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Effects of zinc on epithelial barrier properties and viability in a human and a porcine intestinal cell culture model

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ABSTRACT

Zinc is an essential trace element with a variety of physiological and biochemical functions. Piglets are commonly supplemented, during the weaning period, with doses of zinc above dietary requirements with positive effects on health and performance that might be attributed to anti-secretory and barrier-enhancing effects in the intestine. For a better understanding of these observations increasing zinc sulfate ($ZnSO_4$; 0–200 μM) concentrations were used in an *in vitro* culture model of porcine (IPEC-J2) and human (Caco-2) intestinal epithelial cells and effects on barrier function, viability, and the mRNA expression of one selected heat shock protein (Hsp) were assessed. When treated apically with zinc sulfate, the transepithelial electrical resistance (TEER) did not change significantly. In contrast, cell viability measured by lactate dehydrogenase (LDH) leakage, by ATP and by WST-1 conversion in postconfluent IPEC-J2 monolayers was affected after a 24-h treatment with 200 μM $ZnSO_4$. Caco-2 cells were more resistant to Zn. $ZnSO_4$ did not induce any effect on viability, except when it was used at the highest concentration (200 μM), and only in preconfluent cells. Furthermore, $ZnSO_4$ induced Hsp70 mRNA expression at 200 μM and was more pronounced in preconfluent cells. The observed dose-related effects of zinc are cell-line specific and depended on the differentiation status of the cells. The IPEC-J2 cell line appears to be a suitable *in vitro* model to characterize specific effects on porcine intestinal cells.

1. Introduction

Zinc is necessary for a variety of physiological and biochemical functions. It is a known constituent of important metalloenzymes, is involved in major metabolic pathways and in DNA synthesis, helps to maintain intestinal barrier function and gut-associated immune functions, reduces oxidative stress, and inhibits apoptosis (Blanchard et al., 2001; Hering and Schulzke, 2009; Prasad, 2009). It is also important for the regulation of gene expression and is involved in various cellular signaling pathways by the mobilization of intracellular Ca^{2+} (Hershfinkel et al., 2001).

Zinc supplementation above dietary requirements has been shown to improve performance in piglets after weaning by increasing their growth rate, daily feed intake, and feed conversion (Hahn and Baker, 1993; Hill et al., 2001; Smith et al., 1997; Zhang and Guo, 2009) and to prevent enteric infection and diarrhoea (Carlson et al., 2004; Feng et al., 2006; Owusu-Asiedu et al., 2003). Positive effects of zinc supplementation have also been observed in acute diarrhea in malnourished children (Lukacik et al., 2008; Scrimgeour and Lukaski, 2008).

The underlying mechanisms of zinc are only partly known, but studies in animal models or human cell culture models indicate that the observed positive effects on diarrheal diseases can be attributed to its anti-secretory and barrier-enhancing effects (Canani et al., 2005; Carlson et al., 2008; Rohweder et al., 1998; Sturniolo et al., 2002). For example, in an *in vivo* model of colitis, zinc supplementation reduced the paracellular permeability in the intestine (Rohweder et al., 1998; Sturniolo et al., 2002). However, high zinc concentrations can also have a toxic effect on enterocytes (Zodl et al., 2003).

Corresponding studies are lacking for cell lines of pigs, despite the worldwide application of high doses of zinc in this species, particularly after weaning. The aim of this study has therefore been to investigate a porcine intestinal cell culture model to study effects of increasing zinc concentrations on transepithelial electrical resistance (TEER), as a parameter for barrier function, and viability. The well-established human colon carcinoma cell model (Caco-2) has been used in parallel.

2. Methods and materials

2.1. Cells and culture conditions

The piglet intestinal epithelial cell line (IPEC-J2) was established from the jejunum of a newborn pig (Kandil et al., 1995; Rhoads

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et al., 1994) and was kindly provided by Prof. Anthony Blikslager (North Carolina State University, USA). The IPEC-J2 cells were maintained in Dulbecco's modified eagle medium (DMEM)/Ham's F-12 medium (1:1) supplemented with 5% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 2.5 mmol/l L-glutamine (Biochrom, Berlin, Germany), insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml) (ITS, Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany), epidermal growth factor (EGF, 5 ng/ml, Biochrom, Berlin, Germany) and Penicillin–Streptomycin (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany). IPEC-J2 cells were passaged by trypsinization (0.15 g/l porcine trypsin, 0.06 g/l EDTA). Cells from passages 72–77 were used in the experiments.

Human epithelial intestinal cells from colorectal adenocarcinoma, Caco-2 (ATCC Catalog No. HTB-37, ATCC, Manassas, USA), were maintained in Eagle's Minimum Essential medium with Earle's buffered saline solution (BSS) and 2 mmol/l L-glutamine, as modified by ATCC and containing: 1.0 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, 1.5 g/l sodium bicarbonate, supplemented with 20% FBS and Penicillin–Streptomycin. Cells were studied between passages 36 and 41 and subcultured every 4–5 days, after trypsin treatment (2.5 g porcine trypsin and 0.2 g/l EDTA).

Both cell lines were grown at 37 °C in a humidified atmosphere of 5% CO_2. Medium was changed 3 times per week. Cell cultures were routinely tested and found to be free of mycoplasma contamination.

2.2. Incubation with $ZnSO_4$

$ZnSO_4$ (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) was solubilized in sterile water to a final concentration of 4 mM (stock solution). This stock solution was added to the cell culture media directly before the setup of the experiments. On the day of the experiments the FBS concentration in the media was 10% for both cell lines. Application of Zn was achieved *via* replacement of the media with the corresponding Zn concentrations at the beginning of the experiments.

2.3. Transepithelial electrical resistance (TEER) measurements

For TEER measurements, the cells were seeded on clear polyester membrane cell culture inserts (Snapwell®, 12 mm diameter, 1.12 cm^2 area, 0.4 μm pore size; Corning B.V., Schiphol-Rijk, The Netherlands) at a density of 10^5 cells/1.12 cm^2 and were allowed to differentiate for 9–12 days (IPEC-J2) or 21 days (Caco-2). TEER measurements were performed by using a Millicell-ERS (Electrical Resistance System; Millipore GmbH, Schwalbach, Germany).

2.4. Viability tests

For viability assays, the cells were seeded at a density of 10^4 in 96-well plates (Greiner, Frickenhausen, Germany). Experiments on postconfluent, 'differentiated' cells were conducted at 7–10 days (IPEC-J2) or 21 days (Caco-2) after seeding. The studies with preconfluent, rapidly dividing cells were conducted when the cells reached a confluency of approx. 80%.

2.4.1. WST-1 assay

Cell proliferation and cell viability was detected by using the Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). Following the indicated treatments with $ZnSO_4$, 10 μl WST-1 reagent was added per well (1:10 final dilution). After a 1-h incubation at 37 °C, the absorbance at 450/630 nm was measured by using an ELISA reader (Bio-Rad Laboratories GmbH, München, Germany). As a background control, the absorbance of the culture medium plus WST-1 in the absence of cells was subtracted from the experimental values.

2.4.2. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity was determined by using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega GmbH, Mannheim, Germany), a fluorimetric method for measuring the release of LDH from cells with damaged membranes. All reagents were prepared according to the manufacturer's instructions. CytoTox-ONE™ reagent (100 μl) was added following treatments to each well and incubated for a further 10 min. Then, 50 μl stop solution was added, the plate was shaken for 10 s, and the fluorescent signal was recorded at the 560/590-nm excitation/emission wavelength pair by using Fluostar Optima (BMG Labtech GmbH, Offenburg, Germany). Sample triplicates were treated with 5 μl lysis solution to perform a 100% cell lysis control in order to determine the maximum amount of LDH. To calculate background fluorescence, triplicate wells without cells were set up, and the medium fluorescence was subtracted from the experimental values.

2.4.3. ATP assay

ATP as an indicator of viable cells was quantified by CellTiter-GloLuminescent Cell Viability Assay (Promega GmbH, Mannheim, Germany). The reagents were prepared according to the manufacturer's instructions. Cells were plated at defined cell number in a final volume of 50 μl in half-area opaque-walled 96-well plates with clear bottoms (Costar). CellTiter-Glo reagent (50 μl) was added following treatments to each well, the cells were mixed for two minutes on an orbital shaker, and incubated for a further 10 min. The luminescence signal was recorded by the Fluostar Optima (BMG Labtech GmbH, Offenburg, Germany) with an integration time of 1 s per well. Control wells containing medium without cells were prepared to obtain a value for the background luminescence; the signal for this blank reaction was subtracted from experimental values.

2.5. Real-time quantitative polymerase chain reaction (mRNA expression of Hsp70)

Cells were seeded at a density of 10^5 on 24-well cell culture plates (TPP, Biochrom, Berlin, Germany), allowed to differentiate for 1–2 days (IPEC-J2, preconfluent), 7–10 days (IPEC-J2, postconfluent) and 3–4 (Caco-, preconfluent) and 21 days (Caco-2, postconfluent) and then treated with increasing concentrations of $ZnSO_4$ (0–200 μM) for 6 or 24 h.

Cells from 24-well culture plates were rinsed twice with phosphate-buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany), harvested by scraping, centrifuged, and the cell pellet was stored immediately in RNA*later* RNA Stabilization Reagent (Qiagen GmbH, Hilden, Germany) and frozen at −20 °C until use. Six wells were pooled per concentration and experiment. We used the nucleospin RNA II Kit to isolate total RNA including a step to digest DNA with DNAse I (Macherey–Nagel GmbH & Co. KG, Düren, Germany).

RNA concentration was spectrophotometrically quantified, and its quality was determined by a 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). Only samples with a RIN (RNA integrity number) above 7 were included in the experiments. 100 ng total RNA was reverse-transcribed to cDNA in a final volume of 200 μl with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, München, Germany) according to the manufacturer's instructions. The iScript™ cDNA synthesis kit is a system for first-strand cDNA synthesis. It contains RNase H^+ MMLV (Moloney murine leukemia virus) reverse transcriptase, a premixed RNase inhibitor to prevent indiscriminate degradation of RNA template,