Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells

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Abstract Trace metals influence the absorption of each other from the diet and it has been suggested that the divalent metal transporter (DMT1) represents a common uptake pathway for these important micronutrients. However, compelling evidence from our laboratory suggests that DMT1 is predominantly an iron transporter, with lower affinity for other metals. Several studies have shown that increasing dietary iron downregulates DMT1. Interestingly, our current data indicate that zinc upregulates DMT1 protein and mRNA expression and also pH-dependent iron uptake. Transepithelial flux of iron was also increased and was associated with a rise in IREG1 mRNA expression.

Key words: Divalent metal transporter; IREG1; Iron transport; Caco-2 cell; Zinc

1. Introduction

Zinc and iron are essential dietary trace metals required for a number of physiological and biochemical functions in the body. It is well recognised that there are interactions between these metals in the intestinal lumen, affecting their dietary bioavailability. For example, high concentrations of iron can inhibit zinc absorption [1–6] and likewise high zinc content can reduce iron absorption [7]. However, relatively little is known regarding the effects of prolonged high dietary metal intake on the transport machinery required for the absorption of these essential micronutrients. Recent cloning strategies have greatly improved our knowledge concerning the mechanisms by which iron crosses the intestinal barrier from the diet into the blood. At the apical membrane, the divalent metal transporter DMT1 – previously DCT1/Nramp2 [8,9] – takes iron into enterocytes via a pH-dependent mechanism [8,10]. Basolateral efflux of iron occurs via IREG1 [11] – also known as ferroportin-1 and MTP1 [12,13]. The situation for zinc, however, is not so clear. DMT1 was proposed to act as a common entry pathway for a number of divalent metals. However, evidence from our laboratory [10] and others [14,15] suggests that zinc is not a major substrate for DMT1, and thus the apical membrane transport system for zinc remains elusive. At the basolateral surface of intestinal enterocytes, efflux of absorbed zinc is thought to be mediated by ZnT1 [16]. The purpose of our current study, using the Caco-2 cell model of human small intestinal enterocytes, was to gain a better understanding of the interaction between zinc and iron in the intestinal mucosa, in terms of uptake of these essential nutrients, and in turn to determine how high intakes of these metals might influence the transporters involved in transepithelial iron transfer.

2. Materials and methods

2.1. Cell culture

Stock cultures of Caco-2 TC7 cells were maintained in 25 cm^2 plastic flasks and cultured in a 95% air/5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium, supplemented with 20% heat-inactivated foetal bovine serum. All experiments were carried out on cells at passage numbers 30–40. For experiments, cells were seeded at a density of 1×10^4 cells/cm^2 onto Transwell inserts (Costar UK, Buckinghamshire, UK) to measure metal ion transport, or 75 cm^2 tissue culture flasks for isolation of plasma membrane protein and total RNA. In some experiments, cells were incubated with 50 or 100 μM iron or zinc for the final 24 h of the culture period to determine the effects of high levels of these divalent metals on DMT1 and IREG1 function and expression.

2.2. Metal uptake by Caco-2 TC7 cell monolayers

The measurement of metal ion uptake by Caco-2 TC7 cells has been described previously [10]. Briefly, transepithelial pH gradients were produced using *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffered salt solution (HBSS, pH 7.5; 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, 0.2% bovine serum albumin) in the basolateral chamber, with either HBSS or MBSS (pH 5.5, substituting 2-(*N*-morpholino)ethanesulphonic acid for HEPES) added to the apical chamber. Uptake was initiated by the addition of either 10 μM Fe^{2+} complexed with 1 mM ascorbic acid (prepared fresh prior to the start of each experiment), or 10 μM Zn^{2+} and 37 kBq/ml $^{55}FeCl_3$ or $^{65}ZnCl_2$ respectively, to the apical chamber and terminated after 60 min. Aliquots of the Caco-2 cells (solubilised in 0.2 M NaOH) and the basolateral incubation medium were taken at the end of each experiment to determine apical metal uptake and transepithelial metal transport, respectively.

2.3. Western blot analysis

Following removal of culture medium, cell monolayers were washed

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Abbreviations: DMT1, divalent metal transporter; IREG1, iron-regulated mRNA; RT-PCR, reverse transcription polymerase chain reaction; IRE, iron responsive element; IRP, iron regulatory protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid

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twice in phosphate buffer and harvested using a cell scraper. Total plasma membranes (apical and basolateral pooled) were prepared as described previously [17] and cell membranes (20 μg) subjected to 10% SDS–PAGE. Proteins were transferred onto nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated with a polyclonal antibody (1:250 dilution) raised in rabbit against a synthetic peptide corresponding to amino acids 310–330 of the human DMT1 sequence. Following incubation with a secondary anti-IgG antibody (HRP-labelled) cross-reactivity was visualised using ECL Plus and Hyperfilm ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Band densities were semi-quantified by densitometric analysis using Scion Image software (Scion Corporation, MD, USA).

2.4. RT-PCR for DMT1 and IREG1 mRNA

Total RNA was isolated from Caco-2 cells using Trizol reagent (Life Technologies, Paisley, UK) and stored at −70°C in 75% ethanol until required. Reverse transcription polymerase chain reaction (RT-PCR) was performed in a single step reaction, using Ready-to-go RT-PCR Beads (Amersham Pharmacia Biotech) on total RNA samples (1 μg per tube) using the following primer sequences: GAPDH 5′-GCCATCAATGACCCCTTCAT-3′ (forward) and 5′-GAGGGGG-CAGAGATGATGAC-3′ (reverse); DMT1 5′-GGTGTTGTGCT-GGGATGTTA-3′ (forward) and 5′-AGTACATATTGATGGAA-CAG-3′ (reverse); IREG1 5′-ATTGCTGCTAGAATCGGTCT-3′ (forward) and 5′-AGACTGAAATCAATACGAGC-3′ (reverse). The cDNA transcript was produced by incubation at 42°C for 30 min. PCR was performed by 28 cycles of 95°C for 30 s, 57°C for 30 s (50°C for IREG1), 72°C for 1 min, followed by a final single extension at 72°C for 10 min in a PTC-100 thermal cycler (MJ Research, NV, USA). PCR products were stained with ethidium bromide on a 2.5% agarose gel and visualised using Fluor-S MultiImager (Bio-Rad Laboratories, Hertfordshire, UK), and bands were analysed using MultiAnalyst (Bio-Rad) image analysis software. DMT1 and IREG1 mRNA levels were normalised to GAPDH expression.

2.5. Data analysis

Data are presented as the mean ± S.E.M. Statistical analysis was carried out using the SPSS statistics package, and utilised one-way ANOVA followed by Scheffé's post-hoc analysis, or Student's unpaired *t*-test where appropriate. Differences were considered significant at $P < 0.05$.

2.6. Materials

Radiochemicals and materials for Western blotting were supplied by Amersham Pharmacia Biotech UK, Buckinghamshire, UK. Cell culture medium and plasticware were purchased from Life Technologies (Paisley, UK) unless stated. Heat-inactivated foetal bovine serum was from Sigma Chemical Company (Poole, UK). All other chemicals were of highest grade available and bought from Sigma, Merck or Fluka.

3. Results and discussion

Direct interference, i.e. similar to that which could occur in the intestinal lumen, between zinc and iron was determined by measuring the uptake of 1 μM $^{55}Fe^{2+}$ or $^{65}Zn^{2+}$ in the presence or absence of 100 μM unlabelled metal. Radiolabelled iron uptake in the presence of an inwardly directed pH gradient was significantly inhibited by addition of a 100-fold excess of cold iron but was not reduced by high zinc (Fig. 1a). Interestingly, uptake of $^{65}Zn^{2+}$ was significantly inhibited by both unlabelled zinc and iron, to the same degree (50–60%) compared with control (Fig. 1b). Taken together, these data suggest that there may be a common transport pathway for iron and zinc located in the apical membrane of Caco-2 TC7 cells. Previous work gave rise to the assumption that DMT1 transported a broad range of metals including zinc and iron and therefore might represent this common pathway [8]. However, evidence from our laboratory suggests that a zinc/iron transporter is likely to be distinct from DMT1 since, in contrast to iron, zinc uptake across the apical membrane of Caco-2 TC7 cells is not pH- or membrane potential-dependent [10]. Furthermore, in the presence of a neutralising antibody against DMT1, iron uptake was significantly inhibited, whereas zinc uptake was unaltered [10]. A number of other zinc uptake pathways are present in the apical membrane of Caco-2 cells, which are not affected by metabolic inhibitors [18] or are linear and non-saturable [19] and these may represent the common transport pathway for zinc and iron. However, the nature of these transporters and their substrate specificity remains to be elucidated.

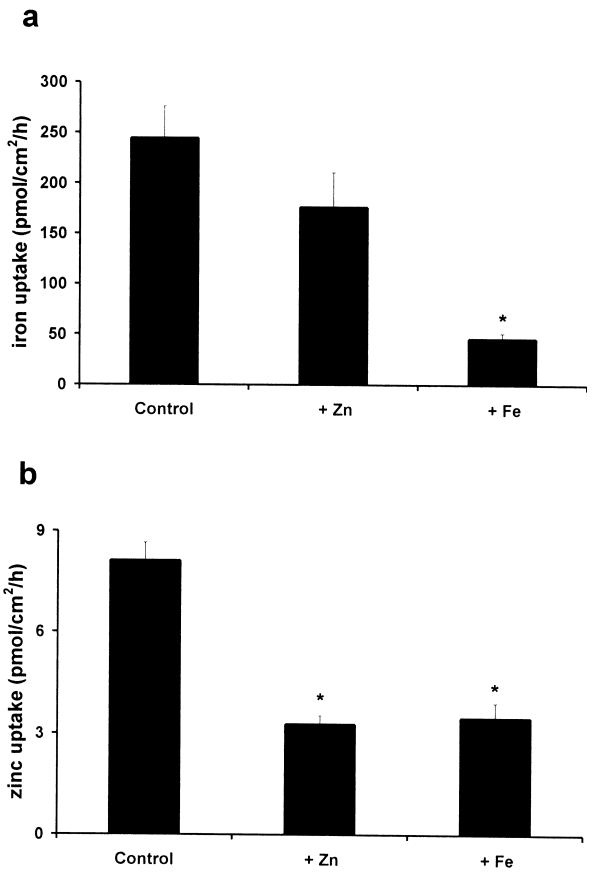

Fig. 1. Competition for uptake between zinc and iron at the apical membrane of Caco-2 TC7 cells. Uptake of 1 μM $^{55}Fe^{2+}$ was significantly inhibited (ANOVA and Scheffé's post-hoc test, $*P < 0.001$) by 100 μM unlabelled iron, but was unaffected by 100 μM zinc (a). In contrast, 1 μM $^{65}Zn^{2+}$ uptake was significantly inhibited (ANOVA and Scheffé's post-hoc test, $*P < 0.01$) in the presence of both zinc and iron compared with control (b). Data are means ± S.E.M. of four observations.

Interestingly, recent evidence suggests that zinc can directly interact with the DMT1 transporter by binding and inducing a proton conductance through the transporter [15]. Therefore, to determine whether zinc could also influence the expression or function of DMT1, we measured DMT1 protein and mRNA expression following exposure to high zinc for 24 h. In agreement with our previous findings [10], exposure to iron decreased DMT1 protein expression in Caco-2 TC7 cell membranes (Fig. 2a). Intriguingly, exposure to zinc (100 μM) for 24 h significantly increased membrane expression of DMT1 (Fig. 2b). DMT1 mRNA was similarly increased by exposure to zinc (Fig. 2c), whereas iron induced a small, statistically

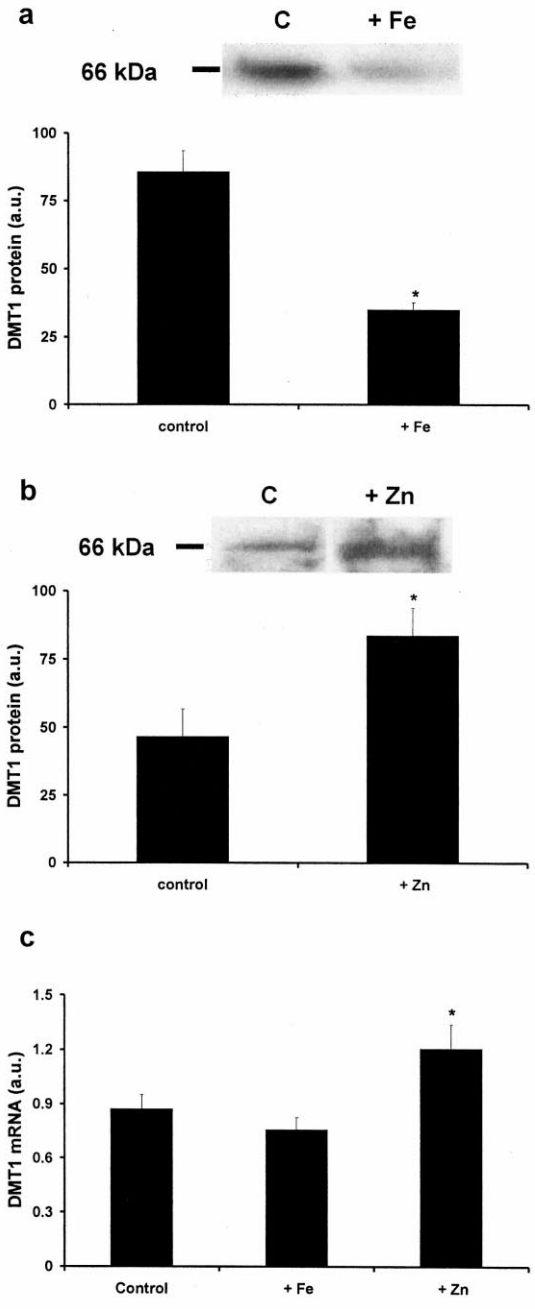

Fig. 2. Effects of dietary metals on DMT1 expression. DMT1 protein was detected on Western blots as a 66 kDa band. Membrane expression of DMT1 was decreased significantly (Student's unpaired *t*-test, $*P < 0.01$) by 100 μM iron (a) but increased (Student's unpaired *t*-test, $*P < 0.05$) following exposure to 100 μM zinc (b). Data are means ± S.E.M. derived from four membrane isolations per condition. DMT1 mRNA, normalised to GAPDH levels and expressed in arbitrary units, was significantly increased in zinc-treated cells compared with iron-treated and control groups (ANOVA and Scheffé's post-hoc test, $*P < 0.005$) (c). Data are presented as means ± S.E.M. of 12 separate determinations in each group.

insignificant, decrease in transporter mRNA (the iron effect was significant after 72 h exposure; data not shown). These changes in DMT1 protein and mRNA expression were mirrored when measuring pH-dependent iron uptake following exposure to high zinc or iron (Fig. 3a). Uptake was significantly decreased by exposure to 50 and 100 μM iron but significantly increased by 100 μM zinc. In contrast, exposure to 50 μM or 100 μM zinc or iron was without effect on ^{65}Zn uptake (Fig. 3b).

With regard to the regulation of DMT1 by dietary metals, it has been demonstrated that an iron responsive element (IRE) is present in the 3′-untranslated region of DMT1, which can bind iron regulatory proteins (IRPs) in vitro [7,20], and – by analogy with the transferrin receptor – high iron is thought to reduce IRP/IRE binding and thereby decrease DMT1 mRNA. Interestingly, recent evidence suggests that high iron may have direct effects on the cellular localisation and abundance of DMT1 protein by either causing transporter destruction [21] or internalisation of the transport protein from the plasma membrane into the cytosol [22]. Either of these mechanisms could partly explain the lack of correlation between changes in DMT1 protein (Fig. 2a) and mRNA (Fig. 2c) following high iron observed in our study.

There is no evidence that IRPs or IREs are sensitive to changes in the cellular levels of zinc. However, the 5′-pro-

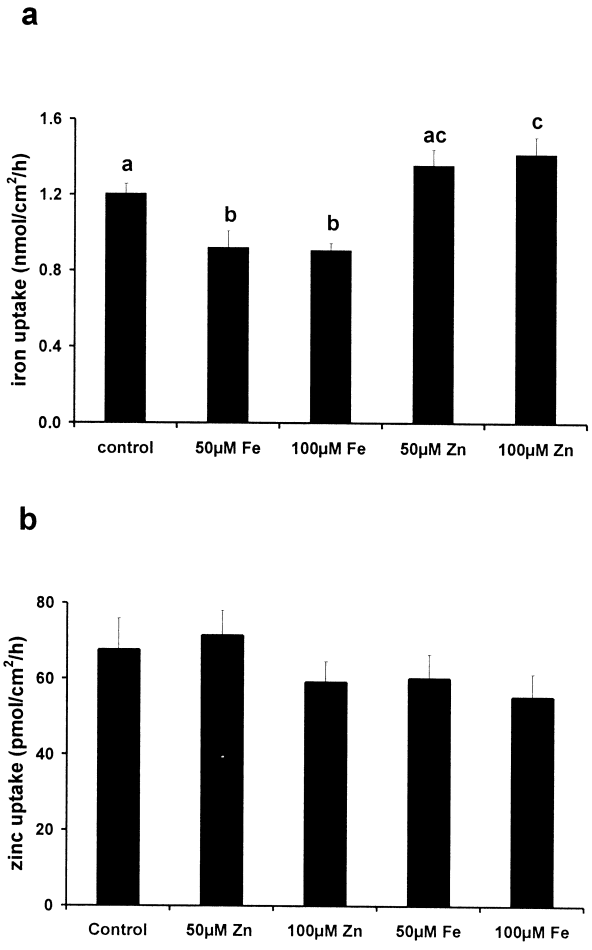

Fig. 3. Metal ion uptake following exposure to zinc or iron. Iron uptake (10 μM Fe^{2+}) was significantly decreased by previous exposure to iron (50 and 100 μM) but significantly increased by exposure to 100 μM zinc (ANOVA and Scheffé's post-hoc test, $P < 0.001$) (a). The presence of common letters above data bars indicates that these groups are not statistically different from each other. Data are means ± S.E.M. of four to six observations in each group. Zinc uptake (10 μM Zn^{2+}) across the apical membrane was not affected by exposure to 50 or 100 μM zinc or iron (b). Data are means ± S.E.M. of four observations in each group.

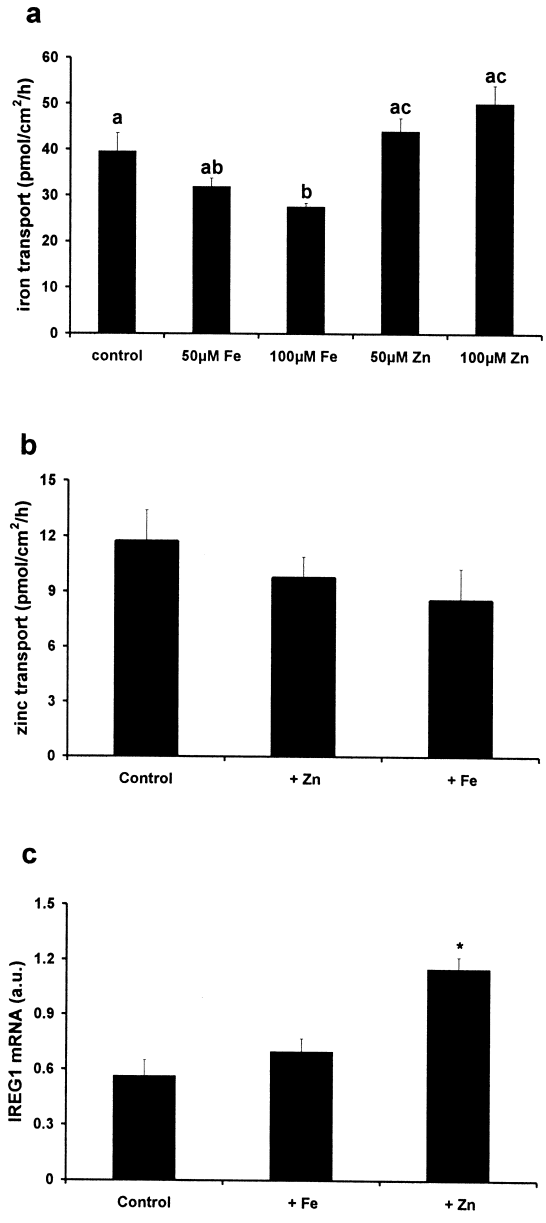

Fig. 4. Metal ion transport across Caco-2 TC7 cell monolayers and IREG1 expression. Iron transport from apical to basal was significantly higher following exposure to zinc (100 μM) compared with cells exposed to high iron (100 μM) (ANOVA and Scheffé's post-hoc test, $P < 0.002$) (a). The presence of common letters above data bars indicates that these groups are not statistically different from each other. Data are means ± S.E.M. of four to six observations in each group. Zinc transport across the epithelial monolayer into the basolateral chamber, however, was not affected by exposure to zinc (100 μM) or iron (100 μM) (b). Data are means ± S.E.M. of four observations in each group. Expression of the basolateral iron transporter IREG1 (normalised to GAPDH mRNA levels) was significantly increased by exposure to zinc, but unaltered (compared with control) by high iron (ANOVA and Scheffé's post-hoc test, $*P < 0.0005$) (c). Data are presented as means ± S.E.M. of 12 separate determinations in each group.

moter region of DMT1 contains several metal responsive element consensus sequences [23]. Similar sequences are present in the promoter of the metallothionein II_A gene [24] and are implicated in increased metallothionein production upon exposure to high zinc levels. Interestingly, expression of other proteins involved in zinc metabolism are also upregulated by high zinc status, including the intestinal basolateral zinc exporter ZnT1 [25]. However, within the timescale of our study, zinc transport (apical to basal) was unaffected by exposure to zinc or iron (Fig. 4b).

Apical to basolateral iron transport across the Caco-2 TC7 cell monolayer was significantly lower in cells exposed to high iron compared to those exposed to high zinc (ANOVA, $P < 0.002$, Fig. 4a). Intriguingly, the mRNA expression of the basolateral iron transporter, IREG1, whilst not altered by exposure to high iron (compared with control cells), was significantly increased by zinc (Fig. 4c). Previous studies have shown that IREG1 mRNA is increased in response to iron deficiency anaemia and hereditary haemochromatosis [11,26] but is unaffected by dietary iron loading [11]. However, our study provides the first evidence that IREG1 expression can be influenced by other dietary metals. The mechanism involved in zinc stimulation is unclear but it is possible that metal responsive elements may also be present in the IREG1 promoter.

In conclusion, we have demonstrated that there may be a common uptake pathway for zinc and iron located in the apical membrane of Caco-2 TC7 cells. Taken together with our previous data [10] the evidence suggests that this transporter is distinct from DMT1 and its nature remains to be determined. Whilst zinc is not transported via DMT1 it does directly influence the function and expression of the transporter, elevating both protein and mRNA levels and increasing pH-dependent uptake of iron. In addition, we also found that the expression of the basolateral iron transporter IREG1 was increased in zinc-treated cells. The mechanisms involved in zinc-dependent regulation are unclear but could be related to metal responsive elements in the promoter regions of these key iron transport proteins.

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