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Inhibition of Iron Uptake from Iron Salts and Chelates by Divalent Metal Cations in Intestinal Epithelial Cells

CHI KONG YEUNG,† RAYMOND P. GLAHN,‡ AND DENNIS D. MILLER*,†

Department of Food Science, Cornell University, Ithaca, New York 14853, and U.S. Plant, Soil, and Nutrition Laboratory, United States Department of Agriculture/Agricultural Research Service, Ithaca, New York 14853

Iron chelates, namely, ferrous bisglycinate and ferric EDTA, are promising alternatives to iron salts for food fortification. The objectives of this study were to compare iron uptake from radiolabeled ferrous sulfate, ferrous ascorbate, ferrous bisglycinate, ferric chloride, ferric citrate, and ferric EDTA by Caco-2 cells with different iron status and in the presence of divalent metal cations. Iron-loaded Caco-2 cells, with reduced DMT-1 and elevated HFE mRNA levels, down-regulated uptake from ferrous ascorbate and bisglycinate but not from ferric compounds. Nevertheless, iron uptake from all compounds was markedly inhibited in the presence of 100-fold molar excess of Co^{2+} and Mn^{2+} cations, with ferrous compounds showing a greater percent reduction. Our results suggest that ferrous iron is the predominant form of iron taken up by intestinal epithelial cells and the DMT-1 pathway is the major pathway for uptake. Iron uptake from chelates appears to follow the same pathway as uptake from salts.

KEYWORDS: Iron uptake; Caco-2; DMT-1; HFE; EDTA; bisglycinate; chelates

INTRODUCTION

Iron homeostasis is regulated at the site of absorption in the intestine. Most mammals, including humans, lack an effective mechanism for excreting excess iron (*1*). Uptake of nonheme iron by intestinal epithelial cells involves a divalent metal transporter protein, DMT-1 (also called Nramp2 or DCT-1), which is located at the apical surface of the cells (*2*). DMT-1 is a transmembrane protein that transports ferrous iron across the absorptive surface of enterocytes in a proton-dependent process (*2*). DMT-1 gene expression is up-regulated in humans with iron deficiency and down-regulated in humans with secondary iron overload (*3*). Patients with hereditary hemochromatosis (an autosomal recessive disease that leads to iron overloading) have a 10-fold greater duodenal expression of DMT-1 compared to healthy individuals (*4*). In addition, iron overloading can be induced in mice with the hemochromatosis gene (HFE) knocked out, suggesting that HFE is another regulatory gene involved in the down regulation of iron absorption (*5*). Han et al. (*6*) reported that HFE mRNA was increased and DMT-1 mRNA was decreased in Caco-2 cells grown on media containing an excess of iron. These findings suggest that both HFE and DMT-1 are involved in homeostatic regulation of human intestinal iron absorption, presumably in a reciprocal manner.

DMT-1 is not iron-specific. As the name implies, DMT-1 mediates cell uptake of a broad range of divalent metal cations besides ferrous iron, including Cd^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, etc. (*2*) Uptake of radiolabeled ferrous iron by Caco-2 cells is reduced by about 50% in the presence of a 100-fold molar excess of unlabeled Co^{2+}, arguably one of the strongest competitors of ferrous iron for DMT-1 (*7*). It has also been shown that, while Mn^{2+} substantially inhibits uptake of ferrous iron by human erythroleukemia cells through the DMT-1 pathway, uptake of ferric iron is not affected (*8*).

Apart from the presence of DMT-1 competitors, iron uptake by Caco-2 cells is also affected by the iron status of the cells, as well as by the valence state of the metal iron. Alvarez-Hernandez et al. (*9*) showed that uptake of both ferrous and ferric iron (as ferrous ascorbate and ferric nitrilotriacetate, respectively) by Caco-2 cells was significantly reduced when the cells were pretreated in iron-enriched medium, and this regulation in response to the iron status was more sensitive for ferrous iron uptake. Their results suggest that uptake of iron, particularly ferrous iron, by Caco-2 cells is inversely related to cell iron status. In a similar manner, Gangloff et al. (*10*) reported that an increase in ferritin levels of Caco-2 cells was coupled with a decrease in ferrous iron uptake, but such a decrease was not noticed in ferric iron uptake. It should be emphasized that uptake for ferrous iron by Caco-2 cells was markedly higher than ferric iron in both studies.

Iron chelates, namely, NaFeEDTA and ferrous bisglycinate, are promising alternatives to ferrous sulfate for food fortification because they are more stable when added to foods and are less affected by iron absorption inhibitors (*11*, *12*). A number of human studies have shown that iron absorption from NaFeEDTA is higher than ferrous sulfate in meals containing iron absorption

* To whom correspondence should be addressed: Phone: (607) 255-2895. Fax: (607) 254-4868. E-mail: ddm2@cornell.edu.
† Cornell University.
‡ United States Department of Agriculture/Agricultural Research Service.

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inhibitors (*13*, *14*). Field studies have also verified that NaFeEDTA is efficacious when added to curry powder, fish sauce, or soy sauce (*15*). Ferrous bisglycinate, although not as widely tested as NaFeEDTA, was more bioavailable than ferrous sulfate in maize and wheat porridge meals fed to human subjects (*16*, *17*). It also induces less gastrointestinal discomfort than ferrous sulfate when used as an iron supplement for pregnant women (*18*). Despite these advantages, it is critically important to evaluate whether iron absorption from these chelates is regulated effectively before they are considered for national fortification programs.

The objectives of this study were to compare uptake of iron from iron salts and chelates by Caco-2 cells with different iron status and to compare uptake of iron from iron salts and chelates by Caco-2 cells in the presence of DMT-1 competitors.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Mallinckrodt Chemicals (Paris, KY) unless stated otherwise. Ingredients and supplements for cell culture media were obtained from GIBCO, Life Technologies (Rockville, MD). Water used in the preparation of reagents was double-deionized. Glassware and utensils were soaked in 10% HCl for no less than 4 h and rinsed with deionized water prior to all experiments.

Cell Culture. Caco-2 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD) at passage 17, were maintained in Dulbecco's modified Eagle medium supplemented with 10% v/v fetal bovine serum, 1% antibiotic antimycotic solution, and 25 mmol/L HEPES. For uptake experiments, cells at passages 29−35 were seeded in collagen-treated 24-well plates (Costar Corp., Cambridge, MA) at a density of 50 000 cells/cm^2. The cells were cultured at 37 °C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity. Previous studies indicated that the seeded cells reached confluence after 4−5 days of growth (*10*). Uptake experiments were conducted 13 days postseeding. To achieve iron-loading, cells were given growth medium supplemented with 20 μmol/L iron in the form of ferric nitrilotriacetate (Fe/nitrilotriacetate molar ratio at 1:5) 72 h prior to the start of the uptake experiments.

Determination of the Ferritin Content in Caco-2 Cells. The procedures used in the determination of ferritin and total protein contents of Caco-2 cells were similar to those described by Etcheverry et al. (*19*) Briefly, growth medium was first aspirated off the culture well, and the cell monolayer was harvested by adding deionized water and placing in a sonicator (Lab-Line Instruments, Melrose Park, IL) for 15 min at 4 °C to disrupt the cells. Ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FER-IRON II Ferritin Assay, RAMCO Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA), respectively. As Caco-2 cells synthesized ferritin in response to increases in the intracellular iron concentration (*10*), the ferritin/protein level of Caco-2 cells, in unit of ng/mg, was used as an index of the cellular iron status.

Determination of HFE and DMT-1 mRNA Levels in Caco-2 Cells. To determine the effect of iron loading on the expression of iron regulatory genes, mRNA levels of HFE and DMT-1 in normal and iron-loaded Caco-2 cells were measured semiquantitatively by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was first extracted from the cells using the RNEasy extraction kit (Qiagen Inc., Valencia, CA). The RNA extract was then subjected to reverse transcription to synthesize first strands of cDNA using the Superscript First-Strand Synthesis System (GIBCO, Rockville, MD). The resulting cDNA mixture was used as the template for PCR amplification. On the basis of the primer sequences published by Han et al. (*6*), primer sets for HFE, DMT-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at the Cornell Bioresource Center (Ithaca, NY). The time−temperature combinations for amplification with Taq polymerase (Perkin−Elmer, Norwalk, CT) were 95 °C for 15 s, 55 °C for 85 s, and 72 °C for 45 s. The number of amplification cycles for HFE, DMT-1, and GAPDH were 29, 22, and 22, respectively. The final PCR products (amplified cDNA of HFE, DMT-1, and GAPDH) were visualized using gel electrophoresis. Photographic negatives of ethidium-bromide-stained gels were quantified by laser densitometry. To minimize intra- and interassay variability caused by differences in reverse transcriptase efficiency, the amounts of HFE and DMT-1 mRNA were normalized to the amount of GAPDH mRNA (*20*).

Table 1. Summary of Experimental Treatments in a 2 × 3 × 6 Design

iron status[a]	DMT-1 competitor[b]	iron compound[c]
normal	no competitor	ferrous sulfate
iron-loaded	Co^{2+}	ferrous ascorbate
	Mn^{2+}	ferrous bisglycinate
		ferric chloride
		ferric citrate
		ferric EDTA

[a] Normal cells received growth medium with no supplemental iron, whereas iron-loaded cells received medium with supplemental iron at 20 μmol/L for 72 h prior to the uptake experiments. [b] DMT-1 competitors were added to the uptake media at 100 μmol/L. [c] Iron compounds were added to the uptake media at 1 μmol/L.

Preparation of ^{59}Fe-Labeled Uptake Media. Radiolabeled solutions of different iron compounds were prepared immediately before use. Radiolabeled ferrous sulfate, ferric chloride, and ferrous bisglycinate (Albion Laboratories, Clearfield, UT) were prepared by spiking solutions of these compounds at pH 1 with carrier-free ^{59}Fe (Perkin−Elmer, Norwalk, CT) in 0.1 mol/L HCl. For the preparation of radiolabeled ferrous ascorbate, ^{59}Fe-labeled ferrous sulfate was mixed with a solution of ascorbic acid (Fe/ascorbic acid molar ratio at 1:20). For the preparation of radiolabeled ferric citrate and ferric EDTA, ^{59}Fe-labeled ferric chloride was mixed with a solution of sodium citrate (Fe/citrate molar ratio at 1:5) and a solution of disodium EDTA (Fe/EDTA molar ratio at 1:1), respectively. Aliquots of these radiolabeled iron solutions were then added to serum-free minimum essential medium (buffered with PIPES at pH 7) to produce uptake media with the required iron compounds constituting a concentration of 1 μmol of Fe/L. When appropriate, 100 μmol/L of unlabeled Co^{2+} or Mn^{2+} cations in the form of cobalt chloride or manganese chloride dissolved in 0.1 mol/L HCl were added to the ^{59}Fe-labeled uptake medium for DMT-1 competition.

Measurement of Iron Uptake by Caco-2 Cells. On the day of the uptake experiment, growth medium was aspirated off from each well and the cells were rinsed 3 times with 500 μL of serum-free minimum essential medium at 37 °C. A 500-μL aliquot of the radiolabeled uptake medium was then transferred into each well of the 24-well plate. The plate was placed on a rocking platform shaker in an incubator at 37 °C with a 5% CO_2/95% air atmosphere at constant humidity for 2 h. The uptake experiment was terminated by aspirating off the uptake medium and immediately rinsing with 500 μL of a "stop" solution (140 mmol/L NaCl and 10 mmol/L PIPES at pH 6.7), followed by 500 μL of a "removal" solution (140 mmol/L NaCl, 10 mmol/L PIPES, 5 mmol/L bathophenanthroline disulfonic acid, and 5 mmol/L sodium dithionite at pH 6.7). Our previous study showed that this "removal" solution can effectively remove surface-bound iron from Caco-2 cells without damaging the brush border membrane (*21*). After a final rinse with phosphate-buffered saline, the cells were solubilized in 1 mL of 0.5 mol/L NaOH. The cell suspension was transferred to a scintillation vial for ^{59}Fe counting. The ^{59}Fe in the radiolabeled uptake medium (500 μL) was also counted so that the percent uptake of iron by Caco-2 cells could be calculated.

Statistical Analysis. A 2 × 3 × 6 design was used in the present study, hence a total of 36 experimental treatments was tested (**Table 1**). All measurements of iron uptake were replicated 6 times for each treatment. RT-PCR, cellular ferritin, and total protein assays were also replicated 6 times. Data were analyzed by ANOVA or Student's *t* test using Minitab Release 12 (Minitab Inc., State College, PA). Significance was defined at $p < 0.05$. If appropriate, means were compared and separated by Fisher least significant difference (LSD) procedures.

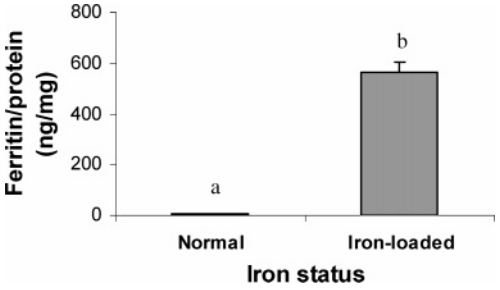

Figure 1. Ferritin/protein levels of normal and iron-loaded Caco-2 cells. Bars with different letters are significantly different. Error bars indicate standard error of the mean ($n = 6$).

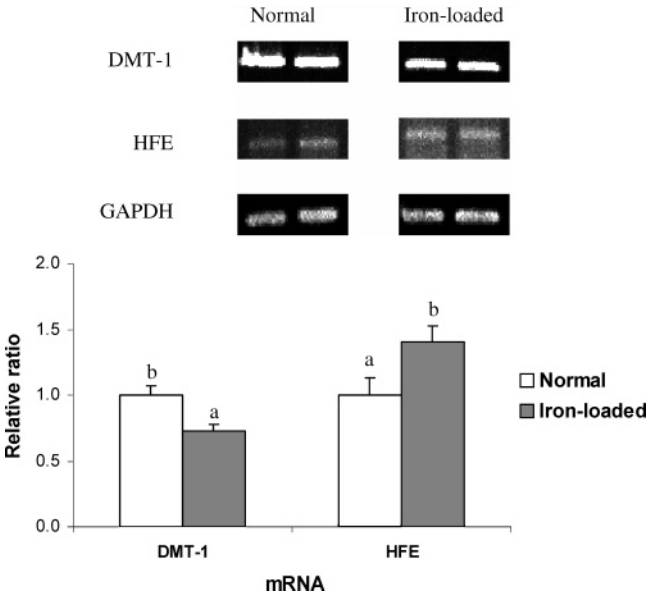

Figure 2. DMT-1 and HFE mRNA levels in normal and iron-loaded Caco-2 cells. Representative blots from ethidium-bromide-stained gels are shown in duplicate at the top. Results were normalized to the GAPDH mRNA level, which was not altered by iron treatments. Bars within the same mRNA group with different letters are significantly different. Error bars indicate standard error of the mean ($n = 6$). Messenger RNA levels of iron-loaded cells were expressed as fractions relative to the levels of normal cells.

RESULTS AND DISCUSSION

Iron Status of Caco-2 Cells. To confirm the iron status of Caco-2 cells prior to the uptake experiments, the ferritin/protein levels of the normal and iron-loaded cells were determined (**Figure 1**). The ferritin/protein level was 100-fold higher in cells given growth medium supplemented with 20 μmol/L ferric nitrilotriacetate for 72 h, indicating that ferric nitrilotriacetate was effective in elevating the iron status of Caco-2 cells. Such a change in cell iron status was accompanied by significant changes in iron regulatory gene expression. DMT-1 and HFE mRNA levels in normal and iron-loaded Caco-2 cells are shown in **Figure 2**. Iron-loading of Caco-2 cells increased the HFE mRNA level by 40% and decreased the DMT-1 mRNA level by 28%, whereas the expression of GAPDH remained unchanged (**Figure 2**).

It is well-documented that the iron status of Caco-2 cells can be altered through iron supplementation (*9*, *10*), as indicated by changes in cellular ferritin content. Besides increased cellular ferritin content, Caco-2 cells given supplemental iron in this study also showed an increased HFE mRNA level and a decreased DMT-1 mRNA level. These trends are consistent with those reported by Han et al. (*6*), although the magnitudes of changes in mRNA levels were greater in the study by Han et al., presumably because of a higher concentration of supplemental iron and longer supplementation time. Our results, nevertheless, verify that gene expression of DMT-1 and HFE is responsive to iron status in a reciprocal manner.

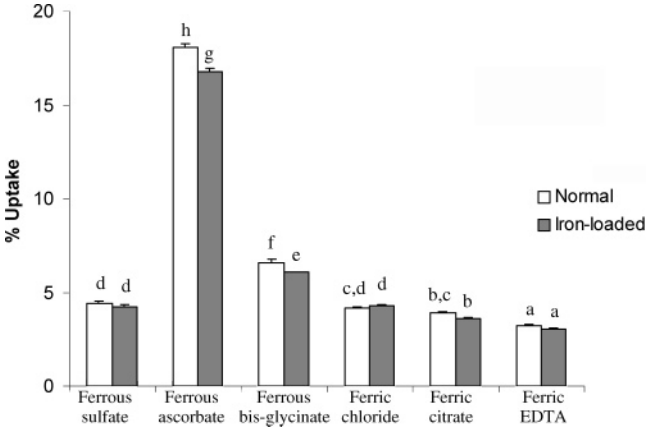

Figure 3. Effect of iron loading on iron uptake by Caco-2 cells. Bars with no letters in common are significantly different. Error bars indicate standard error of the mean ($n = 6$). Iron compounds were added to the uptake medium at 1 μmol/L.

Iron Uptake by Caco-2 Cells. Iron uptake from iron salts and chelates by normal and iron-loaded Caco-2 cells was measured by counting intracellular ^{59}Fe activity after the cells were exposed to ^{59}Fe-labeled media enriched with 1 μmol of Fe/L for 2 h. **Figure 3** shows the effect of iron loading on the percent iron uptake from different compounds. There were significant decreases in iron uptake by iron-loaded cells from ferrous ascorbate (−7.4%) and ferrous bisglycinate (−8.0%) when compared to normal cells, while iron uptake from ferrous sulfate and other ferric compounds was not significantly affected. Iron uptake from ferrous ascorbate was about 2−5-fold higher than other compounds. Ferrous ascorbate was followed by ferrous bisglycinate, whereas ferrous sulfate, ferric chloride, and ferric citrate were about the same in terms of percent iron uptake. Iron uptake from ferric EDTA was the lowest among all of the compounds tested.

Caco-2 cells, with elevated iron status, should have down-regulated iron uptake because iron uptake is inversely related to iron status (*22*). Nonetheless, the effect of iron-loading shown in this study was mild. One possible explanation is that down regulation of iron uptake in Caco-2 cells is less than that in intact humans because the cell culture system lacks hepcidin, which is predominantly expressed in liver cells; hepcidin is a recently identified peptide that may play a role in regulating intestinal iron absorption (*23*). The fact that only ferrous ascorbate and ferrous bisglycinate showed significant decreases suggests that ferrous iron uptake may be more sensitive to changes in the expression of DMT-1 and HFE than ferric iron uptake.

Iron uptake values for ferrous sulfate and ferric chloride were essentially identical despite the difference in the valence state of iron (**Figure 3**). At near neutral pH of the uptake medium and without an effective reducing agent such as ascorbic acid, oxidation of iron from ferrous to ferric is favored. Ascorbic acid reduces ferric iron to its ferrous state at low pH and remains effective up to a limit, somewhere between pH 6.0−6.8 (*24*). It is possible that the iron added to the uptake medium in the form of ferrous sulfate was rapidly oxidized and became available to Caco-2 cells only in the ferric state, resulting in

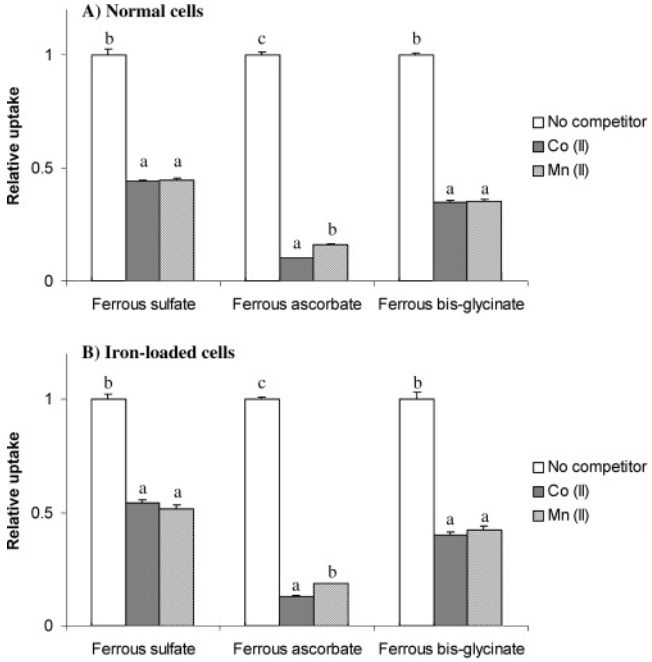

Figure 4. Effect of DMT-1 competition on ferrous iron uptake by (A) normal and (B) iron-loaded Caco-2 cells. For each graph, bars within an iron compound with different letters are significantly different. Error bars indicate standard error of the mean ($n = 6$). Uptake values with Co^{2+} and Mn^{2+} cations were expressed as fractions relative to the value with no competitor. DMT-1 competitors and iron compounds were added to the uptake medium at 100 and 1 μmol/L, respectively.

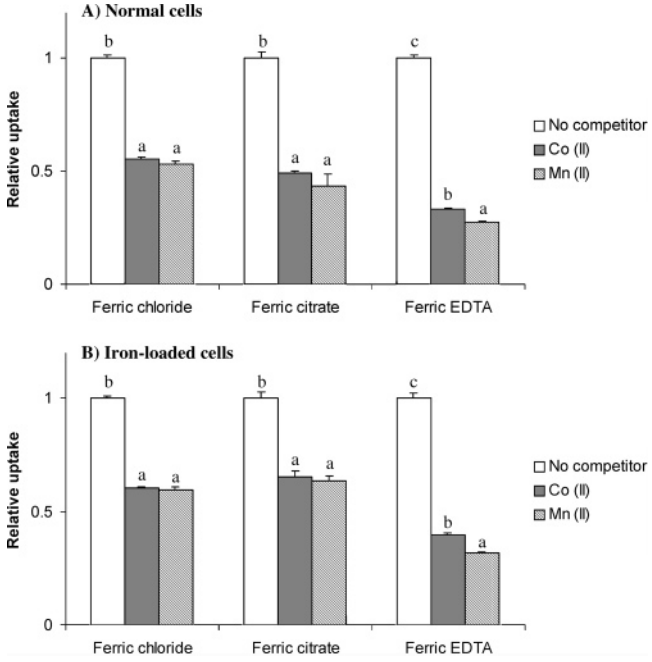

Figure 5. Effect of DMT-1 competition on ferric iron uptake by (A) normal and (B) iron-loaded Caco-2 cells. For each graph, bars within an iron compound with different letters are significantly different. Error bars indicate standard error of the mean ($n = 6$). Uptake values with Co^{2+} and Mn^{2+} cations were expressed as fractions relative to the value with no competitor. DMT-1 competitors and iron compounds were added to the uptake medium at 100 and 1 μmol/L, respectively.

comparable uptake for ferrous sulfate and ferric chloride. However, in the case of ferrous ascorbate (Fe/ascorbic acid molar ratio at 1:20), the iron added to the uptake medium should remain in the ferrous state.

Figure 4 shows iron uptake from ferrous compounds in the presence of 100-fold molar excess of Co^{2+} and Mn^{2+} cations. Co^{2+} and Mn^{2+} are potent DMT-1 competitors, and both cations inhibited ferrous sulfate and ferrous bisglycinate to similar extents, with Co^{2+} being more inhibiting to ferrous ascorbate than Mn^{2+}. In addition, these DMT-1 competition effects were found in both normal and iron-loaded cells, and the inhibition patterns were qualitatively comparable between these two types of cells, although the magnitudes of inhibition were smaller in iron-loaded cells than in normal cells. Ferrous ascorbate showed the greatest reduction in percent uptake (in both types of cells), followed by ferrous bisglycinate and ferrous sulfate.

Similar DMT-1 competition effects on iron uptake were observed in normal and iron-loaded Caco-2 cells given ferric compounds (**Figure 5**). Nonetheless, the inhibition was more pronounced in ferrous iron uptake when compared to ferric iron uptake. While the two cations were equally inhibiting to ferric chloride and ferric citrate, Mn^{2+} was more inhibiting to ferric EDTA than Co^{2+}.

Although iron is the preferred substrate for DMT-1 (*7*), our results showed that a large molar excess of DMT-1 competitors was capable of reducing uptake at least by 40%, regardless of the valence state of iron. However, the reduction was more pronounced in ferrous iron uptake than in ferric iron uptake, with ferrous ascorbate showing a decrease by as much as 90% (**Figure 4**). This observation, together with the fact that iron uptake from ferrous ascorbate was markedly higher than that from ferric iron compounds, raises two implications: first, ferrous iron when available is the predominant form of iron being taken up; and second, the DMT-1 pathway is the major pathway for iron uptake because, once the pathway is blocked by other divalent metal cations, uptake of ferrous iron (as maintained by the presence of ascorbic acid) decreases dramatically.

DMT-1 supposedly mediates the uptake of divalent metal cations only. However, uptake of ferric iron was also affected by DMT-1 competitors as shown in **Figure 5**. This could be best explained by the presence of ferrireductase at the brush border membrane of intestinal epithelial cells (*25*). As ferric iron reaches the brush border membrane, it is reduced to ferrous iron by ferrireductase and subsequently taken up by the cell through the DMT-1 pathway. As a result of this ferrireductase−DMT-1 mechanism, uptake of ferric iron should also be dependent on DMT-1 and therefore susceptible to the competition effects of other divalent metal cations, such as Co^{2+} and Mn^{2+}. It should be noted that the existence of ferrireductase at the brush border membrane does not eliminate the possibility of a separate pathway for ferric iron uptake, such as the β_3-integrin−mobilferrin pathway suggested by Conrad and Umbreit (*26*), although the majority of iron appears to be taken up in the ferrous state through the DMT-1 pathway.

In an iron chelate (such as ferric EDTA or ferrous bisglycinate), the ligand (EDTA or glycine) donates lone-pair electrons to participate in coordinate covalent bonding as a Lewis base (EDTA can donate 6 lone pairs, and each glycine can donate 2 lone pairs). To form the chelates, iron as a transition metal with unfulfilled d orbitals acts as a Lewis acid to accept these lone-pair electrons (*27*). As a result, chelate rings are formed (5 rings in ferric EDTA and 2 rings in ferrous bisglycinate), and the stability of the complex increases with the number of rings it contains (*28*). Apart from their exceptional stability, NaFeEDTA and ferrous bisglycinate are both soluble in aqueous medium at pH values ranging from 2 to 6, suggesting that they are soluble in the gastrointestinal pH environment (*29*). These favorable

properties of NaFeEDTA and ferrous bisglycinate render them suitable fortificants for staple foods that require long storage under adverse conditions or high-temperature processing (*30*).

DMT-1 competitors such as Co^{2+} and Mn^{2+} cations inhibited iron uptake from both salts and chelates. It appeared that iron from ferrous bisglycinate and ferric EDTA was taken up by Caco-2 cells in a similar fashion as from iron salts such as ferrous sulfate. Regardless of the form of iron (whether chelate or salt), ferrous iron when available was the predominant form of iron being taken up and the DMT-1 pathway was the major pathway for iron uptake. Cellular ferritin and iron regulatory gene expression levels of Caco-2 cells could be altered by iron pretreatment, reinforcing the premise that Caco-2 cells are a useful and convenient model for studying the mechanism involved in the regulation of iron uptake.

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