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Copper uptake and intracellular distribution in the human intestinal Caco-2 cell line

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Abstract

The apical uptake of $^{64}CuCl_2$ was investigated in human differentiated intestinal Caco-2 cells grown on permeable supports. At pH 6.0 in the apical compartment, the uptake of copper was linear over the first 6 min and between 10 and 80 μM $CuCl_2$ exhibited non-saturable transport kinetics. In addition, copper uptake was energy-independent, affected by the valency state of copper, preferring Cu(II) over Cu(I), and not influenced by high (10 mM) extracellular calcium. The intracellular distribution of copper was investigated by FPLC at different times of uptake ('pulse') and of 'chase'. Intracellular copper initially bound predominantly to low molecular weight components (i.e., glutathione), and subsequently shifted to higher molecular weight components such as metallothionein and Cu,Zn superoxide dismutase.

Abbreviations: AP – apical; BL – basal; BSA – bovine serum albumin; 2-DOG – 2-deoxy-glucose; FPLC – fast protein liquid chromatography; GSH – glutathione; HBSS – Hanks balanced salt solution; HEPES – N-2-hydroxyethyl piperazine-N-4-butanesulfonic acid; MES – morpholinoethane sulfonic acid.

Introduction

Copper plays several important functional roles in the body. It is a trace essential element capable to adopt two distinct redox states, Cu(II)/Cu(I), which characterize its catalytic function as a cofactor for proteins involved in a variety of biological processes. However, copper is also cytotoxic due to its ability to participate in reactions producing highly reactive oxygen species and to displace other essential metal cofactors from their natural ligands.

In mammals, dietary copper is mainly absorbed in the small intestine but the molecular mechanisms for this uptake have not yet been fully elucidated. Two human genes, homologous to the yeast high-affinity copper transporter gene CTR1, namely hCTR1 and hCTR2 (Zhou & Gitschier 1997) are expressed in many tissues including small intestine and colon, with highest expression in liver, heart and pancreas. hCTR1 has been suggested as a good candidate for high affinity copper uptake in human tissues (Peña *et al.* 1999); however, due to its low expression in the intestine, its role in intestinal copper uptake is still debated. In addition, a divalent cation transporter DMT1, originally cloned from rat duodenum (Gunshin *et al.* 1997) and later identified in humans (Nramp2) (Fleming *et al.* 1997), displays a broad substrate specificity with highest transport capacity for Fe(II), with copper ranking low in the transport order.

Many proteins are involved in intracellular copper distribution and storage, namely metallothionein, Cu,Zn superoxide dismutase and other copper enzymes (Linder & Hazegh-Azam 1996). Recently, a number of small cytosolic copper binding proteins,

copper chaperones, have also been identified, delivering copper to specific compartments and to copper-requiring proteins (Peña *et al.* 1999).

The Caco-2 cell line, derived from a human colon adenocarcinoma, spontaneously differentiates in culture exhibiting several morphological and functional characteristics of mature enterocytes (Pinto *et al.* 1983). Caco-2 cells grown and differentiated on permeable filter supports represent a well-established model for the study of intestinal transport and toxicity of nutrients and xenobiotics, including trace elements (Han *et al.* 1995; Rossi *et al.* 1996; Tapia *et al.* 1996; Alvarez-Hernandez *et al.* 1998; Duizer *et al.* 1999; Ferruzza *et al.* 1999a, b). It has been reported that copper uptake and transport in Caco-2 cells is affected by the zinc concentration in the medium (Reeves *et al.* 1996, 1998). In addition, we have shown that copper effects on tight junctional permeability are related to the apical (AP) uptake of copper (Ferruzza *et al.* 1999a, b). Recently, it has also been reported that in Caco-2TC7 cells the expression of DMT1/Nramp2 is associated with proton-dependent AP uptake of iron (Tandy *et al.* 2000).

The aim of this study was to characterize copper uptake and intracellular distribution in cultured human differentiated intestinal Caco-2 cells.

Materials and methods

The human intestinal Caco-2 cell line, obtained from Prof. Alan Zweibaum (INSERM, Villejuif, Paris, France) was grown and maintained as previously described (Ferruzza *et al.* 1999b) in Dulbecco Modified Minimum Essential Medium containing 25 Mm glucose, 3.7 g/l $NaHCO_3$and supplemented with 4 mM L-glutamine, 1% non-essential amino acids, 1×10^5U/l penicillin, 100 mg/l streptomycin and 10% heat inactivated fetal calf serum (complete culture medium). For copper uptake experiments, cells were seeded on Transwell filters (Transwell, area 4.7 cm^2, pore diameter 0.45 μm; Costar Europe, Badhoevedorp, The Netherlands). For intracellular copper distribution experiments, cells were grown on plastic dishes. Cells were seeded at a density of 4×10^5 cells/cm^2 and were left to differentiate for 15–17 days after confluence; the medium was regularly changed three times a week.

For uptake experiments, differentiated Caco-2 cells were washed three times with Hank's balanced salt solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose) (HBSS) and treated in the AP compartment with $^{64}CuCl_2$ (10–80 μM) in HBSS containing 10 mM morpholinoethane sulfonic acid (MES) at pH 6.0 for 2–240 min in a water bath at 37 °C. The basal (BL) compartment contained HBSS additioned with 10 mM N-2-hydroxyethyl piperazine-N-4-butanesulfonic acid (HEPES) at pH 7.4, with 0.4% copper-free bovine serum albumin (BSA) that had been extensively dialyzed against 0.2 M acetate buffer pH 5.0 to remove bound copper, and with 120 μM reduced glutathione (GSH). For each experiment, about 13 mg of ultra pure $CuCl_2$ were irradiated for 3 h in a TRIGA reactor (1 MWatt) at a flux of around 2.4×10^{12} neutrons /sec. The specific activity of the ^{64}Cu used in the uptake experiments was between 1–2 Ci/g of $CuCl_2$. At the end of the incubation period the AP and BL medium were collected and the cells were transferred on ice and rapidly washed three times with 10 mM EDTA in 150 mM NaCl, 10 mM HEPES, pH 7.4 at 4 °C. Filters and standards were placed in polyethilene boxes with screw caps and copper was detected by counting the total ^{64}Cu. The γ-induced radioactivity of the standards and cells was determined by γ-spectrometry, using a hyperpure germanium crystal at liquid nitrogen temperature and a counting apparatus Ortec G and G. The uptake of Cu(I) was investigated using a copper-ascorbate complex prepared just before the experiment by drop-wise addition of $^{64}CuCl_2$ to ascorbic acid (1:2 v/v) in HBSS at pH 6.0, under constant stirring. For energy depletion, cells were preincubated for 30 min at 37 °C with 1 mM NaN_3 and 50 mM 2-deoxy-glucose (2-DOG) in HBSS in both the AP and BL compartment. Copper uptake in energy-depleted conditions was investigated in HBSS with 50 mM 2-DOG at pH 6.0 in the AP compartment, maintaining in the BL compartment 1 mM NaN_3 and 50 mM 2-DOG in HBSS at pH 7.4.

For total protein determination, filter-grown Caco-2 cells were dissolved in 1 M NaOH and the protein assayed by a colorimetric method (Lowry *et al.* 1951). Differentiated Caco-2 cells on filter had a total protein content of 0.280 ± 0.006 mg/cm^2.

The intracellular distribution of copper was analyzed in differentiated Caco-2 cells grown on plastic after treatment with 30 μM $CuCl_2$ in HBSS at pH 6.0 for 6 and 60 min. At the end of the incubation, the uptake medium was removed, cells were washed and resuspended in 150 mM NaCl, 10 mM EDTA, 10 mM HEPES, pH 7.4 at 4 °C. The cell suspension was lysed by repeated cycles of freezing and thawing in liquid nitrogen. The lysate was centrifuged at $23,000 \times g$

for 15 min at 5 ° C, and the supernatant was collected. To determine the intracellular distribution of copper, aliquots of the supernatant were fractionated by gel filtration chromatography using a 1.6 × 50-cm Superose 12B Fast Protein Liquid Chromatography (FPLC) column equilibrated with 0.15 M NaCl, 10 mM HEPES buffer, pH 7.4. Molecular weights of the material eluted by gel filtration chromatography were determined by comparing their elution volume with those of molecular weight standards. Measurements of copper levels in the FPLC fractions were performed by an atomic absorption spectrophotometer (Perkin Elmer 2100), equipped with a graphite furnace.

In addition, cells were treated with 30 μM $CuCl_2$ for 30 min ('pulse'), washed and fresh HBSS was added and the cells were left in the absence of added copper for different times ('chase'). The distribution of intracellular copper was determined in FPLC fractions during the 'chase' at 3 and 6 h as described above. Intracellular GSH was assayed in FPLC fractions upon formation of S-carboxymethyl derivatives of free thiols with iodoacetic acid followed by conversion of free amino groups to 2,4-dinitrophenyl derivatives by reaction with 1-fluoro-2,4-dinitrobenzene as previously described (Reed *et al.* 1980).

Cu,Zn superoxide dismutase activity was assayed in FPLC fractions by a polarographic method as previously described (Rigo & Rotilio 1977).

Statistical analysis was performed with Statview 4.01 software (Abacus Concepts, Berkley, CA) by one-way ANOVA followed by Scheffè F-test to determine significant differences among means.

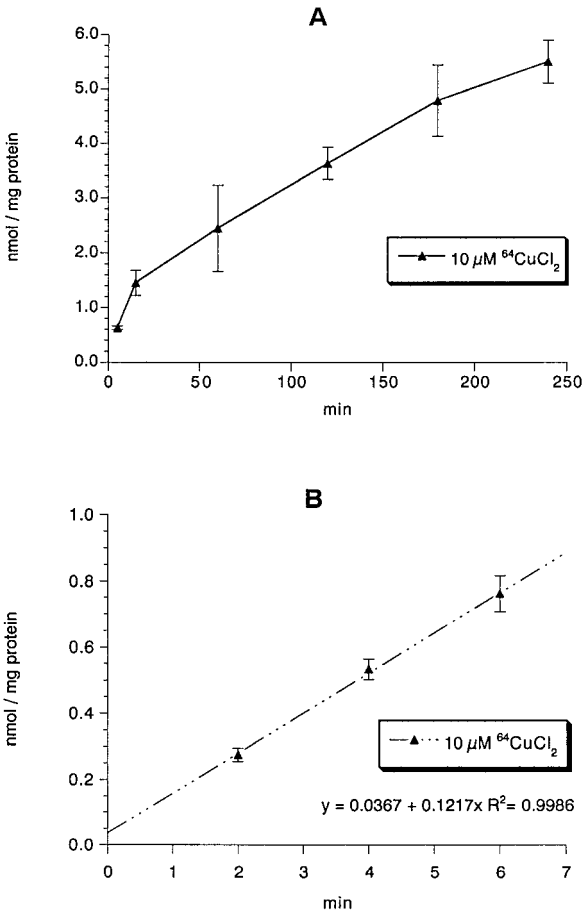

Fig. 1. Time-course of the AP uptake of 10 μM $CuCl_2$ in differentiated Caco-2 cells. (A) Uptake from 5 to 240 min was not linear with time. Each point represents data from two experiments performed in duplicate ($n = 4$) ± SD. (B) Linear copper uptake between 2–6 min from a representative experiment performed in triplicate ± SD. Linear regression analysis was performed to obtain non-specific binding by extrapolation at t_0.

Results and discussion

The characteristics of copper uptake from the apical (AP) surface of differentiated Caco-2 cells were investigated maintaining the AP pH at 6.0 and the basolateral (BL) pH at 7.4 to reproduce the pH conditions existing *in vivo* in the microenvironment in proximity of the surface of the villi of the small intestine (Ranaldi *et al.* 1994). In addition, we had previously shown that maximal rates of copper uptake, from the AP surface, are observed in the pH range pH 6.0 to pH 6.5 (Ferruzza *et al.* 1999b). The AP uptake of 10 μM $^{64}CuCl_2$ at pH 6.0 was followed over time from 5 to 240 min. As shown in Figure 1A the uptake increased progressively but was not linear with time. Since the AP compartment contained 1.5 ml of 10 μM $CuCl_2$, corresponding to a total of 15 nmoles, after 15 min of uptake approximately 10% of the copper had already been taken up. Deviation from linearity is therefore likely to derive from the decrease in copper concentration in the donor compartment. For transport studies it is in fact important to operate under 'sink conditions' (e.g., before $<10\%$ of the substance in the donor compartment has been transported) (Artursson 1990). In addition, Figure 1A shows that Caco-2 cells strongly concentrated copper intracellularly: after 4 h approximately one third of the copper initially present in the AP compartment was found associated with the cells.

The AP uptake of 10 μM $^{64}CuCl_2$ was therefore measured in the linear range of uptake, between 2 and 6 min, (Figure 1B).

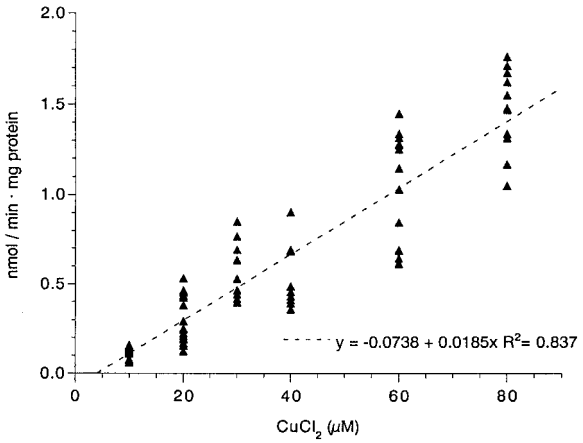

Fig. 2. Kinetics of AP copper uptake by differentiated Caco-2 cells. Initial rates of uptake were calculated in the linear range, between 2 and 6 min. Each point represents the rate of uptake from one filter. At each concentration $n = 12$–15 filters from duplicate experiments.

Kinetics of copper uptake were investigated in the range 10–80 μM $^{64}CuCl_2$ by calculating initial rates of uptake in the first 6 min after subtraction of non-specific binding calculated by extrapolation from the curves. Figure 2 shows that the rates of copper uptake increased linearly with the concentration up to 80 μM. Higher copper concentrations were not used since we had previously shown that $CuCl_2$ can alter the permeability of tight junctions allowing paracellular passage of copper from the AP to the BL compartment (Ferruzza *et al.* 1999a, b). Computer fitting of the data to the equation $v = Kd$ [Cu], representative of a diffusion component, where v is the velocity of uptake, [Cu] is the copper concentration and Kd is the diffusion constant for a non-saturable component, gave an apparent $Kd = 0.0185$ nmol/(min · mg · protein μm).

To the best of our knowledge, copper uptake in Caco-2 cells has previously only been studied at low copper concentrations (0.5–12 μM $CuCl_2$) as a function of zinc concentration in the medium (Reeves *et al.* 1996, 1998). At low zinc concentration (5 μM), close to the zinc concentration in culture medium (Rossi *et al.* 1996), a saturable component of copper uptake was observed exhibiting a Km of 4.4 μM and a V_{max} of 0.98 pmol/(min · mg protein) (Reeves *et al.* 1998). Although the conditions of copper uptake reported by Reeves and co-workers are different from those used in this work, the presence of a saturable component of high affinity copper uptake in Caco-2 cells would not have been detected under the conditions employed in our study.

The uptake of 20 μM $^{64}CuCl_2$ was investigated under different experimental conditions as shown in Table 1. The presence of an active component of copper uptake was investigated under reduced energy conditions obtained by pre-incubating cells with NaN_3 and 2-DOG to lower intracellular ATP levels as previously described (Ranaldi *et al.* 1994; Ferruzza *et al.* 1997). Energy depletion resulted in a significant increase in the rate of copper uptake. This indicates that at this concentration copper uptake occurs by a passive mechanism; the reasons for the increase in the rate of uptake may suggest the presence of an active copper extrusion mechanism that remains, however, to be established. When copper was given in the presence of ascorbate that reduces it to Cu(I) the rate of uptake was significantly lower than that of $CuCl_2$ [Cu(II)]. This is in accord to previous reports demonstrating that ascorbate reduces intestinal copper uptake (van Campen & Gross 1968; van den Berg *et al.* 1994; Wapnir 1998).

Increasing the extracellular concentration of calcium in the transport buffer from 1 mM to 10 mM $CaCl_2$ did not significantly affect the initial rate of copper uptake. Among the candidate transporters for intestinal copper uptake is DMT1/Nramp2 a proton-dependent intestinal divalent cations transporter with relative broad specificity, also expressed in Caco-2 cells (Tandy *et al.* 2000). In *Xenopus* oocytes expression studies the cation uptake activity of DMT-1 was decreased in the presence of 10 mM $CaCl_2$ in the extracellular medium (Gunshin *et al.* 1997). The lack of calcium effect on the AP uptake of copper in Caco-2 cells does not clearly point to an involvement of this transporter and agrees with a previous study performed *in vivo* in goats (Schonewille & Beynen 1995) and with the low specificity of this transporter for Cu(II) (Andrews *et al.* 1999).

The intracellular distribution of copper was followed after FPLC chromatography of Caco-2 cells lysates. Figure 3A shows an elution profile at 280 nm of cell lysates treated with 30 μM $CuCl_2$ for 6 min indicating that copper treatment did not affect the protein distribution. A similar protein distribution was observed after copper treatment for 60 min (data not shown). The copper content of each FPLC fraction was determined by atomic absorption spectroscopy and is reported in Figure 3B. The majority of copper was eluted as a single peak between fractions 17 and 20, corresponding to the region where GSH eluted as indicated by GSH determination. Treatment with 30 μM $CuCl_2$ for 6 min resulted in an increase in total copper content eluting in the same region as in con-

Table 1. Factors affecting the rate of AP uptake of copper[a].

Condition	Additions to 20 μM $CuCI_2$	% of control[b]
Energy depletion	+1 mM NaN_3 + 50 mM 2-DOG[c]	136.6 ± 8.4[d]
Valency state: Cu(l)	+40 μM ascorbate	32.3 ± 3.5[d]
High extracellular calcium	+10 mM $CaCl_2$	104.6 ± 15.8

[a] The rate of AP uptake of 20 μM $^{64}CuCl_2$ was calculated from the increase in intracellular ^{64}Cu over the first 6 min of linear uptake.
[b] Values are expressed as the percentage of the control ± SD. from 6–9 determinations. The control value for the AP uptake of 20 μM $^{64}CuCl_2$ was 0.238 ± 0.089 nmol/min mg protein.
[c] 2-deoxy-glucose.
[d] Significantly different ($P < 0.001$) from control (ANOVA followed by Scheffè F-Test).

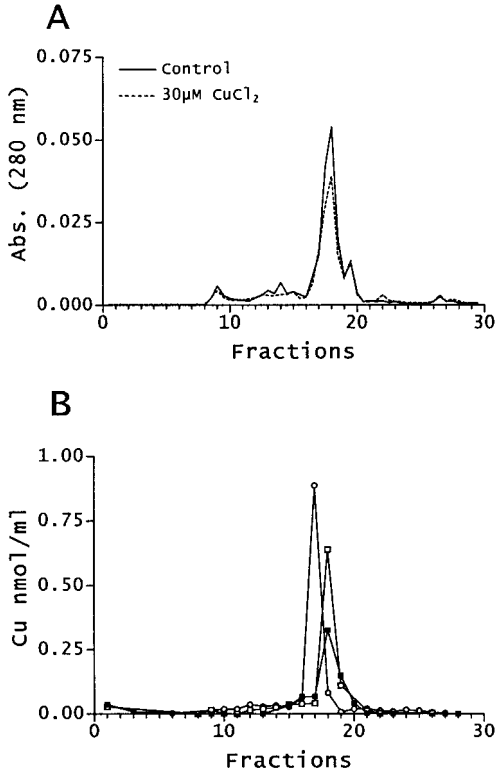

Fig. 3. Copper distribution into subcellular pools of differentiated Caco-2 cells. Cells were incubated with 30 μM $CuCl_2$ under standard conditions. Cell lysates were prepared and equal amounts of proteins fractionated by gel filtration FPLC as described under Materials and methods. (A) Elution profile at 280 nm of cell lysates (B) Copper profile of cell lysates. Data are from a representative experiment. Control cells (solid squares); cells treated with $CuCl_2$ for 6 min (open squares); cells treated with $CuCl_2$ for 60 min (open circles).

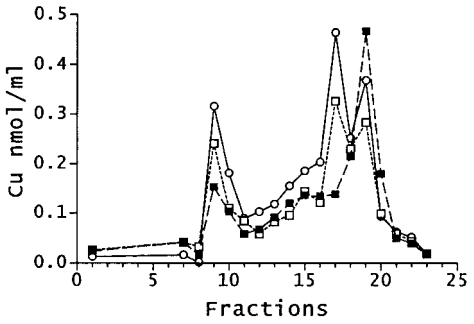

Fig. 4. Kinetics of the distribution of copper into subcellular pools in Caco-2 cells. Cells were incubated with 30 μM $CuCl_2$ for 30 min and then 'chased' for 3 and 6 h. Cell lysates were prepared and equal amounts of proteins fractionated by gel filtration FPLC as described under Materials and methods. Data are from a representative experiment. Cells treated with $CuCl_2$ for 30 min (solid squares); cells 'chased' for 3 h (open squares); cells chased for 6 h (open circles).

trol cells. Longer treatment (60 min) lead to a further increase in total copper content and to a shift in the elution of the major copper peak to fraction 16, corresponding to higher molecular weight components. Previous reports had shown that this region corresponds to where metallothionein elutes (Freedman *et al.* 1989).

To investigate copper distribution into subcellular pools, copper was followed after a 'pulse' treatment. Figure 4 shows the FPLC elution profile of Caco-2 cell lysates treated for 30 min with 30 μM $CuCl_2$ and 'chased' in the absence of added copper for 3 and 6 h. After treatment, the major copper peak localized, as also shown in Figure 3B, in the region where GSH elutes. During 'chase', copper eluted as two peaks progressively shifting from the GSH fractions to higher molecular weight fractions where metallothionein elutes. An increase in copper content of fractions 14 and 15 was also observed during the

'chase' and it is interesting to note that in this region also elutes Cu,Zn superoxide dismutase, as assayed by a polarographic method (data not shown). Furthermore, other higher molecular weight components (fractions 8–10) also bound copper during the chase period, as already reported in hepatoma cells (Freedman *et al.* 1989). Incubation of hepatoma cells with ^{67}Cu resulted in fact in an initial localization of the radiolabel in the GSH peak, and, after longer incubation times, in a decrease in the levels of GSH-^{67}Cu with a concomitant increase in ^{67}Cu-labeled metallothionein (Freedman *et al.* 1989). Our results are in good agreement with those findings and may confirm that copper is transferred from GSH to metallothionein. Conversely, the incorporation of copper into Cu,Zn superoxide dismutase has been shown to occur by a different mechanism involving a specific copper chaperone (Culotta *et al.* 1997).

In conclusion, the results presented indicate that the AP uptake of copper in Caco-2 cells occurs by a passive mechanism, strongly affected by the redox state of the metal, while they do not suggest, at least under the conditions employed in the study, the involvement of specific transporters such as DMT1 or hCTR1. Following uptake, intracellular copper distribution follows a canonical sequence, with copper predominantly bound to low molecular weight components shortly after uptake, and subsequently donated to higher molecular weight components such as metallothionein and Cu,Zn superoxide dismutase.

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