BioMetals (2005) 18:243–253
DOI 10.1007/s10534-005-1247-y

Effects of increased cellular zinc levels on gene and protein expression in HT-29 cells[☆]

Birgit Kindermann[1], Frank Döring[2], Dagmar Fuchs[1], Michael W. Pfaffl[3] & Hannelore Daniel[1,*]
[1]*Molecular Nutrition Unit, Technical University of Munich, 85350 Freising-Weihenstephan, Germany;* [2]*Research Group Molecular Nutrition, University of Kiel, 24103 Kiel, Germany;* [3]*Physiology, Technical University of Munich, 85350 Freising-Weihenstephan, Germany; *Author for correspondence (E-mail: daniel@wzw.tum.de).*

Received 28 September 2004; accepted 26 January 2005; Published online: March 2005

Key words: zinc excess, gene expression, protein expression, HT-29 cells

Abstract

High cellular zinc concentrations lead to impairments in ATP synthesis and cell cycle control particularly in neurons and epithelial cells. The molecular basis for these dysfunctions is still not fully elucidated. Here we analyzed the effects of a high zinc exposure (10 ppm) on gene and protein expression in the human epithelial cell line HT-29. Of the 1176 genes analyzed with cDNA arrays, nine differentially expressed genes were identified. Proteome analysis based on 1310 detected proteins identified 11 molecular targets. Most of the identified genes/proteins have not been linked to cellular zinc status before (e.g. PEC-60, R-ras3). More than half of the targets participate in ATP production or stress response. Therefore, it appears that higher zinc concentrations mediate their effects mainly via impairments in cellular energy metabolism and stress response.

Abbreviations: CDF – cation diffusion facilitator; cDNA – complementary DNA; 2D-PAGE – two-dimensional polyacrylamide gel electrophoresis; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; MALDI-TOF-MS – matrix assisted laser desorption ionization-time of flight-mass spectrometry; MT – metallothionein; NADH – nicotinamide adenine dinucleotide; ROS – reactive oxygen species; ZIP – Zrt-, Irt-like protein

Introduction

Zinc is an essential element for living organisms. It plays an essential role as a cofactor for more than 300 enzymes, including carbonic anhydrase, superoxide dismutase, alcohol dehydrogenase and many proteases (Vallee & Auld 1990). Zinc is also important for the correct folding of specific protein domains and for protein stability (Berg & Shi 1996). The largest group of proteins that require zinc as a structural cofactor are transcription factors. For example, in the eukaryotic model organism *Saccharomyces cerevisiae* almost 2% of the genes in the genome encode proteins with zinc-dependent DNA binding domains, participating in transcriptional control (Schjerling & Holmberg 1996, Bohm *et al.* 1997). Moreover, it is estimated that zinc is required for the function of more than 3% of the eukaryotic proteome (Maret 2001). Even though dietary zinc levels vary substantially, eukaryotic cells must maintain a fairly stable

[☆]This work was in part supported by the Degussa Bio Actives GmbH, Freising-Weihenstephan, Germany

intracellular free zinc concentration for proper gene and protein functions. Zinc homeostasis in mammalian cells is maintained through influx and efflux processes, sequestration of zinc into vesicles (e.g. so called zincosomes) and buffering by zinc binding proteins (e.g. metallothioneins, MTs) (Haase & Beyersmann 2002, Harris 2002). Among transport proteins, two families, the ZIP (Zrt-, Irt-like protein) and the CDF (cation diffusion facilitator) family are of particular importance for zinc transport (reviewed in Liuzzi & Cousins 2004). The ZIP family transports zinc (besides other divalent cations) into cells (Harris 2002). From the 15 known ZIP members in the human genome, four are proposed to participate in zinc uptake (hZip1–4). Members of the mammalian CDF family are involved in zinc efflux or the transport of zinc into intracellular vesicles and thereby control free zinc levels and protect cells from zinc toxicity. To date, 9 members of this family (ZnT1–9) have been identified. Besides transport processes, zinc homeostasis can also be maintained through binding or releasing zinc from zinc binding proteins such as MTs or cysteine-rich intestinal protein (Hempe & Cousins 1992).

Although the cellular zinc concentration in mammalian cells seems tightly controlled, zinc is capable of producing irreversible changes leading to cell death at high levels (Rudolf *et al.* 2003). The underlying mechanisms causing these effects are not fully elucidated, but zinc may bind to inhibitory sites in proteins or may compete with other metal ions for binding sites in proteins. For example, zinc appears to interfere with enzymes of the glycolytic chain (Maret *et al.* 1999) and to form complexes with proteins of the tricarboxylic acid cycle (Brown *et al.* 2000) and the mitochondrial electron transport chain (Dineley *et al.* 2003). In addition, a cellular zinc overload increases the production of reactive oxygen species (ROS) (Dineley *et al.* 2003; Song *et al.* 2004). This all could in turn cause major impairments in energy metabolism and increase the ROS burden that finally may submit cells to apoptosis or necrosis (Provinciali *et al.* 2002; Rudolf *et al.* 2003). Since epithelial cells are primarily affected by an excess intake of zinc *in vivo* (Hogstrand *et al.* 2002) we have used the human colon adenocarcinoma cell line HT-29 as an *in vitro* model to study the response to a high zinc exposure. This cell line has also been used recently to identify differentially expressed genes under zinc-deficient conditions (Kindermann *et al.* 2004). By use of the cDNA array and proteomic technologies we identified genes and proteins with changed steady state expression levels upon exposure of HT-29 cells to a high zinc concentration (10 ppm). It is known that zinc above a certain concentration leads to global perturbations in cellular metabolism resulting in cytotoxicity. Therefore we first determined a suitable zinc concentration, which did not cause impairments in cell viability and cellular growth in our cell culture model, followed by the analysis of changes in gene and protein expression levels under these conditions.

Materials and methods

Cell culture

HT-29 cells were provided by American Type Culture Collection (ATCC) and were used between passage 150 and 200. Cells were cultured as described by Wenzel *et al.* (2000). The zinc concentration in the medium after addition of the serum was ~0.24 ppm (~3.7 μM) as determined by atomic absorption spectrometry. To increase the zinc concentration in the medium, adequate amounts of $ZnCl_2$ were added. Medium was generally replaced after 48 h.

Proliferation and cell viability

Methods to assess proliferation and cell integrity were described previously (Wenzel *et al.* 2000). HT-29 cells were seeded onto 24 well plates and grown for 72 h in medium with either normal (~0.24 ppm; ~3.7 μM respectively) or high zinc concentrations (10, 20, 30, 40, 50, 100 ppm; 153, 306, 459, 612, 765, 1530 μM respectively). Cell proliferation and cell viability were assayed by using SYTOX-Green (Bioprobes), which becomes fluorescent after DNA binding. Cells were exposed to SYTOX-Green to determine the number of cells with impaired integrity based on a calibration curve. Afterwards cells were lyzed by 6% Triton X-100 in isotonic NaCl and total cell numbers were determined. The percentage of cells with impaired integrity (based on permeability for SYTOX-Green) in a given cell population was determined in relation to the fluorescence

measured after the solubilization of cells that assessed the total cell count.

Intracellular zinc concentration

Changes in intracellular free zinc concentrations were measured using the zinc-sensitive dye NewportGreen™ diacetate (Bioprobes). Cells were seeded onto 24 well plates and allowed to adhere. After washing twice with Hepes-buffered medium (HSS, composition in mM: 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 20 Hepes, 15 glucose, 1.8 $CaCl_2$, 10 NaOH, pH 7.4), cells were loaded in the dark with 5 μM Newport green diacetate in HSS for 30 min at 37 °C. Cells were again washed twice with HSS and were kept in the dark for an additional 30 min at 37 °C. Control medium containing ~0.24 ppm (~3.7 μM) and zinc-supplemented medium containing 10 ppm (153 μM) zinc were added and the increase in the fluorescence emission intensity due to the binding of intracellular free zinc was measured at 530 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent, Labsystems) for 48 h.

Atlas™ cDNA array

cDNA array analysis was performed using the Atlas™ Human 1.2 Array III (Clontech) containing 1176 unique cDNAs spotted on a nylon membrane. Total RNA isolations, probe syntheses and array hybridizations were done according to manufacturer's protocol. Cells were grown for 72 h under normal (~0.24 ppm; ~3.7 μM respectively) or high zinc (10 ppm; 153 μM respectively) conditions. Total RNA from either normal or zinc-supplemented cells was reverse transcribed in the presence of [α^{33}P]-dATP (ICN Biomedicals) using a pooled set of primers complementary to the genes represented on the array. After hybridization the membranes were washed and the intensity of the signals was recorded for 96 h (Packard Cyclone Imager, Packard Bioscience). For quantitative analysis the AIDA Array Evaluation software (Raytest) was used. Three independent hybridizations were carried out. Genes were identified as significantly modulated in expression, when the averaged ratio of signal intensities of supplementation to control experiment was > 1.5.

Northern Blot analysis

Northern blot analysis was carried out according to an established capillary blotting method (Sambrook *et al.* 1989). Total RNA (5 μg/lane) was size-fractionated on a denaturating formaldehyde gel and transferred onto a Hybond-N+ nylon membrane (Amersham Bioscience Europe). The cDNA fragments representing unique open reading frames of the following genes were used for hybridization: MT-1 (X64177, 24-302), antigen NY-CO-38 (AF039699, 732-1176), hypothetical 40.0 kD protein (D29810, 309-995) and ferritin light chain (M11147, 174-678). cDNA fragments were amplified using HT-29 total RNA as template. The cDNA fragments were randomly labeled with [α^{32}P]-dATP (ICN Biomedicals). Hybridization and further blot processing was carried out as described elsewhere (tom Dieck *et al.* 2003). Blots were reprobed for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (NM_002046, 600-1052) and the resulting signal was used for normalization.

LightCycler based real-time RT-PCR

Quantitative RT-PCR was performed as described previously (Pfaffl *et al.* 2002a). Briefly, 1 μg of total RNA from HT-29 cells, grown under normal (~0.24 ppm; ~3.7 μM respectively) and high zinc (10 ppm; 153 μM respectively) conditions, was reverse transcribed using random hexamer primers to generate cDNA pools and 25 ng reverse-transcribed total RNA was added to each PCR reaction. After initial denaturation at 95 °C for 10 min, reactions were cycled 45 times using the following parameters: 95 °C for 15 s, 62 °C for 10 s and 72 °C for 20 s. The relative amount of target mRNA normalized to GAPDH was calculated according to the method described by Pfaffl *et al.* (2002b). Primer design was done with regard to primer dimer formation, self-priming formation and primer melting temperature using the HUSAR® software at DKFZ. Blast search in the published sequence database GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that primers are gene-specific and if possible, those primers which span at least one intron were chosen. Based on these criteria, the following primers were used: ferritin light chain (forward primer 23–43 (position in the open reading frame), reverse

primer 527–507), antigen NY-CO-38 (494–511, 635–614), ribonuclease type 4 (161–179, 389–373), creatine kinase (843–862, 1131–1116), gastrointestinal peptide (117–140, 318–295), hypothetical 40 kD protein (1276–1303, 1493–1470), hepatitis A virus cellular receptor 1 (366–389, 662–639), complement factor B (1213–1230, 1538–1522), Ras-related protein R-ras3 (265–286, 392–373) and GAPDH (603–626, 871–848).

2D-PAGE

Sample preparation and 2D-PAGE were carried out as described by Herzog *et al.* (2004). HT-29 cells at 50-60% confluency were incubated for 16 h with either control (~0.24 ppm; ~3.7 μM respectively) or zinc-supplemented (10 ppm; 153 μM respectively) medium. Protein extracts were subsequently collected and stored at −80 °C. Protein concentrations were determined by optical density (OD) at 600 nm using Bio-Rad protein assay (Bio-Rad). Three independent experiments were carried out, each was run in duplicate (600 μg protein/gel). Gels were scanned and analyzed using the ProteomWeaver software (Definiens Imaging GmbH), which combined the spot detection with automatic background subtraction and normalization of the spot volumes. Changes in protein levels were calculated based on the averaged volume of a given spot in all zinc-supplemented gels in comparison to control gels and spots which differed at least 2-fold were picked for MALDI-TOF-MS.

Enzymatic digestion of protein spots and MALDI-TOF-MS

Methods described in this section have been described elsewhere (Herzog *et al.* 2004). Briefly, protein spots were picked, destained and in-gel digestion was performed using sequencing grade modified trypsin (Promega). The resulting peptide fragments were extracted and measured by MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of flight-mass spectrometry) using the Autoflex mass spectrometer (Bruker Daltonics). Proteins were identified with Mascot Server 1.9 (Bruker Daltonics) based on mass searches within human sequences only. The search parameters allowed for carboxyamidomethylation of cysteine and one missing cleavage. The criteria for positive identification of proteins were as follows: (i) a minimum score of 62; (ii) a mass accuracy of ± 100 ppm and (iii) at least a 2-fold analysis from two independent gels.

Determination of lactate levels

Relative lactate levels in the cytosolic extract of HT-29 cells were measured with an UV-test (Boehringer) according to the manufacturer's instructions, using lactate dehydrogenase (LDH) to produce pyruvate and NADH. The increase in NADH, which is stoichiometric to the amount of lactate was measured in a multiwell-plate photometer (Multiskan Ascent, Labsystems) at 340 nm. Cells were cultured under the same conditions as those used for proteome analysis. For preparation of cytosolic extracts cells were trypsinized and centrifuged at $2500 \times g$ for 10 min. 750 μl of a buffer containing 2 mM EDTA, 0.1% w/v CHAPS, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM Hepes/KOH, pH 7.4 were added to each pellet and homogenized by 10 strokes. The homogenate was centrifuged at $14{,}000 \times g$ at 4 °C for 30 min and the supernatant was used for lactate analysis.

Statistical analysis

Calculations were done using the software Prism 2.01 (GraphPad Software). Results for intracellular zinc concentrations and lactate levels were analyzed using unpaired Student's t test, results for proliferation and cell integrity were analyzed with one-way ANOVA and were considered statistically significant at a P value < 0.05. Data are given as means ± SEM.

Results

Effects of increased zinc concentrations on cell viability, proliferation, intracellular free zinc and MT mRNA levels

To establish culture conditions that significantly increase the cellular zinc status in HT-29 cells without alterations in cell viability and proliferation, cells were exposed to increasing zinc concentrations (10–100 ppm). When cells were cultured in those media for 72 h, only a zinc

concentration of 10 ppm was found not to significantly affect cell viability and proliferation (Figure 1 a, b). Subsequently, this concentration was used for further studies. The resulting intracellular free zinc concentration was determined using the zinc-sensitive dye Newport Green. As shown in Figure 2a, intracellular free zinc levels were significantly increased. It is interesting to note that total cellular zinc concentrations raised nearly 7-fold as determined by atomic absorption spectrometry (data not shown) when cells were exposed to 10 ppm zinc, whereas intracellular free zinc levels increased only less than 2-fold (~1.6-fold). In addition, the expression of the metallothionein gene I (MT-1), frequently considered as a valid indicator of intracellular zinc status (Davis & Cousins 2000, Maret 2000), responded with a markedly increase in mRNA levels in cells cultured at 10 ppm medium zinc (Figure 2b). Membrane permeability as an early apoptosis marker and nuclear fragmentation as a late apoptosis marker were assessed according to the method described by Wenzel *et al*. (2003). Both were found to be not affected by the high zinc concentration (data not shown). Taken together, the cell culture conditions used here simulate a high cellular zinc concentration which not yet causes side effects such as cytotoxicity, inhibition of cell proliferation or apoptosis. These conditions therefore should allow the identification of early markers of a zinc action at elevated intracellular ion levels.

Identification of genes responsive to increased cellular zinc levels

In order to identify genes responsive to elevated cellular zinc levels, we carried out cDNA array analysis of cells exposed to a normal or a high zinc concentration for 72 h. Radiolabeled cDNAs synthesized from extracted mRNAs were hybridized to Atlas™ Human 1.2 Array III (Clontech). Analysis of our acquired data set with the software AIDA (Raytest) showed that 57 % ($n = 671$) of the 1176 genes on the arrays yielded signal intensities significantly above background levels. Among these, only 17 genes showed differences in mRNA steady state levels at a threshold ratio of ± 1.5 (average of all hybridizations). Independent confirmation of these changes in gene expression was performed by quantitative RT-PCR (Table 1) and Northern blot analysis (Figure 3). In the case of two genes, a specific PCR product could not be obtained and six transcripts did not show corresponding changes in expression level. For the remaining nine genes mRNA steady state levels showed changes in the same direction as indicated on the array and those are compiled in Table 1. A high intracellular zinc concentration altered the expression of genes important for stress response (antigen NY-CO-38, ribonuclease type 4), energy metabolism (creatine kinase) and other cellular functions (e.g. complement factor B, ferritin light chain).

Identification of proteins responsive to increased cellular zinc levels

To identify proteins responsive to elevated zinc levels we employed proteome analysis. HT-29 cells were grown in a medium with normal or high zinc concentration. 1310 proteins spots were obtained by 2D-PAGE. Comparing protein spot densities from cells treated with high zinc and control cells, 17 proteins were found to differ at least 2-fold in

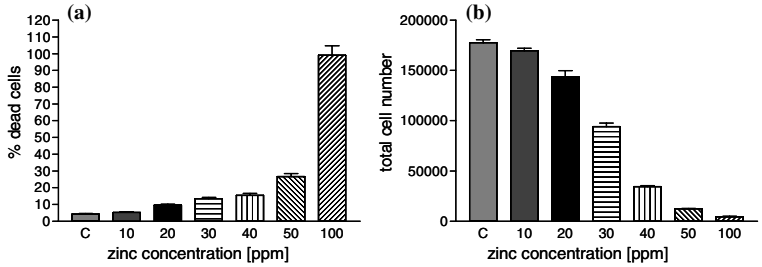

Figure 1. Effects of medium zinc concentration on cell viability (a) and proliferation (b) in HT-29 cells. Cells were cultured for 72 h in media with normal (~0.24 ppm; ~3.7 μM respectively) or increased zinc concentration (10, 20, 30, 40, 50, 100 ppm; 153, 306, 459, 612, 765, 1530 μM respectively). (a) Cell integrity was assessed by SYTOX-fluorescence. The percentage of dead cells was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after the solubilization of cells. (b) Proliferation was determined by cell counting using SYTOX-Green nucleic acid stain. Values are means ± SEM, $n = 4$.

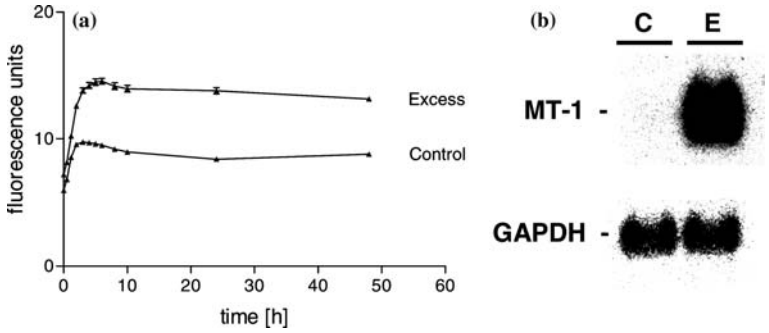

Figure 2. Intracellular free zinc levels (a) and MT mRNA levels (b) in HT-29 cells. (a) Cells were grown under normal (Control) or high (Excess) zinc conditions and cellular free zinc concentrations were assayed for 48 h using the zinc sensitive dye Newport Green, values are means ± SEM, $n = 3$. (b) Northern blot analysis of MT-1 mRNA levels in cells cultured for 72 h under normal (C, control) or high (E, excess) zinc conditions. Human MT-1 mRNA levels were compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control on the same blot.

steady state protein levels. Of these, 4 proteins showed increased and 13 proteins reduced spot densities. Among those, 11 proteins were identified by MALDI-TOF-MS analysis (Table 1). Most of the identified proteins could be linked to energy metabolism ($n = 5$) or stress induction ($n = 3$).

Assessment of cellular lactate levels

Among proteins which showed the most pronounced changes in steady state levels was Pyruvate kinase type M2, a key enzyme of the glycolytic pathway. This protein is of particular interest as tumor cells gain ATP mainly through glycolysis. Therefore we measured cellular lactate levels and those were found to be significantly reduced about 40% in HT-29 cells exposed to high zinc conditions in comparison to control cells (Figure 4).

Discussion

Numerous studies have demonstrated that the intracellular zinc concentration is of critical importance for cell growth, energy metabolism and survival in many cell types. Zinc, however, can also be toxic to cells. In particular epithelial tissues of the intestine are exposed to high concentrations of orally administered zinc. The side effects of a high zinc load are of growing importance in view of the upper safe limits of intake and the fact that zinc is a frequently used supplement and is widely available in the form of many over-the-counter products. Over the last few years, effects of high levels of zinc were the focus of a number of studies, both *in vivo* (Nielsen & Milne 2004) and *in vitro* (Cario *et al.* 2000; Zodl *et al.* 2003). At the cellular level, zinc concentrations above 100 μM were found to be toxic to Hep-2 cells, a human epithelioid cell line derived from carcinoma of larynx (Rudolf *et al.* 2003). In our study we showed that extracellular zinc concentrations of 150 μM are not toxic to HT-29 cells. In both studies, the toxic effect of zinc was defined as inhibition of cell proliferation, decrease in cell viability and induction of apoptosis. Of course, this read out is a relatively indirect effect of high cellular zinc concentrations. To identify molecular targets which are more directly regulated by zinc, an elevated but yet non-toxic zinc concentration was employed. As a model for the intestinal epithelium we have used the human colonic epithelial cell line HT-29. A medium zinc concentration of 10 ppm significantly increased total cellular as well as intracellular free zinc levels and induced transcription of MT-1. On the other hand it is not too high to cause side effects such as changes in cell proliferation and cell viability or induction of apoptosis. In a recent study the same zinc concentration was also shown to have no adverse effect on cell viability and proliferation of human intestinal CaCo-2 cells (Zodl *et al.* 2003). This relatively high zinc concentration was also selected, as intestinal cells are confronted with such high levels of zinc when given as a supplement.

Quantitative analysis of global mRNA levels is the currently preferred method for the analysis of the state of a biological system (cells, tissues etc.) in response to a given experimental condition or perturbation (Fraser & Fleischmann 1997).

Table 1. List of genes and proteins responsive to zinc excess[a].

Accession no.[b]	Encoded protein[c]	Function[d]	Identified by[e]	Δ-fold change at high zinc[f]		
				mRNA level		protein level
				Array	RT-PCR	2D-PAGE
J04469	Creatine kinase, mitochondrial	Energy metabolism	A	−1.7	−1.4	
KPY2_HUMAN	Pyruvate kinase, muscle	Energy metabolism	P			−4.0
XNHUDM	Aspartate transaminase, mitochondrial	Energy metabolism	P			−3.2
A30113	NADH2 dehydrogenase 24K chain	Energy metabolism	P			−2.6
BAA87913	Adenylate kinase 3 alpha	Energy metabolism	P			−2.3
Q9P033	HSPC124, H + −translocating pyrophosphatase	Energy metabolism	P			−2.2
AF039699	Antigen NY-CO-38	Stress-induced	A	−1.6	−1.5	
D37931	Ribonuclease type 4	Stress-induced	A	−1.5	−1.5	
S59075	Pre-mRNA splicing factor SRp30c	Stress-induced	P			−2.4
AAH15529	Ribose 5-phosphate isomerase A	Stress-induced	P			−2.1
AAC96011	Chaperonin containing t-complex, eta subunit	Stress-induced	P			−2.0
AF048700	Gastrointestinal peptide (PEC-60)	Insulin secretion	A	−2.3	−6.7	
AAH14623	Serine proteinase inhibitor, clade H, member 1	Protein degradation	P			−2.2
Q96C36	Similar to pyrroline 5-carboxylate reductase isoform	Amino acid metabolism	P			−2.1
X72875	Complement factor B	Immune system	A	+1.6	+2.0	
M11147	Ferritin light chain	Iron metabolism	A	+1.6	+1.5	
AF022080	Ras-related protein R-ras3	Signal transduction	A	+1.5	+2.2	
D29810	Hypothetical 40 kD protein	Unknown	A	+1.6	+1.5	
AF043724	Hepatitis A virus cellular receptor 1	Unknown	A	−1.5	−2.6	
AAH32123	Hypothetical protein MGC29649	Unknown	P			+7.5

[a]Increased (+) or decreased (−) expression level of genes and proteins in response to excess zinc in HT-29 cells. Genes were originally identified by cDNA array analysis and were confirmed by quantitative RT-PCR. Proteins were identified by 2D-PAGE/MALDI-TOF-MS.
[b]GenBank accession number.
[c]Name of encoded protein.
[d]Proposed function of the encoded protein.
[e]System used for identification (A: array analysis, P: proteome analysis).
[f]Zinc excess induced changes in mRNA and protein levels respectively observed by array analysis, real-time RT-PCR or proteome analysis. Values are means (n = 3 for array analysis and real-time RT-PCR, $n = 6$ for proteome analysis).

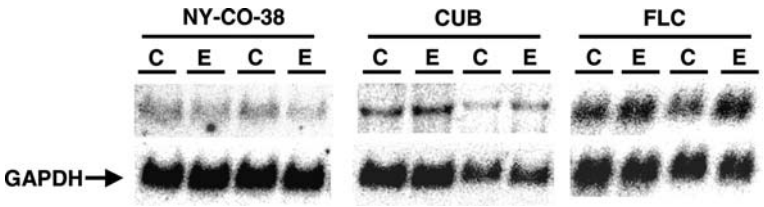

Figure 3. mRNA levels of selected genes analyzed by Northern blot analysis. Total RNA was isolated from HT-29 cells cultured for 72 h in media containing normal (C, control) or high (E, excess) zinc concentrations. Blots were probed for NY-CO-38 (antigen NY-CO-38), CUB (hypothetical 40 kD protein) and FLC (ferritin light chain). Each blot was re-probed for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) serving as an internal control.

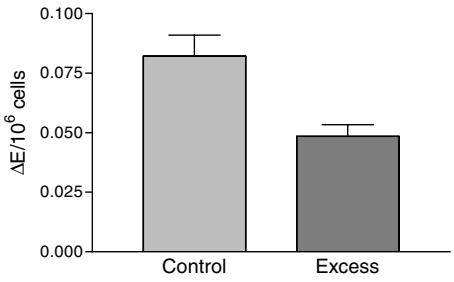

Figure 4. Lactate levels under zinc excess conditions. HT-29 cells were cultured for 16 h in media containing normal (control) or high (excess) zinc concentrations and cellular lactate levels were determined enzymatically as described in the method section. Values are means ± SEM, $n = 5$.

Although transcriptome analysis has proven to be very powerful, it was shown by several authors that in many cases the amount of mRNA is not a reliable indicator of corresponding protein abundance (Anderson & Seilhamer 1997; Gygi *et al*. 1999; Chen *et al*. 2002). This is because changes in protein abundance based on post-transcriptional mechanisms are not predictable from measurement of the mRNA level. Such mechanisms include control of protein translation, protein stability or protein modification. Therefore 2D-PAGE was performed as a complementary approach in addition to DNA array analysis to elucidate how zinc influences different levels of gene expression. Transcriptome and proteome analysis revealed 20 potential molecular targets that responded with changes in steady state expression level to excess amounts of zinc. In contrast to a recent microarray study about the effects of zinc deprivation and excess on gene expression in human THP-1 mononuclear cells (Cousins *et al*. 2003) we have found only a few genes affected by zinc supplementation in our cell model. Cousins *et al*. revealed that ~5% of the genes represented on the microarray were zinc responsive, even though their extracellular zinc concentration was significantly lower (40 μM). This is far more than in our study (which identified < 1% as zinc responsive), even if considered that they measured the transcriptional response to zinc depletion and zinc supplementation. It seems that dependence of gene expression to zinc supplementation strongly depends on the cell type and its sensitivity to this micronutrient. Another reason for this small number of molecular targets in response to zinc supplementation could be that total cellular zinc in our study increased only about 7-fold and that intracellular free zinc levels responded only with an increase of about 1.6-fold to media zinc levels that were 40-fold higher than at normal growth conditions. It is known that exposure of cells to high concentration of zinc activates several protective mechanisms including the down-regulation of zinc import proteins and the up-regulation of zinc efflux proteins (Cousins & McMahon 2000; Gaither & Eide 2001, Cousins *et al*. 2003) which in turn prevent a zinc overload. In addition, high zinc levels lead to an induction of zinc binding proteins such as MT (Hamer 1986; Cousins & McMahon 2000) which serve as a "metal sponge", thereby counteracting an increase in intracellular free zinc levels. Consistent with this, we also observed a drastic increase in MT-1 mRNA levels in zinc-supplemented cells. Moreover, a number of studies with various cell culture models suggested that the effects of high intracellular zinc concentrations are mediated in part by the generation of ROS (Dineley *et al*. 2003; Song *et al*. 2004). MT is capable of scavening ROS by its high cysteine content (Thornalley & Vasak 1985) and the increased MT expression observed could therefore represent a mechanism for cellular protection against ROS. So, when exposed to high amounts of zinc, cells activate several protective mechanisms that result in only a modest increase in intracellular free zinc which than exerts its effects on transcription, translation and protein functions. The modest increase in intracellular free zinc however altered steady state levels of mRNA and proteins that so far have not been described before as regulated by the cellular zinc status and which appear to be particularly sensitive to elevations in free zinc in colonic cancer cells.

Five of the molecular targets, consistently down-regulated by the higher intracellular zinc level were the colon cancer antigen NY-CO-38, ribonuclease type 4, pre-mRNA splicing factor SRp30c, ribose 5-phosphate isomerase A and chaperonin containing t-complex. Although their molecular functions are quite different, all targets have been reported to respond to various stress factors such as heat shock (Kubota *et al*. 1995), viral infections (Egesten *et al*. 1997, Moore *et al*. 2002) or accumulation of nuclear bodies (Denegri *et al*. 2001). Therefore, a reduced expression of these targets as caused by a high zinc concentration may increase the sensitivity of the cells to

toxic effects of environmental stress stimuli. A decrease in the mRNA or protein levels were also observed for pyruate kinase, creatine kinase, adenylate kinase, $NADH_2$ dehydrogenase, H+-translocating pyrophosphatase and aspartate transaminase. These enzymes are known to have a function in cellular ATP production and reduced cellular ATP levels in response to a high zinc exposure have been indeed demonstrated in a number of studies with different cell types as well as in mitochondrial preparations (Kleiner & von Jagow 1972, Kleiner 1974, Sheline *et al*. 2000). As a result from these studies, it seems that the reduction in cellular ATP levels is mediated mainly via inhibition of the electron transport chain, a reduced ATP production by loss of mitochondrial membrane potential and increased mitochondria permeability transition (reviewed in Dineley *et al*. 2003). Enzymes of the tricarboxylic acid cycle such as aconitase were also shown to be inhibited by zinc (Costello *et al*. 1997). Here we provide additional evidence for an impaired cellular ATP status under high zinc conditions, having found a decrease in the expression of a number of genes and proteins that are important for ATP generation. We also identified pyruvate kinase (muscle type), a key protein of the glycolytic pathway with a markedly reduced steady state level under high zinc conditions. A fall of ATP generally triggers glycolysis by activating fructose-6-P kinase. Furthermore NADH, generated by enhanced activity of glyceraldehyde-3-P-dehydrogenase, increases the reducing power that together with increased activity of LDH leads to high cellular lactate formation. A reduced level of pyruvate kinase protein levels therefore could reduce the capability of the cells to gain energy from glycolysis. When cellular lactate levels were determined in zinc-supplemented cells, levels were indeed decreased by about 40% as compared to that of control cells.

For most of the other identified targets, a link to particular cellular processes could not be established. However, the down-regulation of the protease inhibitor Kazal type 4 (gastrointestinal peptide, PEC-60) in HT-29 cells in response to elevated zinc levels is interesting with respect to its proposed functions. This 60 amino-acid peptide has been originally isolated form the gastrointestinal tract but is also expressed in neurons (Fuxe et al. 1994, Norberg *et al*. 2003). The primary function of PEC-60 derived from the gastrointestinal tract is thought to be the inhibition of insulin secretion (Agerberth *et al*. 1989, Ahren *et al*. 1992).

In summary, DNA array and proteome analysis performed in the epithelial cell line HT-29 exposed to a zinc concentration that increased intracellular free zinc but did not cause toxicity, identified an unexpected small number of molecular targets. For most of the identified mRNA and proteins lower steady state expression levels were observed. Most of the targets could be linked to an impaired state of cellular ATP production and cellular stress response. The marked down-regulation of pyruvate kinase protein level suggests that even a modest increase in intracellular free zinc (1.6-fold) can cause a reduction in cell glycolysis and energy production. This in turn could initiate a pleiotropic cell response and could also be the basis for the more toxic effects of zinc, observed when provided in higher concentrations.

References

Agerberth B, Soderling-Barros J, Jornvall H *et al*. 1989 Isolation and characterization of a 60-residue intestinal peptide structurally related to the pancreatic secretory type of trypsin inhibitor: Influence on insulin secretion. *Proc Natl Acad Sci* USA **86**, 8590–8594.

Ahren B, Ostenson CG, Efendic S. 1992 The intestinal peptide PEC-60 inhibits insulin secretion in the mouse and the rat. *Pancreas* **7**, 443–446.

Anderson L, Seilhamer J. 1997 A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* **18**, 533–537.

Berg JM, Shi Y. 1996 The galvanization of biology: a growing appreciation for the roles of zinc. *Science* **271**, 1081–1085.

Bohm S, Frishman D, Mewes HW. 1997 Variations of the C2H2 zinc finger motif in the yeast genome and classification of yeast zinc finger proteins. *Nucleic Acids Res* **25**, 2464–2469.

Brown AM, Kristal BS, Effron MS *et al*. 2000 Zn^{2+} inhibits alpha-ketoglutarate-stimulated mitochondrial respiration and the isolated alpha-ketoglutarate dehydrogenase complex. *J Biol Chem* **275**, 13441–13447.

Cario E, Jung S, Harder D'Heureuse J *et al*. 2000 Effects of exogenous zinc supplementation on intestinal epithelial repair in vitro. *Eur J Clin Invest* **30**, 419–428.

Chen G, Gharib TG, Huang CC *et al*. 2002 Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* **1**, 304–313.

Costello LC, Liu Y, Franklin RB, Kennedy MC. 1997 Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. *J Biol Chem* **272**, 28875–28881.

Cousins RJ, Blanchard RK, Popp MP *et al*. 2003 A global view of the selectivity of zinc deprivation and excess on genes

expressed in human THP-1 mononuclear cells. *Proc Natl Acad Sci USA* **100**, 6952–6957.
Cousins RJ, McMahon RJ. 2000 Integrative aspects of zinc transporters. *J Nutr* **130**, 1384S–1387S.
Davis SR, Cousins RJ. 2000 Metallothionein expression in animals: a physiological perspective on function. *J Nutr* **130**, 1085–1088.
Denegri M, Chiodi I, Corioni M, Cobianchi F, Riva S, Biamonti G. 2001 Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors. *Mol Biol Cell* **12**, 3502–3514.
Dineley KE, Votyakova TV, Reynolds IJ. 2003 Zinc inhibition of cellular energy production: Implications for mitochondria and neurodegeneration. *J Neurochem* **85**, 563–570.
Egesten A, Dyer KD, Batten D, Domachowske JB, Rosenberg HF. 1997 Ribonucleases and host defense: identification, localization and gene expression in adherent monocytes in vitro. *Biochim Biophys Acta* **1358**, 255–260.
Fraser CM, Fleischmann RD. 1997 Strategies for whole microbial genome sequencing and analysis. *Electrophoresis* **18**, 1207–1216.
Fuxe K, Tinner B, Metsis M *et al*. 1994 Generalized presence of a PEC-60-like peptide in catecholamine neurones. *Neuroreport* **5**, 1817–1821.
Gaither LA, Eide DJ. 2001 Eukaryotic zinc transporters and their regulation. *Biometals* **14**, 251–270.
Gygi SP, Rochon Y, Franza BR, Aebersold R. 1999 Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**, 1720–1730.
Haase H, Beyersmann D. 2002 Intracellular zinc distribution and transport in C6 rat glioma cells. *Biochem Biophys Res Commun* **296**, 923–928.
Hamer DH. 1986 Metallothionein. *Annu Rev Biochem* **55**, 913–951.
Harris ED. 2002 Cellular transporters for zinc. *Nutr Rev* **60**, 121–124.
Hempe JM, Cousins RJ. 1992 Cysteine-rich intestinal protein and intestinal metallothionein: an inverse relationship as a conceptual model for zinc absorption in rats. *J Nutr* **122**, 89–95.
Herzog A, Kuntz S, Daniel H, Wenzel U. 2004 Identification of biomarkers for the initiation of apoptosis in human preneoplastic colonocytes by proteome analysis. *Int J Cancer* **109**, 220–229.
Hogstrand C, Balesaria S, Glover CN. 2002 Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. *Comp Biochem Physiol B Biochem Mol Biol* **133**, 523–535.
Kindermann B, Doring F, Pfaffl M, Daniel H. 2004 Identification of Genes Responsive to intracellular zinc depletion in the human colon adenocarcinoma cell line HT-29. *J Nutr* **134**, 57–62.
Kleiner D. 1974 The effect of Zn^{2+} ions on mitochondrial electron transport. *Arch Biochem Biophys* **165**, 121–125.
Kleiner D, von Jagow G. 1972 On the inhibition of mitochondrial electron transport by Zn(2+) ions. *FEBS Lett* **20**, 229–232.
Kubota H, Hynes G, Willison K. 1995 The chaperonin containing t-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur J Biochem* **230**, 3–16.
Liuzzi JP, Cousins RJ. 2004 Mammalian zinc transporters. *Annu Rev Nutr* **24**, 151–172.
Maret W. 2000 The function of zinc metallothionein: a link between cellular zinc and redox state. *J Nutr* **130**, 1455S–1458S.
Maret W. 2001 Zinc biochemistry, physiology, and homeostasis – recent insights and current trends. *Biometals* **14**, 187–190.
Maret W, Jacob C, Vallee BL, Fischer EH. 1999 Inhibitory sites in enzymes: zinc removal and reactivation by thionein. *Proc Natl Acad Sci* USA **96**, 1936–1940.
Moore MM, Fernandez DL, Thune RL. 2002 Cloning and characterization of Edwardsiella ictaluri proteins expressed and recognized by the channel catfish Ictalurus punctatus immune response during infection. *Dis Aquat Organ* **52**, 93–107.
Nielsen FH, Milne DB. 2004 A moderately high intake compared to a low intake of zinc depresses magnesium balance and alters indices of bone turnover in postmenopausal women. *Eur J Clin Nutr* **58**, 703–710.
Norberg A, Gruber S, Angelucci F *et al*. 2003 Identification of the bioactive peptide PEC-60 in brain. *Cell Mol Life Sci* **60**, 378–381.
Pfaffl MW, Georgieva TM, Georgiev IP, Ontsouka E, Hageleit M, Blum JW. 2002a Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. *Domest Anim Endocrinol* **22**, 91–102.
Pfaffl MW, Horgan GW, Dempfle L. 2002b Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**, e36.
Provinciali M, Donnini A, Argentati K, Di Stasio G, Bartozzi B, Bernardini G. 2002 Reactive oxygen species modulate Zn(2+)-induced apoptosis in cancer cells. *Free Radic Biol Med* **32**, 431–445.
Rudolf E, Rudolf K, Radocha J, Peychl J, Cervinka M. 2003 The role of intracellular zinc in modulation of life and death of Hep-2 cells. *Biometals* **16**, 295–309.
Sambrook J, Fritsch EF, Maniatis T. 1989 *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
Schjerling P, Holmberg S. 1996 Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. *Nucleic Acids Res* **24**, 4599–4607.
Sheline CT, Behrens MM, Choi DW. 2000 Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. *J Neurosci* **20**, 3139–3146.
Song J, Lee SC, Kim SS *et al*. 2004 Zn^{2+}-induced cell death is mediated by the induction of intracellular ROS in ARPE-19 cells. *Curr Eye Res* **28**, 195–201.
Thornalley PJ, Vasak M. 1985 Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim Biophys Acta* **827**, 36–44.
tom Dieck H, Doring F, Roth HP, Daniel H. 2003 Changes in rat hepatic gene expression in response to zinc deficiency as assessed by DNA arrays. *J Nutr* **133**, 1004–1010.
Vallee BL, Auld DS. 1990 Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* **29**, 5647–5659.
Wenzel U, Kuntz S, Brendel MD, Daniel H. 2000 Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* **60**, 3823–3831.

Wenzel U, Kuntz S, De Sousa UJ, Daniel H. 2003 Nitric oxide suppresses apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions. *Int J Cancer* **106**, 666–675.

Zodl B, Zeiner M, Sargazi M *et al*. 2003 Toxic and biochemical effects of zinc in Caco-2 cells. *J Inorg Biochem* **97**, 324–330.