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Pre-treatment of Caco-2 cells with zinc during the differentiation phase alters the kinetics of zinc uptake and transport

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Abstract

The Caco-2 cell model was used to study the efficiency of absorption and endogenous excretion of zinc (Zn) regulated by dietary Zn concentration. Cells were seeded onto high pore-density membranes and maintained in medium supplemented with 10% FBS. After confluence, cells were treated with 5 or 25 μmol Zn/L for 7 d, and Zn uptake and transport were measured in both apical (AP) and basolateral (BL) directions by using ^{65}Zn. Similar cells were labeled with ^{65}Zn and the release of Zn to the AP and BL sides was measured. The AP uptake of Zn in cells exposed to 25 μmol Zn/L was slower ($p < 0.05$) than that in cells exposed to 5 μmol Zn/L. The AP to BL transport rate in the 25 μmol Zn/L group was only 40% ($p < 0.05$) of that in the 5 μM group. In contrast, the rate of BL Zn uptake was 4-fold higher in cells treated with 25 μmol Zn/L than in those treated with 5 μmol Zn/L ($p < 0.05$). The BL to AP transport rate was 2-fold higher in cells treated with 25 μmol Zn/L than in those treated with 5 μmol Zn/L ($p < 0.05$). Basolateral uptake was 6 to 25 times greater ($p < 0.05$) than AP uptake for cells treated with 5 and 25 μmol Zn/L, respectively. The rate of Zn release was enhanced about 4-fold ($p < 0.05$) by 25 μmol Zn/L treatment. Release to the BL side was 10 times greater than to the AP side. Zn-induced metallothionein (MT), thought to down-regulate AP to BL Zn transport, was 4-fold higher ($p < 0.001$) in the 25 μmol Zn/L group than in the 5 μM group, but the rate of BL Zn release was higher in cells treated with 25 μmol Zn/L than in those treated with 5 μmol Zn/L ($p < 0.05$). Induced changes in transport rates by media Zn concentrations could involve the up- and/or down-regulation of Zn influx and efflux proteins such as the ZIP and ZnT families of Zn transporters.

Keywords: Caco-2 cells; Zinc transport; Transport kinetics; Zinc uptake; Absorption; Trace elements

1. Introduction

Zn is an essential nutrient that acts as a cofactor for myriad enzymes in biological systems. In animals, the circulating concentration of Zn is dependent upon the dietary intake of Zn. Feeding Zn deficient diets for only a few hours quickly leads to low plasma Zn concentration, and if the deficient diet is continued for a few days or weeks, organ Zn concentrations begin to fall [1,2]. Thus, an available supply of dietary Zn and an efficient rate of absorption from the gut are essential for maintaining normal body Zn status.

Although numerous attempts have been made to determine the mechanism of Zn absorption from the gut, none has clearly defined the process [3–6]. It is known, however, that at least one component of the absorption might be regulated, i.e., the higher the amount of dietary Zn intake the lower the fractional rate of absorption and vice versa [7,8]. The process also seems to be adaptable because the amount of Zn in the mucosal cells and in the blood is kept at near normal concentrations following continuous consumption of high dietary Zn [9]. To further assess this adaptative response of the enterocyte to high Zn concentrations, we have used a cell culture model of the enterocyte, the Caco-2 cell. This model exhibits similar structural and functional characteristics of the enterocytes, including polarity and the induction of specific enzymes during differentiation. Here we show that the exposure of confluent, differentiated cells to high, but physiologic concentrations of media Zn for 7 d, reduces the rate of apical uptake and transport of Zn.

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2. Materials and methods

Caco-2 cells were obtained from the American Type Culture Collection, Rockville, MD, at passage 17. Falcon polyethylene terephthalate high-density (PET-HD; 1.0×10^8 pores/cm^2, 0.45 μm pore size) membrane inserts were purchased from Becton-Dickinson Labware, Lincoln Park, NJ. Cell culture media and chemicals were obtained from Sigma Chemical Co., St. Louis, MO, and/or Gibco Laboratories, Grand Island, NY. Radioactive Zn (^{65}Zn; Specific Activity, 9.6 MBq/μg Zn) was purchased from NEN® Life Science Products, Inc., Boston, MA. Non-radioactive Zn was added to the cell media as a solution of $ZnSO_4 \cdot 7H_2O$.

2.1. Cell culture procedures

Cells between passages 26 to 37 were seeded (58,000 cell/cm^2) onto PET-HD membrane inserts that hung inside the chambers of six-well plates. One and one-half mL of growth medium were placed inside the insert (apical side; AP) and 2.5 mL were placed in each chamber (basolateral side; BL). The growth medium was composed of Dulbecco's modified Eagle's medium (DME) to which 10% FBS, 25 μmol glucose/L, 4 mmol glutamine/L, and 0.1 mmol nonessential amino acids/L were added. The medium was replaced at 2-day intervals throughout the growth, differentiation, and experimental periods.

2.2. Time-dependent uptake and transport of ^{65}Zn

On the day of the experiment (d 21), 1.5 mL of fresh medium were placed in each insert and 2.5 mL in each chamber. After equilibration for 4 h, the experiment began. To determine uptake and transport in the AP to BL direction, an additional 0.1 mL of medium containing 14 kBq of ^{65}Zn was added to the inserts. When studying uptake and transport in the BL to AP direction, the 0.1 mL of labeled medium was added to the chamber. Both AP and BL media contained 5 or 25 μmol Zn/L. All labeling procedures were carried out at 37°C with the plates on a plate warmer. For long intervals ($>$5 min), the labeled plates were returned to the incubators; for short intervals ($\leq$5 min), the plates remained on the warmer.

After incubation with ^{65}Zn for prescribed intervals, the medium was aspirated and each insert was put through a series of ice-cold washes to remove residual ^{65}Zn. These washes consisted of 5s in HEPES buffer (10 mM HEPES, 140 mM NaCl, 7 mM KCl, and 5.6 mM glucose, pH 7.4), 5s in EDTA-HEPES (10 mM EDTA in HEPES buffer), and again for 5s in HEPES buffer. Excess buffer was aspirated from the inserts and the membranes were cut out, placed individually in small tubes and assayed for radioactivity by using a gamma counter (Packard Instruments, Cobra Auto-Gamma, Meriden, CT). After the cells were assayed for ^{65}Zn, they were solubilized in a solution (2 mL/filter) containing 200 mmol NaOH and 7 mmol sodium dodecylsulfate (NaOH-SDS)/L. Cellular protein was determined by the bicinchoninic acid method (Sigma Chemical Co., St. Louis, MO). Counts per minute were converted to pmol of Zn based on the specific activity of the original medium, and expressed as uptake in pmol/mg cellular protein. Medium remaining in each chamber was quantitatively pipetted into small tubes and assayed for ^{65}Zn. These values were used to determine the amount of Zn traversing the cell monolayer, and were expressed as pmoles of Zn/mg cellular protein.

2.3. Time-dependent release of ^{65}Zn

On the 20th day after seeding, the growth medium was replaced with a similar medium containing 37 kBq of ^{65}Zn/mL. Twenty-four h later, the inserts were removed, washed for 10 s in warm 2% FBS/98% DME, and placed in new chambers. Two and ½ mL of DME-FBS without ^{65}Zn were placed in each chamber and 1.5 mL in each insert. The plates were incubated at 37°C. At prescribed intervals, the inserts were removed, the AP media were quantitatively transferred to individual counting tubes, and the inserts were washed and treated as described above. The BL media were quantitatively transferred to separate counting tubes. Both AP and BL media were assayed for ^{65}Zn and the values were used to calculate the amount of Zn released from the cells with time. Total cellular Zn was determined as described below, and the values were used to calculate the specific activity of cellular Zn at the time efflux began. The values were expressed as pmoles of Zn/mg of cellular protein.

2.4. Concentration-dependent uptake and transport of ^{65}Zn

The preparation for this portion of the study was similar to that for the time course of ^{65}Zn uptake and transport, except that the AP or BL media contained a series of added Zn concentrations ranging from 5 to 140 μM. Thirty-seven kBq of ^{65}Zn/mL of either AP or BL media were added to each insert at each Zn concentration. In this case, the specific activity of ^{65}Zn decreased as the amount of unlabeled Zn increased. However, because the amount of ^{65}Zn taken up and/or transported by the cell increased as the Zn concentrations increased, there was enough ^{65}Zn to be efficiently detected at all points. Fifteen min after adding the radioactive Zn, the cells were harvested as described above and the amounts of ^{65}Zn entering the cells and crossing the cell monolayer were determined. The values were expressed as pmoles of Zn/min/mg cellular protein.

2.5. Pretreatment of cells with Zn

The effect of media Zn concentration on the rate of Zn uptake and transport during the differentiation phase was determined. The cell preparations were similar to those described above, except that from d 14 to 21 after seeding,

the cells were incubated in media containing 5 or 25 μmol Zn/L on both sides of the cell layer.

2.6. Zn assay in cells and culture media

Zn concentrations in the media were determined by inductively coupled argon plasma (ICAP) procedures after the samples had been diluted appropriately with deionized water. When cellular Zn was determined, aliquots of cells were placed in HNO_3 (3.2 mol/L) in mineral-free polypropylene tubes, capped, and digested overnight in a waterbath at 37°C. Samples were diluted appropriately and analyzed for Zn by ICAP. Commercially available internal and external standards of Zn were analyzed simultaneously with each set of samples to ensure adequate quality controls (Sigma Chemical, St. Louis, MO).

2.7. Metallothionein assay in cells

Metallothionein (MT) was determined with a modified method of Eaton and Cherian [10], which depends on the high affinity binding of radiolabeled cadmium. Cells were grown to confluence and treated with Zn as described above. Cells were then scraped from the inserts into 1.0 mL of a solution containing 10 mmol Tris-HCl/L, pH 7.4, and sonicated. Cell sonicates were heated at 95°C, placed in an ice bath to cool, and centrifuged at $3{,}000 \times g$ for 5 min. From this point, the procedures of Eaton and Cherian [10] were followed except that 1.78 μmol ^{109}Cd/L instead of 17.8 μmol/L as recommended, to account for the very low abundance of MT in the cell prep. In addition, 1 mmol 2-mercaptoethanol/L was used in the working solution.

2.8. Statistical analysis

At least 6 replicates were used for each time interval and each concentration. Because the variability of replicate experiments can vary between cells of different ages, we maintained the cell passage numbers in a narrow range between 26 to 37 for all experiments.

Empirical models were used to analyze the progression of Zn influx and efflux over time [11–13]. The linear and nonlinear regression procedures in SAS/STAT (SAS Institute, Cary, NC) were used to estimate the coefficients for all models. Multiple regression techniques were used to test whether the estimated values of the coefficients in the models were significantly different ($p < 0.05$) between the different concentrations of Zn in the media or if a single set of coefficients could be used to model the data for both experimental concentrations of Zn. For the nonlinear models, the standard errors of the regression coefficients were expressed as asymptotic approximations. The amounts of label taken up, released or transported at 160 minutes, and the concentrations of Zn and MT in Caco-2 cells were statistically analyzed by using Student's t-test. The SigmaPlot computer program (SPSS Inc., Chicago, IL) was used to generate the graphs, where individual points are means ± SEM, $n = 6$. In some cases, the error bars are hidden by the plot symbols.

2.9. Data analyses

2.9.1. Uptake studies

The data for the time-dependent uptake of ^{65}Zn during 160 min of exposure were modeled by using the exponential equation:

$$U = U_{eq}(1 - e^{-k_{Ueq}t}) + k_u t, \tag{1}$$

where U_{eq} represents the equilibrium uptake values achieved with the rate constant k_{Ueq}, over time, t. The rate constant k_U, represents the uptake rate of a slow component. Theoretically, this slower component would be better approximated by using a second exponential term. However, the coefficients of a second exponential, including the equilibrium value, could not be adequately estimated over the 160 min time course of these studies, thus a linear approximation to the initial uptake was used.

2.9.2. Transport studies

The data for the time-dependent transport of ^{65}Zn across the monolayer, AP to BL, during 160 min of exposure was analyzed by fitting the data to a logistic equation:

$$T_{AP\rightarrow BL} = \frac{T_{M(AP\rightarrow BL)}}{1 + e^{(M_T - K_T t)}}, \tag{2}$$

where T_M represents the maximal amount of zinc transferred across the monolayer, k_T is a first-order rate constant, and M_T is a scaling parameter that, along with k_T, defines the time at which the maximal rate of zinc transfer occurs.

Zn transport from BL to AP was analyzed by fitting the data to an exponential equation:

$$T_{BL\rightarrow AP} = T_0 + T_{Pool}e^{kt}, \tag{3}$$

where T_{pool} is the size of the pool that doubles at the rate of $\ln(2)/k$, and $T_0 + T_{pool}$ represents the nearly instantaneous amount of zinc transported at $t = 0$.

2.9.3. Kinetic studies

A three-parameter rectangular hyperbola model was used to analyze the concentration-dependent uptake of Zn from the BL side of the cell layer. This model takes the form:

$$J_U = \frac{J_{UMax}[Zn]}{K_U + [Zn]} + k_d[Zn], \tag{4}$$

where $[Zn]$ represents the concentration of ^{65}Zn in the media, J_{UMax} is the maximal rate of Zn uptake (pmol/min/mg protein), K_U is the media concentration at the half-maximal rate of uptake (μmol/L), and k_d is a first-order rate constant for what is considered to be a non-saturable or diffusional component of uptake (μL/min/mg protein). For

this experiment, two concentrations of Zn were used in the medium, 5 and 25 μM. In cases where Zn uptake did not fit this model, the data were fitted to a simple linear model of the form:

$$J_U = K_d[Zn] + J_0, \quad (5)$$

where K_d is a first-order rate constant and J_0 represents the nearly instantaneous rate of uptake at [Zn] = 0.

2.9.4. Release studies

Data for the time-dependent release of Zn over 160 min were modeled by using the exponential equation:

$$R = R_{eq}(1 - e^{-k_{Req}t}) + k_R t \quad (6)$$

where R_{eq} represents the equilibrium uptake or release values achieved with the rate constant k_{Req}, over time, t. The rate constant k_R, represents the initial release rate of a slow component. As in Equation 1, this slower component would be better approximated by using a second exponential term. However, the coefficients of a second exponential, including the equilibrium value, could not be adequately estimated over the 160 min time course of these studies, thus a linear approximation to the initial uptake was used.

2.10. Definition of terms used and assumptions about the experimental system

We used the term "uptake" to define the movement of ^{65}Zn into the cell, the term "release" to define the movement of the label out of the cell and into the medium, and the term "transport" to define the movement of ^{65}Zn across the cell monolayer. However, because of the manner in which the experiments were conducted, these terms do not necessarily imply a net accumulation or loss of Zn. At the beginning of each labeling experiment, the system was in equilibrium with respect to unlabeled Zn. In some experiments, only a small amount of labeled Zn was added to the system, the system was unperturbed, and the tracer followed linear system kinetics [14]. Because there was minimal recycling of the tracer over the short experimental time-frame, the rate of movement of the tracer into and out of the cell was regarded as an actual measure of the behavior of Zn itself.

3. Results

3.1. Time-dependent uptake of ^{65}Zn

Cells were exposed to 5 μmol or 25 μmol Zn/L from d 14 to 21, then time-dependent ^{65}Zn uptakes into the cell from the AP (Fig. 1A) or BL (Fig. 1B) media were determined. The data for both AP and BL uptakes were fitted to the non-linear model shown in Equation 1. The response of cells to Zn uptake from the AP side, during the 160 min time-frame, indicated that there were at least two pools, one

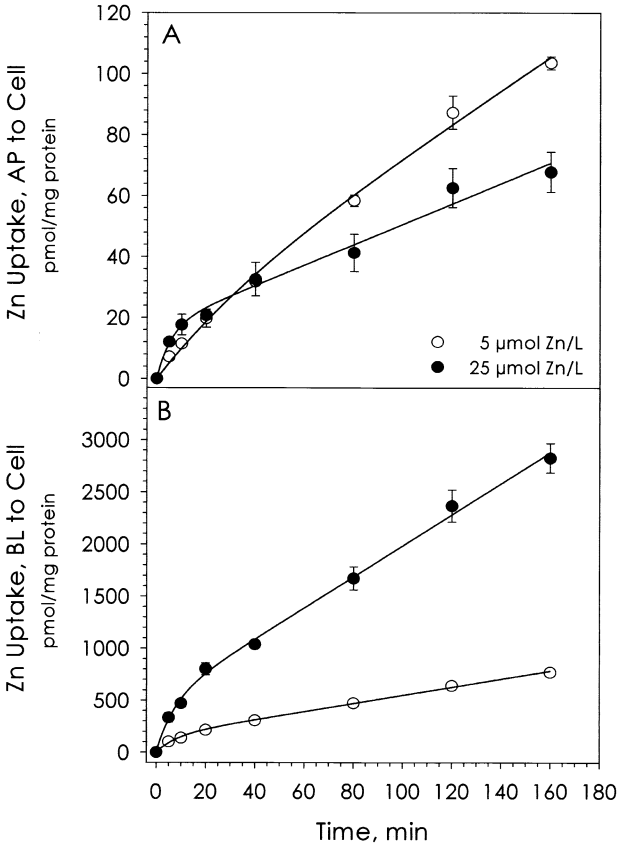

Fig. 1. Media Zn concentration affects the AP (Panel A) and BL (Panel B) uptake of ^{65}Zn into Caco-2 cells. Values are means ± SEM, n = 6. Cells were cultured for 7 days in media containing either 5 or 25 μmol Zn/L. Apical or BL media were then labeled with ^{65}Zn without changing the total concentration, and the amount of label taken up into the cell monolayer with time was determined. The data were fitted to the model in Equation 1, and the coefficients generated are shown in Table 1.

labeled exponentially and the other linearly; however, the shape of the curves differed between media Zn treatments. The fast labeling pool probably represented the initial binding of Zn to ligands on the surface of the AP membrane. The first-order rate constant, k_{Ueq}, for this pool was about 8 times higher for cells treated with 25 μmol Zn/L than for those treated with 5 μmol Zn/L (Table 1). This indicates that the treatment with high media Zn initiated a very high rate of labeling of this pool. The half-time ($\ln(2)/k_{Ueq}$) to equilibrium was only 4 min for cells treated with 25 μmol Zn/L, but greater than 30 min for those treated with 5 μmol Zn/L. However, the pool size (U_{eq}) at equilibrium was not different between the two treatments. The pool that equilibrated at a slower, linear rate probably represented one or more internal cellular ligands. Here we see that the rate constant for cells treated with 25 μmol Zn/L was only about 65% ($p < 0.05$) of the rate in cells treated with 5 μmol Zn/L (Fig. 1A and Table 1).

The Zn uptake response of cells from the BL side also indicated that at least two pools were present (Fig. 1B). However, the effects of Zn treatment were quite different

Table 1
Estimates of coefficients describing the time-dependent uptake of ^{65}Zn into a monolayer of Caco-2 cells from apical (AP) or basolateral (BL) sides over 160 min exposure (Fig. 1)*

Uptake *(Treatment)*	U_{eq}	k_{Ueq}	K_U
AP→Cell†	*pmol/mg protein*	*per min*	*pmol/min/mg protein*
5 μmol Zn/L	22.4 ± 11.6	0.022 ± 0.012[b,‡]	0.521 ± 0.069[a]
25 μmol Zn/L	17.0 ± 2.2	0.174 ± 0.067[a]	0.335 ± 0.021[b]
BL→Cell			
5 μmol Zn/L	153.8 ± 12.0[b]	0.120 ± 0.023	3.92 ± 0.11[b]
25 μmol Zn/L	489.4 ± 46.6[a]	0.134 ± 0.034	14.91 ± 0.44[a]

* The description of each coefficient is given in the text.
† Because the data were fitted to a nonlinear model (Eq. 1), the standard errors for coefficients are asymptotic approximations.
‡ Coefficients with different superscripts within an uptake category are significantly different, $p < 0.05$.

from those for AP uptake. The first-order rate constant (k_{Ueq}) for the fast labeling pool was not different between cells treated with 25 μmol Zn/L and those treated with 5 μmol Zn/L (Table 1). Even though treatment with high-Zn media did not affect the labeling rate of this pool, the pool size appeared to be increased 3-fold. On the other hand, the labeling rate of the slower, linear pool was more than 3.5 times faster in cells treated with 25 μmol Zn/L than in those treated with 5 μmol Zn/L (Fig. 1B and Table 1).

3.2. Time-dependent transport of ^{65}Zn

Time-dependent transport of ^{65}Zn across the monolayer from AP to BA (Fig. 2A) was fitted to the model shown in Equation 2. The maximal transport ($T_{M(AP\rightarrow BL)}$) of Zn during the 160 min time course was significantly ($p < 0.05$) lower in cells treated with 25 μM zinc than in those treated with 5 μmol Zn/L (Table 2). The rate constant (k_T) was not significantly ($p < 0.05$) different between the two treatments. At 160 min, the amount of Zn transported to the BL side by cells treated with 5 μM zinc was about triple that for cells treated with the higher amount of Zn, and the difference was significant ($p < 0.001$).

The level of Zn treatment affected ^{65}Zn transport in the BL to AP direction differently than it did in the AP to BL direction (Fig. 2B). Within the limited time-frame, the data fit the model in Equation 3. We found that the rate constant (k) was significantly ($p < 0.05$) higher in cells treated with 5 μmol Zn/L than in cells treated with 25 μmol Zn/L (Table 2). At 160 min, the amount of Zn transported from the BL to the AP side was about 2-fold higher in cells treated with high Zn. At this period, cells treated with 25 μmol Zn/L seemed to transport similar amounts of Zn whether from AP to BL or from BL to AP. On the other hand, cells treated with 5 μmol Zn/L transported about 4 times more Zn from AP to BL than from BL to AP.

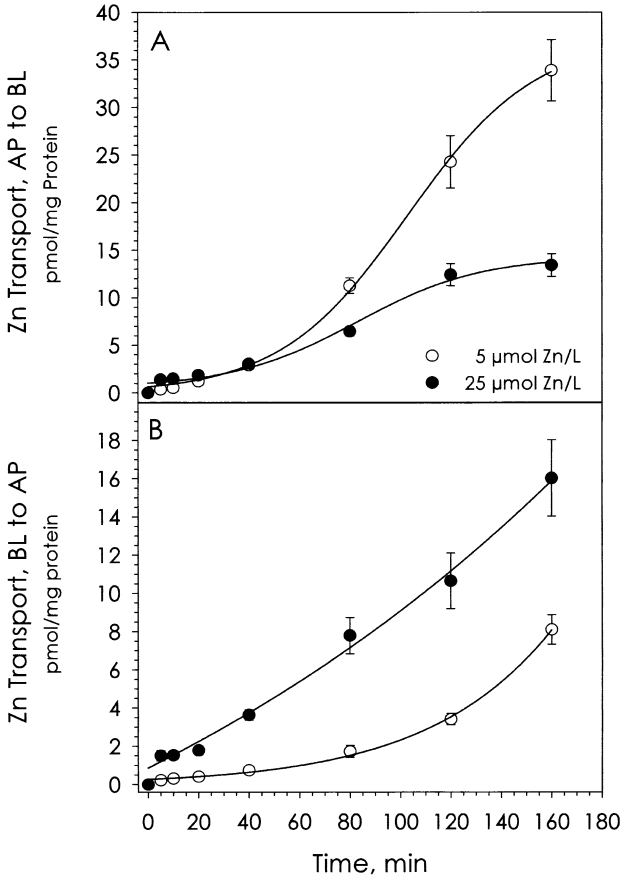

Fig. 2. Media Zn concentration affects AP to BL or BL to AP transport of ^{65}Zn into Caco-2 cells. Values are means ± SEM, n = 6. Cells were cultured for 7 days in media containing either 5 or 25 μmol Zn/L. Apical or BL media were then labeled with ^{65}Zn without changing the total concentration, and the amount of label transported across the cell monolayer with time was determined. The data for AP to BL transport (Panel A) were fitted to the nonlinear model in Equation 2, and the coefficients generated are shown in Table 2. Data for BL to AP transport (Panel B) were fitted to the nonlinear model in Equation 3. The coefficients are shown in Table 2.

3.3. Concentration-dependent uptake and transport of ^{65}Zn

Data for concentration-dependent uptake of ^{65}Zn from the AP media did not follow standard saturation kinetics, thus they were fitted to the linear model in Equation 5, where k_d is the rate constant and J_0 is the Y-axis intercept. Whether cells were pre-treated with 5 or 25 μmol Zn/L, the rates of uptake were elevated with increasing concentrations of assay Zn. However, Zn uptake by cells pre-treated with 25 μmol Zn/L was significantly ($p < 0.05$) lower than uptake in cells pre-treated with 5 μmol Zn/L (Fig. 3A and Table 3).

Data for concentration-dependent ^{65}Zn uptake from the BL media into the cells followed saturation kinetics, and the data were fitted to a 3-parameter hyperbolic model as shown in Equation 4. The maximal rate of uptake was about 30%

Table 2
Estimates of coefficients describing the time-dependent transport of ^{65}Zn across a monolayer of Caco-2 cells from apical (AP) or basolateral (BL) or from BL to AP over 160 min exposure (Fig. 2)*

Transport *(Treatment)*	$T_{eq(AP\rightarrow BL)}$	k_T	k
AP→BL†	*pmol/mg protein*	*per min*	*per min*
5 μmol Zn/L	37.5 ± 1.4[a,‡]	0.039 ± 0.003	—
25 μmol Zn/L	14.9 ± 0.6[b]	0.033 ± 0.002	—
BL→AP¶			
5 μmol Zn/L	—	—	0.020 ± 0.001[a]
25 μmol Zn/L	—	—	0.004 ± 0.002[b]

* The description of each coefficient is given in the text.
† Because the data were fitted to a nonlinear model (Eq. 2), the standard errors for coefficients are asymptotic approximations.
‡ Coefficients with different superscripts within a transport category are significantly different, $p < 0.05$.
¶ Because the data were fitted to a nonlinear model (Eq. 3), the standard errors for coefficients are asymptotic approximations.

higher in cells treated with 25 μmol Zn/L than in cells treated with 5 μmol Zn/L (Fig. 3B and Table 3). The concentration of media Zn that it took to reach half-maximal rate was ~50% higher in cells treated with high-Zn. The linear component of the curve, which we regard as an expression of simple diffusion, was not different ($p > 0.05$) between the two Zn treatments.

The concentration-dependent transport rates of Zn across the cell monolayer from either the AP or the BL side were linear through the Zn concentrations used. There was no difference in AP transport rate between cells treated with 5 or 25 μmol Zn/L (Fig. 4A and Table 4). However, the transport rate from BL was significantly higher in cells treated with 25 μmol Zn/L than 5 μmol Zn/L (Fig. 4B and Table 4).

3.4. Time-dependent release of ^{65}Zn from cells

Caco-2 cells were labeled with ^{65}Zn and the amount released to either side of the cell layer was measured. The equilibration of the initial pool of ^{65}Zn released to the AP side was extremely fast, with 50 to 70% of that maximally released during the 160 min time-frame already released at 5 min (Fig. 5A). The *Y* axis intercepts estimate the values for maximal equilibration (R_{eq}) during the rapid phase of release. The R_{eq} of this pool, in cells treated for 7 days with 25 μmol Zn/L, was about 3-fold higher ($p < 0.05$) than in cells treated with only 5 μmol Zn/L. In addition, the high-Zn treatment elevated ($p < 0.05$) the rate of release (k_R) between 5 and 160 min compared with that in cells treated with 5 μmol Zn/L (Fig. 5A; Table 5).

To analyze the effect of Zn treatment on time-dependent Zn release to the BL side, an exponential model was used, as shown in Equation 6. The results showed that Zn release was composed of two discernible pools at both concentrations of Zn (Fig. 5B). Maximal release (R_{eq}) for the rapid phase was about 5 times greater in cells treated with 25

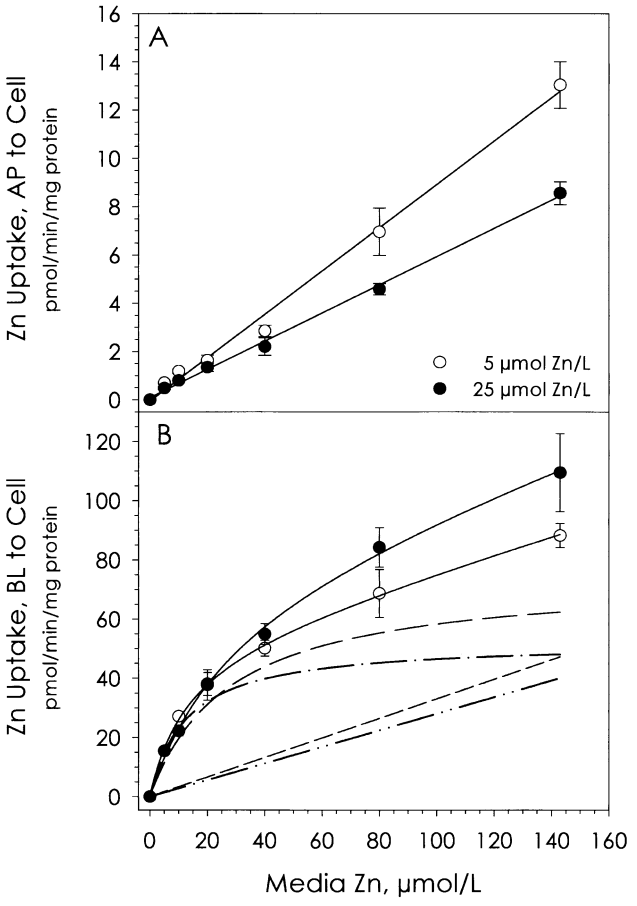

Fig. 3. Concentration dependent uptake of Zn is affected by pretreating cells with variable amount of Zn in the media. Values are means ± SEM, $n = 6$. Confluent cells were cultured for 7 days in media containing either 5 or 25 μmol Zn/L. Cells were then incubated for 15 min in fresh media contain variable amounts of ^{65}Zn. Cells were washed and the amount of ^{65}Zn taken into the cell layer was determined. Uptake of Zn from the AP was linear over the range of Zn concentrations used. The rate constant (K_d) was slightly but significantly ($p < 0.05$) higher in cells treated with 5 μmol Zn/L than with 25 μmol Zn/L (Table 3). Data for uptake from the BL (Panel B) were fitted to a three-parameter hyperbolic model shown in Equation 3, and the coefficients are listed in Table 3. In this case, the maximal rate of uptake (J_{Umax}) was about 40% higher in cells treated with 25 μmol Zn/L (long dash) than those treated with 5 μmol Zn/L (dash-dot). The media Zn concentration at which the rate reached 50% of maximal was over two time higher in the cells treated with the higher amount of Zn. The diffusional component for 5 μmol Zn/L (dash-dot-dot) was not different from that for 25 μmol Zn/L (short dash).

μmol Zn/L than in those treated with 5 μmol Zn/L ($p < 0.05$) (Table 5). On the other hand, the high-Zn treatment had no significant effect on the exponential rate constant (k_{Req}) ($p > 0.05$). The rate constant (k_R) of the linear component was about 3-fold higher in cells treated with 25 μmol Zn/L ($p < 0.05$) than those treated with 5 μmol Zn/L. At 160 min, the amount of label released to the BL side was about 10 times greater than the amount released to the AP side ($p < 0.001$) (Fig. 5A & 5B). The total amount of ^{65}Zn released over 160 min was only 35% of that in the cell at 0 time.

Table 3
Estimates of coefficients describing the concentration-dependent uptake of ^{65}Zn into a monolayer of Caco-2 cells from apical (AP) or basolateral (BL) sides over 15 min exposure (Fig. 3)*

Uptake *(Treatment)*	$J_{Max,\ U}$	K_U	K_d
AP→Cell†	*pmol/min/mg Protein*	*μmol Zn/L*	*μL/min/mg Protein*
5 μmol Zn/L	—	—	0.090 ± 0.004[a,‡]
25 μmol Zn/L	—	—	0.058 ± 0.001[b]
BL→Cell¶			
5 μmol Zn/L	52.3 ± 5.9[b]	12.6 ± 2.9[b]	0.283 ± 0.039
25 μmol Zn/L	74.5 ± 18.9[a]	27.9 ± 10.2[a]	0.334 ± 0.095

* The description of each coefficient is given in the text.
† Data for AP→Cell were fitted to a linear model (Eq. 5).
‡ Coefficients with different superscripts within an uptake category are significantly different, $p < 0.05$.
¶ Because the data for BL→Cell were fitted to a nonlinear model (Eq. 4), the standard errors for coefficients are asymptotic approximations.

The concentrations of Zn and MT in Caco-2 cells were affected by Zn treatment and time of exposure (Table 6). The cells were incubated in media containing 5 or 25 μmol Zn/L for 60 min or 7 d (d 14 to d 21). Cells were then harvested and assayed for Zn and MT content. The results showed that exposing cells to 25 μmol Zn/L for only 60 min had no effect on cell Zn concentration or MT production. However, when cells were treated for 7 d with 25 μmol Zn/L, the amount of cellular Zn increased significantly ($p < 0.001$), and MT concentration was 4-fold higher in cells treated with 25 μmol Zn/L than 5 μmol/L. If we assume that 7 atoms of Zn are bound to 1 mol of MT, this increase in MT only accounted for about 17% of the increase in Zn.

4. Discussion

It is the general conclusion that at least part of the maintenance of Zn homeostasis during bouts of Zn depletion or excess is through regulation of the rate of Zn absorption. For example, Flanagan et al. [15] found that when endogenous Zn concentrations in the small intestine were controlled, the rate of Zn absorption in rats was increased during Zn deficiency. Coyle et al. [16] found similar responses in mice. The amount of Zn absorbed also can be controlled by the amount of Zn administered. Payton et al. [8] showed that human subjects absorbed 56% of a 6 mg dose but only 25% of a 60 mg dose. However, this manner of expressing absorption can be misleading. If the percentages are converted to actual amounts of Zn absorbed, it becomes obvious that subjects receiving the higher dose absorbed more than 4 times the total amount of Zn as those receiving the smaller dose. When the actual amounts of absorbed Zn are plotted against dose, a hyperbolic relationship is revealed [7,8]. This suggests that the absorption mechanism in the gut is saturable, and that the rate of Zn absorption in humans is regulated at the mucosal level.

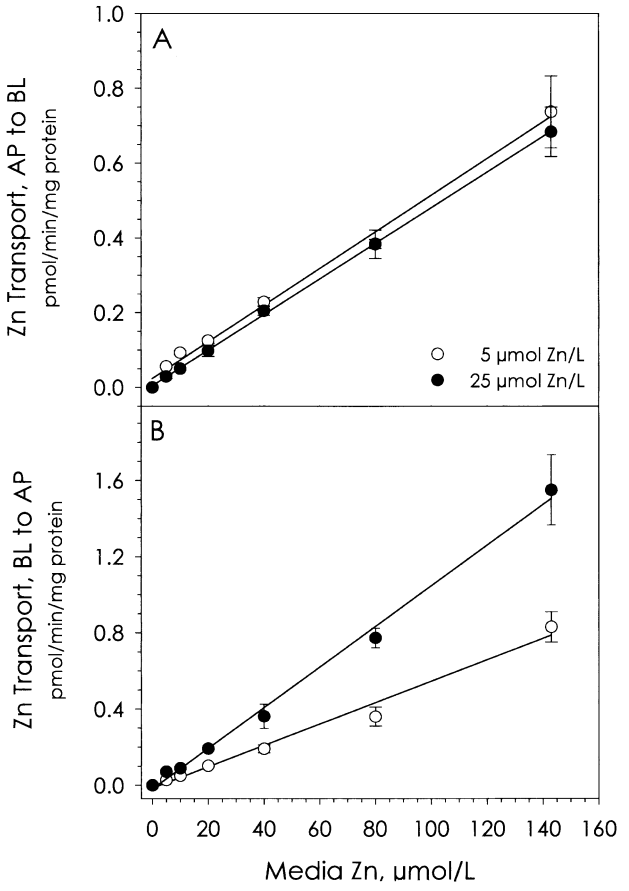

Fig. 4. Concentration dependent transport of Zn was affected by pretreating cells with variable amount of Zn in the media. Values are means ± SEM, $n = 6$. Confluent cells were cultured for 7 days in media containing either 5 or 25 μmol Zn/L. Cells were then incubated for 15 min in fresh media contain variable amounts of ^{65}Zn and the amount transported across the cell monolayer in either direction was determined. Transport of Zn from the AP to BL (Panel A) was linear over the range of Zn concentrations used, and the rate constant (K_d) was not affected by Zn treatment. Transport of Zn from the BL to AP (Panel B) also was linear, but the rate constant was significantly ($p < 0.05$) greater in cells treated with 25 μmol Zn/L than with 5 μmol/L (Table 4).

Table 4
Estimates of coefficients describing the concentration-dependent transport of ^{65}Zn into a monolayer of Caco-2 cells from apical (AP) or basolateral (BL) sides over 15 min exposure (Fig. 4)*

Transport *(Treatment)*	K_d
AP→BL†	*μL/min/mg Protein*
5 μmol Zn/L	0.005 ± 0.001
25 μmol Zn/L	0.005 ± 0.001
BL→AP	
5 μmol Zn/L	0.006 ± 0.001[b,‡]
25 μmol Zn/L	0.011 ± 0.001[a]

* The description of the coefficient is given in the text.
† Data were fitted to a linear equation (Eq. 5).
‡ Different superscripts within a transport category are significantly different, $p < 0.05$.

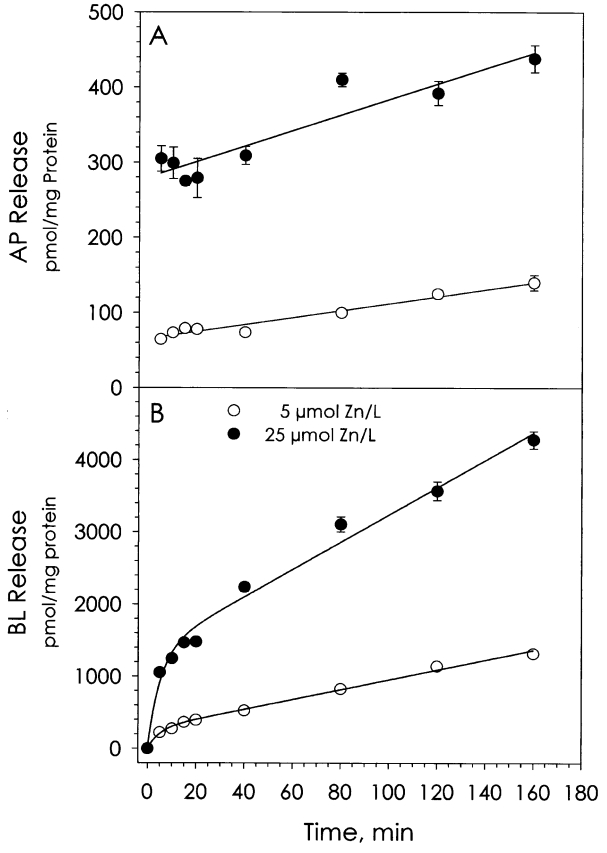

Fig. 5. Time-dependent release of Zn from cells was affected by pretreating the cells with different amounts of media Zn. Values are means ± SEM, $n = 6$. Confluent cells were cultured for 7 days during differentiation in media containing either 5 or 25 μmol Zn/L. Cell were then incubated for 21 h with ^{65}Zn in complete media without changing the total Zn concentration. Cells were washed and placed into fresh media, and the amount of label released from the cell monolayer over time was determined. The rate constant for ^{65}Zn release to the AP side of the cell layer was about 2.5 times higher in cells treated with 25 μmol Zn/L than in those treated with 5 μmol Zn/L, but the total amount released was about 4 times higher in cells treated with the higher amount of Zn. Time-dependent Zn release to the BL side followed an exponential model (Eq. 5). The rate constant (k_{Req}) for release of ^{65}Zn to the BL side was not affected by Zn treatment (Table 5). However, the pool size (R_{eq}) was significantly ($p < 0.05$) greater in cells treated for 7 days with 25 μmol Zn/L than with 5 μmol Zn/L. In addition, the rate constant (K_R) for the linear, slow filling pool was significantly ($p < 0.05$) higher in cells treated with 25 μmol Zn/L than with 5 μmol.

Because it is difficult to control various parameters in the intestinal system of the whole animal, simpler, in vitro systems have been developed to define specific mechanisms of Zn uptake and transport. These systems include brush border and BL membrane vesicles [3,17–19], primary cultures of isolated enterocytes [20], and enterocyte mimics such as Caco-2 cells derived from a human colon adenocarcinoma [4,6]. The rate of Zn uptake in intestinal cell brush border preparations of Zn deficient rats was higher than that of cells derived from Zn adequate rats [3]. Although Raffaniello et al. [4] and Finley et al. [6] found that Zn uptake was a saturable process, and that both uptake and transport through AP and BL membranes could be studied simultaneously, they did not determine the effects of Zn deficiency or Zn excess on the rates of Zn movement into and across the cells. The present series of studies are the first to show the effects of Zn treatment on Zn uptake and transport in this cell type.

The procedures we used were somewhat different than those used by other investigators. In the past, there have been concerns that when using radioactive Zn, the specific activity of the Zn taken up or transported may not be the same as that in the cell; thus erroneous values could be derived. We designed our procedures so that the conditions during uptake-transport were as close as possible to the conditions during cell growth and differentiation. This included a complete medium with 10% fetal bovine serum. Others, with the exception of Finley et al. [6], grow their cells in complete media, but then run the uptake-transport procedures in a serum-free, balanced salt medium.

By using the alternative approach, we demonstrated conclusively that treating Caco-2 cells with higher than normal,

Table 5
Estimates of coefficients describing the time-dependent release of ^{65}Zn from a monolayer of Caco-2 cells to the apical (AP) or basolateral (BL) sides over 160 min exposure, after the cells had been preloaded with labeled Zn for 24 h (Fig. 5)*

Release *(Treatment)*	R_{eq}	k_{Req}	K_R
Cell→AP[†,‡]	*pmol/mg protein*	*per min*	*pmol/min/mg protein*
5 μmol Zn/L	69.6 ± 3.1[b,¶]	—	0.37 ± 0.04[b]
25 μmol Zn/L	278.7 ± 8.1[a]	—	0.90 ± 0.10[a]
Cell→BL[§]			
5 μmol Zn/L	277.5 ± 23.3[b]	0.18 ± 0.05	6.28 ± 0.23[b]
25 μmol Zn/L	1366.7 ± 67.3[a]	0.18 ± 0.03	17.32 ± 0.67[a]

* The description of each coefficient is given in the text.
† Data for Cell→AP were fitted to a linear model (Not shown).
‡ R_{eq} values for Cell→AP are Y axis intercepts (Fig. 5A).
¶ Coefficients with different superscripts within a release category are significantly different, $p < 0.05$.
§ Because the data for Cell→BL were fitted to a nonlinear model (Eq. 6), the standard errors for coefficients are asymptotic approximations.

Table 6
Zn and metallothionein (MT) concentration in Caco-2 cells with short-term (60 min) or long-term (7 d) treatments in media containing either 5 or 25 μmol Zn/L*

Media Zn	Cellular Zn		Cellular MT	
	60 min	7 d	60 min	7 d
μmol/L		*nmol/mg protein*		
5	2.90 ± 0.40	3.89 ± 0.56[b,†]	0.067 ± 0.008	0.064 ± 0.013[b]
25	3.00 ± 0.39	12.71 ± 1.22[a]	0.070 ± 0.008	0.277 ± 0.013[a]

* Values are means ± SD, $n = 12$.
† Column means with different superscripts are significantly different ($p < 0.01$) as determined by Student's t-test. On a molar basis, there was 6 times more Zn in the cell layer than could be accounted for by the increase in MT, assuming 7 atoms of Zn per mole of MT.

but still physiological concentrations of media Zn for a 7-d period during the differentiation phase, significantly decreased the rate of AP uptake and AP to BL transport of Zn across the cell layer. Concentration-dependent uptake of Zn from the AP side of the cell was linear at concentrations of media Zn ranging from 5 to 140 μM, and cells pre-treated with 25 μmol Zn/L for 7 days had lower uptake and transport rates than those treated with 5 μmol/L. One or more specific transporters probably control the uptake of Zn into the mucosal cell from the AP side. This might indicate that treatment with high Zn caused a down-regulation of transport by reducing transporter number, by reducing transporter affinity for Zn, or by affecting other mechanisms for moving the element across the apical membrane. Candidates for cellular uptake of Zn might include the ZIP family of Zn transporters [21,22] and/or DCT-1 [23,24]. However, recent evidence suggests that DCT-1 might not be involved in Zn transport in some systems [25,26]. The coordinated process of Zn transport across the cell layer might involve the ZnT family of transporters [24,27] and intracellular Zn-binding ligands such as MT and CRIP (cysteine-rich intestinal protein) [28,29].

The media composition in which uptake-transport studies are conducted can have a profound effect on outcomes. By using serum-free transport media, Raffaniello et al. [4] had shown previously that the uptake rate of Zn from the AP side was similar to that from the BL side. However, in our experiments where complete media were used, we observed that in non-Zn treated cells, the rate of uptake from the BL side was 6 times faster than from the AP side. Finley et al. [6] who also used a complete medium, showed that the rate of Zn uptake from the BL side of the cell was much higher than from the AP side. Raffaniello et al. [4] also showed that transport across the monolayer was twice as fast from BL to AP as from AP to BL. Our procedures showed the opposite effect, which was faster from the AP side than from the BL side.

In our studies, the concentration-dependent uptake data from the BL side of the cell fit a hyperbolic relationship quite well; however, treating the cells with high-Zn enhanced the rate of Zn uptake instead of lowering it as seen on the AP side. This suggests that Zn uptake on the BL side is regulated by a different factor, possibly a different type of Zn transporter that is up-regulated by high Zn. McMahon and Cousins [27] showed recently that the transporter protein ZnT-1 was localized in the BL membranes of rat duodenal and jejunal enterocytes, and was marginally up-regulated by high dietary Zn. However, this transporter is thought to aid in the release of Zn from the cell, and not involved in uptake. In that regard, our experiments showed that Zn release to both the AP and BL sides of the cell was enhanced in cells treated with 25 μmol Zn/L. This suggests that ZnT-1, or a different efflux protein, could have been up-regulated by this treatment. The rate of release to the BL side was about 18-fold higher than to the AP side.

Is cellular MT involved in the regulation of Zn uptake and transport in the enterocyte? The answer to this question has eluded investigators for many years. Some work suggests that low Zn status lowers intestinal MT, thus reducing the binding capacity of the enterocyte for Zn and allowing more to be absorbed [30]. Other work suggests that intestinal MT might enhance Zn absorption [31]. Recent investigations by Moltedo et al. [32] suggest that the rate of zinc transport in Caco-2 cells is related to the cellular production of MT and its secretion into the AP media. Differentiated cells treated for 20 h with 50 to 200 μM zinc had higher cellular concentrations of MT and secreted more MT into the AP media than cells treated with 5 μmol Zn/L. The rate of Zn transport across the monolayer was positively correlated with both the amount of cellular MT and MT secreted into the AP media. The authors theorized that intracellular and extracellular MT's are acting cooperatively to regulate Zn transport in the polarized enterocyte type cell.

We also observed increases in cellular and AP media concentrations of MT (latter data not shown) when differentiated Caco-2 cells were treated with 25 μmol Zn/L for 7 days. However, because we observed that the rate of Zn uptake into the cell and transport from AP to BL were reduced by this treatment, we can only conclude that elevated MT is not associated with an enhanced AP Zn transport rate, but rather, the opposite effect. But, please recall also that treating the cells with 25 μmol Zn/L for 7 days enhanced Zn uptake from the BL side of the cell layer, and as Moltedo et al. [32], we found that the amount of MT released to the BL side was much less than to the AP side (data not shown).

One of the differences between our approach and that of Moltedo et al. [32] was the length of time the cells were treated. They treated cells for only 20 h and our cells were treated for 7 days. The longer treatment could have allowed the cells to adapt to the elevated Zn environment in a manner that reduced the rate of AP Zn uptake and transport. Cells treated with 25 μmol Zn/L accumulated four times more Zn than those treated with 5 μmol Zn/L, but the amount of MT accumulated by treated cells over the same period accounted for only 17% of the increase in cellular Zn. This suggests that the cells down-regulated their capacity to produce MT, and that they developed another form of Zn storage, and possibly another mechanism to regulate Zn uptake and transport; perhaps a different Zn transporter. Relevant to this discussion, it has been shown that feeding high dietary Zn to rats for long periods down-regulates MT in the enterocyte [9]. Whether or not cellular MT has a direct effect on Zn uptake is still unknown. If so, it may act only as a transient factor that helps the cell cope with high Zn until a more stable and long-term factor(s) can be produced.

Finley et al. [6], Raffaniello et al. [4], and the present study showed that the concentration-dependent uptake of Zn from the AP side of the Caco-2 cell was linear or curvilinear up to at least 200 μM Zn. More than likely, a large part of the increase in Zn transport with an increase in AP Zn concentration observed by Moltedo et al. [32] was simply a

concentration dependent response with little added effect of MT. Nonetheless, when they transfected cells with the mouse MT-I gene and treated the cells with high media Zn, they observed a higher cellular MT concentration, more MT secreted into the AP media, and a higher Zn transport rate than in those without the extra copies of the mouse gene.

In summary, the present studies and those of others point up the complexities of Zn transport regulatory events even in a more simplified cell culture model than the gut itself. Treating Caco-2 cells with higher than normal, but physiological amounts of Zn in the media depressed the rates of AP Zn uptake into the cell and Zn transport across the monolayer. At the same time; however, the treatment enhanced these rates from the BL side of the cell. Although cellular MT concentration was induced during Zn treatment, it seems not to be directly correlated with Zn uptake by the cell. Our conclusion is that the Caco-2 cell is a good model to study the mechanism(s) of Zn transport regulation, and more research is needed to unravel the specific mechanisms that aid Zn uptake and transport in the intestinal cell, including the function of metal transport proteins.

Notes

1. Mention of a trademark or proprietary product does not constitute a guarantee of warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that also may be suitable.

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