Iron-zinc interaction during uptake in human intestinal Caco-2 cell line: Kinetic analyses and possible mechanism

Vasuprada Iyengar, Raghu Pullakhandam and K Madhavan Nair*
Micronutrient Research Group, Biophysics Division, National Institute of Nutrition, Indian Council of Medical Research, Jamai Osmania, Hyderabad 500 604, India

Received 07 January 2009; revised 02 July 2009

Iron and zinc interact at the enterocyte during absorption, but the mechanism(s) remain elusive. The aim was, therefore, to understand the mechanism of interaction using kinetic analyses of iron and zinc uptake, individually and in combination under normal and altered cellular mineral concentrations in human intestinal Caco-2 cell line. Striking differences in kinetic parameters were observed between iron and zinc uptake. Iron uptake followed a two-component model, while zinc uptake followed a three-component model. Iron uptake had a $K_m$ of 3.6 µM and $V_{max}$ of 452 pmol/mg protein/min, while zinc uptake had a $K_m$ of 42 µM and $V_{max}$ of 3.09 pmol/mg protein/min. Zinc dose-dependently inhibited iron uptake through mixed-inhibition but iron marginally increased zinc uptake. Cellular zinc repletion doubled iron uptake and eliminated inhibition, but zinc depletion decreased iron uptake. Iron pre-treatment had no effect on zinc uptake. Based on these results, a two-transporter model of iron uptake, comprising the apical iron uptake transporter divalent metal ion transporter-1 (DMT-1) and an unknown putative transporter was derived. This model for DMT-1 was verified by immunoblotting. These results implied that cellular zinc status profoundly influenced iron uptake and its interactions with zinc during uptake. DMT-1 might not simultaneously transport iron and zinc, providing a mechanistic basis for observed interactions.

Keywords: Caco-2 cells, DMT-1, Interactions, Iron, Kinetics, Zinc

Iron and zinc are essential micronutrients required for growth, development and maintenance of physiological systems. They are similar in physico-chemical properties such as atomic radii (iron 140 pm, zinc 135 pm) and oxidation states ($\text{Fe}^{2+,3+}$; $\text{Zn}^{2+}$). Both are stored predominantly in the liver and maximally absorbed in the proximal duodenum, but only to about 5% (iron) or 15% (zinc). Major food sources are meat, poultry, nuts and legumes and inositol hexaphosphates (phytates) are common absorption inhibitors. These similarities in many properties may lead to interactions at the enterocyte during absorption\(^1\). Subclinical deficiencies of iron and zinc recognized as public health problems in India have been attributed to decreased absorption of these minerals from cereal/pulse-based foods rich in phytates. However, conflicting results have been observed during combined supplementation of iron and zinc, which may be due to enterocyte level interactions. The nature and locus of these interactions are still unclear.

The divalent metal ion transporter-1 (DMT-1) is the principle proton coupled metal-ion symporter for intestinal iron absorption\(^2,3\) and is regulated according to cellular iron status\(^4\). Downregulation or antibody neutralization of DMT-1 in cell culture models\(^5,6\), mutations in DMT-1 in animal models such as the \textit{mk} mouse\(^7\) and \textit{Belgrade} rat\(^8\) or humans\(^9\) decrease iron absorption but does not result in complete loss of iron uptake, indicating the presence of other functional compensatory mechanisms of iron uptake. The role of DMT-1 as an authentic zinc transporter is not established, although heterologous expression in \textit{Xenopus laevis} oocytes evokes intracellular proton currents with divalent cations including $\text{Zn}^{2+}$ ion\(^10\). However, these currents have later been shown to be the result of $\text{H}^+$ and $\text{Cl}^-$ conductance, rather than actual Zinc transport\(^11\). Therefore, the role of DMT-1 in iron-zinc uptake and interactions is controversial.

Caco-2 cells are an extensively tested and widely used model of absorptive enterocytes and have also been used to study bioavailability of micronutrients\(^12\). They are, therefore, a suitable model to study the

---

*Author for correspondence:
Tel: 91-40-27197269; Fax: 91-40-27019074
E-mail: nairthayil@hotmail.com

\textbf{Abbreviation:} Caco-2, colon adenocarcinoma-2 cell line; DFO, desferoxamine; DMEM, dulbecco’s modified eagle’s medium; DMT-1, divalent metal ion transporter-1; GAPDH, glyceraldehyde-6-phosphate dehydrogenase; HRP, horse radish peroxidase; SLC, solute carrier; TPEN,N,N,N’,N'-teterakis [2-pyridylmethyl] ethylene diamine.
elucidated iron-zinc interactions by measuring the kinetics of iron and zinc uptake under normal and altered cellular iron/zinc status in Caco-2 cells.

Materials and Methods

Materials

All chemicals and enzymes were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated. All antibodies (DMT-1, GAPDH) were procured from Ms. Santa Cruz Biotechnology Inc (USA). $^{59}$FeCl$_3$ (carrier free) and $^{65}$ZnCl$_2$ (specific activity 3 Ci/g) were obtained from Board of Radiation and Isotope Technology, Mumbai, India.

Cell culture

Caco-2 cells were obtained from the National Center for Cell Sciences (Pune, India), routinely maintained and used in experiments at passage 25-35 as mentioned elsewhere.

Mineral uptake

In all uptake studies, unless otherwise mentioned, cells were incubated in DMEM with iron or zinc (50 µM) traced with $^{59}$Fe (0.1 µCi) or $^{65}$Zn (0.5 µCi) for 2 h, washed thrice with ice-cold 50 mM PBS buffer (pH 7.4) containing 1 mM EDTA, 1 mM 1, 10-bathophenanthroline, 5 mM sodium dithionite (to remove non-specifically bound iron) or 10 mM HEPES, 1 mM EDTA (to remove non-specifically bound zinc) and scraped into ice-cold 50 mM PBS, pH 7.4. $^{59}$Fe was counted using a Packard Tri-Carb 2100 TR and $^{65}$Zn using a GRS–201L, (PLA electroappliances, Mumbai, India). Actual uptake was calculated from disintegrations per min (dpm) and specific activity of isotope used.

Dose-dependent uptake

Cells were incubated with 0-200 µM iron or zinc for 2 h. Uptake was quantitated as above, plotted with mineral concentration on the X-axis and uptake (mean of all replicates) on the Y-axis, and analyzed ($r^2>0.95$) using non-linear regression equations (Sigma plot ver 7.1). Double-reciprocal plots with 1/concentration (µM)$^{-1}$ on the X-axis and 1/uptake (nmol/mg protein)$^{-1}$ on the Y-axis were plotted to obtain $K_m$ and $V_{\text{max}}$. $K_m$ was defined as the mineral concentration at which the rate of uptake was half its maximal value and represents the collective rate constant of all process(es) involved in mineral uptake. $V_{\text{max}}$ was defined as the overall resultant velocity of all processes involved in mineral uptake. The uptake curve was resolved in to individual linear components by the peeling technique.

Peeling for components

Data points for uptake at concentrations less than $K_m$ of the curve were fitted to a linear curve ($y=mx+c; r^2>0.95$) and extrapolated to the axes. The observed uptake was then subtracted from the corresponding extrapolated data points, curve-fitted, linearized and subtracted till all components were resolved to linearity. The slope and y intercept ($y_0$) of individual linear components were obtained from the graph.

Interaction studies

Iron or zinc uptake in the presence of 1:0-1:4 molar ratios of the other mineral (zinc or iron) for a period of 2 h was quantitated as above and converted to percentage of 1:0 molar ratio (100%).

Kinetic studies

Cells were incubated with 1:0-1:4 molar ratios of iron: zinc either at 5-100 µM iron for 2 h or with 50 µM iron for 5-120 min and uptake was quantitated as above. From a double-reciprocal plot of 1/time (min)$^{-1}$ in the X-axis and 1/uptake (nmol/mg protein)$^{-1}$ in the Y-axis, kinetic constants $U_{\text{max}}$ and $K_a$ were obtained, where $U_{\text{max}}$ was defined as maximum uptake at 50 µM iron concentration and $K_a$ as the rate constant of iron uptake. $K_m$ and $V_{\text{max}}$ were obtained as above. $K_i$ (inhibition constant for zinc) was obtained from a secondary plot of the dose-response data with zinc concentration on the X-axis and 1/$V_{\text{max}}$ on the Y-axis.

Iron-zinc uptake upon altered mineral concentration

Cells were either left untreated or pretreated with 50 µM zinc for 1-4 h, 50 µM iron for 2-6 h, 10 µM TPEN (N, N, N', N'-teterakis [2-pyridilmethyl] ethylene diamine, an intracellular zinc chelator for 2 h or 100 mM desferoxamine (DFO, cell permeable iron chelator) for 8 h in basal DMEM which was replaced after incubation. Subsequently, iron or zinc uptake was quantitated as above in the concomitant presence or absence of the other mineral (zinc or iron) in a 1:1 molar ratio.

DMT-1 protein expression

After treatment with iron, zinc, TPEN or DFO for the indicated time, cells were washed, lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM NaCl with protease inhibitors) and protein
was determined by BCA kit method (Sigma, MO, USA). About 60 µg of protein was resolved on 10% SDS-polyacrylamide gels, electrotransferred, blocked, and incubated with anti-DMT-1 or anti-GAPDH, followed by HRP-conjugated anti-species antibody. Bands were visualized using the tetramethylbenzidine (TMB) liquid substrate system, scanned in a GS-710 densitometric scanner and quantitated using Quantity-one software (Bio-Rad, Hercules, CA, USA).

Statistical analyses

All uptake experiments were performed in triplicates and repeated at least once to generate six independent observations. Data were presented as mean±SD. Means between groups or between time-points within a group were compared by One-way ANOVA and post-hoc ‘t’ test (SPSS software, version 11.0). P<0.05 was considered significant.

Results

Iron and zinc uptake

As iron and zinc uptake saturated at 50 µM concentration, all further experiments were carried out at this concentration. Uptake data were fitted to non-linear regression equations with concentration on the X-axis and uptake in the Y-axis. Iron uptake fitted a two-compartment hyperbolic equation \(Y = ax/b + x\); Fig. 1A, \(r^2 = 0.98\), while zinc uptake fitted a three-component model \(Y = \{(ax/b + x) + cx\};\) Fig. 1B, \(r^2 = 0.99\). Iron uptake was calculated to have \(K_m\) of 3.61 µM and \(V_{max}\) of 452 pmol/min/mg protein (Fig. 1C, \(r^2 = 0.98\), while zinc uptake had \(K_m\) of 42.1 µM and \(V_{max}\) of 3.09 pmol/min/mg protein (Fig. 1D, \(r^2 = 0.98\)). Resolution of two-compartment iron uptake into individual linear components gave slope (nmol/mg protein/2 h/µM), \(y_0\) (nmol/mg protein/2 h) values of 0.12, -0.13 (component I) and 0.024, 4.69 (component II), respectively. Resolution of three-compartment zinc uptake into individual linear components gave slope (pmol/mg protein/2 h/µM) and \(y_0\) (pmol/mg protein/2 h) values of 4.53, 2.55 (I), 0.877, 96.49 (II), -0.304, 156 (III), respectively.

Simultaneous iron-zinc uptake

Zinc significantly decreased iron uptake in a dose-dependent manner upon simultaneous incubation at all ratios tested (Fig. 2A). However, the decrease was not significant above 1:0.5 molar ratio, due to large variation. Maximum inhibition was observed at 1:4 molar ratio (42% of control; 8.74 ± 2.21 to 3.65 ± 0.59 nmol/mg protein). Significant inhibition of iron uptake was observed even at the molar ratio of 1:0.25 of iron: zinc. Equimolar ratio was used for further studies, as there was no difference in iron uptake above 1:1 molar ratio. On the contrary, zinc uptake increased upon concomitant incubation with varying molar ratios of iron (Fig. 2B). This effect was
only marginal and not dose-dependent, but significant (107% of control; 247.7 ± 9.28 vs. 258.5 ± 12.61 pmol/mg protein) at 1:1 molar ratio and above.

Dose-response and kinetics of inhibition

Iron uptake (5-100 µM iron) in the concomitant presence of increasing molar ratios of zinc showed significant inhibition only above 50 µM and at 1:1 ratio and above. No inhibition was observed even at 1:4 molar ratio at the 5 µM and 20 µM iron (Fig. 3A). A double-reciprocal plot of uptake gave ~40% decrease in $V_{\text{max}}$ and $K_m$ at 1:1, compared to 1:0 iron: zinc (Fig. 3B; Table 1). $K_i$ was calculated to be 5.18 µM from the secondary plot (data not shown).

Iron uptake in the presence of increasing molar ratios of zinc for 5-120 min showed significant inhibition only above 1:0.5 molar ratio and at the 15 min time point onwards. No inhibition was observed even at 1:4 molar ratio at 5 and 10 min (Fig. 3C). Double-reciprocal plots of uptake showed a ~50% decrease in $U_{\text{max}}$ and $K_u$ at 1:1, compared to 1:0 molar ratios of iron: zinc (Fig. 3D; Table 1).

Slope and intercept values were obtained from the linear fit of double-reciprocal plots

<table>
<thead>
<tr>
<th>Fe:Zn ratio</th>
<th>Dose-response $K_m$ (µM)</th>
<th>$V_{\text{max}}$ (pmol/mg protein/min)</th>
<th>Time-kinetics $K_u$ (min)</th>
<th>$U_{\text{max}}$ (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>3.61</td>
<td>452</td>
<td>27.14</td>
<td>1.52</td>
</tr>
<tr>
<td>1:0.25</td>
<td>3.11</td>
<td>532</td>
<td>25.65</td>
<td>1.33</td>
</tr>
<tr>
<td>1:0.5</td>
<td>2.07</td>
<td>369</td>
<td>29.15</td>
<td>1.13</td>
</tr>
<tr>
<td>1:1</td>
<td>1.72</td>
<td>182</td>
<td>14.23</td>
<td>0.77</td>
</tr>
<tr>
<td>1:2</td>
<td>0.65</td>
<td>103</td>
<td>15.66</td>
<td>0.72</td>
</tr>
<tr>
<td>1:4</td>
<td>0.18</td>
<td>41</td>
<td>17.46</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Fig. 2—Iron and zinc uptake in the presence of various molar ratios [(A): Iron uptake in the presence of indicated molar ratios of zinc and (B): zinc uptake in the presence of various molar ratios of iron was quantitated. Uptake is expressed as % of 1:0 (control) cells. Bars that do not share a common letter are significantly different at P<0.05]

Fig. 3—Dose-response and kinetics of zinc inhibition of iron uptake [Iron: zinc in various molar ratios at 5-100 µM iron concentration (A) with double-reciprocal plot (B, plots for 1:1, 1:2 and 1:4 are alone given as no inhibition was observed at lower ratios) or for various time points (C) with double-reciprocal plot (D). Iron uptake is mean of three replicates]
Effect of cellular iron-zinc status on uptake

Zinc pre-treatment increased iron uptake per se to greater than 200% of control at all time points tested. Importantly, equi-molar zinc did not inhibit iron uptake and was similar at all time points tested (Fig. 4A). On the contrary, iron pre-treatment did not affect zinc uptake significantly, either in the presence or absence of concomitant equi-molar iron (Fig. 4B). Cellular deficiency with TPEN (zinc) or DFO (iron) significantly decreased iron uptake, which further decreased in the presence of equimolar zinc (Fig. 4C). Under similar conditions, zinc uptake increased and interestingly, concomitant iron further enhanced zinc uptake (Fig. 4D).

Effect of iron-zinc on DMT-1 expression

Zinc pre-treatment significantly increased DMT-1 expression at 3 h and 4 h compared to 0 h or control (Fig. 5A). Iron pretreatment (Fig. 5B) did not affect DMT-1 expression levels after correcting for GAPDH intensity. TPEN (Fig. 5C) and DFO (data not shown) also did not affect total cellular DMT-1 expression.

Discussion

Although various trials in humans on the effects of combined supplementation of iron and zinc have clearly shown their interaction, the direction and mechanism involved are not yet understood. Thus, an insight into the kinetics of iron and zinc uptake at the enterocyte can shed some light on the mechanism(s) of such interactions. There is scanty literature on the kinetics of iron or zinc uptake, individually or in combination at the enterocyte level. In this study, Caco-2 cells, an ex-vivo model of differentiated enterocytes have been used to understand the kinetics of iron and zinc uptake, individually and in combination.

Previous kinetic studies in Caco-2 cells\textsuperscript{14} and human upper small intestine microvillus membrane vesicles\textsuperscript{15} have reported $K_m$ of 7 µM and $V_{\text{max}}$ of 361 pmol Fe$^{3+}$/mg membrane protein/min, respectively. The kinetic constants in this study are in agreement with the above.

Mineral uptake can be membrane transporter-mediated or paracellular. The kinetics of uptake and
its resolution into individual components can provide insights into the transport process. Component I of iron uptake contributes to about 45% of total uptake and is found to have a near-zero $y_0$ value and a greater slope (0.12), implying that this component has a high affinity for iron. On the other hand, component II had a higher $y_0$ value and a lower slope compared to component I, indicating comparatively lower affinity for iron. The low $K_m$ value (3.6 µM) combined with negligible iron uptake at iron concentrations below 10 µM preclude the presence of a paracellular route. Thus, iron uptake entails two transporters that contribute equally to total uptake, but with differing affinities. It is possible that the transporter corresponding to component II is more abundant than the other, thus enabling equal iron uptake in spite of lesser affinity.

Two-component models for iron uptake in Caco-2 cells have been reported earlier, either with specific uptake and paracellular transport or specific binding, followed by transcellular transport. Consistent with the proposed two-transporter model, it has been previously shown that DMT-1 knockdown or neutralization still results in substantial iron uptake, indicating the presence of other iron transporter(s). Therefore, it is possible that component I represents DMT-1 and component II, another putative iron transporter.

Zinc uptake, unlike iron uptake, is a three-compartment saturable process. Of the two kinetic constants, $K_m$ is in good agreement with a similar study that reported $K_m$ of 41 µM. However, the same study reported a $V_{max}$ of ~300 pmol/mg protein/min (0.3 nmol/cm$^2$/10 min) which is not in agreement, probably because the earlier study measured only initial uptake for 10 min (initial velocity) as against 2 h (steady-state velocity).

Compared to iron uptake, zinc uptake is more complex, as it involves three components. Resolution of this triphasic zinc uptake into individual components shows substantial uptake at concentrations below $K_m$. The uptake data fit an equation that incorporates a non-mediated component. Component I has a steep slope (4.53 units) and low $y_0$ value which are characteristic of paracellular uptake and contributes substantially (~30%) to total uptake. Component II has a slope of 0.8 units and is responsible for the remaining zinc uptake, suggesting that it represents mediated uptake. Component III has a negative slope and a high $y_0$ value (156) and is, therefore, involved in maintaining steady-state concentrations of cellular zinc by efflux of excess zinc from the cell.

Based on the above, we hypothesize the following: component I represents diffusion limited paracellular uptake, component II represents zinc influx and component III an efflux transporter which serves to maintain requisite steady-state cellular zinc concentrations. Zinc influx could be mediated by the Zip family of transporters (SLC39A), possibly Zip4 (SLC39A4), which is most responsive to cellular zinc status while efflux could be through the ZnT (SLC30) family of efflux transporters, most plausibly ZnT-1, as it protects cells from excessive zinc and is reciprocally regulated with the Zip transporters. The possibility of a paracellular uptake route has been previously reported.

There are striking differences in $K_m$, $V_{max}$ and uptake of iron and zinc. This negates the possibility of direct competition for a single transporter, when presented together at the enterocyte. Moreover, it suggests the existence of more than one transporter responsible for simultaneous iron and zinc transport. When iron and zinc are presented together to the cells, under identical conditions, zinc halves iron uptake, but iron marginally (7%) increases zinc uptake. The physiological significance of this increase is $K_u$ known. It is, however, possible that the interactions become physiologically relevant under altered mineral status, especially that of zinc. Another possibility is that the kinetic nature of these interactions is non-competitive, as competitive inhibition would have resulted in a marginal decrease in zinc uptake, at least at four-fold molar excess of iron. To the best of our knowledge, this is the first report that has determined the effect of iron on zinc uptake, although zinc inhibition of iron uptake in Caco-2 cells has been previously reported.

In order to further characterize the non-competitive inhibition, concentration and time-dependent kinetic constants, $K_m$, $V_{max}$ and $U_{max}$, $K_u$ have been derived. All constants show a decrease upon increasing molar ratios of zinc and, therefore, it is proposed that zinc inhibits iron uptake through mixed-inhibition. Mixed-inhibition necessitates the presence of more than one iron transporter and this is consistent with our two-transporter model of iron uptake.

Acute alterations in cellular mineral concentration can influence interactions to a great extent. Zinc pre-treatment and chelation (TPEN, binding constant for...
zinc $K_a = 10^{16} \text{M}^{-1}$ reciprocally modulates iron uptake, highlighting the importance of cellular zinc as a determinant of iron uptake and its interactions with zinc during uptake. These results are in agreement with previously published observations of increased iron uptake upon zinc pre-treatment in Caco-2 cells and decreased iron status in the zinc-deficient rat. On the contrary, cellular iron status is probably not of major importance to zinc uptake, as iron pre-treatment does not significantly affect zinc uptake or its interactions with iron during uptake. However, DFO increases zinc uptake even in the presence of concomitant iron. It is possible that DFO induces cellular zinc deficiency similar to that of TPEN, as it can also marginally chelate zinc ($\log \beta_1 = 11.1$), apart from primarily chelating iron ($\log \beta_1 = 30.6$). Although it is expected that DFO treatment should result in increased iron uptake, iron uptake decreases probably due to comparatively marginal zinc chelation, further reiterating the importance of cellular zinc in iron uptake and interactions during uptake.

Taking into account the proposed two-transporter model of iron uptake and the patterns of iron-zinc uptake under normal and altered mineral concentrations, we propose the following to explain our observations. There are two transporters capable of iron transport, of which DMT-1 is already established. Based on the low $K_i$ value which indicates high affinity for zinc, the second transporter can be a zinc influx transporter. In the presence of iron alone, both transporters contribute equally to iron uptake. In the presence of concomitant zinc, the second transporter is unable to transport iron, thus halving iron uptake. Upon zinc pre-treatment, the second transporter becomes non-functional, eliminating negative interactions. In support of this, Zip-14 has been shown to transport non-transferrin bound iron along with zinc when expressed in human embryonic kidney (HEK) and insect SF9 cells and zinc inhibits iron uptake upon overexpression of this transporter. Moreover, Zip-2 is also important in liver iron homeostasis and during murine embryo development. These transporters are, therefore, ideal candidates for the predicted putative iron transporter. However, we have not tested the role of these transporters in the present study.

In order to test the above model, DMT-1 protein expression has been analyzed by Western blotting. Zinc increases DMT-1 expression which is probably responsible for the observed increase in iron uptake per se upon zinc pre-treatment. If DMT-1 is simultaneously transporting iron and zinc, increased DMT-1 expression upon zinc pre-treatment should result in persistent zinc inhibition of iron uptake, but we have observed elimination of negative interactions. This implies that DMT-1 cannot simultaneously transport iron and zinc. Moreover, TPEN and DFO increase zinc uptake without altering DMT-1 expression, further suggesting DMT-1 may not transport zinc. However, both TPEN and DFO decrease iron uptake, possibly through DMT-1 translocation from the apical membrane or inhibition. Such translocation and possible recycling of DMT-1 due to iron treatment has been reported and is currently under investigation. Our inference that DMT-1 may not simultaneously transport iron and zinc and is, therefore, not the locus of negative interactions observed during uptake, is in agreement with earlier report of limited zinc transport by DMT-1.

In conclusion, kinetic data analyses reveal that unlike zinc, iron uptake occurs through two transporters rather than just DMT-1. We report for the first time that zinc inhibits iron uptake through mixed-inhibition. Cellular zinc status profoundly affects iron uptake and its interactions with zinc during uptake without a reciprocal effect of iron on zinc uptake. DMT-1 protein expression under varied cellular mineral conditions supports our two-transporter mechanism for iron uptake and proves that it cannot simultaneously transport iron and zinc. These results thus provide a mechanistic basis for enterocyte-level interaction of iron and zinc during uptake.

Acknowledgement

The project is supported by a grant from DBT, grant no. BT/PR/6728/AGR/02/334/2005, Department of Biotechnology, India. VI is supported by a research fellowship from the University Grants Commission, India.

References