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ARTICLE

Inhibitory Effect of Calcium on Non-heme Iron Absorption May Be Related to Translocation of DMT-1 at the Apical Membrane of Enterocytes

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Many studies show that calcium reduces iron absorption from single meals, but the underlying mechanism is not known. We tested the hypothesis that calcium alters the expression and/or functionality of iron transport proteins. Differentiated Caco-2 cells were treated with ferric ammonium citrate and calcium chloride, and ferritin, DMT-1, and ferroportin were quantified in whole-cell lysate and cell-membrane fractions. Calcium attenuated the iron-induced increase in cell ferritin levels in a dose-dependent manner; a significant decrease was seen at calcium concentrations of 1.25 and 2.5 mM but was only evident after a 16−24 h incubation period. Calcium and iron treatments decreased DMT-1 protein in Caco-2 cell membranes, although total DMT-1 in whole cell lysates was unchanged by either iron or calcium. No change was seen in ferroportin expression. Our data suggest that calcium reduces iron bioavailability by decreasing DMT-1 expression at the apical cell membrane, thereby downregulating iron transport into the cell.

KEYWORDS: Iron absorption; calcium; interactions; DMT-1; ferritin; ferroportin; Caco-2 cells

INTRODUCTION

Calcium is a well-documented inhibitor of iron absorption from single meals (*1*−*5*) and initially was thought to exert its effect in the lumen of the gastrointestinal tract (*4*). However, results of more recent studies suggest that inhibition may occur at both the apical and basolateral membranes of the enterocytes (*6*, *7*). Potential mechanisms of action include alterations in the balance of intraluminal ligands, changes in gastrointestinal transit time, decreased iron uptake by receptor competition, and/or interference with the transport of iron through the mucosal cells (*3*). Although calcium and iron have to be consumed together for an effect on iron absorption to be seen (*8*, *9*), only small quantities of calcium are needed to cause an inhibition; therefore, it is unlikely that the effect is a simple direct competition between calcium and iron ions for transporter proteins at the apical membrane of the enterocytes (*7*, *10*).

In contrast to the results from single meals, there is consistent evidence from longitudinal studies that calcium does not interfere with long-term iron absorption from whole diets (*11*) or significantly alter iron status in men and women (*12*−*15*). Although several explanations have been proposed, the most attractive hypothesis is that homeostatic mechanisms have evolved in mammals that are designed to cope with fluctuations in the supply of dietary iron and that these are manifest through changes in the efficiency of absorption (*12*, *14*). In support of this homeostatic adaptation hypothesis, studies have shown that a high calcium intake did not affect either iron absorption or status in piglets consuming a high calcium diet. It was suggested by the authors that the high calcium intake might have induced upregulation of iron transfer across the epithelial membrane (possibly via ferroportin) (*16*).

The objective of our present study was to test the hypothesis that calcium alters the expression and/or functionality of the iron transport proteins, DMT-1 and ferroportin, in a manner that would result in a lower intestinal iron transport. Caco-2 cells were used as a model system in which to explore the possible modes of action of calcium. A series of experiments were performed to measure the time course and dose−response effect of calcium on iron absorption, using ferritin as a surrogate measure of iron uptake into cells (*17*). Experiments were also undertaken to examine the effect of different iron and/or calcium treatments on subcellular localization of DMT-1 and ferroportin.

MATERIALS AND METHODS

Cell Culture. Caco-2 cells (HTB37) were purchased from American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% (v/v) fetal calf serum. Medium was changed every 3 days. Cells were seeded at a density of 3×10^4 cell/cm^2 and used for experiments 12 days post-seeding. At 1 day prior to experiments, cells were placed in serum-free medium.

To determine the temporal effects of calcium on iron absorption, cells were incubated for up to 24 h in serum-free medium supplemented with either 2.5 mM calcium chloride ($CaCl_2$), 30 μM ferric ammonium citrate,

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or 2.5 mM $CaCl_2$ plus 30 μM ferric ammonium citrate. A dose−response experiment was performed to determine the concentrations of calcium required to exert an inhibitory effect on iron absorption. Caco-2 cells were incubated in serum-free medium supplemented with 30 μM ferric ammonium citrate and 0, 0.156, 0.313, 0.625, 1.25, and 2.5 mM $CaCl_2$.

Cell Ferritin Concentration. At the end of each experiment, the incubation medium was aspirated and the cells were washed twice with piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (130 mM NaCl, 5 mM KCl, and 5 mM PIPES at pH 6.7). A total of 2 mL of Milli-Q water was then added, and the monolayers were scraped using an inverted 200 μL pipet tip. The resulting lysate was suspended evenly, sonicated, and stored at −20 °C for analysis. The total protein content of the cell lysates was analyzed using the BCA protein assay kit (Pierce, U.K.) according to the protocol of the manufacturer.

The total ferritin content of the cell lysates was analyzed using the spectroferritin enzyme-linked immunosorbent assay (ELISA) kit (Ramco, Stafford, TX) according to the protocol of the manufacturer. A standard curve was created using 0, 6, 20, 60, 150, and 200 ng of standard/mL, and 30 μL of the unknown samples was loaded into the 96-well plate in triplicate.

Subcellular Fractionation. To determine the effect of calcium on subcellular localization of DMT-1 and ferroportin, Caco-2 cells were incubated in serum-free media containing either 30 μM ferric ammonium citrate, 2.5 mM $CaCl_2$, or 30 μM ferric ammonium citrate plus 2.5 mM $CaCl_2$. After 4 h, the medium was removed and the cells were rinsed twice with phosphate-buffered saline (PBS) before being scraped gently from the flask. Cells were pelleted by centrifugation, and each pellet was resuspended in 1 mL of homogenization buffer [50 mM mannitol and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.2−7.4] and homogenized using a T25 UltraTurax (Janke and Kunkel, Germany) with a 0.5 mm probe attached on three-quarters speed for 2× 30 s, with a 10 s pause between each interval. After homogenization, a 250 μL aliquot was taken as a whole-cell lysate sample and the remainder was centrifuged at 1500*g* for 15 min. The supernatant was then removed and centrifuged at 15000*g* for 30 min. The plasma-membrane-rich pellet was resuspended in PBS. Protein pellets were stored at −20 °C until used for western blotting studies.

Western Blot Analysis Protocol. The cell protein lysates from the ferritin protein time course were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions. After the total cell protein was quantified using the BCA assay (Pierce, U.K.), the samples were loaded onto a 10% Bis-Tris gel, along with 25% sample loading buffer and 10% 0.5 M D,L-dithiothreitol as a reducing agent (Invitrogen, U.K.). Where necessary, 2 μL of biotinylated marker (Sigma, U.K.) was also added, and the gel was run for 55 min at 200 V in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (Invitrogen, U.K.). After separation, the protein was transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) at 30 V for 1 h before being blocked with protein-free blocking buffer (Pierce, U.K.) for 1 h at room temperature. The membrane was then incubated in protein-free blocking buffer (Pierce, UK) with the primary antibody for 1 h at room temperature, with rocking. After this, the membrane was washed briefly 3 times in TBS-Tween solution before being incubated with the secondary antibody, again in protein-free blocking buffer (Pierce, U.K.) for 1 h at room temperature, with rocking. To this second incubation, if biotinylated markers were used, 0.5 μL of ExtrAvidin−peroxidase conjugate (Sigma, U.K.) was added to attach to the biotinylated marker. After the second incubation, to prepare the membrane for chemiluminescence, the membrane was again washed briefly 3 times in TBS-Tween before a quick rinse in Milli-Q water. The bands were visualized using a SuperSignal West Pico chemiluminescent substrate kit (Pierce, U.K.) according to the instructions of the manufacturer and a Fluor MultiImager (BioRad, U.K.) along with Quantity One (BioRad, U.K.) band analysis software.

Statistical Analysis. All statistical analyses were performed with SPSS version 16.0.0 software (SPSS, Inc., Chicago, IL), using the analysis of variation (ANOVA) general linear model with Tukey's post hoc analysis. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

Calcium Inhibits Iron-Induced Ferritin Formation. Incubation with iron (30 μM) significantly increased cell ferritin levels in a time-dependent manner (**Figure 1a**). The addition of calcium (2.5 mM) attenuated this response; calcium reduced ferritin formation at both the 16 and 24 h time points by approximately half ($p < 0.001$ and $p < 0.05$, respectively). There was no significant effect of calcium alone on cell ferritin formation over the 24 h incubation period.

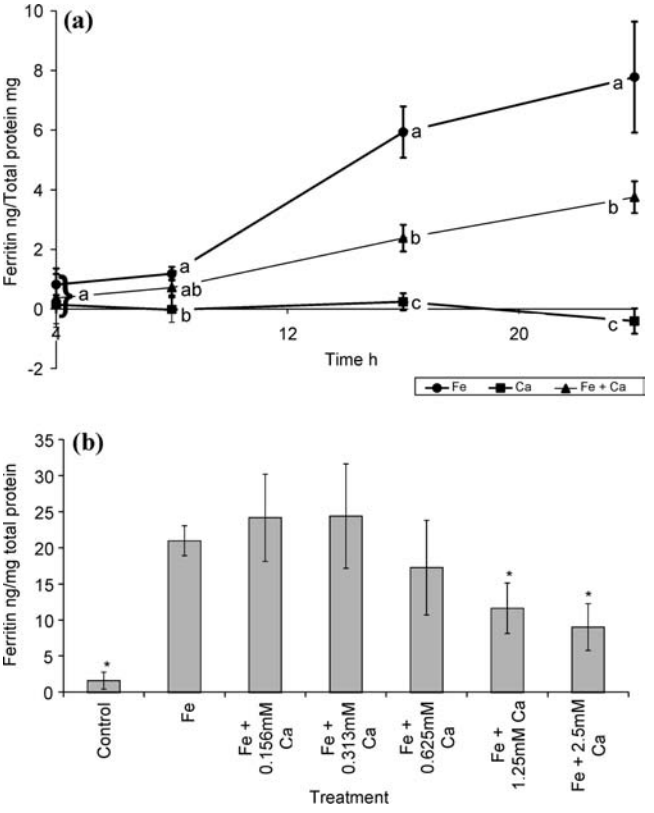

Figure 1. (a) Effect of calcium on ferritin formation over 24 h in cells exposed for 2 h to 30 μM ferric ammonium citrate (●), 2.5 mM calcium chloride (■) or calcium chloride plus ferric ammonium citrate in combination (▲). Error bars indicate mean values ± standard deviation (SD) (n = 6). (a, b, and c) Significant differences ($p < 0.05$) between groups at each time point. (b) Dose−response effect of calcium on ferritin formation for 24 h after 2 h of exposure to 30 μM ferric ammonium citrate. Error bars indicate mean ± SD (n = 6). (*) Values that are significantly different to the Fe positive control ($p < 0.05$).

To determine the dynamics of the inhibitory effects of calcium on iron-induced ferritin formation, studies were carried out using a range of calcium concentrations (**Figure 1b**). Inhibition of ferritin formation was only observed at the highest calcium concentrations employed (1.25 and 2.5 mM; $p < 0.05$ and $p < 0.005$, respectively).

Effect of Calcium on Iron Transporter Expression. The data presented in panels **a** and **b** of **Figure 1** suggest that calcium may compete with iron for uptake into the cell. However, we have shown previously that even a 100-fold excess of calcium does not inhibit iron transport through DMT-1 (*18*). We therefore examined the possibility that calcium might alter Caco-2 cell iron transporter expression. Cells were exposed to iron, calcium, or calcium plus iron for 4 h and processed to produce a whole-cell lysate and a crude plasma-membrane fraction. DMT-1 levels determined in the subsequent Western blots were normalized to the structural protein villin.

There was no effect of any of the metal treatments on total cellular DMT-1 expression determined in whole-cell lysates (**Figure 2a**). However, in the cell-membrane fraction samples, DMT-1 levels were significant decreased following exposure to

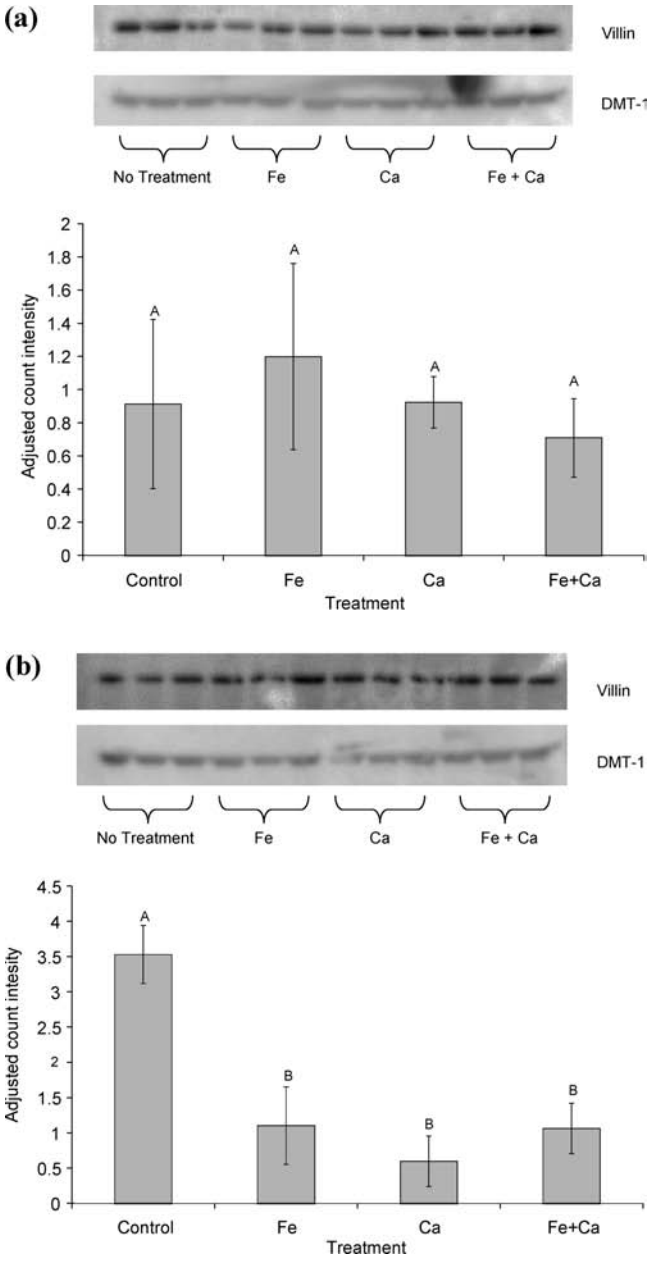

Figure 2. (a) Western blot showing the effect of different 30 μM ferric ammonium citrate and/or 2.5 mM calcium chloride treatments on whole-cell lysate DMT-1 levels. Error bars indicate mean values ± SD (n = 3). (A) There were no significant differences between the treatment groups. (b) Western blot showing the effect of different 30 μM ferric ammonium citrate and/or 2.5 mM calcium chloride treatments on cell-membrane fraction DMT-1 levels. Error bars indicate mean values ± SD (n = 3). (A and B) Significant differences ($p < 0.05$) between treatment groups.

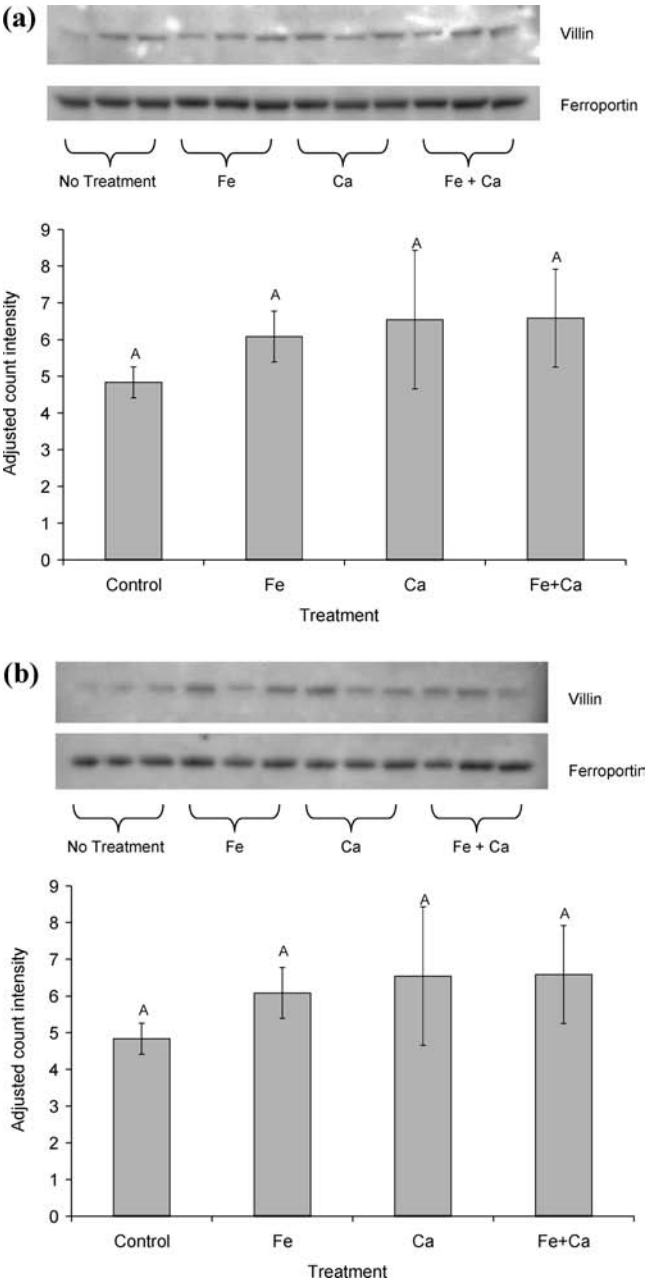

Figure 3. (a) Western blot showing the effect of different 30 μM ferric ammonium citrate and/or 2.5 mM calcium chloride treatments on whole-cell lysate ferroportin levels. Error bars indicate mean values ± SD (n = 3). (A) There were no significant differences between the treatment groups. (b) Western blot showing the effect of different 30 μM ferric ammonium citrate and/or 2.5 mM calcium chloride treatments on cell-membrane fraction ferroportin levels. Error bars indicate mean values ± SD (n = 3). (A) Significant differences ($p < 0.05$) between treatment groups.

iron, calcium alone, or iron plus calcium ($p < 0.05$, < 0.005, and < 0.05, respectively) (**Figure 2b**).

The effects of calcium and iron on iron transporter expression were restricted to DMT-1; western blots probed with ferroportin antibodies revealed no changes in ferroportin levels following exposure to iron, calcium, or iron plus calcium in whole-cell lysates ($p = 0.72$, 0.42, and 0.40) or in cell-membrane fractions ($p = 1.00$, 0.90, and 0.75) compared to untreated controls (panels **a** and **b** of **Figure 3**).

DISCUSSION

Calcium has repeatedly been shown to be an inhibitor of nonheme iron absorption, but the mechanisms underlying these observations are not fully understood. In the present study, we have demonstrated that calcium significantly decreased iron-induced ferritin formation (a surrogate marker of iron absorption) in Caco-2 cells. The calcium effect was dose-dependent with a threshold concentration of 1.25 mM and was only evident after a 16−24 h incubation period; shorter time treatments had no effect on ferritin formation. The inhibitory effect of calcium on iron absorption supports data from single-meal studies in human volunteers (*18*−*20*). Recent *in vitro* Caco-2 cell data (*21*) also show a significant inhibitory effect of calcium on cell ferritin; however, this contrasts with a previous study, where

the addition of different calcium salts to human milk, including calcium glycerophosphate and calcium gluconate, had no effect on ferritin levels in Caco-2 cells (*22*).

Because calcium has been reported to influence iron transport across both apical and basolateral membranes of enterocytes, we investigated the effects of calcium on the expression of the iron transporters DMT-1 and ferroportin. In whole-cell lysates, exposure to calcium, iron, or calcium plus iron did not alter the total cellular protein levels of either DMT-1 or ferroportin. However, all treatments resulted in a significant decrease in DMT-1 levels in the Caco-2 cell-membrane fraction compared to untreated control cells, indicating that DMT-1 was removed from the cell surface to reduce subsequent iron uptake. In contrast, there was no effect of any treatment on ferroportin levels in the same membrane samples. This contrasts with a study in rats, in which calcium loading did not influence iron uptake into intestinal epithelial cells, whereas transfer across the basolateral membrane of the cells was reduced (*7*). Conversely, results from a different study using electron microscopy and quantitative measurements of iron revealed that calcium reduces the uptake of iron at the apical membrane of the microvilli (*6*). Our data suggest that calcium exerts its inhibitory effect on intestinal iron absorption by altering the cellular localization of DMT-1. While exposure to iron has previously been shown to cause rapid internalization of DMT-1 protein from the apical membrane of rat intestinal enterocytes (*23*−*25*) and Caco-2 cells (*26*), this is the first report of changes in DMT-1 subcellular localization in response to calcium. While no significant changes were seen in the levels of ferroportin protein following exposure to metals, we cannot rule out an effect of calcium on the function of this transporter, which might in turn explain the inhibitory effects of calcium on iron efflux observed in earlier studies (*7*).

In summary, the data from the present study support the hypothesis that calcium exerts an inhibitory effect at the apical cell membrane by decreasing DMT-1 expression at the cell membrane and thereby downregulating iron transport into the cell.

LITERATURE CITED

(1) Dunn, J. A. The effects of dietary calcium salts and fat on iron absorption in the rat. *S. Afr. J. Med. Sci.* **1968**, *33*, 65–70.

(2) Freeman, S.; Ivy, A. C. The influence of antacids upon iron retention by the anemic rat. *Am. J. Physiol.* **1942**, *137*, 706–709.

(3) Hallberg, L.; Brune, M.; Erlandsson, M.; Sandberg, A. S.; Rossander-Hulten, L. Calcium: Effect of different amounts on nonheme− and heme−iron absorption in humans. *Am. J. Clin. Nutr.* **1991**, *53*, 112–119.

(4) Kletzien, S. W. Iron metabolism I. The role of calcium in iron assimilation. *J. Nutr.* **1940**, *19*, 187–197.

(5) Preziosi, P.; Hercberg, S.; Galan, P.; Devanlay, M.; Cherouvrier, F.; Dupin, H. Iron status of a healthy French population: Factors determining biochemical markers. *Ann. Nutr. Metab.* **1994**, *38*, 192–202.

(6) Barton, J. C.; Conrad, M. E.; Parmley, R. T. Calcium inhibition of inorganic iron absorption in rats. *Gastroenterology* **1983**, *84*, 90–101.

(7) Wienk, K. J.; Marx, J. J.; Lemmens, A. G.; Brink, E. J.; Van Der Meer, R.; Beynen, A. C. Mechanism underlying the inhibitory effect of high calcium carbonate intake on iron bioavailability from ferrous sulphate in anaemic rats. *Br. J. Nutr.* **1996**, *75*, 109–120.

(8) Gleerup, A; Rossander-Hulten, L.; Hallberg, L. Duration of the inhibitory effect of calcium on non-haem iron absorption in man. *Eur. J. Clin. Nutr.* **1993**, *47*, 875–879.

(9) Gleerup, A.; Rossander-Hulthen, L.; Gramatkovski, E.; Hallberg, L. Iron absorption from the whole diet: Comparison of the effect of two different distributions of daily calcium intake. *Am. J. Clin. Nutr.* **1995**, *61*, 97–104.

(10) Hallberg, L.; Rossander-Hulten, L.; Brune, M.; Gleerup, A. Calcium and iron absorption: Mechanism of action and nutritional importance. *Eur. J. Clin. Nutr.* **1992**, *46*, 317–327.

(11) Reddy, M. B.; Cook, J. D. Effect of calcium intake on nonheme−iron absorption from a complete diet. *Am. J. Clin. Nutr.* **1997**, *65*, 1820–1825.

(12) Minihane, A. M.; Fairweather-Tait, S. J. Effect of calcium supplementation on daily nonheme−iron absorption and long-term iron status. *Am. J. Clin. Nutr.* **1998**, *68*, 96–102.

(13) Snedeker, S. M.; Smith, S. A.; Greger, J. L. Effect of dietary calcium and phosphorus levels on the utilization of iron, copper, and zinc by adult males. *J. Nutr.* **1982**, *112*, 136–143.

(14) Bendich, A. Calcium supplementation and iron status of females. *Nutrition* **2001**, *17*, 46–51.

(15) Sokoll, L. J.; Dawson-Hughes, B. Calcium supplementation and plasma ferritin concentrations in premenopausal women. *Am. J. Clin. Nutr.* **1992**, *56*, 1045–1048.

(16) Wauben, I. P.; Atkinson, S. A. Calcium does not inhibit iron absorption or alter iron status in infant piglets adapted to a high calcium diet. *J. Nutr.* **1999**, *129*, 707–711.

(17) Glahn, R. P.; Lee, O. A.; Yeung, A.; Goldman, M. I.; Miller, D. D. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* **1998**, *128*, 1555–1561.

(18) Tandy, S.; Williams, M.; Leggett, A.; Lopez-Jimenez, M.; Dedes, M.; Ramesh, B.; Srai, S. K.; Sharp, P. Nramp2 expression is associated with pH-dependent iron uptake across the apical membrane of human intestinal Caco-2 cells. *J. Biol. Chem.* **2000**, *275*, 1023–1029.

(19) Cook, J. D.; Dassenko, S. A.; Whittaker, P. Calcium supplementation: Effect on iron absorption. *Am. J. Clin. Nutr.* **1991**, *53*, 106–111.

(20) Monsen, E. R.; Cook, J. D. Food iron absorption in human subjects. IV. The effects of calcium and phosphate salts on the absorption of nonheme iron. *Am. J. Clin. Nutr.* **1976**, *29*, 1142–1148.

(21) Zhu, L.; Glahn, R. P.; Nelson, D.; Miller, D. D. Comparing soluble ferric pyrophosphate to common iron salts and chelates as sources of bioavailable iron in a Caco-2 cell culture model. *J. Agric. Food Chem.* **2009**, *57*, 5014–5019.

(22) Etcheverry, P.; Wallingford, J. C.; Mille, D. D.; Glahn, R. P. The effect of calcium salts, ascorbic acid and peptic pH on calcium, zinc and iron bioavailabilities from fortified human milk using an in vitro digestion/Caco-2 cell model. *Int. J. Vitam. Nutr. Res.* **2005**, *75*, 171–178.

(23) Yeh, K. Y.; Yeh, M.; Watkins, J. A.; Rodriguez-Paris, J.; Glass, J. Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2000**, *279*, G1070–G1079.

(24) Oates, P. S.; Trinder, D.; Morgan, E. H. Gastrointestinal function, divalent metal transporter-1 expression and intestinal iron absorption. *Pflugers Arch.* **2000**, *440*, 496–502.

(25) Frazer, D. M.; Wilkins, S. J.; Becker, E. M.; Murphy, T. L.; Vulpe, C. D.; McKie, A. T.; Anderson, G. J. A rapid decrease in the expression of DMT1 and Dcytb but not Ireg1 or hephaestin explains the mucosal block phenomenon of iron absorption. *Gut* **2003**, *52*, 340–346.

(26) Johnson, D. M.; Yamaji, S.; Tennant, J.; Srai, S. K.; Sharp, P. A. Regulation of divalent metal expression in human intestinal epithelial cells following exposure to non-haem iron. *FEBS Lett.* **2005**, *579*, 1923–1929.

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