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Inulin Affects Iron Dialyzability from $FeSO_4$ and FeEDTA Solutions but Does Not Alter Fe Uptake by Caco-2 Cells

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The in vitro effects of inulin on the fluxes of Fe (F_{Fe}) and uptake by Caco-2 cells from $FeSO_4$ and FeEDTA were evaluated. Cell ferritin formation was used as a measure of Fe uptake. Mitochondrial (MTT test) and lysosomal activities were monitored as biomarkers of the changes of cellular metabolism. Changes in mRNA expression of Fe transporters, DMT1 and Dcytb, were evaluated. Inulin decreased dialyzability and F_{Fe} from $FeSO_4$ solution, suggesting a mineral binding effect, but increased those from FeEDTA. Cultures exposed to FeEDTA solutions exhibited higher ferritin values and MTT conversion percentages. Regardless of Fe source, cell Fe uptake and mRNA expression of Fe transporters were similar with or without inulin, suggesting that inulin did not impair Fe uptake. These observations might indicate a faster cellular Fe internalization from FeEDTA solutions. From a physiological perspective, the decreased F_{Fe} from $FeSO_4$ might be reflected in a decreased Fe uptake.

KEYWORDS: Prebiotic; inulin; iron bioavailability; iron uptake; ferritin; Caco-2 cells

INTRODUCTION

Iron (Fe) is one of the most critical nutrients for infants and children, because it is essential for several biological processes. As such, Fe deficiency is a leading nutritional concern worldwide, affecting more than a third of the world's population (*1*). One of the key causes of Fe deficiency is poor bioavailability of Fe in the diet, due primarily to the presence of phytates and polyphenols (found in most plant foods) and to the lack of highly available forms of Fe such as heme Fe (present in meat). The lack of dietary promoters of Fe uptake, such as ascorbic acid, is also thought to limit dietary iron availability (*2*). Strategies for combating Fe deficiency include supplementation, dietary diversification, and food fortification. The nonheme Fe sources that have had widespread use in fortification include, among others, FeEDTA and $FeSO_4$ (*3*). NaFeEDTA has been considered to be safe (*4*) and has been shown to be effective in human studies (*5*). In the same way, $FeSO_4$ has been successfully used to fortify infant formula and several other foods (*6*).

In the past few years, a number of food constituents have attracted attention as potential enhancers of mineral absorption, among them nondigestible oligosaccharides such as inulin-type fructans (*7, 8*). In addition, inulin has many interesting functional attributes that meet the needs of the food industry in formulating healthy foods by improving the fiber content. However, dietary soluble fiber increases viscosity of the gut contents, and this may slow gastric emptying and small intestine transit rates (*9*). Also, nonfermented soluble dietary fiber have mineral-binding capacities (*10*). Thus, it could be expected that soluble fiber may negatively influence mineral bioavailability in the duodenum—the main site of mineral absorption. However, data available in the literature characterizing the negative effects on mineral absorption or the enhancing properties of prebiotics on Fe absorption are contradictory and mechanisms of action involved are poorly understood. An in vitro study (*11*) showed that supplemental 3% inulin impaired the dialyzability of Fe. In contrast, a human in vivo study showed that nondigestible fructooligosaccharides do not interfere with calcium and nonheme-Fe absorption in young healthy men (*12*). Yasuda et al. (*13*) showed that supplemental 4% inulin added to a corn/soybean-based meal increased Fe absorption in young pigs. The authors also reported higher soluble Fe levels in the digesta of the proximal, mid, and distal colon (*13*). In light of these observations, understanding the interactions and mechanisms of the possible enhancing effects of prebiotics on Fe absorption is important. However, less information exists on the interactions of inulin with different sources of Fe.

The Caco-2 cell line is a human adenocarcinoma cell line that has proven to be a useful model for studying Fe absorption (*14, 15*). Under appropriate conditions, these cells

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differentiate into polarized enterocyte-like monolayers. Its applications in the case of Fe have made it possible to study mechanisms of uptake (*16, 17*) and interaction with several enhancers and inhibitors (*2, 18*). The low cost, ease of use, and widespread acceptance of the Caco-2 cell line make this model system an attractive alternative to animal studies and an available tool for use in conjunction with human trials (*18*).

In consideration of the above, the aim of the present study was to evaluate the effects of inulin on the flux of common sources of fortificant Fe across a semipermeable membrane and on Fe uptake by Caco-2 cells.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All glassware used in the sample preparation and analyses was treated with 10% (v/v) concentrated HCl (37%) for 24 h and then rinsed with 18 MΩ deionized water before use.

Instruments. Iron concentration was determined with an inductively coupled argon plasma emission spectrometer (ICP-ES, model 61E Trace Analyzer, Thermo Jarrell Ash Corp., Franklin, MA). Other equipment used included a spectrophotometer (DU 520 UV/vis, Beckman Coulter, Palo Alto, CA) and an automatic gamma counter Wizard 3 Wallac 1480 (Perkin-Elmer, Norwalk, CT).

Iron Standards. Ferrous sulfate, $FeSO_4$ (catalog no. 5056, Mallinckrodt AR, Germany), and ethylenediaminetetraacetate, NaFeEDTA (EDFS, Sigma), were used. Working solutions of 12 μM as Fe were prepared by dissolving appropiate amounts of the standards in 0.1% (v/v) HCl aqueous solutions. The working solutions were mixed with inulin (0.041 ± 0.001 g). Inulin (Synergy, Orafti, Belgium) used in our study was a mixture of short- (polymerization degree, PD = 3) and long-chain (PD = 30) oligofructose polymers.

In Vitro Digestion. A method to simulate the human gastrointestinal digestion process was applied (*14*). Porcine pepsin (P-7000) (800–2500 units/mg of protein), pancreatin (P1750) (activity, 4 × USP specifications), and bile extract (B8631) (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were demineralized with Chelex-100 (Bio-Rad Laboratories, Hercules, CA) before use.

Briefly, peptic and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37 °C/5% CO_2/95% relative humidity). After the gastric step (pepsin in 0.1 M HCl/pH 2/1 h), the intestinal digestion (pancreatin−bile extract in 0.1 $NaHCO_3$/pH 6.9–7/10, 20, 40 or 120 min) was carried out in the upper chamber of a two-chamber system in six-well plates. The upper chamber was formed by fitting the bottom of an appropriately sized Transwell insert ring (Corning) with a 15000 molecular weight cutoff dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). Total incubation time for the in vitro digestion procedure was 3 h. The cell cultures were incubated for up to 24 h prior to ferritin quantification.

Evaluation of Fluxes of Fe and Dialyzability Percentages. To evaluate the dialyzable Fe, the inserts were placed in six-well culture plates without cells. Two identical sets of samples were prepared as follows: an aliquot (1.5 mL) of the intestinal digest was pipetted into the upper chamber, and 1 mL of an isotonic solution (140 mM NaCl, 5 mM KCl) was added to the bottom compartment. One of them (A) was allowed to stand over the benchtop for 1 h at room temperature. From the other (B), samples (300 μL) from the bottom compartment were collected every 10 min for 60 min. Aliquots of the isotonic solution were added to replace the volumes removed during sampling. Fluxes of Fe (F_{Fe}, cm/s) were calculated from the linear slope of the Fe concentration in the bottom chamber according to the following equation: $F_{Fe} = (dC/dt)(V_a/AC_0)$, where V_a is the volume of upper compartment (upper, 1.5 mL; bottom, 1 mL), A is the surface area of the dialysis membrane placed in the insert, C_0 is the initial concentration in the upper compartment (μM), and (dC/dt) is the flux (μM/s) determined by the linear slope of the iron concentration in the bottom chamber corrected for dilution versus time (*19*).

In the first set of samples (A), the total Fe that diffussed to the bottom chamber was used to calculate the Fe dialyzability percentages relative to the initial amount added to the upper chamber as (dialyzable Fe/Fe in the upper chamber) × 100.The experiment was repeated in triplicate on two different days for each sample.

Total Iron Determination. Aliquots (0.3 mL) of media from the bottom chamber were acid digested in 1.0 mL of HNO_3 with 1.5 mL of $HClO_4$ at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. The samples were diluted with 5% HNO_3 to 6 mL. The instrument was calibrated with 10% $HClO_4$ as the low standard and 1 μg/g Fe in a multielement standard as the high standard. The Fe was determined using the 238.2 nm line.

Cell Cultures. Caco-2 cells were obtained from the American type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 25–33. Cells were seeded at a density of 50000 cells/cm^2 in collagen-treated six-well plates (Costar, Cambridge, MA) and maintained with Dulbecco's modified Eagle's medium (DMEM) under conditons previously described (*14*). The cells were used for iron bioavailability experiments at 13 days postseeding. On the day prior to the in vitro digestion experiment, the DMEM medium was removed and washed with 2 mL of minimal essential medium (MEM, GIBCO), and then 2 mL of fresh MEM was added and the plates were returned to the incubator.

Ferritin Analysis. The procedures used in the determination of ferritin and total protein contents of Caco-2 cells were as previously described (*14*). The ferritin concentration and total protein concentration were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FERIRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular iron concentration. Therefore, the ratio of ferritin/total cell protein expressed as nanograms of ferritin per milligram of protein was used as an index of the cellular iron uptake.

Total RNA Isolation and mRNA Analysis of the Iron Transporters. Cells were harvested and immidiately transferred to liquid nitrogen. Total RNA was isolated from the harvested cells using a comercial kit (Rneasy Midi-Maxi kit, Qiagen, CA) and reverse-transcribed using oligo (dT) and Superscript II reverse transcriptase (MBI, fermentase).

First strand cDNAs were synthesized from 5 μg of total RNA from cell cultures using oligo $(dT)_{18}$ as primers in the presence of MLV reverse transcriptase (fermentase), for 1 h at 42 °C. PCR was carried out with primers chosen from the fragment of the human duodenal DMT1 (forward, 5′-GGT GTT GTG CTG GGA TGT TA-3′; reverse, 5′-AGT ACA TAT TGA TGG AAC AG-3′), Dcytb (forward, 5′-CCA TGG GCT CCG CCC TCT CTC CGG G-3′; reverse, 5′-TAG GGC GTT TCC ATT GGG GCC TGG T-3′), and ferritin gene. Ribosomal 18S was used to normalize the results, with primers from the human small intestine ribosomal 18S mRNA (forward, 5′- GAA CTA CGA CGG TAT CTG ATC GTC T-3′; reverse, 5′- CCG CCC GTC CCC GCC GGT TGC CTC T-3′). Determination of the linear phase of the PCR amplification was performed with Tfi-DNA polymerase (Access RT-PCR system, Promega) with pooled aliquots removed at 15, 20, 25, 30, 35, 40, 45, 50, and 55 cycles. Amplification of the human duodenal DMT1/Dcytb genes was performed for 32 and 35 cycles respectively, which consisted of denaturation (95 °C, 30 s), annealing (48 °C, 1 min), and extension (72 °C, 1 min); ribosomal 18S was amplified at 32 cycles under identical conditions in a different tube. Ribosomal 18S (350 bp) and human duodenal DMT1/Dcytb (350 bp) PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using a Gel-Pro analyzer version 3.0 (Media Cybernetics, Bethesda, MD).

Mitochondrial Enzyme Activities. These activities were evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazolium bromide) conversion on exposed cultures after an incubation period (*20*). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases coupled to the phosphorylation process (*21*), yielding a blue formazan product that can be measured spectrophotometrically. The amount of formazan produced is proportional to the number of viable cells. Then, 2 mL of acidic (0.1% HCl, v/v) isopropanol was added to redissolve insoluble

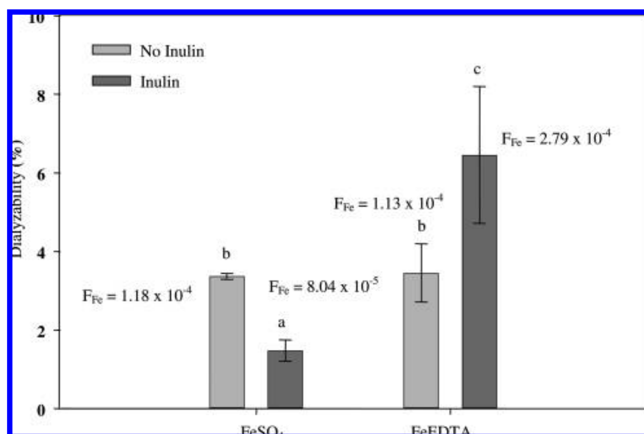

Figure 1. Effects of inulin on iron fluxes (F_{Fe}, cm/s) and dialyzability (%) up to 60 min. Values are expressed as mean ± standard deviation (n = 5). Experiments were conducted in triplicate on two different days. Bars within an iron compound with no letters in common are significantly (p < 0.05) different.

formazan, and the absorbance was measured at 570 nm with background subtraction at 690 nm. Control cells exposed to MEM but not to iron were analyzed with every assay.

Lysosomal Activity. This activity was investigated by using the neutral red (toluylene red; 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay (*22*). The medium was removed, and cells were washed twice with PBS. The uptake of toluylene red was measured using a commercial kit (Sigma, no. 7H092), and the absorbance was measured at 540 nm with background subtraction at 690 nm. Control cells exposed to MEM but not to iron were analyzed with every assay.

Statistical Analysis. Two independent trials with all treatments were conducted on separate days. Each treatment was performed in triplicate on both days. A one-factor analysis of variance (ANOVA) and the Tukey test (*23*) were applied to determine statistical differences. All experiments were conducted in triplicate on two different days. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 5.1 (Rockville, MD) was used for the statistical analysis.

RESULTS AND DISCUSSION

Total Fe concentrations in the solutions added to cell cultures were quantified by ICP-ES. Although all of the experiments were conducted on different days and freshly prepared Fe-containing working solutions were used, no signifficant ($p > 0.05$) differences in Fe concentrations were quantified. Iron concentrations in the solutions loaded in the upper chamber, containing $FeSO_4$ or FeEDTA solutions were 12.6 ± 0.9 and 12.3 ± 0.6 μM (values obtained with $n = 9$, triplicate analysis of three independent prepared Fe-containing working solutions), respectively. When challenged Fe-deficient Caco-2 cultures to these concentrations levels, Fe uptake from the diffused content in the bottom chamber was complete (*14*). Similar concentrations were successfully used in a previous study by our group, in which different factors [ascorbic acid, pH, and Fe(II) chelating agent] affecting the Fe uptake from both $FeSO_4$ and FeEDTA solutions (10 μM) were evaluated (*24*).

Effect of Inulin on Dialyzable Fe and Fluxes. Dialyzability percentages and fluxes of Fe (F_{Fe}) from the $FeSO_4$ and FeEDTA solutions, both with and without inulin, are shown in **Figure 1**. Dialyzability (%) of Fe was not statistically ($p > 0.05$) different among the two Fe compounds in the absence of inulin. However, after the addition of inulin, the dialyzability of Fe from the $FeSO_4$ solution was significantly ($p < 0.05$) decreased. These data correlated with the decreased F_{Fe} when inulin was added to the $FeSO_4$ solution. In contrast, the dialyzability of FeEDTA was increased ($p < 0.05$) 2-fold in the presence of inulin with respect to the value obtained with the same source of Fe without inulin. The observation was accompanied by a higher F_{Fe}in the in vitro system.

These data suggest a possible interaction between the dissociable Fe from $FeSO_4$ and inulin, forming a complex and decreasing the dialyzability. In contrast, the nondissociable Fe of the FeEDTA solution may not readily exchange with inulin. We have no direct evidence to explain the higher F_{Fe} from FeEDTA solution. Perhaps the high solubility of complexed Fe with EDTA solution interactswith inulin, produced by their high molecular size,causing the physical repulsion between these two compounds, which explain the increased dialyzability and F_{Fe} calculated for FeEDTA (I+) solution (**Figure 1**). The decreased dialyzability of $FeSO_4$ in the presence of inulin is in good agreement with several in vitro studies, which have reported mineral-binding capacities for nonfermented soluble dietary fibers (*25*). These findings could explain the previously reported decrease in dialyzability of Fe in a standard infant formula (cow's milk-based) supplemented with soluble dietary fiber fractions such as inulin (3% w/w in dry basis) (*11*). However, a recent study showed an increased soluble Fe content in the proximal, mid,and distal colon of young pigs fed a corn and soybean meal supplemented with 4% inulin (*13*). In addition, a greater dietary Fe absorption in rats fed fructooligosaccharides such as inulin (100 g/k in the diet) with respect to the control diet has been reported (*26*). Taken together, these studies do not rule out the possibility that inulin could influence the Fe diffusion toward the brush border membrane without impairing the uptake in the intestinal epithelia. Therefore, we investigated the ferritin formation response in Caco-2 cell cultures as an estimator of the Fe uptake.

Effect of Inulin on Ferritin Formation. It is widely accepted that ferritin formation by Caco-2 cells occurs in response to Fe that has been taken up by the cell (*27*). From a physiological point of view, mineral binding ability of nonfermented soluble dietary fibers could impair Fe uptake in the upper intestine by reducing the diffusion toward brush border membrane. As stated before, inulin exhibited a mineral binding ability to decrease Fe dialyzability from $FeSO_4$ solution (**Figure 1**). In contrast, we might expect that Fe uptake from FeEDTA solution will be increased due to higher iron concentrations in the lower chamber. To verify this hypothesis,several incubation times (10, 20, 40, or 120 min) for intestinal step were compared to estimate Fe uptake from $FeSO_4$ and FeEDTA solutions with or without added inulin (**Figure 2**).

Marked differences in the cellular response for ferritin formation between cultures exposed to FeEDTA and $FeSO_4$ can be observed. The higher ferritin concentration in cell cultures exposed to FeEDTA reflects a higher Fe uptake relative to $FeSO_4$ without added inulin solution. Indeed, at earlier intestinal incubation times (10 and 20 min) Fe uptake in cultures exposed to FeEDTA was 2-fold higher than that in cultures exposed to $FeSO_4$. These data support our findings on F_{Fe} from the different sources used (**Figure 1**). However, these differences in ferritin formation were lower in cultures subjected to incubation of 40 min, and after an incubation period of 120 min, the cellular response in ferritin formation was closer for both compounds. The ferritin formation response observed from both sources of Fe without inulin is in agreement with the ferritin formation response from $FeSO_4$ and NaFeEDTA solutions at pH 7.2 previously reported by our group (*24*). Furthermore, the data also suggest that inulin did not impair Fe absorption processes

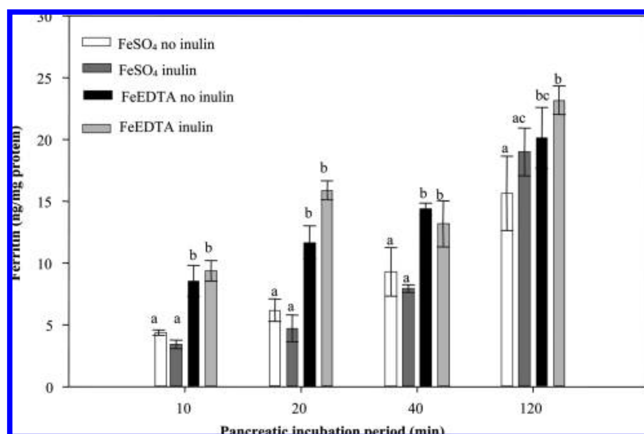

Figure 2. Caco-2 cell ferritin formation in cultures exposed for several intestinal incubation periods (10, 20, 40, and 120 min). Values are expressed as mean ± standard deviation ($n = 6$). Experiments were conducted in triplicate on two different days. The cells grown in MEM alone had baseline ferritin of 4.3 ± 0.9 ng/mg of protein. Bars within an incubation time and iron compound with no letters in common are significantly ($p < 0.05$) different.

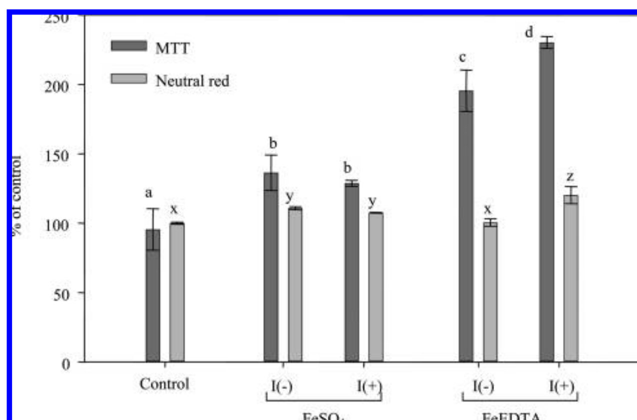

Figure 3. MTT conversion and neutral red uptake by Caco-2 cells. Values are expressed as mean ± standard deviation ($n = 5$). Experiments were conducted in triplicate on two different days. Bars within an iron compound with no letters in common are significantly ($p < 0.05$) different (dark gray bars compared to dark gray bars, and light gray bars compared to light gray bars).

at the brush border membrane and that the effect could occur via modification of the kinetics of diffusion of iron across the membrane as evidenced from the different calculated F_{Fe} (**Figure 1**). This observation that inulin does not impair Fe uptake at the brush border membrane is in agreement with a study using nonanemic humans, which reported that nondigestible oligosaccharides do not interfere with nonheme-iron absorption (*12*). It also agrees with the previously reported data from animal studies mentioned above (*13, 26*).

To explain the higher ferritin formation response from FeEDTA solution than from $FeSO_4$, both without added inulin, we should consider many previous results. It is well-known that Fe uptake at the intestinal level is a saturable process (*16, 28*) that involves the divalent metal transporter-1 (DMT1) (*17*). Arredondo et al. (*28*) showed that high levels of Fe present in the apical surface of intestinal epithelia caused the relocalization of the DMT1 transporter from the brush border membrane, thus reducing Fe uptake (*17*). Another important aspect of Fe metabolism, to be taken into account, is that the absorption of Fe and iron regulatory protein (IRP) activities are regulated by the intracellular pool of Fe (*28*). We must bear in mind that ferritin expression is translationally regulated by the cellular levels of Fe (*29, 30*). Fe homeostasis at the intestinal level is unique, because the cells must regulate their intracellular Fe levels and transcellular transport. In this sense, previous studies reported that changes in the ferritin concentrations were restricted to a limited range of cellular Fe concentrations, showing an exponential distribution as a function of the intracellular Fe concentration (*14, 28*). From the latter studies, a rapid initial ferritin synthesis would be expected, followed by a plateau with increasing intracellular Fe concentrations. This fact would imply a limited ferritin formation response in in vitro systems where Fe trafficking to the basolateral side is not allowed. Thus, from all of the observations mentioned above, we might speculate that a faster intracellular flux of Fe from FeEDTA solutions might be responsible for the higher ferritin formation response detected.

Cellular Metabolic Responses. Changes in mitochondrial enzyme and lysosomal activities could be a fairly sensitive biomarker of the changes of cellular metabolism in response to Fe uptake. In this way, an increased MTT conversion has been previously reported in HuTu-80 cells transfected with Dcytb, which constitutes an iron-regulated ferric reductase with a heme containing b-type cytochrome associated with DMT1 and the absorption of dietary Fe (*31*). In addition, DMT1 relocalization from brush border membrane has been associated with a late endosomal/lysosomal compartment (*17*). Thus, the earlier cellular metabolic responses, mitochondrial and lysosomal activities, obtained by using MTT and neutral red uptake assays, respectively, were monitored in Caco-2 cultures exposed to an incubation time of 2 h (**Figure 3**).

When the mitochondrial enzyme activities were monitored, no significant differences in MTT conversion were observed between cell cultures exposed to $FeSO_4$ solution, with or without added inulin. In contrast, cultures exposed to FeEDTA exhibited a significantly ($p < 0.05$) higher MTT conversion with respect to those exposed to $FeSO_4$. These results indicate a markedly earlier cellular metabolic response, which would reflect a more effective Fe internalization into cell from FeEDTA than $FeSO_4$ solution. The latter observation would explain higher ($p < 0.05$) ferritin values when shorter incubation times were used (**Figure 2**).

As for the influence of inulin present in the media on cellular metabolism, the similar MTT conversion values estimated when cultures were challenged by $FeSO_4$ were in accordance with the similar ferritin formation levels detected for each source of Fe. In contrast, higher MTT conversion in cultures incubated with FeEDTA solutions was highest from FeEDTA (I+) solution. This observation might be reflecting higher Fe concentrations at the apical surface of Caco-2 cultures caused by the increased F_{Fe} in the presence of inulin. However, the increased MTT conversion was not proportional to the higher Fe flux from FeEDTA solution (2-fold higher), which is explained by the saturable nature of the mechanism proposed for Fe uptake in mammalian cells (*16*).

Another aspect that should be taken into account, and has been previously reported, is the trafficking of DMT1(+IRE) transporter protein away from the apical membrane of intestinal epithelial cells (*17*). The latter authors demonstrated that this fact was caused by high levels of Fe (100 μM) presented to the apical membrane and can occur within a few hours (4 h). It was suggested that this was associated with an increase in transporter levels within an endosomal/lysosomal compartment (*17*), an event that has been also shown to occur in the endosomal fraction of Caco-2 cells (*32*). Thus, we monitored

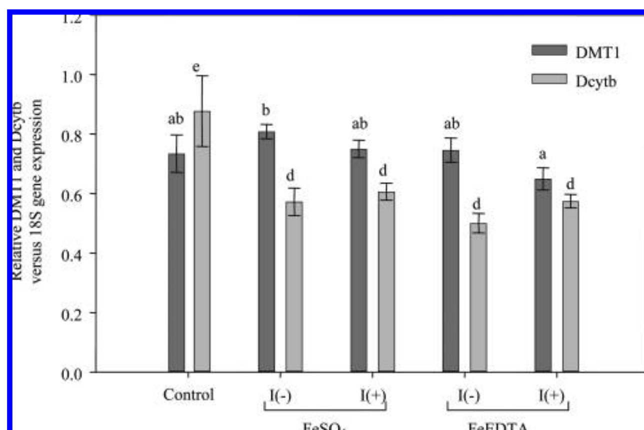

Figure 4. Caco-2 DMT1 and Dcytb relative gene expression versus 18S conversion. Values are expressed as mean ± standard deviation ($n = 5$). Experiments were conducted in triplicate on two different days. Bars within an iron compound with no letters in common are significantly ($p < 0.05$) different (dark gray bars compared to dark gray bars, and light gray bars compared to light gray bars).

the neutral red uptake as an estimator of the lysosomal activity (*22*) (**Figure 3**). Regardless of whether inulin is added or not, cultures exposed to $FeSO_4$ solutions showed similar but slightly significant ($p < 0.05$) increases in neutral red uptake with respect to the controls. In contrast, for FeEDTA solutions a significant ($p < 0.05$) increase in neutral red uptake was detected only in the presence of inulin. This observation would be in accordance with the DMT1 redistribution effect reported by Johnson et al. (*17*). It must be pointed out that this solution exhibited the highest F_{Fe} (**Figure 1**).

From all of these scenarios in regard to the cellular metabolic response, the data might also suggest that for both Fe sources assayed, the mitochondrial activities are sensing differences in Fe uptake, which are translated in different ferritin levels. In this sense, we can speculate that variations observed in MTT conversion might be caused by an increased iron−sulfur cluster biosynthesis inside the mitochondria (*33*). The latter is a key process in regulating the enzymatic activity of mitochondrial aconitases and controlling the iron regulatory protein IRP-1 activity in mammalian cells (*33*).

mRNA Expression of Fe Transporters. Caco-2 relative DMT1 and Dcytb mRNA expression changes are shown in **Figure 4**. In the present study, mRNA expression of Fe transporters was monitored after an incubation period of 24 h in cultures exposed to gastrointestinal digests for 2 h. PCR analysis revealed that there was no change in DMT1 mRNA expression with respect to the control. The higher ($p < 0.05$) DMT1 mRNA expression in cultures exposed to $FeSO_4$ (I−) than FeEDTA (I+) solution is in accordance with the low Fe uptake quantified in cultures exposed to this source of Fe (**Figure 2**). Otherwise, the addition of inulin caused no significant ($p > 0.05$) differences in DMT1 mRNA expression relative to nonadded Fe-containing solutions. It is important to mention that, although higher Fe uptake values have been quantified for FeEDTA solutions, there were no statistically significant ($p < 0.05$) differences for DMT1 mRNA expression relative to the controls. The data presented for FeEDTA may suggest a nondirect relationship between Fe uptake and DMT1 mRNA expression. The latter effect is in accordance with those data reported previously by other authors (*17, 34*), which showed a rapid regulation of DMT1 protein but not mRNA expression in response to nonheme Fe uptake in Caco-2 cells. However, Johnson et al. (*17*) have reported that high levels of Fe present in the apical surface of Caco-2 cells caused a reversive relocalization into cells of the DMT1 transporter. Although, if this took place, in our study differences in F_{Fe} calculated for FeEDTA (I+) and FeEDTA (I−) would cause higher Fe concentration from FeEDTA (I+). Thus, when previous data (*17*) are taken into consideration, a lower Fe uptake should be observed in cultures exposed to FeEDTA (I+). In contrast, in the present study the highest mean value estimated for Fe uptake from FeEDTA (I+) solution suggests that DMT1 has not been removed from the apical membrane, although a faster Fe diffusion toward brush border was calculated (**Figure 1**). The lower Fe concentration used in our study (12 vs 100 μM) may be responsible for differences in DMT1 mRNA expression between our results and those reported by Johnson et al. (*17*).

The analysis of relative Dcytb mRNA expression in cultures exposed to $FeSO_4$ and FeEDTA solutions, with or without added inulin, revealed a similar ($p > 0.05$) down-regulation in all cases. It is interesting to mention that an increased MTT conversion has been reported in HuTu-80 cells transfected with Dcytb (*31*). However, in the present study the higher MTT conversion observed in cultures exposed to FeEDTA solutions rather than $FeSO_4$ was not reflected in the overexpression of Dcytb mRNA. A recent paper (*35*) has described a new isoform of ascorbate-reducible cytochrome b561, which could be also expressed in the intestine and might be a candidate for the ferric reductase facilitating Nramp2/DMT1-mediated transfer of Fe into the cytoplasm. However, further studies are needed to demonstrate this activity in Caco-2 cells.

In summary, dietary inulin may exhibit a mineral-binding effect on $FeSO_4$ solution, thereby lowering the diffusion of Fe toward the brush border membrane. In contrast, inulin increased the diffusion of nondissociable Fe such as FeEDTA. From a physiological perspective, the decreased F_{Fe} from dissociable Fe sources in the brush border membrane might be reflected in an impaired Fe absorption in the upper intestine, the main site of absorption. However, it appears that Fe absorption from nondissociable dietary supplements would be improved in the upper intestine by the addition of soluble fibers such as inulin. Otherwise, inulin does not influence the Fe uptake at the intestinal epithelia, as concluded from the closer ($p > 0.05$) cellular responses in ferritin formation and cell metabolism measurements for each source of Fe. The potential effects of soluble fiber presented should be considered in the design of food fortification strategies; however, it is important to confirm these results with in vivo studies. Further studies along this line would be helpful to design rational guidelines for more efficient Fe supplementation programs. The data presented in the present study in conjunction with human trials could be a useful tool to attain a better knowledge of the mechanism of the soluble fibers that could influence Fe absorption in vivo.

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