A High Amount of Dietary Zinc Changes the Expression of Zinc Transporters and Metallothionein in Jejunal Epithelial Cells in Vitro and in Vivo but Does Not Prevent Zinc Accumulation in Jejunal Tissue of Piglets

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Abstract

High dietary zinc concentrations are used to prevent or treat diarrhea in piglets and humans, but long-term adaptation to high zinc supply has yet not been assessed. Intestinal zinc uptake is facilitated through members of zinc transporter families SLC30 (ZnT) and SLC39 (ZIP). Whereas in rodents, regulation of zinc homeostasis at low or adequate zinc supply has been described, such mechanisms are unclear in piglets. A total of 54 piglets were fed diets containing 57 [low dietary zinc (LZn)], 164 [normal dietary zinc (NZn)], or 2425 [high dietary zinc (HZn)] mg/kg dry matter zinc. After 4 wk, 10 piglets/group were killed and jejunal tissues taken for analysis of zinc transporters SLC30A1 (ZnT1), SLC30A2 (ZnT2), SLC30A5 (ZnT8), SLC39A4 (ZIP4), divalent metal transporter 1 (DMT1), and metallothionein-1 (MT). Weight gain was higher (P < 0.05) in pigs fed HZn than in the LZn and NZn groups during the first 2 wk. Food intake did not differ between groups. The digesta and jejunal tissue zinc concentrations were higher (P < 0.05) in the HZn pigs than in NZn and LZn pigs. Expression of ZnT1 was higher (P < 0.05) and ZIP4 lower (P < 0.05) in HZn pigs than in the 2 other groups, whereas expression of ZnT5 and DMT1 did not differ between treatments. Expression of ZnT2 was lower (P < 0.05) in the LZn group than in the HZn and NZn groups. The mRNA expression and protein abundance of MT was higher (P < 0.05) in the HZn group than in the NZn and LZn groups. Studies with intestinal porcine cell line intestinal epithelial cell-J2 confirmed the dose-dependent downregulation of ZIP4 and upregulation of ZnT1 and MT (P < 0.05) with increasing zinc concentration within 24 h. In conclusion, high dietary zinc concentrations increase intracellular zinc, promote increased zinc export from intestinal tissues into extracellular compartments, and decrease zinc uptake from the gut lumen. The adaptive process appears to be established within 24 h; however, it does not prevent tissue zinc accumulation.

Introduction

Zinc is an essential trace element that is involved in numerous processes in the body (1). As with other essential elements, zinc uptake and excretion underlies homeostatic regulation and zinc ions are mainly transported actively across biological membranes. In recent years, several zinc transporters have been identified and to date, 2 families of zinc transporters have been described. The first is the ZnT-like family (SLC30), which mainly decreases intracellular zinc concentrations by transporting zinc ions from the cytoplasm into the extracellular matrix or into cell organelles (2). The second family is the ZIP (or Zrt, Irt-like) proteins (SLC39), whose members mainly increase the intracellular zinc concentration (2). Different transport proteins from both families can be found in different organs of the body. In the small intestine, these include zinc transporters SLC30A1 (ZnT1), SLC30A2 (ZnT2), SLC30A5 (ZnT8), SLC39A4 (ZIP4), and ZIP5 (SLC39A5) (2). The divalent metal-ion transporter 1 (DMT1) appears to play a minor role in zinc homeostasis (3).
To date, most studies on zinc transporters and their role in homeostasis of the body zinc pool have been performed in rodent models and by using adequate compared with marginal zinc supply. For example, ZIP4 expression is upregulated in enterocytes under marginal zinc supply, likely to increase zinc uptake from the gut lumen, whereas ZnT1 and ZnT2 expression is downregulated (4,5). Similarly, the expression of metallothionein (MT), the main zinc-binding protein in the cytosol of the cell, is downregulated in intestinal tissue under marginal zinc supply (4,5). Balance studies with rodents demonstrated that zinc homeostasis is mainly regulated via intestinal uptake and fecal excretion under marginal or excessive zinc supply (6–8). According to Fujimura et al. (9), high zinc supply leads to downregulation of ZIP4 and ZnT1 in the jejunum of the rat. However, only very few data are yet available in humans or their omnivorous model, the pig (10). On the other hand, high (pharmacological) doses of zinc oxide are commonly used for humans, especially children, in developing countries (11,12) and for young piglets (13,14) to prevent or treat gastrointestinal disorders. Whether homeostatic regulation of zinc in the body is still maintained at these high concentrations is questionable due to reports on increasing zinc concentration in various tissues (15,16). To date, it can only be assumed that different dietary zinc amounts will change the expression of zinc transport and binding proteins in the pig intestine to maintain homeostasis. However, an outbalanced homeostasis can have significant consequences with respect to health and organ function under long-term zinc supply.

The current study thus aimed at determining the influence of low, optimal, or pharmacological amounts of zinc supply on expression of small intestinal zinc transporters and MT in weaned pigs and in vitro in an intestinal porcine epithelial cell line (IPEC-J2).

Materials and Methods

Pigs, diets, and sampling. All procedures involving pig handling and treatments were approved by the local state office of occupational health and technical safety ‘Landesamt für Gesundheit und Soziales Berlin’ (LaGeSo Reg. Nr. 0347/09).

A total of 54 purebred Landrace piglets (born and raised at the Institute of Animal Nutrition, Freie Universität Berlin, Germany) were weaned at the age of 26 ± 1 d (mean body weight: 8.1 ± 0.9 kg) and randomly allocated into 3 groups with 18 piglets each balanced for litter and gender. Piglets were housed in commercial flatdeck pens (n = 2/pig) with stainless steel framings. Piglets in each group received 1 of 3 experimental diets (Supplemental Table 1) based on wheat, barley, and soybean meal. The analyzed zinc concentration in the basal diets was 35 mg/kg and the zinc concentration of the 3 diets was adjusted by replacing corn starch with analytical-grade zinc oxide (Sigma). The analyzed zinc concentration of the 3 diets was 57 [low dietary zinc (LZn)], 164 [normal dietary zinc (NZn)], and 2425 [high dietary zinc (HZn)] mg/kg, respectively. Water and feed were provided ad libitum. Room temperature was maintained at 16 h light, 8 h dark with lights switched on at 04:00 h.

Body weight and food intake were recorded on a weekly basis. After 4 wk, 10 pigs/group were randomly selected and killed for tissue sampling. 4 h after the morning meal. Digesta was collected from the distal jejunum (starting at 3 m from duodenum until 1 m before the ileum) and a tissue section (10 cm) was obtained from the mid jejunum, immediately snap-frozen in liquid nitrogen, and stored at −80°C until further analyses.

Chemical analyses. Weende proximate nutrients (ash, crude protein, ether extract, crude fiber) and starch in feed were determined using standard procedures (17). Feed and intestinal and jejunal whole tissue samples were subsequently solubilized with hydrochloric acid and zinc concentration was analyzed by atomic absorption spectrometry in an AAS vario 6 spectrometer (Analytik Jena).

Response of IPEC-J2 cells to varying zinc concentrations. The in vitro cell culture experiments were performed using porcine IPEC-J2 initially derived from the jejunum of a newborn pig (18,19). The cells were maintained in DMEM/Ham’s F-12 medium (1:1) supplemented with 5% FBS (Biochrom), 2.5 mmol/L L-glutamine (Biochrom), insulin (5 mg/L), transferrin (5 mg/L), sodium selenite (5 μg/L) (ITS, Sigma–Aldrich Chemie), epidermal growth factor (5 μg/L, Biochrom), and penicillin-streptomycin (Sigma Aldrich Chemie). The cells were seeded at a concentration of 10^5 in 24-well cell culture plates and grown at 37°C in a humidified atmosphere of 5% CO₂.

Postconfluent cells (7–9 d old) from passages 72–77 were used for dose–response studies with increasing concentrations of ZnSO₄ (Sigma–Aldrich Chemie) to adjust for final zinc concentrations in the medium of 0, 50, 100, and 200 μmol/L. We used zinc sulfate due to its better solubility in the media compared with zinc oxide. However, the lowest and highest zinc concentrations in the media were chosen based on own data about the concentration of free zinc ions with low or very high dietary zinc in jejunal digesta of piglets (20).

Cells were harvested after 24 h of treatment for measurements of mRNA expression. The cells were rinsed twice with PBS solution without Ca²⁺ and Mg²⁺ (Biochrom), harvested by scraping, and the centrifuged cell pellets were immediately stored in RNAlater RNA Stabilization Reagent (Qiagen) at −20°C until use. The cells from 6 wells were pooled per concentration and experiment. The mRNA was extracted from the cell suspensions and 100 ng total RNA was reverse-transcribed into cDNA in a final volume of 200 μL using an iScript cDNA Synthesis kit (Bio-Rad Laboratories). The complete reaction mix was incubated for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C in an iCycler iQ (Bio-Rad Laboratories). Real-time qPCR was performed as described below.

Gene expression. Analysis of jejunal gene expression was accomplished as previously described (21). The mRNA quality and quantity were determined on an Agilent 2100 Bioanalyzer (Agilent). Primers for zinc transporters ZnT1, ZnT2, ZnT5 (SLC10a family), ZIP4 (SLC39 family), DMT1 (SLC31A2), metallothionein-1 (MT), 60S ribosomal protein L19 (RPL19), β₂-microglobulin, and succinate dehydrogenase subunit A (SDHA) were designed based on published sequences. Primer information and annealing temperatures are given in Supplemental Table 2. The Ct values were normalized using the values for the housekeeping gene and arbitrary values were calculated and used for statistical comparisons. All primer sets were initially validated for single amplicon generation and standard curves tested prior to analysis. Only primer pairs with efficiency rates of >90 and ≤110% were considered for gene expression analyses. Melt curves and PCR efficiency were used as standard quality criteria for each RT-PCR run.

MT protein abundance. MT expression was determined by Western-blot analysis. For preparation of IPEC-J2 cell extracts, cultured cells were washed twice with ice-cold PBS and lysed in lysis buffer (20 mmol/L HEPES, pH 7.8; 0.35 mmol/L NaCl; 20% glycerol; 1% NP 40; 1 mmol/L MgCl₂; 1 mmol/L dithiothreitol; 1 mmol/L phenylmethylsulfonyl fluoride; 0.5 mmol/L EDTA; 0.5 mmol/L EGTA; 0.5 g/L aprotinin, and 1 g/L leupeptin) for 30 min at 4°C. Cell lysates were centrifuged at 16,000 g for 5 min and the supernatants were used for Western blotting. Protein extraction from intestinal samples was performed as previously described (22). The protein concentration was determined using a 2-D Quant kit (GE Healthcare, Life Sciences). Western-blot analysis was performed according to Eklund et al. (23) with some modifications. Briefly, sample proteins (30 μg intestinal mucosa extract or 70 μg cell extract) and a prestained protein molecular weight marker (Fermentas) were resolved by SDS-PAGE in 15% polyacrylamide gels and transferred at 6.8 mA/cm² for 1 h onto nitrocellulose membranes (pore size 0.2 μm, GE Healthcare, Life Sciences) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Equal transfer of proteins was confirmed by Ponceau staining. Following transfer, to detect MT in cell extracts, membranes were incubated in 2.5% glutaraldehyde in water for 1 h and...
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then washed 3 times for 5 min each in phosphate buffer with 50 mmol/L monoethanolamine according to the protocol of Mizzen et al. (24). Generally, the membrane pieces used for detecting metallothionein (proteins with a molecular weight <20,000 D) were blocked with 0.5% Tween20 in PBS-T, pH 7.5 and then incubated for 2 h with primary monoclonal MT antibody (Clone E9, Dako) diluted 1:150 in PBS-T (23). The membrane pieces for detection of GAPDH and β-actin (proteins with a molecular weight >20,000 D) were blocked with 5% (w/v) nonfat milk powder (Carl Roth) prepared in Tris-buffered saline containing 0.1% Tween20 for 1 h at room temperature and then incubated with either a 1:2500 dilution of mouse monoclonal anti-GAPDH antibody (Acris Antibodies) or a 1:500 dilution of mouse monoclonal anti-β-actin antibody (Santa Cruz) overnight at 4°C. After several washes, membranes were incubated in a 1:25,000 dilution of an anti-mouse HRP-conjugated secondary antibody (GE Healthcare, Life Sciences). The signals were detected by chemiluminescence with ECL Advance (GE Healthcare, Life Sciences) according to the manufacturer’s instructions.

Statistical analysis. Normally distributed data were analyzed by 1-way ANOVA followed by a Tukey’s honestly significant difference test using SPSS (version 19.0). For not normally distributed data, the Kruskal-Wallis test was applied to test for group differences and groups were separated using the Mann-Whitney test. Differences at P < 0.05 were considered significant. Data were given as mean ± SE unless otherwise stated.

Results
All piglets remained in good health throughout the experimental period and diarrhea occurred only occasionally. The body weight of the piglets receiving the HZn diets was greater (P < 0.05) at the end of the experimental period than in the NZn and LZn groups (Supplemental Table 3). Weight gain was greater (P < 0.05) for the HZn group than for the LZn group in wk 1 and greater than for the NZn group in wk 2 but did not differ from the other groups thereafter. Food intake did not differ between groups throughout the entire period (Supplemental Table 3).

The jejunal digesta zinc concentration was higher (P < 0.05) in the HZn group than in the other 2 groups. Similarly, jejunal tissue zinc concentration was higher (P < 0.05) in the HZn group than in the other 2 groups (Supplemental Table 3). Expression of ZnT1 was higher (P < 0.05) and that of ZIP4 lower (P < 0.05) in the HZn group than in the other groups, whereas ZnT5 and DMT1 expression did not differ between groups (Table 1). Expression of ZnT2 was lower (P < 0.05) in the LZn group than in the 2 other groups. The expression of MT was higher (P < 0.05) with high zinc supply than in the other groups (Fig. 1A). Similarly, the protein abundance of MT was higher in jejunal tissue of pigs fed high compared with low amounts of dietary zinc (Fig. 1B).

Using IPEC-J2 cells, the dose-dependent response of expression of ZIP4, ZnT1, and MT was studied. Confirmatory with the in vivo results, a dose-dependent increase in the expression of ZnT1 (Fig. 2A) and a dose-dependent decrease in the expression of ZIP4 were demonstrated after 24 h of zinc exposure (Fig. 2B). The mRNA expression (Fig. 3A) and protein abundance (Fig. 3B) of MT was upregulated (P < 0.05) with 200 μmol zinc in the incubation medium (P < 0.05).

Discussion
Zinc oxide has been used for more than 2 decades to treat or prevent diarrhea in humans (25) and pigs (13,14). The current study confirms that the latter is additionally linked to a growth-promoting effect during the first 2 wk after weaning, but the effect was absent thereafter (Supplemental Table 3). Some studies in the literature also revealed a growth-promoting effect of high zinc concentrations (14,26), whereas others did not (15,16,27). Usually, the growth-promoting effect is observed rather in short-term periods during the first 2 wk after weaning (i.e., after the start of feeding surplus zinc). Buff et al. (28) also observed improved weight gain during wk 1 and 2 after supplementation of 2000 mg/kg zinc as zinc oxide but did not determine such effects between wk 3 and 5. The reasons for the absence of the growth-promoting effect during longer periods of feeding high amounts are yet unclear but could be related to the fact that pigs have to cope with a high zinc concentration in various organs. It can be hypothesized that the high dietary zinc supplementation causes an imbalance in zinc homeostasis after periods longer than 2 wk. For example, recent data from our laboratory indicate 3- to 4-fold more zinc and an upregulation of

### TABLE 1 Relative mRNA expression of zinc transporters and DMT1 in the jejenum of piglets fed LZn (67 mg zinc/kg), NZn (164 mg zinc/kg), or HZn (2425 mg zinc/kg) diets

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>LZn</th>
<th>NZn</th>
<th>HZn</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnT1</td>
<td>1.06 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.32 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ZnT2</td>
<td>0.48 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ZnT5</td>
<td>1.41 ± 0.07</td>
<td>1.22 ± 0.12</td>
<td>1.16 ± 0.13</td>
<td>0.24</td>
</tr>
<tr>
<td>ZIP4</td>
<td>1.32 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DMT1</td>
<td>2.34 ± 0.93</td>
<td>1.48 ± 0.26</td>
<td>1.41 ± 0.34</td>
<td>0.11</td>
</tr>
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<sup>a</sup> Data are means ± SEs, n = 10/group. Labeled means in a row without a common letter differ, P < 0.05. DMT1, divalent metal transporter 1; HZn, high dietary zinc; LZn, low dietary zinc; NZn, normal dietary zinc; ZIP4, zinc transporter SLC39A4; ZnT1, zinc transporter SLC30A1; ZnT2, zinc transporter SLC30A2; ZnT5, zinc transporter SLC30A5.
stress response molecules in hepatic and pancreatic tissue of pigs after long-term supplementation with high dietary amounts of zinc oxide (29,30).

It has been shown in rodent and pig models that the zinc homeostasis is influenced by dietary zinc amounts by a reduced absorption from and an increased secretion into the gut lumen (6–8). The availability of free zinc ions is an important factor for zinc uptake from the gut lumen. Zinc oxide is relatively insoluble at neutral pH, but the solubility increases with decreasing pH. The proportion of free zinc in jejunal digesta of pigs is ~13% with normal zinc supply from dietary zinc oxide (31). This is in good agreement with recent findings by Pieper et al. (20), who reported similar values in jejunal digesta with normal (150 mg/kg) or very high (2500 mg/kg) zinc supply, respectively. The total amount of zinc ions in the jejunal digesta is substantially increased with high dietary zinc supply and might promote the regulation of zinc-specific transport and intracellular binding mechanisms. It has been hypothesized that the zinc uptake by ZIP proteins is ATP independent and driven by a concentration gradient (10). However, the zinc concentration in jejunal tissue was tripled with very high dietary zinc amounts compared with the suboptimal or normal amounts (Supplemental Table 3), suggesting outbalanced homeostatic regulation. Similarly, expression and protein abundance of metallothionein increased in the jejunal tissue, reflecting increased tissue zinc-binding capacity. This is in concordance with previous findings by Martinez et al. (15,32), who reported increased abundance of MT mRNA in pigs fed very high amounts of dietary zinc for 2 wk. Similar to the in vivo findings in the piglets of the present study, expression of MT mRNA and protein increased with increasing zinc concentration in IPEC-J2 cells in vitro (Fig. 3A). Detection of MT by Western blotting is difficult (24). Nevertheless, our results on the in vivo findings in the piglets of the present study, expression of MT mRNA and protein increased with increasing zinc concentration in IPEC-J2 cells in vitro (Fig. 3A). Detection of MT by Western blotting is difficult (24). Nevertheless, our results on
groups. Recently, Sargeant et al. (40) showed that the ZIP4 gene is downregulated in the pig small intestine at a high zinc supply of 3100 g/kg diet and under enterotoxigenic Escherichia coli challenge. The ZIP4 transporter seems to be a crucial factor for zinc uptake as evidenced by severe deficiency symptoms caused by mutations of the gene in various species, including humans (2). In pigs, a nucleotide exchange in the ZIP4 gene with respective replacement of the corresponding amino acid is related to changes in pancreatic zinc concentration in different pig breeds (41). In agreement with the results obtained in vivo, ZIP4 expression decreased in vitro in IPEC-J2 cells in a dose-dependent manner. Expression significantly differed between the 4 varying zinc concentrations in the incubation medium after 24 h. The expression of the ZIP4 gene is regulated by a number of zinc-dependent transcription factors, including Krüppel-like factor (KLF) 4 (42). This may require further analyses.

The expression of ZnT1 increased with the very high zinc supply and did not differ between the other 2 groups, which is similar to previous studies in rodents (43). This might reflect increased zinc accumulation in the epithelium as a response to the high intestinal zinc concentration. Interestingly, expression of ZnT2, which is mainly involved in zinc transport into intracellular vesicles (2), was only downregulated with low dietary zinc, suggesting that this mechanism plays only a minor role when intracellular zinc concentrations are high.

We observed no changes in ZnT5 expression with different amounts of dietary zinc. This is in line with previous findings by Cragg et al. (44), who observed no changes in ZnT5 with varying zinc supply in humans and Caco-2 cells. The ZnT5 protein seems to be involved in both import and export from the cell, but several interactions with other genes and factors can occur (2).

The expression of the DMT1 was not significantly altered with different dietary zinc concentrations, but a tendency toward increased mRNA abundance was observed with the low zinc supply. Whether DMT1 plays a significant role in zinc uptake has been intensively discussed (3,45). Our data support the hypothesis that DMT1 plays only a minor role in zinc homeostasis when zinc supplementation is very high, but it might be involved in zinc recovery from the gut lumen when the dietary zinc supply is low.

In conclusion, the current study reveals altered intestinal expression of different zinc transporters due to increasing amounts of dietary zinc as an attempt to reduce the intestinal uptake and increase the zinc export from the jejunal epithelium of the pig. However, this did not protect from an increased zinc concentration in the jejunal tissue and increased MT expression. Studies using the IPEC-J2 cell line confirmed the regulation of specific zinc transporters and MT and furthermore indicated dose-dependent regulation within the first 24 h after exposure to different zinc concentrations. As such, the present study evidenced that a very fast response of the intestinal epithelium targeted at preventing tissue accumulation of Zn becomes inefficient at high Zn intake, which may have toxicological implications for pigs, as well as humans, on prolonged antidiarrheal zinc supplementation.

Acknowledgments

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Literature Cited


