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Effects of copper and ceruloplasmin on iron transport in the Caco 2 cell intestinal model

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Abstract

Previous studies have implicated copper proteins, including ceruloplasmin, in intestinal iron transport. Polarized Caco2 cells with tight junctions were used to examine the possibilities that (a) ceruloplasmin promotes iron absorption by enhancing release at the basolateral cell surface and (b) copper deficiency reduces intestinal iron transport. Iron uptake and overall transport were followed for 90 min with 1 μM ^{59}Fe(II) applied to the apical surface of Caco2 cell monolayers. Apotransferrin (38 μM) was in the basolateral chamber. Induction of iron deficiency with desferrioxamine (100 μM; 18 h) markedly increased uptake and overall transport of iron. Uptake increased from about 20% to about 65% of dose, and overall ^{59}Fe transport from $<$1% to 60% of dose. On the basis of actual iron released into the basal chamber (measured with bathophenanthroline), transport increased 8-fold. Desferrioxamine pretreatment reduced cellular Fe by 55%. The addition of freshly isolated, enzymatically active human ceruloplasmin to the basolateral chamber during absorption had no effect on uptake or transport of iron by the cells. Unexpectedly, pretreatment with three different chelators of copper (18 h), which reduced cellular levels about 40%, more than doubled iron uptake and raised overall transport to 20%. This was so, whether or not cells were also made iron deficient with desferrioxamine. Acute addition of 1 μM Cu(II) to the apical chamber had no significant effect upon iron uptake, retention, or transport in iron deficient or normal cells, in the presence of absence of ascorbate. We conclude that intestinal absorption of Fe(II) is unlikely to depend upon plasma ceruloplasmin, and that cuproproteins involved in this form of iron transport must be binding copper tightly.

Keywords: Iron absorption; Copper; Ceruloplasmin; Caco 2 cells; Iron uptake; Iron transport

1. Introduction

Many aspects of the mechanisms involved in intestinal iron transport still remain unknown. Most studies agree that ferrous iron is preferentially absorbed by the intestine, although iron administered as Fe(III) is also absorbed [1–9]; and the ferrireductase involved appears to have been identified and cloned [10–12]. A divalent metal transporter (DMT1/Nramp2/DCT1) in the brush border of intestinal cells is likely to be responsible for most (or all) of the Fe(II) uptake [13–16]. The protein expression of this transporter (as well as of the ferrireductase), and its deployment, are inversely related to levels of iron within the enterocyte. Together, changes in brush border concentrations of DMT1 and the ferrireductase would thus account for the well known phenomenon that nutritional iron status is inversely related to the rate of intestinal iron uptake and absorption [2,4–6,17]. The mechanisms controlling expression of these proteins are still being studied; but in the case of DMT1, one mRNA form contains an iron responsive element (IRE) in the 3'UTR [3,6,14,18,19]. This appears to bind IRPs and thus be stabilized, resulting in enhanced production of the transporter when intracellular iron concentrations are low. (The regulation is similar but not identical to that of transferrin receptor mRNA, which has 5 IREs in the 3'UTR [20,21].)

Despite these important and interesting findings about brush border uptake, there has been evidence for some time that the main control over iron absorption is exerted at the basolateral end of the enterocyte in connection with transfer of iron to the blood [2]. Thus, there can be considerable uptake across the brush border accompanied by little transfer across the basolateral surface. Since DMT1 is required not just for Fe(II) uptake but also for uptake of other divalent metal ions [13–16], and since there is a non iron-regulated form of DMT1 mRNA [6,14,18,19], large

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amounts of iron in the diet would be expected to result in a large iron uptake by mucosal cells, even when this is not needed by the body. Nevertheless, that does not mean that most of it will be transferred into the body per se, since basolateral transfer is more stringently controlled. However, even here control is not perfect, and continuous exposure to excess iron in the diet can lead to iron overload [23,24].

Just how iron crosses the basolateral surface, and just how control is exerted there, are matters still not fully understood. Recent findings suggest that at least two mechanisms of basolateral transport may occur. One would include the activity of an iron transporter, IREG1/ferroportin, first detected in zebrafish [25] and hypotransferrinemic mice [26] and established as present in the serosal membrane of the intestinal epithelium [26]. Second, there is good evidence for an endo/exocytic cycling mechanism involving apotransferrin [27–29]. Based on studies with Caco2 cells, apotransferrin appears to be absorbed from the blood/interstitial fluid and travel in vesicles to a compartment above the nucleus [28,29], before returning to the serosal surface. During its transit (and perhaps in the trans-Golgi network), it might pick up iron, forming diferric-transferrin, which is then released into the blood. Iron-containing transferrin can also enter enterocytes from the blood, but it goes to a different vesicular compartment [28,29], and primarily to crypt cells [29], thus perhaps being a signal to the developing mucosa about the body's iron status [9,30]. The copper-containing protein, hephaestin, originally identified through a sex linked anemia in mice [31] and with homology to ceruloplasmin, could be involved in the transfer of iron to apotransferrin during vesicular cycling. The product of the gene (HFE), that when defective results in hemochromatosis [32,33], might be involved in inhibiting the transfer of iron to the apotransferrin/transferrin receptor complex, or in slowing exocytosis or cycling of these factors, especially in crypt cells [30,34].

During severe copper deficiency, it has long been known that iron transport within the body is adversely affected, and that iron tends to accumulate in many tissues [35–37]. Generally but not always [38], there also is a hypochromic, microcytic anemia similar to that produced by iron deficiency [37]. Severe copper deficiency virtually eliminates the presence of copper-containing ceruloplasmin in the blood circulation [37], although apoceruloplasmin is still present [39]. Normally, this α_2-globulin binds 6 atoms of copper per molecule [40] and accounts for at least two thirds of the copper in human and rat plasma [41,42]. The copper attached to ceruloplasmin is not dialyzable and is required for its characteristic ferroxidase activity [37,43]. In copper deficiency, much of the protein is still made and secreted, but it has little or no ferroxidase (or other oxidase) activity [39]. A lack of active blood ceruloplasmin has been implicated in the tissue iron accumulation that occurs in copper deficiency. This is because (a) the infusion of copper-containing ceruloplasmin into the circulation of copper deficient livers results in immediate release of iron into the blood [44–46]; (b) genetic aceruloplasminemia (detected in some humans [47, 48]) results (in time) in an accumulation of tissue iron similar to that seen in severe copper deficiency.

There is one report of a promotional effect of ceruloplasmin on iron release into the blood by the intestine. Wollenberg et al [49] followed the rate of appearance of ^{59}Fe in the portal blood after its intubation into the intestinal lumen of copper deficient rats and provided evidence that this markedly increased immediately after i.v. infusion of ceruloplasmin. The increase failed to occur when a copper-albumin complex was infused instead. (In similar studies by others with liver [44–46], copper salts also failed to stimulate iron release.) Thus, it seemed likely that the intestine might (at least in part) depend upon plasma ceruloplasmin to release iron from the enterocyte, a matter we examined in the current studies. However, there were mixed reports with regard to whether iron accumulates in the intestine in copper deficiency. Gubler and colleagues [50,51] found it did and reported decreases in transfer of iron from the intestine into the blood. Other groups found no accumulation of iron in the intestines of copper deficient animals [52–54], and Coppen and Davies [55] found no effects of ceruloplasmin infusion on iron release, using isolated intestinal loops.

As already indicated, an additional involvement of copper in intestinal iron absorption was recently identified, based upon the cloning of the gene responsible for murine sex linked anemia [31]. The resulting normal gene product, named hephaestin, proved to be a copper binding protein with some homology to ceruloplasmin and to the yeast membrane protein (FET3) necessary for uptake of iron in those organisms [56,57]. The location of this transmembrane protein appears to be the trans-Golgi network rather than the basolateral membrane of the enterocyte [58], suggesting (as already indicated) that it is involved in a vesicular mechanism that allows iron to cross the basolateral membrane. If that were the case, we hypothesized that copper deficiency would compromise the rate of basolateral iron transport, which was also examined here.

Our results show that, at least in the Caco2 cell model, ceruloplasmin does not appear to be involved in promoting intestinal iron transport. However, copper may have other, unexpected and potentially regulatory roles in modulating intestinal iron absorption.

2. Materials and methods

2.1. Caco2 cell culturing and measurements of iron absorption

Caco2 cells were cultured and used for measurements of iron absorption as previously described [59]. We are grateful to Dr. Jonathan Glass and his colleague Dr. Juan Rodriguez (Feist-Weiller Cancer Center, LSU Medical Center, Shreveport, LA) for teaching us the technique and for other helpful advice. Briefly, cells were cultured in DMEM, sup-

plemented with 20% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM Na pyruvate, and antibiotics (100 units/mL penicillin-G and streptomycin, and 250 units/mL Fungisone), in T75 flasks, until almost confluent. After a second pass, cells were transferred to collagen coated Transwell plates (CoStar, Corning, MA) (10^6 cells per 6.5 mm diameter well containing 4 ug rat tail collagen). When trans epithelial electrical resistance reached about 250 Ohms/cm^2, cells were used for uptake and transport studies. Some cells were pretreated with 100 uM desferrioxamine (for 18 h), to induce iron deficiency. Copper deficiency was induced with triethylenetraamine (teta; 1 mM; Fluka, Milwaukee, WI), or bathocuproine sulfonate (BCS; 40 μM; Sigma Chemical Corp., St. Louis, MO), or N,N-bis(2-aminoethyl)-1,3-propanediamine (tet; 20 μM; Acros Oganics, Geel, Belgium), for 18 h. Iron uptake and overall transport was followed with $^{59}FeSO_4$ (1 μM) added to the apical (brush border) chamber in Hepes-buffered saline (130 mM NaCl, 10 mM KCl, 50 mM Hepes, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4), that contained fresh 1 mM ascorbate. Human apotransferrin (3 mg/mL; 38 μM; Sigma) was added to the basal medium, which consisted of the same Hepes-buffered saline. In some cases, freshly purified human ceruloplasmin (about 144 μg/mL) was added as well. Samples were removed from the basolateral chamber at 30, 60 and 90 min, for determination of overall transport (as percent of ^{59}Fe dose). At the 90 min time point, overall uptake was calculated from (a) the radioactivity remaining in the apical chamber (including washes—with Hepes-buffered saline/1 mM ascorbate—collected from the cells during harvest), and (b) from the recovery of ^{59}Fe in cells and basal medium, which was also determined. [Washing the cells with the ascorbate solution removed residual ^{59}Fe at the apical surface.] Radioactivity was detected by gamma counter (Cobra II, Packard Instruments, Downer's Grove, IL). In one experiment, cells were preloaded for 24 h with tracer $^{64}CuCl_2$ (about 0.014 μCi/well) in culture medium, to study release of copper from the cells by teta. The $^{64}CuCl_2$ was obtained from the MIR facility at Washington University (St. Louis, MO), courtesy of Dr. Debra McCarthy.

2.2. Purification of ceruloplasmin

Previously frozen human serum, dialysed for 1.5–2 h into 100 mM K phosphate buffer, pH 6.8, was subjected to chromatography on DEAE-Sepharose CL6B, as previously described [60]. In the three peak fractions, ceruloplasmin accounted for about 25% of the total protein. The entire purification was completed in 5 h, and the ceruloplasmin (which was enzymatically active in oxidation of Fe(II) and p-phenylene diamine) was immediately used in the absorption experiments.

2.3. Copper and iron analyses

Copper in the cells and basal media was determined by furnace atomic absorption spectrometry, after wet ashing, as previously described [60]. Actual iron in cells and media was detected with bathophenanthroline disulfonate (Sigma) [61] on the same samples.

2.4. Cell protein determinations

To correct results for numbers of cells, the cells in each well were dissolved in 500 μL 0.2 M KOH after washing (with Hepes-buffered saline) to remove loosely bound ^{59}Fe. The resulting extracts were assayed for protein with the Bradford dye binding method, using the reagents and protocol of the manufacturer (BioRad, Richmond, CA), and bovine serum albumin as the standard.

2.5. Statistical analysis

Results are expressed as Means $\pm$ standard deviation, for the number of determinations in the parentheses. Statistical analysis of the data was by one way ANOVA. Probability (p) values of <0.05 were considered significant.

3. Results

3.1. Effects of iron deficiency on transport of iron by Caco2 cells

Caco2 cell monolayers with tight junctions, that had and had not been pretreated with desferrioxamine to induce iron deficiency, were examined for their abilities to take up and transport iron from the apical to the basolateral chamber. As shown in Fig. 1A, pretreatment with desferrioxamine for 18 h had a very marked effect on overall transport of iron. Without pretreatment, only a small percentage of the ^{59}Fe entered the basolateral chamber. With pretreatment, overall transport of radioactivity was many fold higher, reaching about 60% by 90 min. Desferrioxamine treatment did not affect the tightness of cell junctions; it caused no change in the resistance across the cell monolayer, which was always measured. The amounts of iron transported in the iron-normal cells (not pretreated with desferrioxamine) were of the same order of magnitude as those previously reported by Glass, Nunez and their colleagues [27–29,59]. values being in the range of 1–2% of dose, which translates into 1–2 pmol Cu/insert or 3.3–6.7 pmol Cu/mg cell protein, in 90 min. (Coefficients of variation on the means were $<25\%$.) It should be noted that, as previously described by the same groups [28,29], the addition of apotransferrin to the basal chamber during the transport studies was very important. Very little iron was released from the cell into the basal chamber in the absence of apotransferrin (data not shown).

Uptake of iron by the cells (from the apical chamber) was

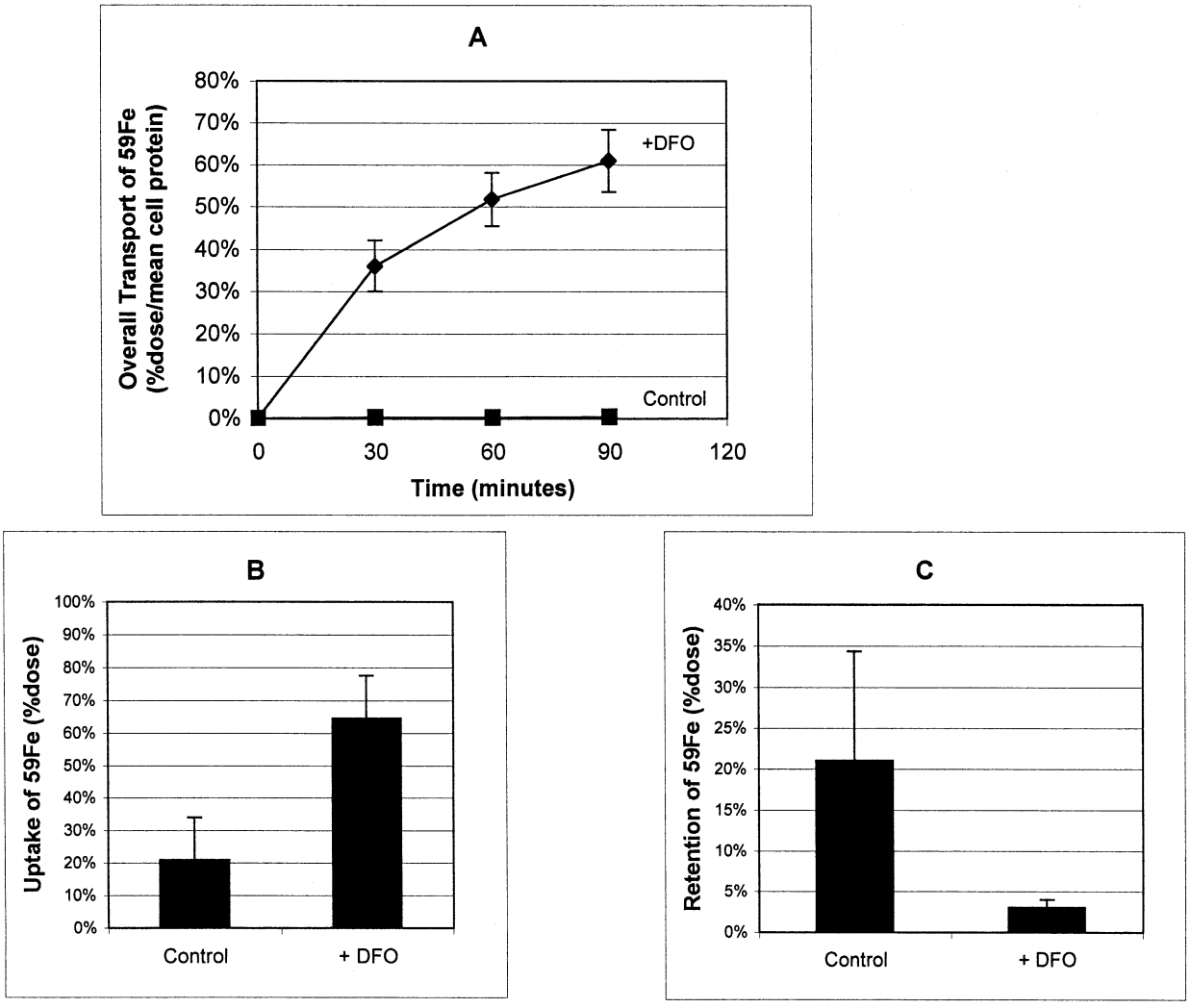

Fig. 1. Effect of iron deficiency on transport of iron by Caco2 cells. Values are Means ± SD for percent of dose of ^{59}Fe taken up, transported, or retained, corrected for cell protein, for 12 determinations (triplicate sets of cells in 4 experiments). A. Overall transport of ^{59}Fe, over time, for cells that were (diamonds; +DFO) and were not (squares; Control) pretreated with desferrioxamine (DFO) for 18 h. B. Uptake of ^{59}Fe(II) by the cells from the apical medium, determined from the radioactivity remaining in the apical fluid. C. ^{59}Fe retained by the cells.

also markedly affected by desferrioxamine pretreatment. Fig. 1B summarizes how much ^{59}Fe was removed from the apical solution by the end of the 90 min absorption period. Without pretreatment, about 20% was taken across the apical membrane; with pretreatment, uptake increased to about 65%. Retention of radioactive iron by the cells was also altered (Fig. 1C). Without desferrioxamine pretreatment, most of the ^{59}Fe taken up at the apical surface was retained; almost none was retained when the cells were first depleted of iron.

Measurements of actual iron in the cells (with bathophenanthroline, after wet ashing) confirmed that desferrioxamine had reduced cellular iron levels more than 50% in 18 h (Table 1). Concentrations of actual iron in the basal and apical media were also measured and were used to confirm that iron depletion enhanced uptake as well as basolateral iron transport. As in the case of calculations based on radioactivity, Table 2 shows that more than twice as much actual iron was removed from the apical medium by cells made deficient with desferrioxamine, compared with controls. More importantly, the amount of actual iron transferred to the basal chamber of the deficient cells was 8-fold higher than in the case of untreated cells. This compares with a >50-fold increase in overall transport of ^{59}Fe (described earlier), and reflects differences in the dilution of the radioisotope by non radioactive iron within the cells prior to basolateral transfer. The Caco2 cells thus behaved entirely as expected for absorptive cells of the intestinal mucosa, increasing their uptake and overall transport of dietary iron in response to iron deficiency, and decreasing cellular iron retention.

3.2. Effect of ceruloplasmin on iron transport

To test whether ceruloplasmin might play a role in the release of iron from intestinal mucosal cells into the blood,

Table 1
Effects of iron and copper chelators on the iron and copper contents of the Caco2 cells

Treatment	Cellular Iron		Cellular Copper	
	(ng/mg cell protein)	(% of control)	(ng/mg cell protein)	(% of control)
None (Control)	769 ± 65		145 ± 1	
Desferrioxamine	349 ± 7*	45	126 ± 3*	87
Desferrioxamine + teta	314 ± 61*	41	99 ± 15*°	68
Triethylenetetraamine alone (teta)	685 ± 64	89	82 ± 4*	57

Cells were treated with desferrioxamine and/or triethylenetetramine (teta) for 18 h, just prior to the uptake and absorption studies. Washed and harvested cells were wet ashed with nitric acid and peroxide prior to metal analysis. Values are given as ng Fe or Cu per mg cell protein (Mean ± SD) for 4 determinations.

* Significantly different from values for untreated cells ($p < 0.001$).

° Significantly different from values for desferrioxamine treatment alone ($p < 0.025$).

Caco2 cell monolayers were exposed to freshly purified human ceruloplasmin in the basal chamber during the iron transport phase. The ceruloplasmin was enzymatically highly active, as determined by measuring its ferroxidase and p-phenylene diamine oxidase activities. As shown in Fig. 2A, the presence of ceruloplasmin failed to enhance or significantly alter the rate of appearance of ^{59}Fe in the basal medium; and this was so, whether or not the cells were depleted of iron prior to the studies. Acute ceruloplasmin addition also made no difference to the apical uptake or cellular retention of iron (Fig. 2B and C).

3.3. Effect of copper depletion on iron transport

Since at least one copper containing protein within intestinal cells has been implicated in intestinal iron absorption, the effects of copper depletion were examined. Cells were pretreated with triethylenetetraamine (teta) overnight. This resulted in a 40% reduction in the cellular copper content (Table 1), with no significant change in cellular iron. There was no change in electrical resistance across the monolayers. As concerns iron transport, copper depletion unexpectedly enhanced rather than depressed it (Fig. 3A; +teta). The appearance of ^{59}Fe in the basal medium reached about 20% of dose by 90 min as compared with about 1% in controls. The effect of copper removal was thus about half that obtained with iron depletion, in the same (see later) and earlier (Fig. 1A) experiments. The increase in iron transport with copper depletion was attributable to iron uptake (which more than doubled; Fig. 3B) as well as increased basolateral transport (Fig. 3A). There was little or no change in cellular ^{59}Fe retention (Fig. 3C). Again, acute addition of fresh ceruloplasmin made no difference to the results.

Potential interactions of iron and copper deficiencies in iron transport were also examined. The effects of copper and iron depletion on iron uptake, separately as well as together, were similar: uptake went from 21 ± 12% of dose (Mean ± SD, N = 6) in controls, to 50–64% of dose (±9–13) in the case of the copper and iron-depleted cells. As before, overall transport was markedly enhanced by both forms of metal depletion (from 1% of dose in controls to 21% by copper depletion, and to 61% of dose by iron depletion, alone). The effect of double depletion (+ DFO + teta) was indistinguishable from that of iron depletion alone (58 versus 61% of dose), and the same was true for cellular iron retention, which was 20–25% of dose in the normal and copper depleted cells but fell to 5–8% of dose with iron depletion.

The findings just described were confirmed by actual iron analysis of the cells and fluids (Table 2). Iron uptake was equally stimulated (doubled) by iron or copper depletion (or both). Iron depletion (with or without copper depletion) had a greater effect than copper depletion on baso-

Table 2
Uptake and overall transport of actual iron by normal, iron deficient and copper deficient Caco2 cells

Pretreatment	Iron Uptake		Overall Transport	
	(ngFe/mg cell prot.)	(% of control)	(ngFe/mg cell prot.)	(% of control)
None (Control)	53 ± 6	100	21 ± 2	100
Desferrioxamine	115 ± 9*	217	176 ± 12*	838
Desferrioxamine + teta	115 ± 10*	217	165 ± 14*	785
Triethylenetetraamine alone (teta)	112 ± 8*	211	85 ± 6*	405

Cells were treated with desferrioxamine and/or triethylenetetramine (teta) for 18 h, just prior to the uptake and absorption studies. Values are Fe removed from the apical fluid (Iron Uptake) and Fe released into the basal medium (Overall Transport) after 90 min, measured directly with bathophenanthroline after wet ashing, and given in terms of ng Fe per mg cell protein (Mean ± SD, for 4 determinations).

* Significantly different from values for untreated cells ($p < 0.01 - <0.001$).

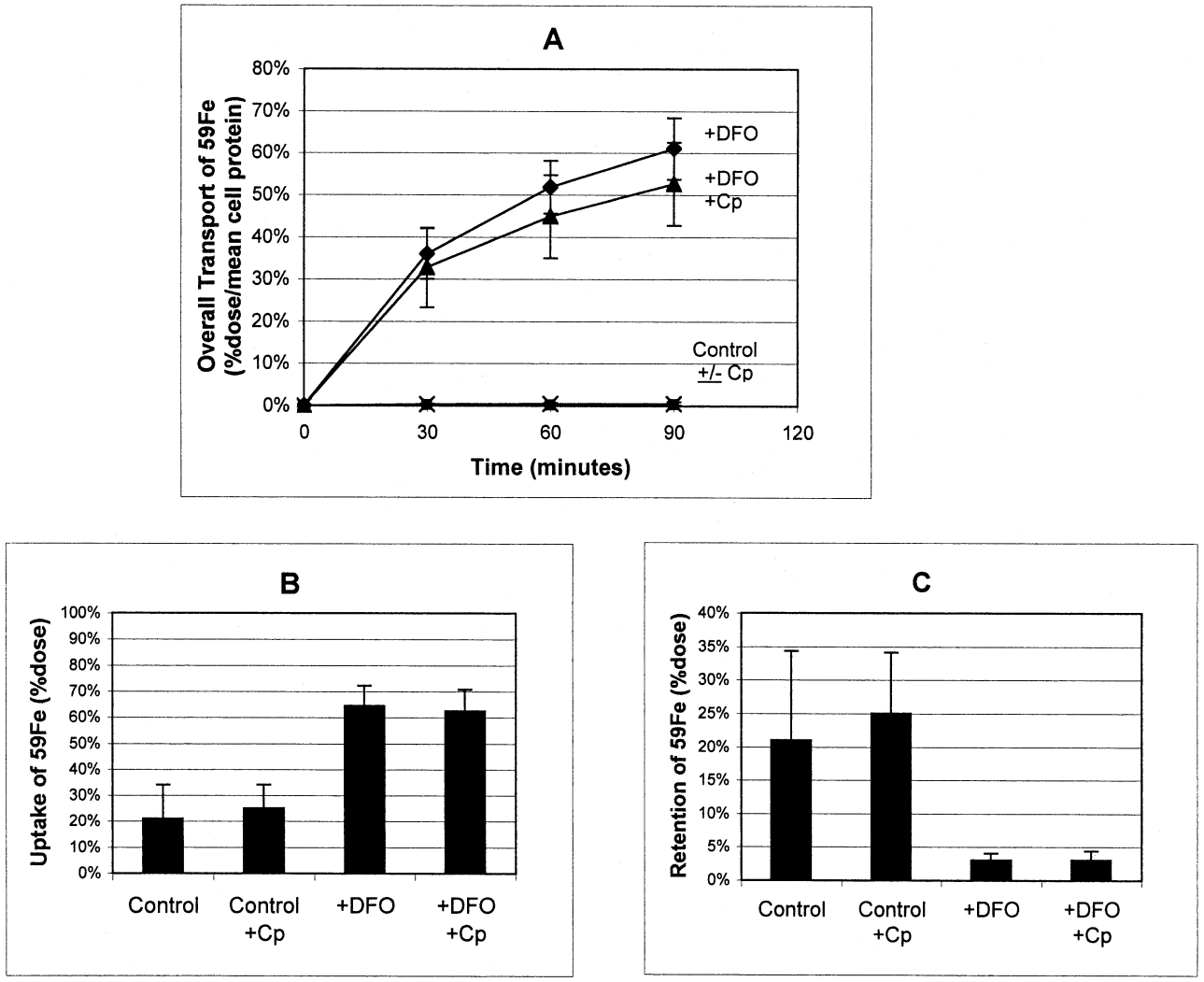

Fig. 2. Effect of ceruloplasmin on iron absorption. Overall transport (A), uptake (B), and cellular retention (C) of ^{59}Fe was followed in Caco2 cell monolayers, that were (+DFO) and were not (Control) pretreated with desferrioxamine (DFO) to induce iron deficiency, in the presence (+Cp) and absence of freshly purified human ceruloplasmin (Cp) added to the basal medium during the absorption studies. Points and bars indicate mean values (percent dose per mean cell protein), and the error bars, standard deviations, for triplicate sets of cells in 4 experiments (N = 12).

lateral transport ("Overall Transport"). However, again, copper depletion alone markedly stimulated overall transport (4-fold, compared to untreated controls).

The effects on cellular copper concentrations of the copper chelator (teta) (Table 1) were about the same whether or not cells were also treated with desferrioxamine, and vice versa. The desferrioxamine appeared to have only a small (lowering) effect on cellular copper concentrations, and the teta no significant effect on cellular iron. [Since desferrioxamine slightly but significantly reduced cellular copper concentrations, it cannot be excluded this effect also contributed to the effectiveness of the iron chelator to enhance iron absorption.] The 18 h teta treatment reduced cellular copper levels about 40% (Table 1). The effect of the chelator on release of ^{64}Cu from preloaded cells was also examined. Cells were preincubated for 24 h in normal culture medium to which fresh 1 μM $CuCl_2$, labeled with ^{64}Cu(II), had been added. Subsequent treatment of the preloaded and rinsed cells with teta for 18 h released 67% of the radioactivity ($\pm$6; Mean $\pm$ SD, N = 4). This indicates that the chelator was removing a larger proportion of copper that had recently entered the cells (as compared with copper that had been there for a longer time).

The iron absorption-enhancing effect of copper depletion (teta pretreatment) did not occur immediately and required time to manifest. As shown in Fig. 4, 8 h of chelation was not sufficient to increase the rate of ^{59}Fe uptake, although it did significantly increase overall transport, from 0.3 $\pm$ 0.2 to 4 $\pm$ 0.2% (Mean $\pm$ SD; N = 4; $p < 0.001$). The maximum effect of copper depletion on iron uptake (at this teta concentration) was achieved by 18 h; it did not change further from 18 to 48 h. Most of the increase in overall iron transport also occurred between 8 and 18 h of the start of copper chelation; but there appeared to be an additional

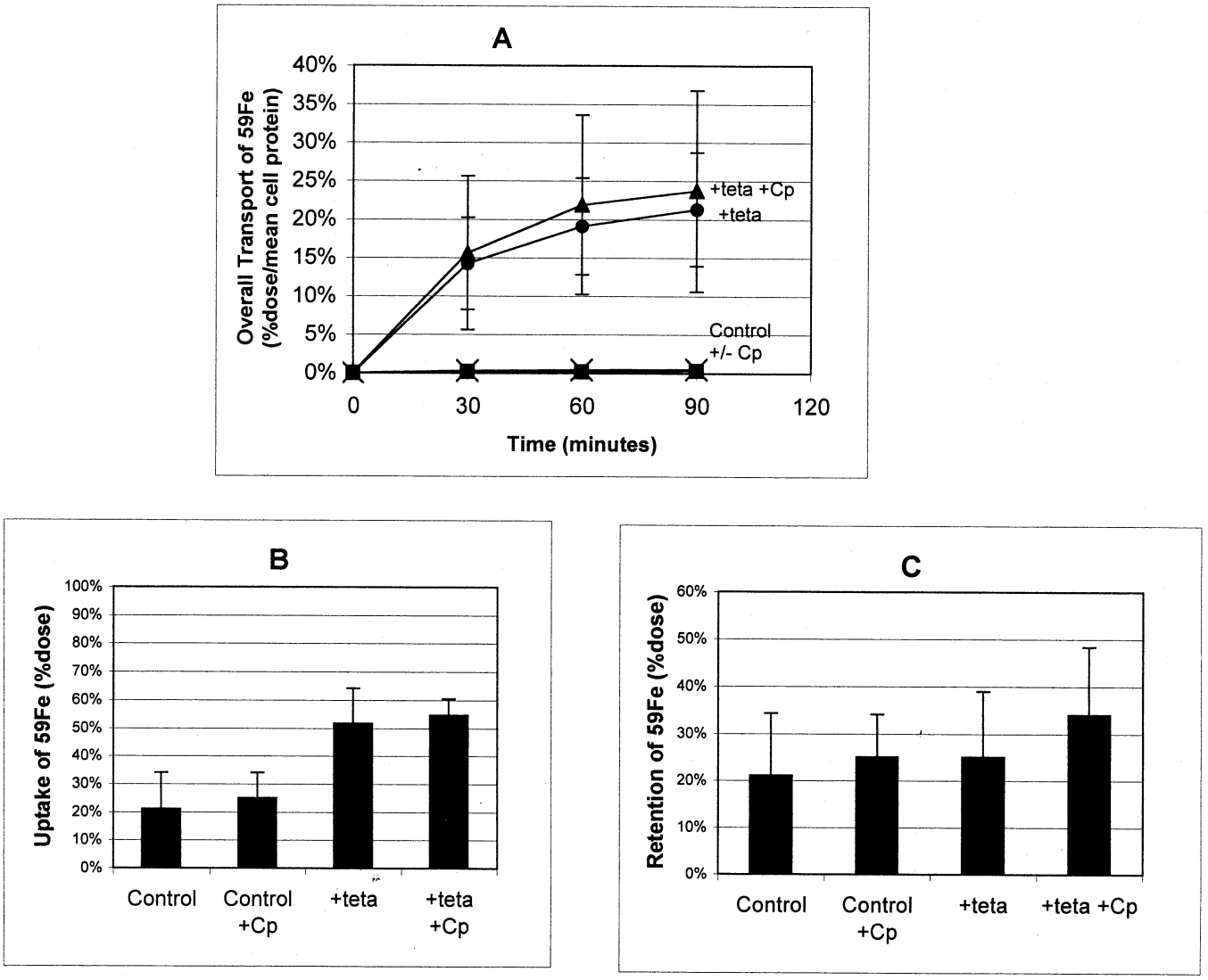

Fig. 3. Effect of copper depletion on iron absorption. Overall transport (A), uptake (B), and cellular retention (C) of ^{59}Fe was followed in Caco2 cell monolayers, that were (+teta) and were not (Control) pretreated with triethylenetetraamine (teta) for 18 h to induce copper deficiency, in the presence (+Cp) and absence of freshly purified human ceruloplasmin (Cp) added to the basal medium. Points and bars indicate mean values (percent dose per mean cell protein), and the error bars, standard deviations, for triplicate sets of cells in 4 experiments (N = 12).

increase from 18 to 48 h, balanced by a reduction in cellular ^{59}Fe retention.

The effect of the copper chelator was not specific to teta. Table 3 shows that very similar results were obtained by overnight (18 h) pretreatment of cells with two other copper chelators, bathocuproine sulfonate and N,N-bis(amino-ethyl)-1,3-propanediamine. All three chelators enhanced iron uptake and transport in the Caco2 cells.

3.4. Effects of acute copper administration on iron absorption

To assess whether acute administration of copper might reverse the effects of copper depletion on iron transport, and to begin to ascertain whether copper and iron might be using the same apical transporter, cells depleted of copper overnight were administered 1 μM non radioactive Cu(II) along with the 1 μM ^{59}Fe in the apical solution. The results are shown in Fig. 5. In this case, iron absorption was also examined in the absence of fresh ascorbic acid added to the apical solution, since copper might also be reduced and thus not compete with iron as a divalent ion. The results showed, first that as expected, ascorbic acid enhanced overall iron absorption and transport (Fig. 5A). Second, acute addition of copper in either the absence of presence of ascorbate did not inhibit iron uptake (Fig. 5B), in iron deficient or control cells. Third, acute addition of copper failed to alter overall iron transport in the presence or absence of ascorbate or iron depletion (Fig. 5A), and the same was true for cellular iron retention (Fig. 5C).

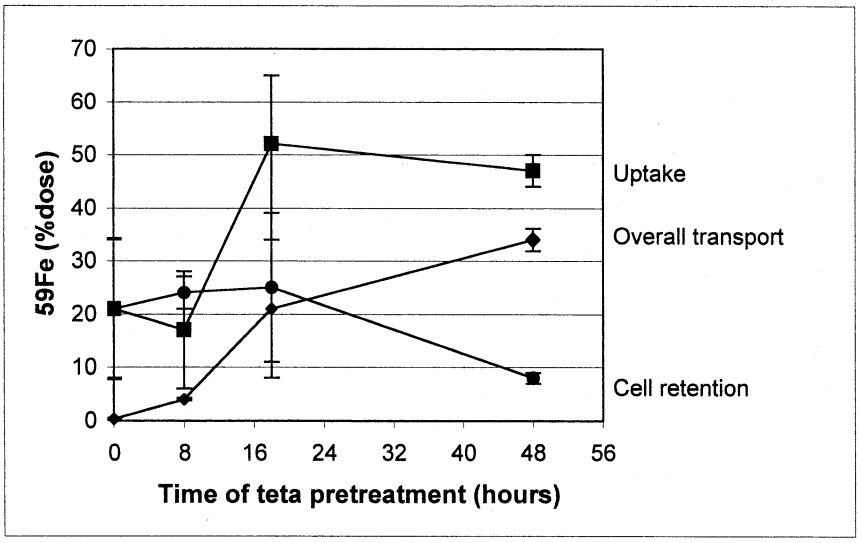

Fig. 4. Time course of the effect of copper chelation on iron transport. Shown are overall transport (diamonds), uptake (squares) and cellular retention (circles) of ^{59}Fe, as % dose in 90 min, in relation length of time of pretreatment with the copper chelating agent, teta. Points and bars indicate mean values (percent dose per mean cell protein), and the error bars, standard deviations, for triplicate sets of cells in 2 experiments (N = 6).

4. Discussion

We have shown that, at least in the Caco2 cell model of the intestinal mucosa, ceruloplasmin availability on the "blood" side of the monolayer plays no role in the release of iron from the enterocytic cells. We found that this was the case whether or not cells had been depleted of copper or iron prior to the absorption studies. Our findings agree with those of Coppen and Davies [55] and not those of Wollenberg et al [49], who examined intestinal release of iron in copper deficient rats, finding no effect of ceruloplasmin and a specific iron-releasing effect of ceruloplasmin, respectively, after i.v. infusion. Our results with the Caco2 cells also agree with those obtained for another polarized transporting cell model. In this case, release of iron from BeWo cell monolayers (models for placental transport) was also unaffected by ceruloplasmin [62]. Overall, therefore, it seems unlikely that blood ceruloplasmin plays any direct role in intestinal iron transport, although it does appear to enhance iron release from liver/hepatocytes [44–46]. Nevertheless, the *intracellular* homologue of ceruloplasmin, hephaestin [31], would appear to play some role in intestinal (and perhaps also placental) iron transport [31].

The Caco2 cell intestinal model behaved as expected of intestinal mucosa, markedly increasing its uptake and overall transport of iron in response to iron deficiency [2,4–6, 17], and increasing its uptake of iron in the presence of ascorbic acid [63,64]. It also behaved as previously reported for Caco2 cells with regard to the stimulatory effect of apotransferrin added to the basal medium [28,29,65]. However, unexpectedly, we found that copper deficiency failed to inhibit iron absorption. Removal of 40% of total cellular copper (or two-thirds of recently-absorbed copper) by pretreatment with specific copper chelators, failed to inhibit iron uptake or overall transport. Thus, if hephaestin is necessary for this mode of iron uptake and/or intestinal absorption, its copper must be bound rather tightly, and turnover of the protein must be sufficiently slow not to have impaired the process during the time periods of treatment examined here.

Not only did the depletion of copper within the cells fail to inhibit iron absorption or uptake, but the opposite phe-

Table 3
Comparative effects of three copper chelators on iron transport by Caco2 cell monolayers

Pretreatment	Iron Uptake	Overall Transport	Cell Retention
None	27 ± 8	0.2 ± 1	17 ± 3
Triethylenetetraamine	61 ± 7*	22 ± 7*	36 ± 11
N,N-Bis(aminoethyl)-1,3-propanediamine	47 ± 16*	24 ± 3*	28 ± 1
Bathocuproinedisulfonic acid	58 ± 3*	23 ± 3*	34 ± 6

Cells were treated with triethylenetetraamine (1 mM), N,N-Bis(2-aminotheyl)-1,3-propanediamine (20 μM) or bathocuproinedisulfonic acid (40 μM) for 18 h, just prior to the ^{59}Fe uptake and absorption studies. Values are percent of dose of ^{59}Fe(II) taken up (Uptake), transported overall (Overall Transport), or retained by cells (Cell Retention), Mean ± SD for 4 determinations.

* Significantly different from values for untreated cells ($p < 0.001$).

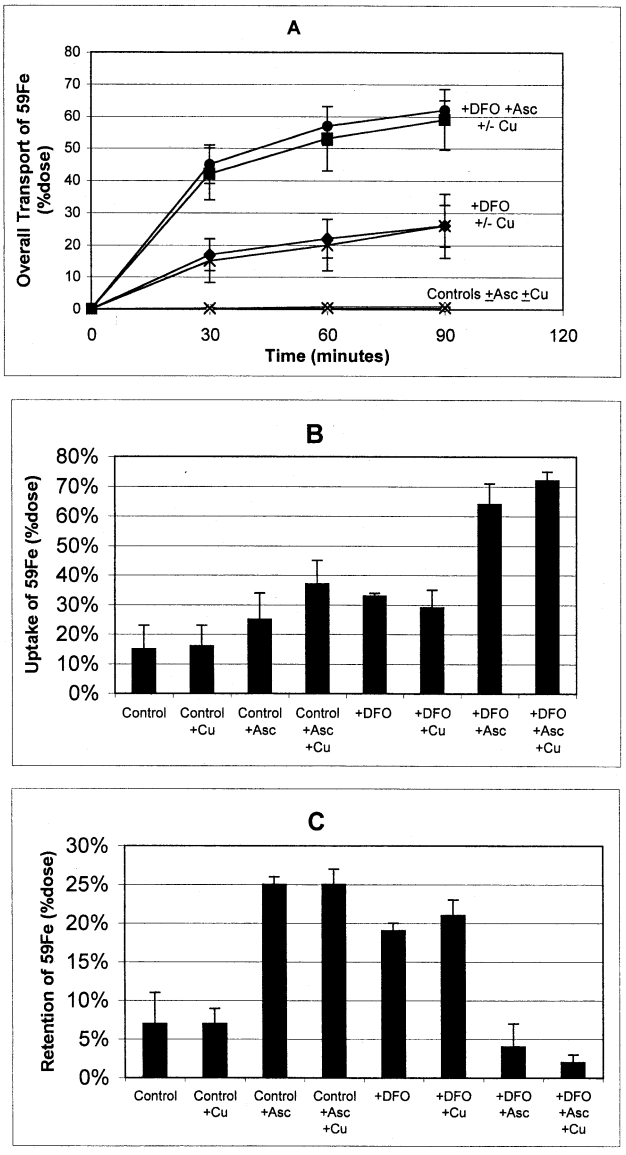

Fig. 5. Effect on iron absorption of adding copper to the apical solution. Overall transport (A), uptake (B), and cellular retention (C) of ^{59}Fe was followed in Caco2 cell monolayers, that were (+DFO) and were not (Control) pretreated with desferrioxamine (DFO) to induce iron deficiency, and then given (+Cu) or not given (−Cu) 1 μM non-radioactive Cu(II) added to the apical solution, in the presence (+Asc) and absence (−Asc) of fresh ascorbic acid. Points and bars indicate mean values (percent of dose), and the error bars, standard deviations, for triplicate sets of cells in 2 experiments (N = 6).

nomenon was observed, namely that both iron uptake and basolateral transfer were enhanced. This occurred in the absence of iron depletion, and the effects of copper depletion were nearly as great as with iron depletion: uptake doubled (as with iron deficiency), and basolateral transport increased 4-fold (versus 8-fold in the case of iron depletion alone). This suggests that copper deficiency had two separate effects on iron absorption.

For most of our studies, the copper depletion period was 18 h. However, we found that even 48 h of copper chelation did not inhibit iron absorption. Indeed, if anything, it further enhanced uptake and basolateral transfer. [As we did not examine the copper content of the 48 h depleted cells, we cannot be certain that it was lower than at 18 h. However, there appeared to be a significant exaggeration of the 18 h copper depletion effect at the later time, and that suggests there was further copper removal.] It would not be surprising if the copper in hephaestin is indeed hard to deplete. This is the case for the copper in its plasma homologue, ceruloplasmin. Ceruloplasmin-copper is deeply buried in its structure [40] and not dialyzable [37], although it is readily absorbed by most cells/tissues [42,66], presumably through the mediation of specific receptors [37,66].

The finding that iron uptake was twice as rapid upon copper depletion suggests that copper may be influencing the expression, deployment, or activity of DMT1, the carrier thought to be involved in ferrous iron entry into the enterocyte, across the brush border/apical cell membrane. The mechanism by which copper depletion might be enhancing *basolateral* iron transfer is not at all clear. However, since the form of basolateral iron transport studied here appears to involve entry and cycling (with iron uptake) of apotransferrin [28,29], it may be that copper deficiency is increasing the rate of apotransferrin entry and/or the rate of Fe_2-transferrin exit to the "blood". These matters will need to be explored. [The apotransferrin mechanism is only one of the possible ways for iron to cross into the blood.] Either way, our studies indicate that copper availability in the intestinal absorptive cell has more than one kind of effect on iron absorption, and adds to the growing evidence for copper involvement in (and perhaps regulation of) many aspects of iron transport [31,35,36,48,56,67–69].

We also observed that, at equimolar concentrations, copper failed to inhibit iron uptake by the Caco2 cells. This was true whether or not ascorbic acid was also present and suggests that the divalent metal transporter (DMT1) is not very active in intestinal copper transport. The finding is consistent with observations made in animals [70], and with determinations that there is a specific copper transporter, CTR1, in most cells [71], including the intestine [72]. However, the affinity of DMT1 for Cu(II) may simply be much lower than that for Fe(II), and the transporter does appear to be involved in the intestinal uptake of other divalent metal ions, notably Zn(II) and Mn(II), as also reported for the Caco2 cell model [73]. More detailed studies will have to be carried to determine with certainty whether or not, and how much (relative to CTR1), DMT1 might be involved in intestinal copper transport, also taking into consideration the expression of isoforms and the nutritional state of the enterocyte.

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