasis in infants. In this study, we used Cu-supplemented suckling rat pups as a model of human infants consuming water where the Cu concentration exceeds the WHO recommendation and hypothesized that the neonate can regulate intestinal Cu absorption through changes in Ctr1, Atp7A and MT expression levels. Caco-2 cells were used to further investigate the effects of varying Cu exposure on Cu uptake and transport, Ctr1, Atp7A and MT expression.

2. Materials and methods

2.1. Suckling rat pup model

This study was approved by Animal Research Services at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Pregnant Sprague Dawley rats 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-hr dark: light cycle and allowed to consume purified, deionized water and a standard rat chow (Ralston Purina, St. Louis, MO) *ad libitum*. Rats were acclimatized to their environment and allowed to deliver normally. Litters were culled to 10 pups/dam at postnatal day (PN) 1 and were randomly assigned to Cu treatment groups ($n = 15$ each). Cu supplementation level was chosen to represent the level of Cu an infant would receive if infant formula was prepared with Cu contaminated water, with the dose adjusted for body weight of the rat pups. Rat pups were allowed to nurse *ad libitum* (Cu concentration of rat milk was 2.5 μg/mL). Pups were randomly assigned to treatment groups and were given a daily dose of either 0 or 25 μg Cu/day as $CuSO_4$ in 10% sucrose solution until PN 10. Pups were weighed daily. On PN 10, pups were killed by asphyxiation with CO_2 and small intestine was perfused with ice-cold saline and collected for mineral analysis or immediately homogenized in Trizol Reagent for RNA analysis (Gibco-Life Technologies, Rockville, MD). Blood was collected by cardiac puncture into heparinized vials, and plasma was separated by centrifugation at 2,000*g* for 15 min at 4°C and stored at −20°C for mineral analysis.

2.1.1. Immunostaining

Fixed tissues from d 10 control rats were embedded in frozen tissue mounting medium (Fisher Scientific, Pittsburgh, PA), sectioned (4 μm) and mounted on positively charged microscope slides. Frozen sections were dried at room temperature and localization of Ctr1 and Atp7A was determined using rabbit polyclonal antiserum (1:1250), detected with 3,3′ diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

2.2. Cell culture model

Caco-2 cells (American Type Tissue Culture Collection, Rockville, MD) were used between sub-culture 40 to 50 and grown in Minimal Essential Medium (Invitrogen Life Sciences, Rockville, MD), containing 10% fetal bovine serum, 1% penicillin and streptomycin (10 units/mL and 1 mg/mL respectively) at 37°C with 5% carbon dioxide. The Cu concentration of basal medium containing serum was 0.4 μM, as measured by AAS. For uptake and transport studies, cells were seeded (225,000 cells/cm^2) onto Transwell permeable polycarbonate filters (1 cm^2, and 0.4 μm pore size) (Costar, Cambridge, MA) and monolayer integrity was monitored by measuring the transepithelial electrical resistance (Millicell Electrical Resistance System, Millipore, Bedford, MA). Monolayers with a resistance $<180\ \Omega/cm^2$ were not used. Post-confluent cultures were Cu-loaded using unsupplemented medium or medium supplemented with Cu as $CuSO_4$ (3 and 94 μM) for 7 days. On the day of the experiment, cells were rinsed with Hanks Buffered Salt

Solution pH 7.4 containing 25 mmol/L HEPES (HBSS, Sigma, St. Louis, MO) and pre-incubated for 45 min at 37°C. HBSS was removed and 0.5 mL fresh HBSS containing ^{67}Cu (0.1 μCi) (Brookhaven National Laboratory, New York, NY) was added to the top chamber, 2.5 mL HBSS was added to the bottom chamber and cells were incubated at 37°C for 3, 6, or 12 h. Buffer was removed from the top and bottom chambers, cells were washed twice in ice-cold HBSS and radioactivity in the top and bottom chambers and cell monolayer was measured in a gamma counter.

2.2.1. Cu analysis

Small intestine and serum were digested in concentrated nitric acid as previously described [20]. Caco-2 cells grown in Transwell chambers were digested in 200 μL ultra-pure 16 N nitric acid, diluted to 1 mL with deionized H_2O and allowed to digest overnight. Cu concentration was determined by flame atomic absorption spectroscopy (Model Smith-Heifjie 4000, Thermo Jarrell Ash, Franklin, MA).

2.2.2. Analysis of Ctr1, Atp7A and MT mRNA levels by Northern blot

cDNA probes for Ctr1, Atp7A, and MT were generated by reverse transcriptase polymerase chain reaction (RT-PCR) from Caco-2 mRNA as previously described followed by gene specific PCR [21]. Probes were designed to detect human and rat sequences, due to sequence homology; 89% for Ctr1 and 88% for Atp7A. Ctr1 primers were forward: 5′-GCT GGA CTT GAC CTG GAA AG-3′ and reverse: 5′-ATT GGA GCA GGA ATC ACG TC-3′. Atp7a primers were forward: 5′-CCA TGG GAC CAT ATC CAA AG-3′ and reverse: 5′-TAT AGC CCC GGT GAT ACA GC-3′. MT primers were forward: 5′-CCA GAT CTC GGA ATG GAC CCC AAC-3′ and reverse: 5′-GTG CAC TTG TCC GAG GCA CCT TTG-3′. Probes were labeled with ^{32}P-dCTP using Rediprime II Random Prime labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) following manufacturer's directions. Total RNA was extracted from small intestine and cells grown on Transwells using Trizol reagent according to the manufacturer's protocol (Gibco-Life Technologies). Equal amounts of total RNA (20 μg) were denatured in formaldehyde and electrophoresed through a 0.8% agarose gel containing 1X MOPS-EDTA sodium acetate buffer (Sigma). RNA was transferred to a nylon membrane (Hybond, Amersham Pharmacia Biotech), cross-linked by baking under vacuum at 80°C for 2 h and hybridized at 68°C with ^{32}P-labeled cDNA probes for 2 h. After hybridization, blots were stringently washed with 2X SSC/0.1% SDS for 30 min at room temperature, and twice with 0.5X SSC/0.1% SDS for 20 min at 65°C. Radiolabeled membranes were exposed to film at −80°C in an autoradiography cassette with 2 enhancing screens (Fisher, Pittsburg, PA). All gels were examined under UV light before and after transfer to membrane to validate transfer of RNA. Relative amounts of mRNA were quantified by densitometry using a Chemi-doc Gel Quantification System (Bio-Rad, Hercules, CA). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and expressed in arbitrary units relative to the control group.

2.2.3. Antibody production

Ctr1 and Atp7A antisera were generated against peptide sequences by Genemed Synthesis Inc (San Francisco, CA) as previously described [20]. Specificity of peptide antibodies was verified by the appearance of specific bands not detected following incubation with pre-immune serum on a Western blot of Caco-2 protein (see below).

2.2.4. Western blots

For membrane protein isolation, samples were homogenized in 20 mM HEPES pH 7.4, 1 mM EDTA, 250 mM sucrose, with protease inhibitors (Sigma). Samples were centrifuged at 300*g* for 15 min at 4°C, and post-nuclear supernatant was centrifuged at 100,000*g* for 30 min at 4°C. The pellet containing the crude membrane fraction was suspended in 1 mL homogenization buffer. Protein concentration was determined by Bradford (Bio-Rad). Membrane proteins (50 μg) were electrophoresed through polyacrylamide gels (Ctr1, 10%; Atp7A, 8%), transferred onto nitrocellulose membrane at 350 mA for 60 min, blocked overnight in phosphate buffered saline/0.1% Tween-20 (PBST) with 5% non-fat milk at 4°C. Blots were incubated with Ctr1 and Atp7A antiserum (1:3,000 in PBST), washed 3 times with PBST, and the secondary antibody used at 1:20,000 in 5% milk. Bands were detected using Super Signal Femto Chemiluminescent Reagent (Pierce).

2.2.5. Confocal immunofluorescence microscopy

For fluorescence microscopy, cells were grown on coverslips and post-confluent cells were exposed to 94 μM Cu for one week. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.4% Triton X-100 for 4 min. Cells were washed with PBS and blocked in 10% goat serum/ 0.1% BSA in PBS at room temperature for 30 min. Ctr1 and Atp7A antibodies were used at a 1:160 dilution in blocking solution. Antibodies were detected using Alexa 488 goat anti-rabbit IgG (2 μg/mL, Molecular Probes, Eugene, OR). The nucleus was stained using 1 μM TO-PRO-3 (Molecular Probes) for 10 min. Endosomal co-localization was determined following incubation with Alexa 567-conjugated transferrin (Tf, 0.5 μg/mL, Molecular Probes) for 10 min prior to fixation. Golgi co-localization was determined using 5 μg anti-Golgi 97 mouse IgG monoclonal CDF4 labeled with Xenon Alexa Fluor 647 mouse IgG labeling kit (Molecular Probes). Coverslips were mounted in ProLong Antifade (Molecular Probes). Confocal microscopy was performed using an Olympus BX50WI (Olympus America Inc, Melville, NY), with UPlanApo 100X oil lens N.A. 1.35 and digital images were captured using Bio-Rad Radiance 2100 confocal system, LaserSharp2000 version 4.1.

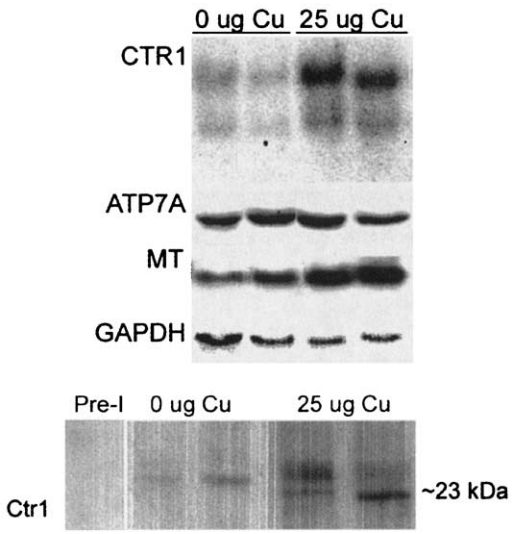

Fig. 1. (A) Messenger RNA levels of CTR1, ATP7A, MT and GAPDH in the small intestine of rat pups supplemented with Cu. Representative Northern blot of total RNA extracted from the small intestine of rats orally supplemented with 0 or 25 μg Cu/day (n = 2/treatment). GAPDH was used as a normalization control. (B) Ctr1 protein expression. Representative Western blot of membrane protein extract (50 μg) from small intestine of rats orally supplemented with 0 or 25 μg Cu/day (n = 2/treatment) incubated with pre-immune serum (Pre-I), or Ctr1 antiserum.

2.3. Statistical analysis

Results are presented as means ± SD. Statistical analysis was performed using GraphPad Prism v 3.02 (San Diego, CA). Effect of Cu supplementation on rat pups was determined using Student's t-test. Effect of Cu exposure on Caco-2 cells was determined using repeated measures one-way analysis of variance (ANOVA) followed by Tukey post-test. Statistical significance was demonstrated at $P < 0.05$.

3. Results

3.1. Cu supplementation of rat pups

Suckling rats supplemented with 25 μg Cu/day had significantly higher small intestine Cu levels than control rats ($P < 0.05$). However, serum Cu concentration remained unchanged with Cu treatment, 0.32 ± 0.02 in control vs. 0.37 ± 0.02 μg/mL in supplemented pups. This suggests that infant rat pups can regulate Cu transfer across the intestine in response to dietary Cu intake. To delineate the mechanism which may be responsible for this regulation, Ctr1, Atp7A, and MT gene expression was assessed. Rat pups treated with 25 μg Cu/day had significantly higher Ctr1 and MT gene expression levels ($P < 0.05$) compared to control pups (Fig. 1A), whereas Atp7A gene expression levels were unchanged. Ctr1 protein expression was also increased with Cu supplementation (Fig. 1B), while Atp7A protein expression remained unchanged (data not shown).

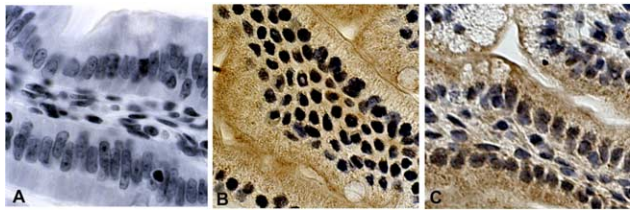

Fig. 2. Immunostaining of fixed small intestine (4-μm sections) from control rats at d 10. (*A*) Negative control; (*B*) Ctr1, (1:1250); (*C*) Atp7A, (1:1250). Rabbit immunoglobulin G–conjugated DAB was used as a secondary antibody (1:5000), sections were counterstained with hematoxylin and visualized at 60X magnification.

3.2. Identification and localization of Cu transporters in small intestine

Immunohistochemistry was used to characterize Cu transporters in small intestine from suckling rat pups. Ctr1 was apically localized to the villus tip, the length of the villi and in crypt cells with Ctr1 staining diffuse within the cell (Fig. 2B). Atp7A also localized to the villus tip, length of villi and in crypt cells with Atp7A staining more distinct, suggesting vesicular localization (Fig. 2C).

3.3. Cu exposure of Caco-2 cells

Following the one week treatment with basal medium, or medium supplemented with 3 or 94 μM $CuSO_4$, intracellular Cu concentrations of cells grown in Transwells increased significantly ($P < 0.01$) with increased Cu supplementation (Fig. 3); however, exposure of Caco-2 cells to 94 μM Cu resulted in lower cellular Cu uptake and export ($P < 0.05$) compared to untreated cells (Fig. 4A and 4B).

3.3.1. Gene and protein expression

The relative gene expression of Ctr1, Atp7A and MT was measured using Northern blot analysis in Caco-2 cells grown in Transwell chambers and pretreated for one week with Cu supplemented media (Fig. 5). There were no sig-

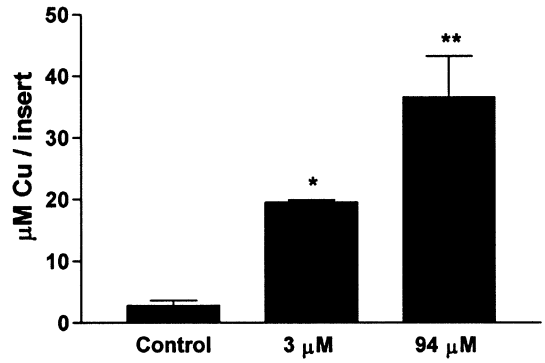

Fig. 3. Intracellular Cu concentrations of Caco-2 cell monolayers grown in Transwell chambers with Cu supplemented media for 7 days. Values represent mean ± SD, n = 3 inserts/concentration, Significant effect of Cu exposure, *$P < 0.01$, **$P < 0.001$.

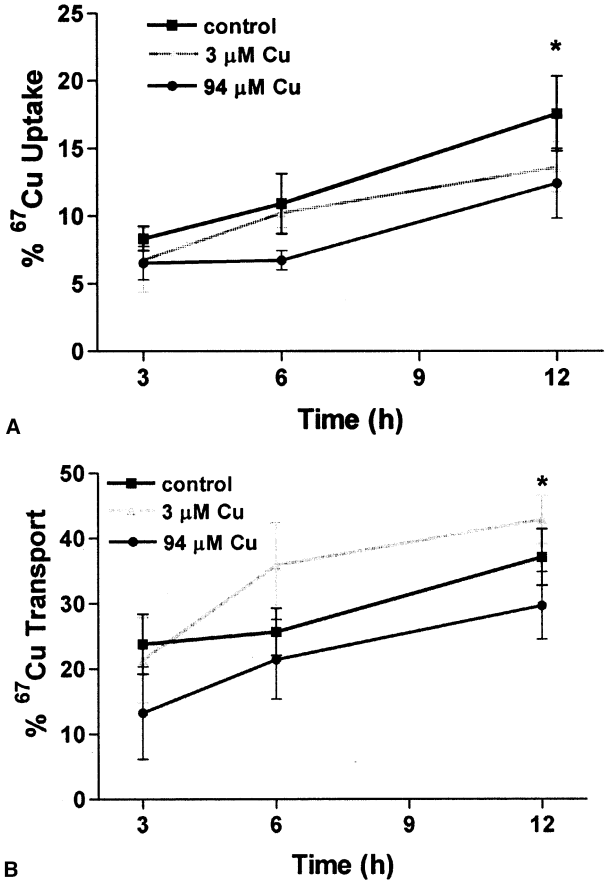

Fig. 4. ^{67}Cu uptake and transport in Caco-2 cells grown in Transwells and treated for one week with Cu supplemented media. (A) Uptake of ^{67}Cu into the cell monolayer. Values represent mean ± SD, $n = 3$ inserts/time. *Significant effect of Cu treatment on ^{67}Cu uptake into the cell layer in cells treated with 94 μM Cu. (B) Transepithelial transport of ^{67}Cu into the bottom chamber. Values represent mean ± SD, $n = 3$ inserts/time. *Significant effect of Cu treatment on ^{67}Cu transport into the bottom chamber.

nificant differences in Ctr1 or Atp7A gene expression as an effect of Cu exposure; however, MT gene expression was increased but only in the cells treated with 94 μM Cu ($P < 0.05$). There was no significant effect of length of Cu exposure on gene expression (data not shown).

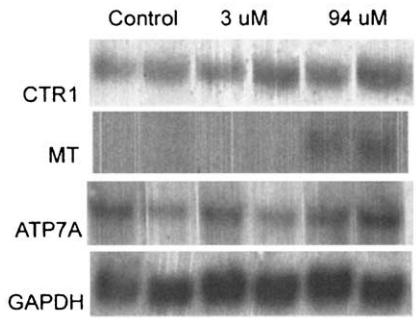

Fig. 5. Effect of Cu and length of exposure on mRNA levels of CTR1, MT, and ATP7A from Caco-2 cells grown in Transwells. Representative Northern blot of total RNA extracted from Caco-2 cells treated for one week with Cu supplemented media. GAPDH expression was used as a normalization control.

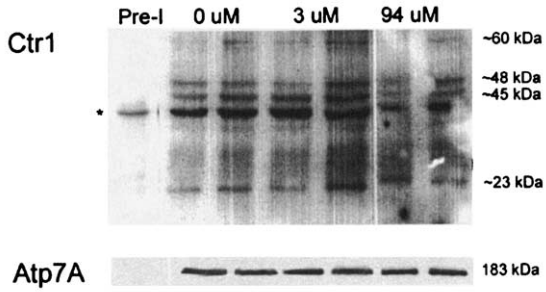

Fig. 6. Ctr1 and Atp7A protein levels in Caco-2 cells grown in Transwells and treated for one week with Cu supplemented media. Representative Western blot of membrane protein extract (50 μg) from Caco-2 cells exposed to 0, 3 or 94 μM Cu incubated with pre-immune serum (Pre-I), Ctr1 antiserum or Atp7A antiserum.

Protein levels of Ctr1 and Atp7A were measured in the isolated membrane protein fraction of Caco-2 cell grown in Transwells by Western analysis. Ctr1 antiserum detected major bands at 23, 45 and 48 kDa and a minor band at 60 kDa (Fig. 6). A band of approximately 40 kDa was observed with exposure to both pre-immune and antiserum (*), and represents nonspecific binding. There was no effect of Cu treatment or length of Cu exposure (data not shown) on Ctr1 protein levels. Atp7A antiserum detected a specific band at 183 kDa and there was no significant effect of Cu treatment (Fig. 6) or length of Cu exposure (data not shown) on Atp7A protein levels.

3.3.2. Confocal immunofluorescence microscopy.

In differentiated Caco-2 cells, Ctr1 was localized on the plasma membrane as well as intracellularly and was shown to co-localize with Tf, indicating endosomal cycling of the Ctr1 protein (Fig. 7A). Ctr1 was predominantly located at the apical side of the cell as determined following a Z-series scan (2 μm sections) of the cell monolayer. Following exposure to 94 μM Cu for one week, Ctr1 localization, as determined by relative distance from the nucleus at the apical surface, was less distinct at the plasma membrane and more prominent intracellularly. There was not an observable overlap between Ctr1 and Golgi markers with or without Cu treatment (data not shown). Atp7A also appeared to be associated with the plasma membrane and intracellularly in Caco-2 cells (Fig. 7B). In contrast to Ctr1 localization, Atp7A was predominantly localized intracellularly. No significant co-localization was seen between Atp7A and Tf or Golgi markers (data not shown) and following Cu treatment; there appeared to be no significant relocalization of Atp7A.

4. Discussion

Dietary Cu intake of infants varies, primarily due to the Cu content of water used to dilute infant formula as a

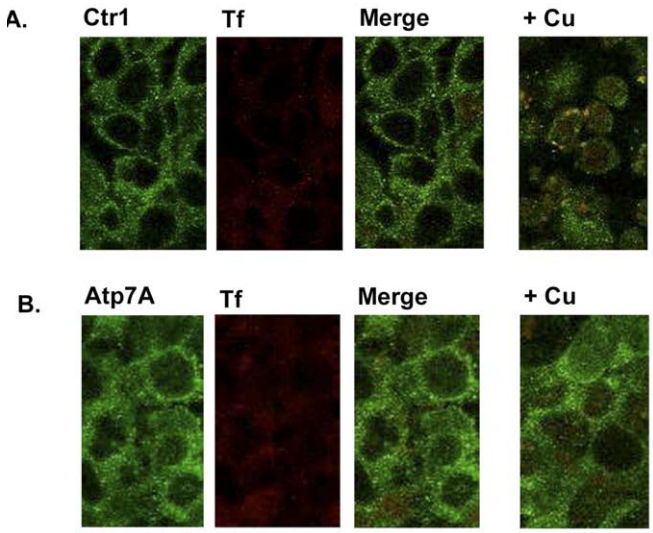

Fig. 7. Subcellular localization of Ctr1 and Atp7A in Caco-2 cells exposed to 0 or 94 μM Cu. (A) Ctr1 (green) was localized primarily to the plasma membrane in control Caco-2 cells and co-localized with transferrin (Tf in red); however, Ctr1 exhibited a more predominant intracellular staining pattern in Caco-2 cells exposed to 94 μM Cu (+Cu). (B) Atp7A (green) was localized primarily intracellulary in control Caco-2 cells, did not co-localize with transferrin (Tf in red) or alter its localization in Caco-2 cells exposed to 94 μM Cu (+Cu).

consequence of Cu mining and plumbing, as Cu has been documented to leach into drinking water. Despite variable Cu intakes, common indicators of Cu status such as serum Cu and ceruloplasmin levels remain unchanged in these infants [22–24] suggesting that Cu homeostasis is tightly regulated. In agreement with human infant studies, serum Cu concentrations remained unchanged with Cu treatment in rat pups. The fact that we observed an increase in intestinal Cu concentration and MT expression concurrently without any affect on Atp7A gene expression in Cu-supplemented rat pups suggests that the intestine does play a role in this homeostasis. We also report that Ctr1 and Atp7A are present in rat small intestine, and both localize along the intestinal villi, with heavy staining in the villus tip and crypt cells. While this animal model is useful for understanding Cu homeostasis during infancy, an intestinal cell culture model was also used to explore the molecular events that may occur in the enterocyte exposed to an elevated Cu concentration and how that exposure may regulate Cu transport.

Very limited research has been conducted on Cu uptake by intestinal cells in culture [25–27]. Arrendondo et al. reported that treatment of Caco-2 cells with Cu up to 20 μM for 14 days resulted in Cu accumulation in the cell layer similar to the results of this current study [25]. An additional study reports Cu accumulation in Caco-2 cells with Cu levels as low as 1 μM [28]. One possible explanation for these observations may be that the basal medium may not provide adequate Cu; thus, supplementation with 1 μM results in an accumulation of Cu in response to Cu deficiency, although this remains to be determined. Alternatively, Cu has been shown to be transported across Caco-2 cells in a saturable, concentration dependent manner reaching a plateau at 4 to 6 μM, suggesting a role for a carrier mediated mechanism [25]. These results, in combination with our observations of lower ^{67}Cu uptake and export in Caco-2 cells treated with 94 μM, support the conclusions of others suggesting that Cu transport is affected by intracellular Cu concentration which may reflect an adaptive response that results in increased intestinal cell storage of Cu, preventing acute changes in plasma Cu concentration [25]. Studies in Caco-2 cells deficient in Cu demonstrate homeostatic regulation of Cu by increased Cu uptake and transport, while less Cu is retained within the cell, indicating increased flux through the cell [27]. Specific mechanisms for this remain unclear, although Cu transporters are believed to play a role. In this study, although results did not reach significance, Caco-2 cells treated with 3 μM had decreased cellular Cu uptake, and increased Cu transport, suggesting increased flux of Cu through the cell, while intracellular Cu concentration increased.

Ctr1 has been shown to be expressed in most tissues analyzed with high levels detected in liver and kidney [5], and shown to be localized to the villi of the small intestine, suggesting a role in the transport of dietary Cu [2]. The essentiality of Ctr1 was demonstrated in a mouse Ctr1 knockout model that resulted in embryonic lethality and defects in tissue Cu accumulation as well as reduced activities of Cu-containing enzymes in heterozygotes [2,5]. Expression of Ctr1 appears to be tissue specific, with plasma membrane, cytoplasmic vesicular, and perinuclear locations depending on cell type [6,29]. Ctr1 gene expression has been shown to not be affected by dietary Cu status in growing rats [5]. However, we determined that in suckling pups Ctr1 gene and protein expression are higher in pups supplemented with 25 μg Cu/day, suggesting that there may be developmental regulation of intestinal Cu absorption. Preliminary results indicate that induction of Ctr1 gene expression by Cu differs during development stages and studies are currently underway to confirm this hypothesis.

In Caco-2 cells, similar to reports of others [30], we determined that Ctr1 gene and protein expression was unaffected by Cu treatment. We observed multiple protein sizes of Ctr1, consistent with others who have shown that Ctr1 proteins exist as multimers [3] and are post-translationally modified by glycosylation [6]. The lack of effect on Ctr1 gene or protein expression levels appears to be inconsistent with our observations of decreased cellular Cu uptake, thus suggesting post-translational regulation. Interestingly, localization of myc-Ctr1 on the plasma membrane in transfected cells is a result of a dynamic process involving endocytosis from the cell surface and Cu has recently been reported to play a role in regulating Ctr1 endocytosis in transfected cells [29]. In our current study documenting effects on native Ctr1 localization following exposure of differentiated Caco-2 cells to Cu, Ctr1 is no longer distinctly present on the plasma membrane, but instead stains more

diffusely within the cell, and co-localizes with Tf, in agreement with observations made by others [29]. However, Klomp et al. reported a lack of effect on Ctr1 localization in response to Cu excess or deficiency in Caco-2 cells [6]. Although the reason for this discrepancy is unknown, our current study utilized Cu-loaded cells in response to elevated Cu exposure for seven days (as determined by measurement of cellular Cu concentration) while the previous study used Caco-2 cells in response to acutely elevated levels of Cu for only 2 h. Thus, the length of Cu exposure may have been a determining factor. Nevertheless, our data suggest that one point of regulation for intestinal Cu absorption may be Ctr1 internalization in response to Cu, thus limiting the amount of Cu taken up by the cell.

The accumulation of Cu in both small intestine and Caco-2 cells increased the expression of MT. MT is a cysteine-rich, low molecular weight protein known to play a role in homeostasis of metals such as zinc and cadmium [11]. Additionally, MT has been suggested to play a role in regulating Cu absorption [25], but no clear evidence exists. Cu loaded rats fed a diet containing 1.5 g Cu/kg had intestinal MT levels that were very high and stained most intensely in the cytosol and nuclei of villous columnar epithelium as well as the Paneth cells in the crypts [31], suggesting that MT may play a protective role, making Cu unavailable for transport across the basolateral membrane. Furthermore, a MT knockout model demonstrated that MT protects against Cd, Cu, and Zn toxicity [32] and the protective effect of MT has also been documented following cross-breeding of Atp7A and MT knockout mice, thus eliminating the ability to store or export excess Cu, which resulted in Cu toxicity and embryonic lethality [33]. Although MT was not induced in response to 3 μM Cu, cells treated with 94 μM Cu had increased intracellular Cu and MT levels, suggesting that MT acts as an intracellular functional trap for excess Cu, making it unavailable for transport across the basolateral membrane.

Atp7A (the Menkes disease gene product) is a Cu efflux protein found in many tissues including intestine, placenta, lung, heart, brain and kidney, but lacking in spleen and liver [34]. Its presence in the intestine suggests a role for regulation of dietary Cu absorption and it may present a rate-limiting step in absorption as we observed Cu accumulation in the intestine following Cu overload. Atp7A gene expression does not appear to be affected by Cu exposure [34]; instead, Atp7A has been shown to be regulated post-translationally, by an alteration of its cellular localization to the plasma membrane in response to Cu to increase Cu efflux [8]. However, the enterocyte is a unique cell type which we hypothesize is involved in regulation of Cu absorption, and it is possible that excess Cu is not simply pumped out of the cell, supplying the body with increased Cu. Instead, it may be responsive to Cu exposure to protect against Cu toxicity by limiting the passage of Cu into circulation. To our knowledge, there have been no studies on Atp7A trafficking in polarized intestinal cells or the effects of Cu exposure on gene expression or protein levels and how these factors might play a role in regulation of Cu absorption. In the present study, Atp7A gene expression and protein levels were unaffected by Cu supplementation and subsequent cellular Cu accumulation. It is possible that it is the lack of effect of Cu on Atp7A expression that results in the trapping of Cu in the intestinal cell, thus protecting the organism from Cu overload. Interestingly, confocal microscopy studies of Atp7A also showed no significant effect of Cu supplementation on its cellular localization in these cells, which may help explain the lack of an effect on ^{67}Cu export across the basolateral membrane despite increased cellular Cu levels. Recent evidence that Cu chaperones may play a regulatory role in delivering Cu to Atp7A for export [35] may provide further understanding of this process.

In summary, our results support the hypothesis that Cu enters the enterocyte via Ctr1 and is exported across the basolateral membrane by Atp7A. Differences between results obtained from our *in vivo* studies and the cell culture model may reflect the fact that other organs (liver) or Cu status may play a role in Cu homeostasis. Nevertheless, Ctr1 and Atp7A gene and protein expression in Caco-2 cells remained unchanged after exposure to moderate amounts of Cu, despite increased cellular Cu levels. However, Ctr1 localization at the plasma membrane is reduced with Cu treatment, which may functionally limit Cu uptake into the cell. In response to increased intracellular Cu content, MT expression is up-regulated, potentially protecting against oxidative damage. Our results further suggest that the enterocyte may serve a unique function to decrease the amount of Cu transported across the basolateral membrane of the cell under high Cu conditions, possibly mediated by Atp7A, although the mechanism remains unclear.

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