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ScienceDirect
Nutrition Research 28 (2008) 406–413
Nutrition Research
www.nrjournal.com

Cooperation of metallothionein and zinc transporters for regulating zinc homeostasis in human intestinal Caco-2 cells

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Received 1 May 2007; received in revised form 20 December 2007; accepted 8 February 2008

Abstract

This investigation examined the effects of zinc status on cell proliferation and the synergic roles of the metallothionein (MT) and zinc transporter (ZnT) in the human colon adenocarcinoma cell line Caco-2. Cells were treated with 0 to 300 μmol/L $ZnSO_4$ or 0 to 10 μmol/L *N*,*N*,*N*′,*N*′-tetrakis (2-phridylmethyl) ethylenediamine (TPEN). Cell proliferation was determined by MTT assay and apoptotic cells detected by flow cytometry (Hoechst 33258 dye). mRNA expression of MT1; ZnT1; zrt, irt-like protein 1, 4 (ZIP1, 4); and divalent metal transporter (DMT1) were determined by the reverse transcription polymerase chain reaction or real-time polymerase chain reaction. The results showed that either high or low zinc could inhibit the cell proliferation. The number of apoptotic cells increased with incremental increases in the concentrations of $ZnSO_4$ and TPEN. The mRNA expression of ZnT1 and MT1 responded significantly after 6 and 12 hours with 200 μmol/L zinc treatment, respectively, and increased gradually with zinc levels from 0 to 200 μmol/L. Compared with the unchanged ZIP1 mRNA expression, ZIP4 was closely dependent on TPEN treatment duration and concentration. The DMT1 mRNA expression was upregulated time-dependently but not concentration-dependently in the late TPEN treatment duration. The results suggest that ZIP4 and DMT1 mRNA expressions are susceptible to low extracellular zinc concentration and upregulated to enhance zinc absorption, whereas the ZnT1 and MT1 act as the key regulators under high zinc conditions to enhance the intracellular zinc efflux to maintain zinc homeostasis. We propose that in response to variations in zinc concentration, the cooperated regulative roles of ZnT1, MT1, DMT1, and ZIP4 contribute to zinc homeostasis.

Keywords: Zinc; Zinc transporter; Metallothionein; Zrt, irt-like protein; Divalent metal transporter; Caco-2 cells

Abbreviations: AE, acrodermatitis enteropathica; ANOVA, analysis of variance; CDF, cation diffusion facilitator; DMT1, divalent metal transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MT, metallothionein; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide; PCR, polymerase chain reaction; PI, propidium iodide; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-phridylmethyl) ethylenediamine; ZIP, zrt, irt-like protein; ZnT, zinc transporter.

1. Introduction

Zinc is an essential trace element that plays a critical role in biochemical processes. Organisms must maintain adequate intracellular zinc concentration even when extracellular or dietary zinc levels are low. A well-recognized disease of zinc metabolism is acrodermatitis enteropathica (AE), a human genetic disorder [1]. This autosomal recessive disorder causes classical symptoms of zinc deficiency, such as dermatological lesions, gastric mucosa changes associated with digestive system problems, lack of weight gain, as well as immune system and reproductive problems [2-4]. Remarkably, these symptoms can be ameliorated by dietary

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doi:10.1016/j.nutres.2008.02.011

zinc supplementation [2,4,5]. Recent genetic mapping localized the *AE* gene to chromosome 8q24.3 and led to its identification as a member of the zrt, irt-like protein (ZIP) family [6]. The gene was named *ZIP4*, which was found to be expressed in enterocytes and to reside in the plasma membrane. In addition, mutations in ZIP4 were detected in AE patients [7,8].

Sufficient absorption of zinc from the small intestine is essential for the body, but a high level of zinc is deleterious and can lead to cytotoxicity. Therefore, maintaining an appropriate zinc level is of critical importance. Zinc homeostasis is coordinated via regulation by proteins involved in uptake, excretion, and intracellular storage or trafficking of zinc. These proteins are metallothioneins (MTs) and transmembrane transporters, which include ZIP and cation diffusion facilitator (CDF) families. Metallothioneins belong to a family of low molecular weight, cysteine-rich intracellular proteins that bind transition metals, including zinc and cadmium [9], and their biological roles include the detoxification of harmful metals and the homeostasis of essential metals [10]. The ZIP family plays prominent roles in zinc uptake and transport of zinc from outside the cell into the cytoplasm. ZIP transporters have also been found to mobilize stored zinc by transporting the metal from an intracellular compartment into the cytoplasm [11-13]. As a member of the ZIP family, ZIP1 plays a role in zinc uptake [14,15]. Recently, *ZIP1* was described as a tumor suppressor gene in prostate cancer [16-18].

Compared with ZIP1, the expression of ZIP4 is robust in tissues involved in nutrient uptake, such as the intestine and embryonic visceral yolk sac, and is dynamically regulated by zinc. Previous results provided compelling evidence that ZIP4 is a zinc transporter (ZnT) that plays an important role in zinc absorption, a process that is defective in AE [19-22]. The CDF family transports zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell or into the lumen of an organelle [23-25].

In mammalian cells, 10 homologous SLC30 proteins, named ZnT1 to ZnT10, have been described [26]. These proteins are members of the CDF family. ZnT1, a ubiquitous ZnT located in the plasma membrane, transports zinc out of cells, and its absence accounts for the increased sensitivity of mutant cells to zinc toxicity [27,28]. In addition, divalent metal transporter (DMT1) is located in the apical membrane of enterocytes and takes iron into enterocytes. It is predominantly an iron transporter, with lower affinity for other metals such as zinc [29,30]. Some data indicate that zinc transport is upregulated by DMT1 based on protein levels and mRNA expression [31].

Much attention has been given to the regulation of a single ZnT in previous studies. The intestine is the first tissue confronted with zinc. It is interesting whether zinc can initiate intestinal cell injury and how zinc homeostasis is maintained by MTs, CDF, and ZIP transporters when the extracellular zinc concentration is high or low. Therefore, we investigated in the human intestinal cell-line Caco-2 the effects of decreasing or increasing zinc levels on Caco-2 cell growth. Moreover, we determined the mRNA expression of MT1, ZnT1, DMT1, ZIP1, and ZIP4 from specific genes in response to the different extracellular zinc levels in Caco-2 cells.

2. Methods and materials

2.1. Cell culture

Caco-2 cells were obtained from the cell bank of the Institute of Biochemistry and Cell Biology in Shanghai. The cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C with a 5% CO_2 atmosphere. Caco-2 cells were used between passages 20 and 40 in the present study [32].

2.2. Proliferation assay of Caco-2 cells

Caco-2 (5×10^3 cells/mL) cells were incubated in 96-well plates in 200 μL of culture medium for 24 hours at 37°C and 5% CO_2 atmosphere. After the designed time, the culture medium was aspirated and cells were incubated with 0 to 300 μmol/L $ZnSO_4$ or 0 to 10 μmol/L *N*,*N*,*N*′,*N*′-tetrakis (2-phridylmethyl) ethylenediamine (TPEN) for 24 hours followed by a 4-hour pulse of 50 μg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) [33]. Color development at a wavelength of 570 nm was measured using an enzyme-linked immunosorbent assay reader (Bio-rad Inc, Hercules, CA).

2.3. Hoechst 33258 dying

Caco-2 cells were treated as described above. Typical nuclear condensation was used as a morphological marker of apoptosis and observed using Hoechst 33258 dye (Beyotime Inc, Jiangsu, China) [34].

2.4. Flow cytometry

Briefly, cells treated as described above were harvested, washed in phosphate-buffered saline, resuspended in prediluted binding buffer, and stained with annexin V/fluorescein iosthiocyanate for 15 minutes at room temperature, protected from light. Cells were then washed and resuspended in binding buffer. Propidium iodide (PI) was added, and cells were analyzed immediately by flow cytometry using ModFit 3.0 LTTM Software [35].

2.5. RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted from Caco-2 cells using Trizol (Invitrogen, Inc., Carlsbad, CA) according to the manufacture's protocol. Total RNA was reverse transcribed by random primer (TaKaRa Inc, Tokyo, Japan). Each cDNA sample was used as a template for a polymerase chain

Table 1
Primers for PCR

Gene name	Sequence		Product size (bp)
Human MT1	Forward	TCAACTTCTTGCTTGGGATC	319
	Reverse	ATACAGTAAATGGGTCAGGGTT	
Human ZnT1	Forward	CAATACCAGCAACTCCAACGG	352
	Reverse	GCAAGGACCAGCCTCATAAAC	
Human ZIP1	Forward	CCACTTGTCTCCTGGACCTGC	476
	Reverse	AGCCACCACCTGTGCCCTAA	
Human DMT1	Forward	CCGGAACAATAAGCAGGAAGTT	419
	Reverse	GGATGATGGCAATAGAGCGAGT	
Human GAPDH	Forward	GTGAAGGTCGGAGTCAACGG	226
	Reverse	CCTGGAAGATGGTGATGGGAT	

reaction (PCR) reaction that was performed according to the method described by the kit (TaKaRa Inc). The reactions of PCR amplification were heated to 95°C for 3 minutes and immediately cycled 30 times through a 30-second denaturing step at 95°C, a 30-second annealing step at 57°C, and a 30-second elongation step at 72°C. After the final cycle, a 5-minute elongation step at 72°C was carried out. All primers used are listed in Table 1. The amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by UV fluorescence. The intensity of stained PCR fragments from photographs was quantified by scanning densitometry (Furi Inc, Shanghai, China).

2.6. Quantification of ZIP4 mRNA levels

The primers and probe of ZIP4 were designed with the assistance of Primer Express TM 1.5 (Applied Biosystems, Foster, CA). The corresponding sequences were as follows: 5′CCAGTGTGTGGGACACGGTAT3′ (forward), 5′ TGTTCCGACAGTCCATATGCA 3′ (reverse), and 5′ CCTGAGTGCCAGGGACGTGAGG3′ (probe). The Taq-Man probe contained 6-carboxyfluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end and was designed to hybridize to a sequence located between the PCR primers. cDNA synthesis was described above. Incubation steps were 94°C for 5 minutes, followed by 60 cycles of 94°C for 30 seconds and 60°C for 30 seconds (Bio-rad Icycler 5; Bio-rad Inc.).

2.7. Statistical analysis

The results of the MTT assay were expressed as the values of absorption at a wavelength of 570 nm. The results of flow cytometric analysis were expressed as the percentage of early apoptotic cells and late apoptotic or necrotic cells. The relative mRNA expressions of the genes were expressed as the ratio of target genes to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data were expressed as means ± SD. A 1-way analysis of variance (ANOVA) was used to perform comparisons using SPSS (version 11.0; SPSS Inc, Chicago, Ill). Group differences resulting in P values of less than .05 were considered to be statistically significant. The Caco-2 cells without $ZnSO_4$ or TPEN treatment were used as the control.

3. Results

3.1. High or low zinc inhibited cell growth of Caco-2 cells

The results of the MTT assay showed that the optimal zinc concentration for Caco-2 cell proliferation was about approximately μmol/L ($P < .05$). Cell proliferation decreased dramatically when the zinc concentration reached 200 and 300 μmol/L compared with the cell viability at 150 μmol/L ($P < .05$; Fig. 1A). When exposed to 0 to 10 μmol/L TPEN, cell proliferation showed a dose-dependent inhibition. There was a significant decrease when the TPEN concentration was >5 μmol/L ($P < .05$; Fig. 1B).

3.2. High or low zinc induced apoptosis in Caco-2 cells

Based on the results of the MTT assay, we conducted other experiments on the morphological features of cells and flow cytometric analysis of the zinc (or TPEN)-treated cells. Using Hoechst 33258 dye, condensed nuclear DNA was

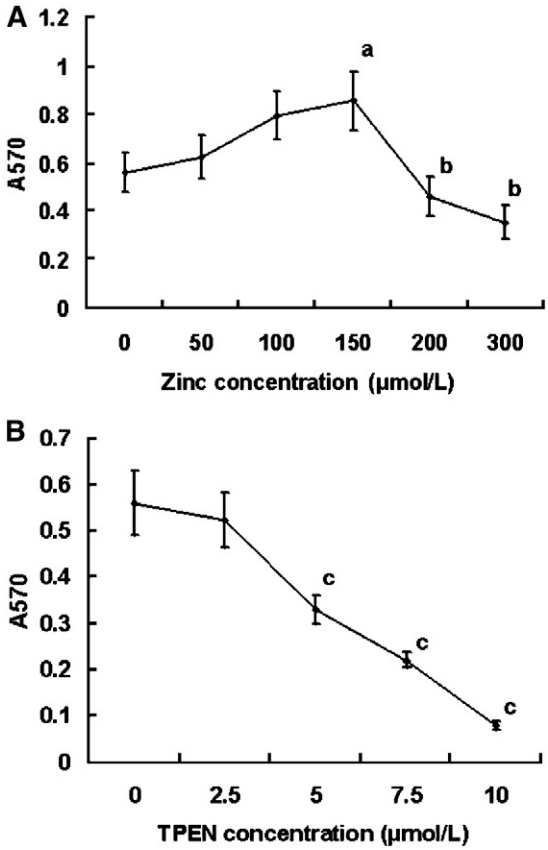

Fig. 1. Effects of $ZnSO_4$ or TPEN administration on Caco-2 cell proliferation. Caco-2 cells were incubated with various concentrations of $ZnSO_4$ (A) or TPEN (B) for 24 hours. Cell proliferation was assessed by MTT assay. Values are expressed as means ± SD, n = 5. The data were analyzed using 1-way ANOVA. The superscript "a" indicates a significant difference between mean values of 150 and 0 μmol/L $ZnSO_4$ administration ($P < .05$). The superscript "b" indicates a significant difference ($P < .05$) between 200 or 300 μmol/L and 150 μmol/L $ZnSO_4$ administration. The superscript "c" indicates a significant difference ($P < .05$) compared with 0 μmol/L TPEN administration.

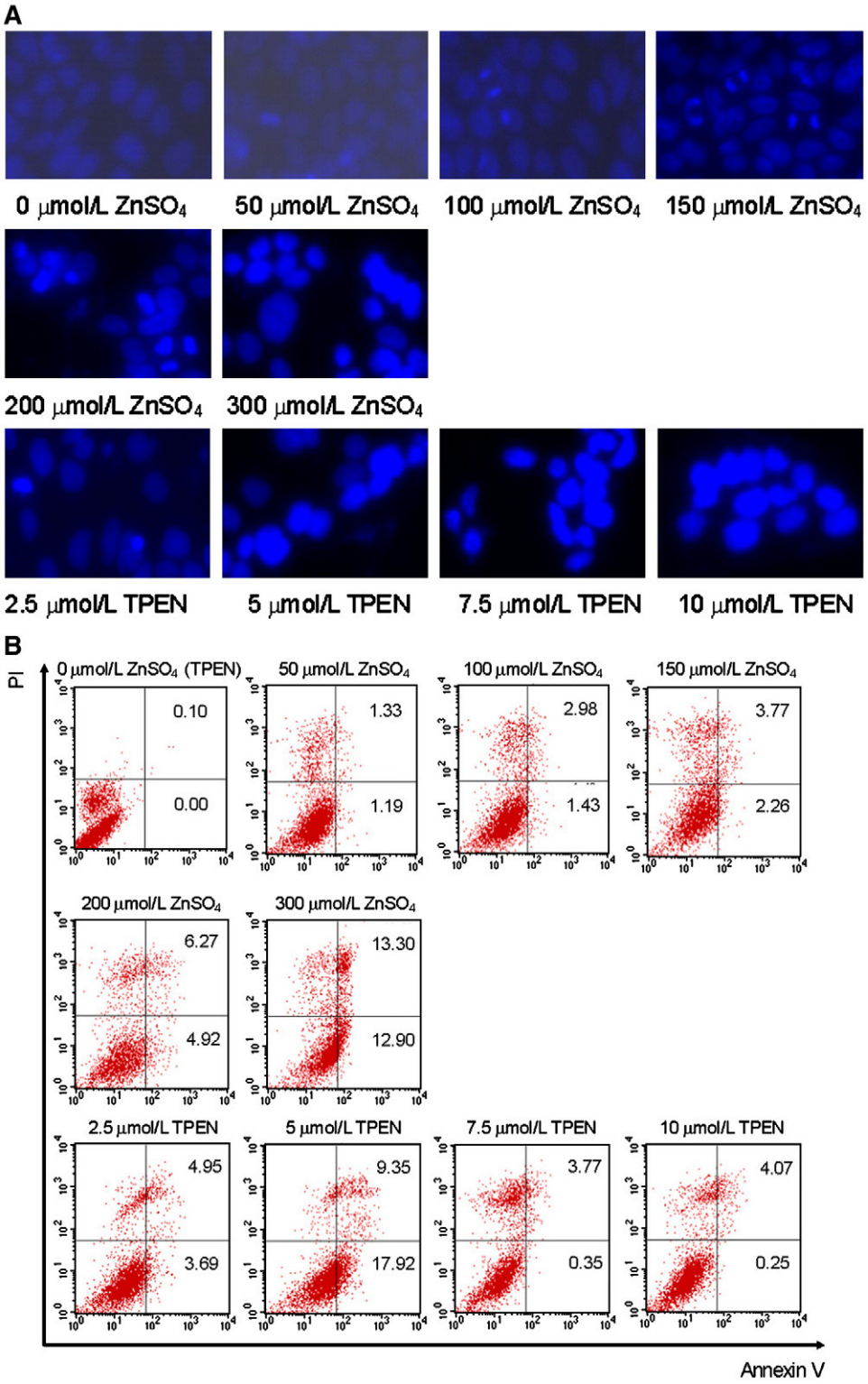

Fig. 2. Apoptosis observed in Caco-2 cells when treated with $ZnSO_4$ or TPEN detected by Hoechst 33258 dying (A) and flow cytometry (B). Cells were harvested 24 hours after treatment with various $ZnSO_4$ or TPEN concentrations. The percentage of early apoptotic cells (annexin V–positive, PI-negative) and late apoptotic or necrotic cells (annexin V/PI-double positive) was calculated. The results shown are representative of 3 independent experiments.

observed under fluorescence microscopy 24 hours after zinc or TPEN administration (Fig. 2A). There was considerable condensed nuclear DNA when the zinc concentration was >200 μmol/L or when TPEN concentration was >5 μmol/L. The number of apoptotic cells increased gradually with the increment increase in zinc or TPEN concentration. In

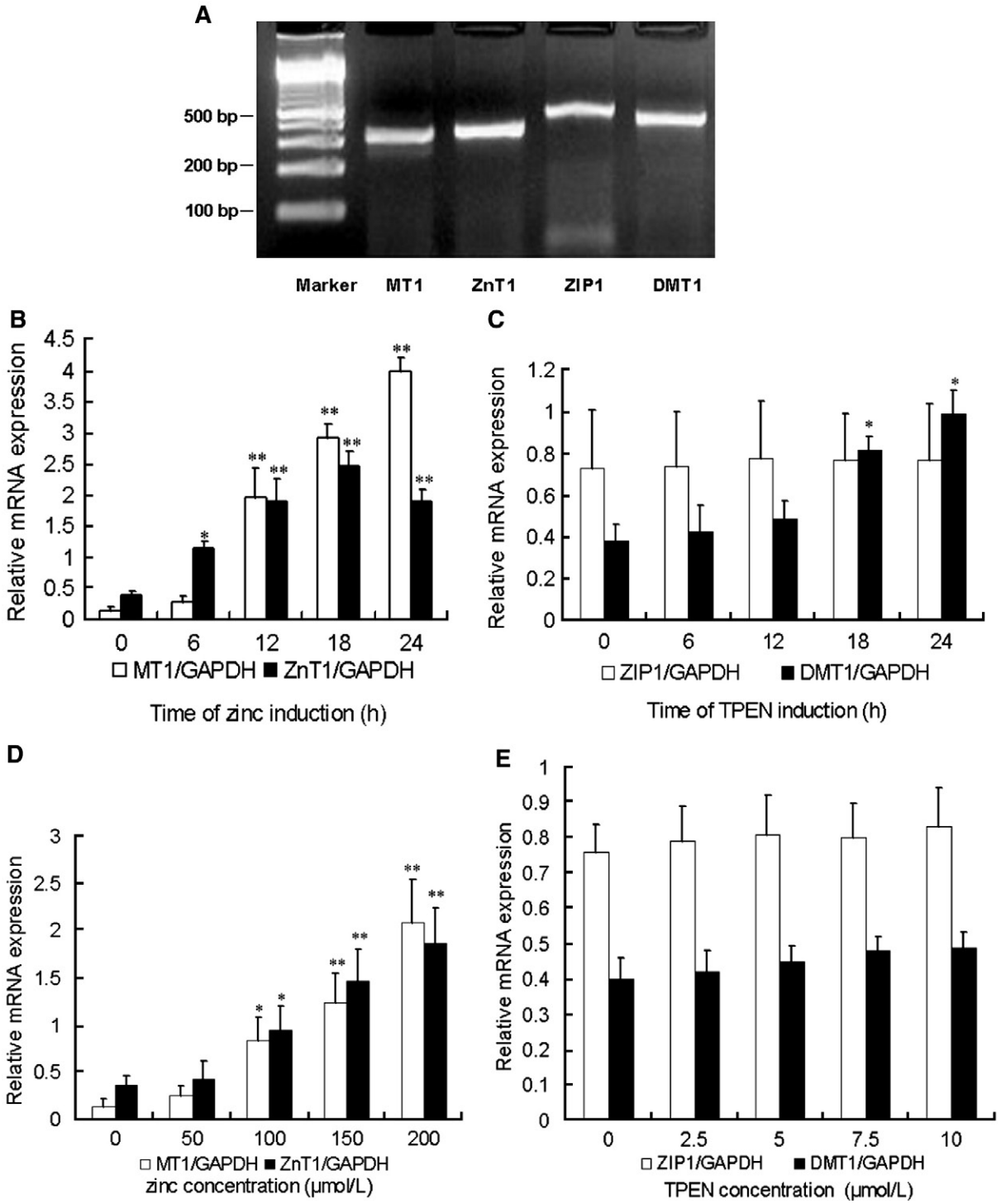

Fig. 3. Effects of zinc on MT1, ZnT1, DMT1, and ZIP1 mRNA expression in Caco-2 cells induced by various $ZnSO_4$ or TPEN concentrations. A, Gene expression of MT1, ZnT1, DMT1, and ZIP1 in Caco-2 cells. Total RNA was isolated from Caco-2 cells. B, Relative mRNA expression of MT1 and ZnT1 (ratio to GAPDH) in Caco-2 cells induced with 200 μmol/L $ZnSO_4$ for 24 hours. C, Relative mRNA expression of DMT1 and ZIP1 (ratio to GAPDH) in Caco-2 cells induced with 10 μmol/L TPEN for 24 hours. D, Relative mRNA expression of MT1 and ZnT1 (ratio to GAPDH) in Caco-2 cells induced with varying $ZnSO_4$ concentrations for 12 hours. E, Relative mRNA expression of DMT1 and ZIP1 (ratio to GAPDH) in Caco-2 cells induced with varying TPEN concentrations for 12 hours. Values are expressed as means ± SD, n = 5. The data have been analyzed using 1-way ANOVA. An asterisk (∗) indicates a significant difference when compared with 0 hour or 0 μmol/L $ZnSO_4$ or TPEN administration ($P < .05$). Two asterisks (∗∗) indicate a significant difference when compared with 0 hour or 0 μmol/L $ZnSO_4$ or TPEN administration ($P < .01$).

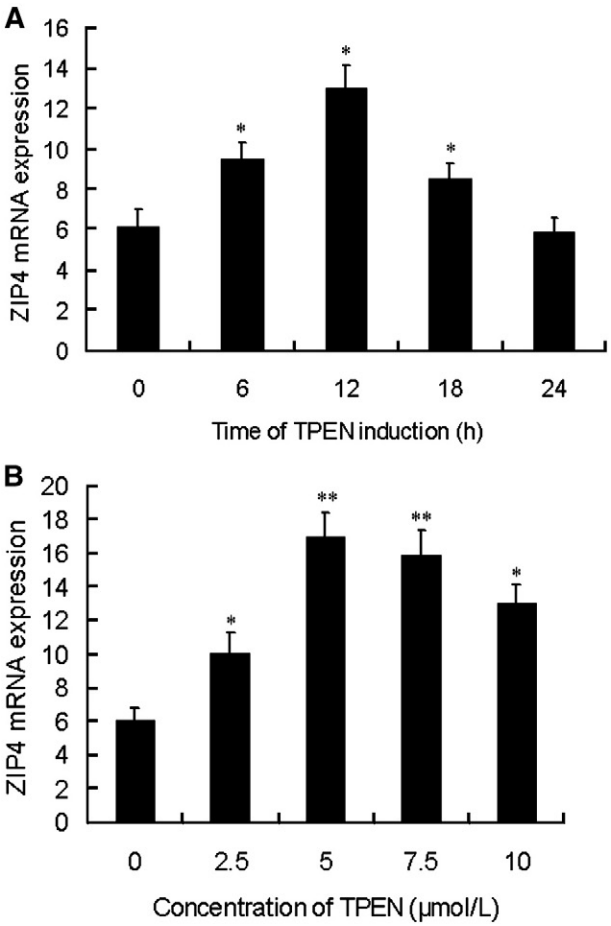

Fig. 4. The expression of ZIP4 mRNA in Caco-2 cells induced by TPEN. A, mRNA expression of ZIP4 in Caco-2 cells induced with 10 μmol/L TPEN for 24 hours by real-time PCR. B, mRNA expression of ZIP4 in Caco-2 cells induced with 0 and increasing TPEN concentrations for 12 hours by real-time PCR. GAPDH was used as the RNA control. Values are expressed as means ± SD, n = 5. The data have been analyzed using 1-way ANOVA. An asterisk (*) indicates a significant difference when compared with 0 hour or 0 μmol/L TPEN administration ($P < .05$). Two asterisks (**) indicate a significant difference when compared with 0 μmol/L TPEN administration ($P < .01$).

addition, the percentages of annexin V–positive cells (apoptotic cells, including PI^+ and PI^-) are shown in Fig. 2B. Early and late apoptotic cells were prominent in Caco-2 cells 24 hours after 300 μmol/L zinc or 5 μmol/L TPEN administrations ($P < .05$).

3.3. Effect of zinc on MT1, ZnT1, DMT1, ZIP1, and ZIP4 mRNA expressions in Caco-2 cells

Reverse transcription PCR results showed that all of the genes studied were expressed in Caco-2 cells (Fig. 3A). We examined the time- and concentration-dependent behavior of these genes. We found that the mRNA expression of MT1 increased with time for the 200 μmol/L zinc treatment; however, ZnT1 mRNA expression revealed a bell-like curve with time for the 200 μmol/L zinc induction. The highest expression of ZnT1 occurred at 18 hours of zinc exposure ($P < .01$; Fig. 3B). The administration of TPEN did not change the mRNA expression of DMT1 until 18 hours of zinc treatment ($P < .05$), but the mRNA expression of ZIP1 was not regulated significantly by TPEN from 0 to 24 hours (Fig. 3C). When Caco-2 cells were exposed to various concentrations of zinc from 0 to 200 μmol/L for 12 hours, the MT1 and ZnT1 mRNA expressions increased at an equal rate for the increment of zinc concentration ($P < .05$; Fig. 3D), whereas the ZIP1 and DMT1 mRNA expressions were resistant to TPEN treatment (Fig. 3E).

We detected ZIP4 mRNA expression using real-time PCR, and ZIP4 mRNA expression was upregulated by TPEN. The peak mRNA level of ZIP4 reached at 12 hours after 10 μmol/L TPEN administration ($P < .05$; Fig. 4A) or 24 hours after 5 μmol/L TPEN administration ($P < .01$; Fig. 4B).

4. Discussion

Caco-2 cells derived from a human colon adenocarcinoma are a good model system for studying intestinal epithelial absorption [36,37]. Caco-2 cells treated with $ZnSO_4$ or TPEN, a kind of special zinc-chelating reagent, were used in current study. We observed that either high or low zinc inhibited cell growth and that apoptosis could be induced in cells by a high zinc or TPEN concentration. An appreciation for moderate increases in intracellular free Zn^{2+}, which are deleterious and can eventually lead to cell death, has only recently emerged. Optimal cell health, growth, and function are achieved only within a narrow window of intracellular free Zn^{2+} concentrations [38]. The current results revealed that cell proliferation and viability depend on appropriate zinc concentration, and there was apoptotic cell death when the extracellular zinc level lapsed from optimal concentration. Zinc-deficient status led to derangements in cell function because Zn^{2+} was no longer available to fulfill its many required roles in association with Zn^{2+}-binding proteins and presumably as a signaling molecule. In addition, if intracellular free Zn^{2+} levels rose above critical levels, even transiently, important cellular functions were inhibited. Moreover, Zn^{2+} may initiate processes that can lead to cell death. Thus, proper Zn^{2+} homeostasis is a requirement for cell survival, and all living organisms have evolved complex and redundant mechanisms to regulate total cellular and intracellular free Zn^{2+}.

In the present study, both ZnT1 and MT1 mRNA expressions were dose-dependent. Although both ZnT1 and MT1 could attenuate zinc toxicity, they may play different roles at different stages. The mRNA expression of ZnT1 was upregulated significantly at 6 hours after zinc administration, which was earlier than that of MT1, and the peak level of ZnT1 mRNA expression was at 18 hours after zinc administration and then it decreased. In contrast, MT1 was expressed in a time-dependent manner. Compared with the MT1 mRNA expression, ZnT1 was more sensitive to a

high zinc concentration and was less durable. The ZnT1 and MT1 mRNA expressions seem to be upregulated at high zinc concentrations to enhance zinc efflux to maintain zinc homeostasis in Caco-2 cells.

The behavior of ZIP4 mRNA expression followed a bell-shape curve for time and the concentration of zinc induction, whereas the mRNA expression of DMT1 was time-dependent but not dose-dependent in the late TPEN treatment duration. It has been proposed that ZIP4 plays a role in zinc absorption at the early stage of zinc deficiency, and the cooperated upregulation of ZIP4 and DMT1 mRNA expression in low zinc condition would contribute to enhancing the zinc absorption in Caco-2 cells at the late stage of zinc deficiency. Barnes and Moynahan first observed that the disorder of AE was caused by the inability to absorb sufficient zinc, and they achieved a complete recovery of all AE symptoms by zinc supplementation [39,40]. Some clinical observations suggest that patients with AE retain some mechanism for intestinal zinc uptake. The previous and present studies showed that one possibility is that the mutations discovered in AE patients may impede ZIP4 function but not completely destroy it. If so, the effects of such hypomorphic mutations might be overcome by increased intake of zinc. A second explanation may lie in the supplemental action of another ZnT, expressed in the intestine. For example, the DMT1 also resides on the apical surface of the enterocyte and transports zinc in vitro.

Although the expression of mRNA from the genes in the present study detected in cell culture reflects zinc transportation in Caco-2 cells, this does not indicate what happens in the body. In addition, the experiments in cell cultures will contribute to a better understanding of intestinal zinc absorption that can be extended to in vivo investigations.

In conclusion, we have established responses to zinc or TPEN treatment of the MT and ZnTs in Caco-2 cells in culture. The present results suggest that mRNA expressions of ZIP4 and DMT are susceptible to low extracellular zinc and are upregulated to enhance zinc absorption, whereas the ZnT1 and MT1 mRNA act as key regulators under high zinc condition to enhance the intracellular zinc efflux to maintain zinc homeostasis in Caco-2 cells. It is proposed that in response to variations in zinc concentration, the cooperation of ZnT1, MT1, DMT1, and ZIP4 provides a functional regulation for cellular zinc homeostasis in Caco-2 cells.

Acknowledgment

This study was supported by the National Natural Science Grants program of China (Nos. 10175085 and 30170805). The authors thank Jiangang Long and Zhengyin Li for their critical reading of this manuscript.

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