Eur J Nutr (2011) 50:379–386
DOI 10.1007/s00394-010-0146-3

ORIGINAL CONTRIBUTION

Differentiation- and polarization-dependent zinc tolerance in Caco-2 cells

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Received: 26 July 2010 / Accepted: 4 November 2010 / Published online: 20 November 2010

Abstract
Purpose Enterocytes are feasibly confronted with enormous zinc concentrations especially as a result of oral zinc supplementation. In the present study, we investigated the mechanisms underlying the exceptional ability to withstand this usually toxic load using the enterocytic cell line Caco-2.
Methods By MTT test analysis, we compared zinc tolerance in undifferentiated Caco-2 cells (udCaco-2) and differentiated Caco-2 cells (dCaco-2). By RT-PCR, we compared the respective baseline expression levels of certain zinc transporters and metallothioneins (MTs) as well as the regulation of these components in response to high zinc concentrations. Moreover, using dCaco-2 cells cultured on porous membranes, we explored zinc tolerance in dependence of the side of zinc administration: apical versus basolateral.
Results dCaco-2 cells tolerate significantly higher levels of zinc compared to udCaco-2 cells. This adaptation was accompanied by upregulated ZnT-1 and downregulated ZIP1 levels. The expression of metallothioneins MT1A and MT1X was also significantly downregulated during differentiation. Moreover, apparent from profound differences in zinc tolerance between apical and basolateral zinc application, polarization was concluded to have substantial impact on cellular zinc tolerance.
Conclusions The profound increase in zinc tolerance we found in differentiated enterocyte-type Caco-2 cells and in particular, the impact of polarization are likely to reflect the physiologically indispensable capability of enterocytes to cope with remarkable concentrations of intestinal zinc.

Keywords Zinc · Caco-2 cells · Zinc transporters · Metallothioneins · Differentiation

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Introduction

The tight regulation of zinc homeostasis represents a fundamental feature for cellular survival, since both zinc overload as well as zinc deficiency is incompatible with regular cellular functions. Enterocytes hold an exceptional position in this context, as these cells are feasibly confronted with remarkable extracellular concentrations of zinc. While plasma levels of zinc are about 15 μM [1], the concentration in gut, especially after oral application of zinc supplements can be calculated to reach values up to the mmolar range, dependent on stomach contents and quantity of supplement intake. The responsibility for the regulation of cellular zinc uptake and release as well as intracellular compartmentalization is shared by certain members of the zinc transporter families ZnT and ZIP [2, 3]. ZIP-transporters are in general responsible for increasing the cytosolic concentration of zinc by importing zinc from the extracellular space as well as from intracellular zinc storing compartments, while ZnT-transporters exert the opposite function [3, 4]. The intracellular metal-binding protein family of metallothioneins (MTs) is capable of sequestering free intracellular zinc, thereby contributing to the zinc regulatory machinery [5].

The colon carcinoma cell line Caco-2 represents a suitable model to study enterocyte-specific details of zinc homeostasis, as these cells are capable of spontaneous differentiation into cells displaying enterocytic characteristics [6]. For cadmium or iron, it was shown that enterocyte-type differentiated Caco-2 cells (dCaco-2) display a significantly higher tolerance toward these compounds compared to undifferentiated Caco-2 cells (udCaco-2) [7, 8]. However, for zinc, a differentiation-dependent adaptation of tolerance levels could not be assured so far [9].

In the present study, we first ascertained that zinc tolerance is increased in the course of Caco-2 cell differentiation. We next determined whether this adaptation is accompanied by changes in the baseline expression levels of the zinc transporters ZnT-1, ZnT-5, ZIP1, or ZIP4 or of the MTs MT1A, MT1X, or MT2A during differentiation. In addition to the baseline expression levels, the ability of cells to adjust these expression levels in accordance with the actual supply and demand is essential for maintaining zinc homeostasis. Therefore, we analyzed whether differences between udCaco-2 cells and dCaco-2 cells regarding the regulation of zinc transporters and MTs in response to elevated zinc levels exist. Finally, by using dCaco-2 cells grown on porous membranes, which allow the separate addition of zinc from either the apical or the basolateral side of the cells, we determined the impact of the side of zinc application on zinc tolerance and on the regulation of zinc transporters and MTs. We thereby provide elementary data on the regulatory equipment responsible for maintaining zinc homeostasis in Caco-2 cells.

Materials and methods

Cell culture

Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO^{TM}), supplemented with 10% FCS (GIBCO^{TM}), 2 mM Glutamin (PAA), and 100 U/mL Penicillin/Streptomycin (PAA) in monolayer culture in 25-cm^2 flasks (Greiner) at 37 °C in a humidified 5% CO_2 atmosphere. For experiments, DMEM was supplemented with 5% FCS. Note that as the zinc concentrations used in the experiments of the present study were considerably high, the basal amount of zinc derived from serum (which is around 1.5 μM for DMEM + 5%FCS) was considered negligible, and no zinc depletion of the medium was performed. Cells were passaged prior to reaching confluence to avoid initiation of the differentiation process and plated out at a density of 1–2 × 10^4 cells/cm^2 into new flasks, 6-well or 12-well plates. Cells were then cultured until reaching about 50% confluence (udCaco-2 cells) or for 18 days after reaching confluence (dCaco-2 cells). For polarization experiments, Caco-2 cells were transferred into Millicell Inserts (Millipore) at a density of 1–2 × 10^4 cells/cm^2 and cultured for 21 days. $ZnCl_2$ stock solution was prepared by dissolving $ZnCl_2$ (200 mM, SIGMA) in 30 mM HCl.

MTT assay and protein content determination

Caco-2 cells were incubated for 24 h with zinc chloride in 12-well plates. For MTT test analysis, cells were thereafter washed and incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide) solution (10% in DMEM) for 1 h. After washing, formazan was extracted with DMSO and transferred into a 96-well plate for measuring the extinction at 595 nm in a plate reader (BIO-RAD). For protein content determination, cells were lysed with lysis buffer (100 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, and 2 mM Na-othovanadate) containing protease inhibitor cocktail (Sigma). Total protein amount was measured using the BCA^{TM} Protein Assay Kit (Pierce). To determine protein content per well, sample concentration was multiplied with sample volume.

Transepithelial electric resistance measurements (TEER)

Caco-2 cells were plated out into Millicell(R) Hanging Cell Culture Inserts (Millipore) in 6-well or 12-well plates (pore size 0.4 μM) and cultured for 21 days. TEER was measured at several timepoints during differentiation to test for monolayer development using a Millicell-ERS-probe (Millipore). Electrical resistance was measured between the apical side and the basolateral side. To validate measurements, TEERs of empty transwell inserts (without cell monolayers, but with medium) were determined as blank values, to correct the TEER calculations. TEER of the cell layers was calculated according to the equation: TEER = (Rtotal − Rblank) × A (Ω cm^2), where Rtotal is the resistance measured, Rblank is resistance of control filters without cells and A is the surface area of filter.

RT-PCR

Total RNA was isolated using TriFast^{TM} reagent (PEQLAB) according to the manufacturers protocol. The RNA content of each sample was measured using a Nano-Drop^{TM} (Thermo Scientific). Samples were excluded when the 260/280 nm ratio was lower than 1.8. Depending on sample RNA content, 2–5 μg RNA per 20 μL preparations were transcribed. Total RNA of the samples was reverse transcribed using, random hexamer primers and RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas). Relative

quantities of transcripts of interest were assessed by PCR of cDNA products (all reagents were purchased from Fermentas). Gene-specific primer sequences (taken from literature or designed using Primer3 software) and annealing temperatures are listed in Table 1.

Results

dCaco-2 cells tolerate profoundly higher levels of zinc compared to udCaco-2 cells as shown by MTT test (Fig. 1a) and determination of protein content (Fig. 1b). By MTT test, we found that a zinc concentration of 1 mM is needed to noticeably reduce the viability of dCaco-2 cells after 72 h. However, complete loss (about 99%) could only be observed after incubating cells with 1.6 mM zinc for 72 h. From these experiments, the LC_{50} value (concentration of zinc that reduces cell viability to 50% of control treated cells) for dCaco-2 cells was concluded to be 1,330 μM. In contrast, udCaco-2 cells only tolerated zinc levels of up to 100 μM for 72 h. With concentrations of 150 μM zinc, the reduction in viability observed was similar in magnitude to the reduction induced by concentrations of 1.2 mM in dCaco-2 cells (65%). With 250 μM zinc, the cell viability was reduced to 12%. For udCaco-2 cells, the LC_{50} value was found to be only about 180 μM. The results from protein content determination (B) were superimposable to the results of the MTT test analysis as apparent from the matching LC_{50} values of 170 μM for udCaco-2 cells and 1,290 μM for dCaco-2 cells. These findings confirm the results of the MTT assay and show that the observed reduction of cell viability is the result of a reduction in total cell number.

Differentiation-dependent changes in mRNA expression levels of zinc transporters and metallothioneins are shown in Fig. 2. ZnT-1 mRNA expression levels significantly increased during differentiation of Caco-2 cells, while ZIP1 mRNA levels significantly decreased. The expression of ZnT-5 as well as ZIP4 mRNA remained stable. Among the metallothioneins, the mRNA expression levels of MT1A and MT1X were significantly downregulated during the differentiation process, while the expression levels of MT2A remained unchanged.

Zinc stimulates the expression of ZnT-1 as well as of MTs in both differentiated and undifferentiated Caco-2 cells (Fig. 3).

Zinc slightly but significantly induced the expression of ZnT-1 mRNA in udCaco-2 cells at a concentration of 100 μM (open bars) after 24 h. In dCaco-2 cells, zinc, applied in a concentration of 100 μM, failed to induce the expression of ZnT-1 mRNA expression, while concentrations from 200 to 800 μM induced a dose-dependent stimulation that was up to twofold compared to control treated cells (gray bars). In contrast, the expression levels of ZnT-5, ZIP1, and ZIP4 were unaffected by zinc at any concentration or differentiation state investigated (Fig. 3a).

The mRNA expression levels of the metallothioneins MT1A, M1X, and MT2A were all stimulated by zinc in

Table 1 PCR conditions

	Primer-sequence (5′–3′)	Product size (bp)	Gene bank number	Annealing temperature	Ref.
GAPDH	f GGA GCC AAA AGG GTC ATC ATC TC	185	NM_002046	62 °C	–
	r GTC ATG AGT CCT TCC ACG ATA CG				
S/I	f CAT CCT ACC ATG TCA AGA GCC A	196	NM_001041	60 °C	[28]
	r GCT TGT TAA GGT GGT CTG GTT T				
ZnT-1	f TCT TCC TGA CTG GCC TCT GTT	491	NM_021194	60 °C	–
	r CAG AAA AAC TCC ACG CAT GTT				
ZnT-5	f AAG CCA TTT TCT TCT GGG AAA	387	NM_022902	60 °C	–
	r TAC TCC ACC CTT GTG ATC TGC				
ZIP1	f CTC CCG ATC TCT GAT TGC TCC	345	NM_014437	63 °C	–
	r CCG CGA AAC AGC TTA CTA GGC				
ZIP4	f GCC CAG AGT TGA GGC TAC TG	350	NM_017767	60 °C	–
	r GAG CAT GTC GCA GAG TGC TA				
MT1A	f CGT GCG CCT TAT AGC CTC TC	327	NM_005946	62 °C	–
	r TGG GTC AGG GTT GTA TGG AA				
MT1X	f TCT CCT TGC CTC GAA ATG GAC	303	NM_005952	62 °C	–
	r AGA TGT AGC AAA CGG GTC AGG				
MT2A	f CCG ACT CTA GCC GCC TCT T	259	NM_005953	61 °C	[12]
	r GTG GAA GTC GCG TTC TTT ACA				

f forward, *r* reverse

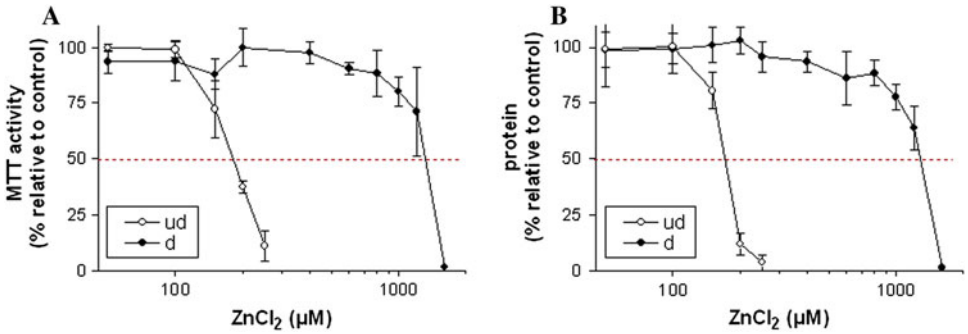

Fig. 1 MTT assay (**a**) and protein content determination (**b**): undifferentiated (ud, *open circle*) Caco-2 cells as well as differentiated (d, *filled circle*) Caco-2 cells were incubated with zinc chloride (concentrations as indicated) for 72 h in DMEM supplemented with 5% FCS. *Points* represent mean ± SD of three independent experiments

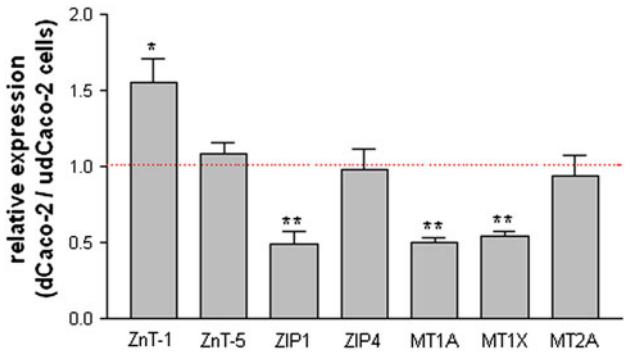

Fig. 2 Baseline expression levels of zinc transporters (ZnT-1, ZnT-5, ZIP1, and ZIP4) and metallothioneins (MT1A, MT1X, and MT2A) in differentiated versus undifferentiated Caco-2 cells determined by RT-PCR. GAPDH was used as loading control. *Bars* represent mean ± SD of three independent experiments. $*p < 0.05$; $**p < 0.01$

both differentiated and undifferentiated Caco-2 cells (Fig. 3b). In detail, 100 µM zinc applied to udCaco-2 cells induced a significant stimulation of MT1A (>6-fold), MT1X (>6-fold) and MT2A (>3-fold) mRNA expression levels. In dCaco-2 cells, the stimulation was dose dependent in the concentration range of 100–800 µM zinc. However, the stimulatory response of differentiated cells to 100 µM zinc was less pronounced compared to the response of undifferentiated cells. On average, an approximately fourfold higher amount of zinc was necessary to induce a stimulation comparable in magnitude to the one found in undifferentiated cells.

Caco-2 cells tolerate higher levels of zinc, when applied from the apical side of the cells (Fig. 4). Measurement of transepithelial electric resistance (TEER) is a widely used tool to assess the integrity of Caco-2 monolayers.

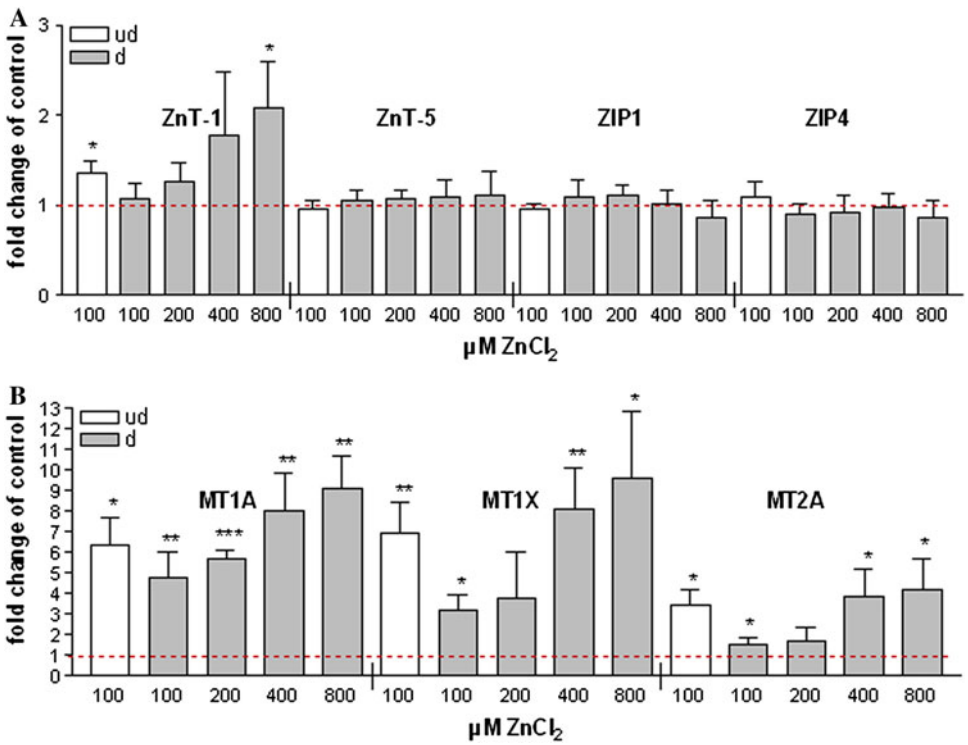

Fig. 3 **a** Expression of zinc transporters (ZnT-1, ZnT-5, ZIP1, and ZIP4) and **b** expression of metallothioniens (MT1A, MT1X, and MT2A) in undifferentiated (ud, *open bars*) as well as differentiated (d, *gray bars*) Caco-2 cells after incubation with different zinc concentrations for 24 h, determined by RT-PCR. GAPDH was used as loading control. *Bars* represent mean ± SD of three independent experiments. $*p < 0.05$

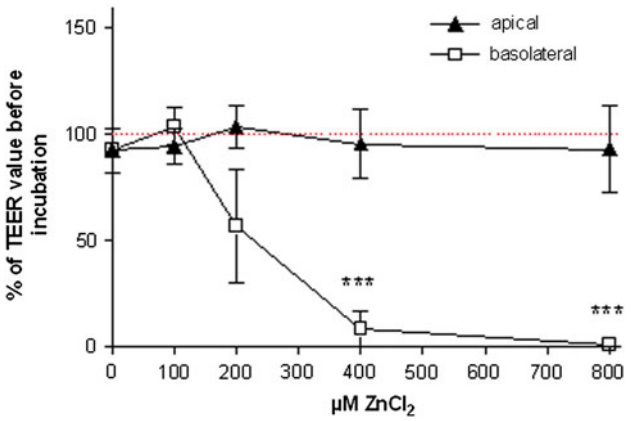

Fig. 4 TEER determination. Differentiated Caco-2 cells (grown in Millicell inserts) were incubated with different concentrations of zinc chloride for 24 h. Data are presented as the ratio of the TEER values after 24 h of incubation/TEER value prior to incubation (0 h) ×100 for the respective concentration. *Plot* shows mean ± SD of three independent experiments. ***$p < 0.001$

In addition, it can be used to measure the relative toxicity of substances added to the cells. Markowska et al. [10] reported that Caco-2 monolayers reach an optimal stable state after 16–26 days. Therefore, cells were cultured for 21 days after plating to assure full differentiation of the cells. Average TEER on day 21 after plating was 429 ± 30 Ω cm^2. The addition of medium supplemented with up to 800 μM zinc from the apical side for 24 h failed to influence monolayer integrity as displayed in unaffected TEER values compared to TEER values of control treated cells. In contrast, the application of 200 μM zinc from the basolateral side for 24 h resulted in a reduction of TEER values to 60% of the initial value. Further increasing the concentration up to 400 or 800 μM resulted in TEER values of 8 and 1%, respectively, indicating the complete disruption of the monolayer.

Impact of the side of zinc application on the mRNA expression levels of zinc transporters is shown in Fig. 5. dCaco-2 cells, grown in Millicell inserts, showed a slight, but not significant, increase in expression levels of ZnT-1 mRNA in response to zinc. However, differences dependent on the side of application (apical vs. basolateral) or a dose response could not be observed. The expression levels of the other transporters investigated (ZnT-5, ZIP1, and ZIP4) remained unaffected by zinc. The application of physiological concentrations of zinc, i.e. 100 μM applied from the apical and 15 μM from the basolateral side, resulted in the significant induction of ZnT-1 mRNA expression. Again, the expression levels of the other transporters remained unchanged.

MT1A mRNA expression is significantly higher stimulated when zinc is applied from the basolateral side of the monolayer (Fig. 6). The magnitude of the stimulation of the mRNA expression of MT1A was dependent on the side of application as well as on the concentration of zinc applied. In general, the response was more profound when zinc was applied from the basolateral side. In detail, the stimulation with 100 μM zinc from the basolateral side resulted in a stimulation comparable to the addition of 200 μM zinc from the apical side (>5-fold). The application of 200 μM from the basolateral resulted in a significantly higher stimulation (>9-fold) compared to the corresponding application from the apical side. Similar results were found for MT1X mRNA expression levels, but without reaching significance. No dependence of MT2A mRNA was detectable on neither the side of application nor on the concentration of zinc. Finally, the addition of physiological concentration of zinc (100 μM apical and 15 μM basolateral) resulted in the stimulation of the expression of all MTs investigated.

Discussion

There is a remarkable shift in zinc tolerance from an initially "normal" tolerance in udCaco-2 cells, similar to that found with cells from other tissues [11, 12], to a significantly higher zinc resistance in dCaco-2 cells. The extent of this change is reflected in a more than sevenfold higher LC_{50} value for dCaco-2 cells compared to udCaco-2 cells. Our initial hypothesis that changes in the baseline expression patterns of zinc transporters and/or MTs or a different regulation of these components in undifferentiated and differentiated Caco-2 cells in response to zinc might accompany this adaptation was partly verified.

ZnT-1 and MT1 are purported to be responsible for maintaining zinc homeostasis under high zinc conditions [13]. Corresponding to a very recent finding of Jou et al. [14], we found upregulation of ZnT-1 expression during the differentiation process of Caco-2 cells, providing a possible explanation for the higher zinc resistance of dCaco-2 cells. However, udCaco-2 cells, which are unable to withstand higher zinc concentrations, also significantly upregulate their ZnT-1 expression levels in response to high doses of zinc. Moreover, we found that both the application of zinc from the basolateral or from the apical side of the cells resulted in upregulated ZnT-1 expression levels after 24 h, which failed to rescue Caco-2 cells challenged with zinc from the basolateral side. Indeed, our experiments revealed that zinc resistance of dCaco-2 cells challenged with zinc from the basolateral side for 24 h was superimposable to the resistance of udCaco-2 cells determined by MTT test after 72 h of incubation.

The expression of ZnT-1 as well as of MTs is regulated by zinc via the metal response element-binding transcription factor 1 (MTF-1) [15, 16]. Four classes of MTs with

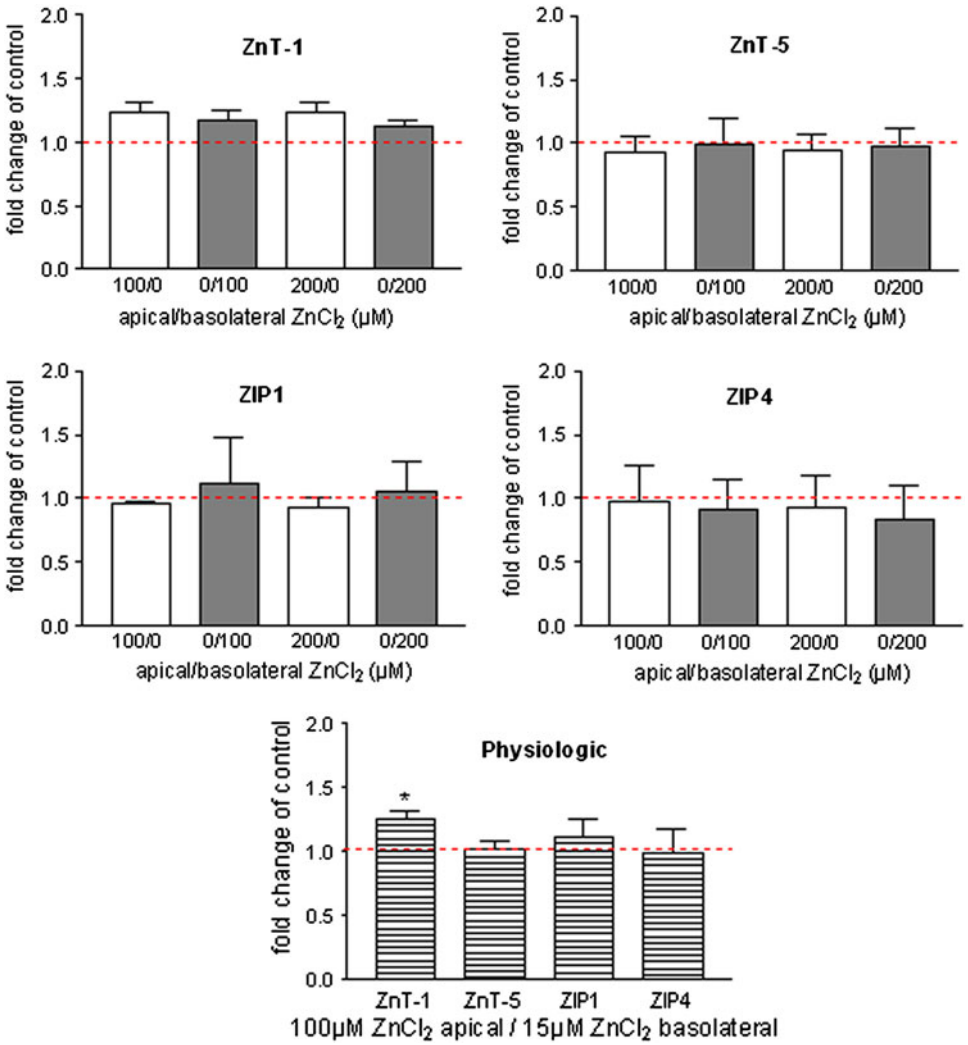

Fig. 5 Expression of zinc transporters (ZnT-1, ZnT-5, ZIP1, and ZIP4) in differentiated Caco-2 cells (grown in Millicell inserts) after incubation with different zinc concentrations from either the apical (*open bars*) or the basolateral (*gray bars*) side of the cells or after incubation with physiologic zinc concentrations (*striped bars*) for 24 h, determined by RT-PCR. GAPDH was used as loading control. *Bars* represent mean ± SD of three independent experiments. $*p < 0.05$ (One-Sample *T* Test)

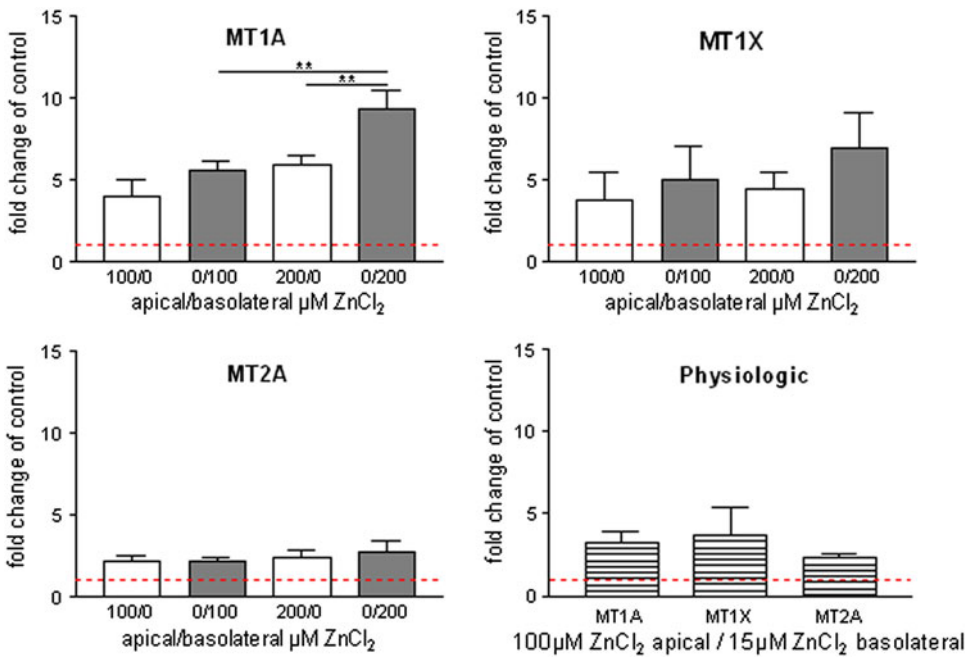

Fig. 6 Expression of metallothioniens (MT1A, MT1X, and MT2A) in differentiated Caco-2 cells (grown in Millicell inserts) after incubation with different zinc concentrations from the apical (*open bars*) or basolateral (*gray bars*) side or after incubation with physiologic zinc concentrations (*striped bars*) for 24 h, determined by RT-PCR. GAPDH was used as loading control. *Bars* represent mean ± SD of three independent experiments. $**p < 0.01$ (Bonferroni's Multiple Comparison Test)

multiple isoforms are known at present, from which MT1 and MT2 are ubiquitously expressed, whereas the expression of MT3 [17–19] and MT4 [20] is limited to certain tissues. Both udCaco-2 cells as well as dCaco-2 cells showed a significant upregulation of MT expression in response to zinc. However, the application of zinc in a concentration of 100 μM induced a greater stimulation of both ZnT-1 and MT mRNA levels in udCaco-2 cells compared to dCaco-2 cells. Apparently, when comparing the LC_{50} values of undifferentiated and differentiated Caco-2 cells, 100 μM zinc does not represent a similar "challenge" for these cells. The zinc concentrations needed to induce a comparable stimulation of ZnT-1 and MT1A mRNA in dCaco-2 cells were more than twofold higher and for MT1X and MT2A even fourfold higher. However, the LC_{50} value for zinc was more than sevenfold higher in dCaco-2 cells. We consequently compared the response of dCaco-2 cells to eightfold higher zinc concentrations (800 μM compared to 100 μM for udCaco-2 cells), which thus should represent an equal level of challenge. Under these conditions, the response of dCaco-2 cells was more profound than the response of udCaco-2 cells, indicating that dCaco-2 cells might have a higher adaptive capacity for zinc.

Our finding that the stimulation of MT2A in response to 100 μM zinc is stronger in udCaco-2 cells is in accordance with the findings of Cardin et al. [7] who also described MT2A to be downregulated during differentiation of Caco-2 cells. This finding could not be confirmed by our data. Nevertheless, the expression of the metallothioneins MT1A and MT1X was downregulated in our study during the process of differentiation, indicating that these proteins are unlikely to be accountable for the higher zinc tolerance of dCaco-2 cells.

The expression levels of ZIP4 and ZnT-5 were unaffected during differentiation and unresponsive to zinc challenge in our study. These findings match the reports purporting that these transporters are particularly important under zinc depletion [11, 13], which was not investigated within our study. The present available data pinpoint toward a predominant role of ZIP4 in the regulation of systemic zinc homeostasis, since a variety of mutations in the SLC39A4 gene encoding ZIP4 protein expression were associated with the severe zinc deficiency disease, acrodermatitis enteropathica [21–24]. However, while the expression of ZIP4 remained constant, the expression levels of ZIP1 (in accordance with Bedrine-Ferran et al. [25]) significantly declined during differentiation. Although the relevance of ZIP1 for the regulation of systemic zinc homeostasis is questioned by now [26], our data indicate that the impact of ZIP1 for cellular zinc homeostasis in Caco-2 cells might deserve further attention.

Taken together our data provide a detailed overview on zinc tolerance of Caco-2 cells. The enterocytic cell line Caco-2 provides an excellent model to explore the mechanisms underlying the ability to withstand a usually toxic load of zinc, as these cells are capable of adjusting their threshold values for zinc toxicity during their differentiation process. However, to resolve the mechanisms underlying this ability, further investigations are needed. In particular, it has to be assessed whether differences in intracellular zinc levels in response to equal zinc concentrations can be detected between udCaco-2 and dCaco-2 cells. In this context, Zödl et al. [9] purported that the uptake levels of zinc are similar in udCaco-2 and dCaco-2 cells. However, the concentrations used in this study were only ranging up to 200 μM, and the differentiation states of Caco-2 cells used in this study are merely described as pre- or postconfluent making a direct comparison difficult.

Finally, our study also emphasizes the impact of the side of zinc administration to the Caco2 cell monolayer on zinc tolerance, strongly advocating that the polarized expression of components of zinc regulation is a key to zinc tolerance. In addition to profound differences of zinc tolerance, significant differences in the regulation of MT1 expression were observable between the apical and the basolateral application of zinc. In sum, the basolateral stimulation with zinc resulted in a more profound activation of MT1 expression. These results indirectly support the findings of Raffaniello et al. [27], that zinc uptake from the basolateral side is unsaturable and faster compared to the uptake from the apical side. The topology of zinc transporters in polarized cells thus might represent a key feature and needs to be established. Such clarification of the details of polarization likely will unravel the entire regulatory machinery of control and maintenance of cellular zinc homeostasis.

Acknowledgments NZ and AZ equally contributed in conducting the study and writing the manuscript. PK was responsible for culturing Caco-2 cells in Millicell inserts. MH and IE supervised the study design. All authors declare that there is no conflict of interest. This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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