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Intracellular zinc is required for intestinal cell survival signals triggered by the inflammatory cytokine TNFα☆

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Abstract

The essential micronutrient zinc has long been known to be a functional component of diverse structural proteins and enzymes. More recently, important roles for free or loosely bound intracellular zinc as a signaling factor have been reported. Insufficient zinc intake was shown to exacerbate symptoms in mouse models of inflammation such as experimental colitis, while zinc supplementation was found to improve intestinal barrier function. Herein, we provide evidence that intracellular zinc is essential for maintaining intestinal epithelial integrity when cells are exposed to the inflammatory cytokine Tumor Necrosis Factor (TNF)α. Using the human intestinal Caco-2/TC7 cell line as an *in vitro* model, we demonstrate that depletion of intracellular zinc affects TNFα-triggered signaling by shifting intestinal cell fate from survival to death. The mechanism underlying this effect was investigated. We show that TNFα promotes a zinc-dependent survival pathway that includes modulation of gene expression of transcription factors and signaling proteins. We have identified multiple regulatory steps regulated by zinc availability which include the induction of cellular Inhibitor of APoptosis (cIAP2) mRNA, possibly through activation of Nuclear Factor-Kappa B (NF-κB), as both nuclear translocation of the p65 subunit of NF-κB and up-regulation of cIAP2 mRNA were impaired following zinc depletion. Moreover, X-linked inhibitor of apoptosis protein level was profoundly reduced by zinc depletion. Our results provide a possible molecular explanation for the clinical observation that zinc supplements ameliorate Crohn's disease symptoms and decrease intestinal permeability in experimental colitis.

Keywords: Apoptosis; Zinc; Inflammatory bowel diseases; Crohn's diseases; Caco-2; Intestinal permeability; Enterocyte

1. Introduction

Zinc is an essential micronutrient acquired from the diet through protein-rich foods such as meat, seafood and legumes [1]. Through its ability to bind a diverse array of proteins, zinc plays essential roles as a catalytic cofactor for enzymatic activity or as a structural element within specific protein domains [2–4]. The role of zinc as a structural and catalytic component of enzymes and other proteins has been known for decades; more recently, robust experimental evidence has highlighted additional functions for free or loosely bound labile zinc ions as intracellular signaling factors [5]. It is not surprising therefore that a great number of biological processes are affected by imbalanced zinc homeostasis, with complex implications for the physiology of different organs and tissues [6].

Severe nutritional zinc deficiency was first described in the 1960s, and it has been extensively studied ever since [7,8]. More subtle and difficult to assess is marginal zinc deficiency, which can potentially arise from several causes: insufficient dietary intake, low bioavailability and/or interaction with other nutrients and zinc losses due either to disease processes or to specific genetic background. Any of these aspects can contribute to the increased predisposition to different pathologies observed in conditions of mild zinc deficiency. Deregulation of zinc metabolism has been associated with a number of chronic diseases including diabetes, Alzheimer's dementia, asthma [3] and inflammatory bowel diseases (IBDs), the latter including both Crohn's disease (CD) and ulcerative colitis [9]. IBDs are complex disorders characterized by chronic inflammation of the gastrointestinal tract. They have a strong genetic component, as indicated by studies on families and twins [10], and are also influenced by dietary and other environmental factors [11]. Results obtained using a diet-based treatment, which would be a desirable approach to control intestinal inflammation, have been promising but so far inconclusive [12]. Dietary modulation of intestinal inflammation can be achieved

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through identification of specific bioactive compounds capable of modulating the intestinal microbiota and/or by eliciting a positive response via the intestinal epithelium. Dietary optimization is currently obtained through elimination of suspect foods and their gradual reintroduction until a response reaction is observed. However, this laborious method only allows identification of harmful, rather than beneficial, foods. It has also been proposed that both incidence and severity of CD are modulated through personalized dietary recommendations tailored to individual genotypes [13], but this approach is hindered by limited information on the factors affecting the intestinal immune system and the intestinal cell response to specific nutritional stimuli.

A balanced rate of proliferation versus apoptosis is crucial to maintain normal gastrointestinal epithelial morphology and function. Excessive intestinal epithelial apoptosis causes villous atrophy and epithelial disruption, leading to loss of epithelial barrier integrity, which represents an early step in the onset of CD and plays a central role in its pathogenesis [14]. Current clinical strategies to ameliorate IBD symptoms are based on anti-inflammatory steroid therapy; positive results in controlling CD symptoms have also been obtained with therapeutic strategies including specific monoclonal antibodies that neutralize the effect of the inflammatory cytokine tumor necrosis factor (TNF)α [15]. TNFα is a pleiotropic cytokine that activates both anti- and proapoptotic signaling pathways; the balance of these pathways determines cell fate. In intestinal cells, TNFα promotes antiapoptotic signaling through a complex pathway that includes activation of the kinase suppressor of Ras as well as nuclear factor-kappa B (NF-κB). Disruption of signaling mediated by either of these regulatory molecules shifts TNFα receptor signaling towards a proapoptotic program [16].

The intestinal epithelium plays a pivotal role in the maintenance of zinc homeostasis, as zinc absorption is tightly regulated by the coordinated expression of specific transporters displaying a polarized distribution within the enterocyte [17,18]. Low zinc concentration has frequently been associated with IBD, a likely result of general malabsorption that arises in such pathologies, but it may also contribute to worsening disease symptoms. This hypothesis is supported by zinc supplementation trials in patients with CD in remission, as well as by studies in animal models of intestinal inflammation. Zinc supplementation was reported to improve intestinal barrier function, contributing to resolution of alterations in permeability and to reduction in the risk of relapse [19–21]. Notably, a marginally zinc-deficient diet was shown in a recent report to exacerbate experimentally induced colitis in rats [22]. Although zinc affects epithelial permeability, a direct effect on tight junctions remains to be demonstrated. The most likely hypothesis is that zinc modulates the inflammatory cascade, which in turn regulates tight junction physiology by yet to be described mechanisms. In this study, we investigated the role of intracellular zinc in the regulation of intestinal epithelial integrity, taking advantage of a well-characterized *in vitro* model, the human Caco-2/TC7 intestinal cell line, under controlled inflammatory stress conditions.

2. Material and methods

2.1. Cell culture and materials

Caco-2/TC7, a cell line of clonal origin derived from high-passage parental Caco-2 cells [23], was kindly donated by Monique Rousset (INSERM, Paris). Cells were grown and differentiated on polycarbonate filters (Transwell, 24-mm diameter; 0.45-μm pore size; Costar Corning Life Sciences, the Netherlands) as previously described [24]. For morphological studies, cells were cultured on transparent filters (polyethylene terephthalate track-etched membrane, 25-mm diameter, 0.4-μm pore size; Becton Dickinson Labware, Italy). Cells were allowed to differentiate for 15–17 days, with regular medium changes three times a week in complete culture medium: Dulbecco's modified Eagle medium containing 25 mM glucose and 3.7 g/L $NaHCO_3$ and supplemented with 4 mM L-glutamine, 1% nonessential amino acids and 10% fetal bovine serum (FBS). Experiments were performed at 37°C in 10% CO_2/air atmosphere in culture medium lacking FBS (experimental medium). All substances employed during the experiments were added to both the apical and the basolateral compartments.

2.2. Experimental design

Prior to each experiment, cells were maintained in experimental medium for 12 h. TNFα-treated cells were placed in fresh experimental medium for a further 2 h and subsequently exposed to 2 ng/ml TNFα (Sigma-Aldrich Co., Italy). Zinc-depleted cells were incubated in experimental medium containing 20 μM *N,N,N',N'*-tetrakis (2-pyridylmethyl)ethylene-diamine (TPEN) (Sigma-Aldrich Co., Italy) for 2 h [25,26]. Following TPEN removal, cells were exposed to fresh medium containing 2 ng/ml TNFα (Sigma-Aldrich Co., Italy) where indicated. To demonstrate specificity of the TPEN effects, 50 μM $ZnSO_4$ (Sigma-Aldrich Co., Italy) was added to a set of filters in the presence of TNFα. To test for cell recovery capacity, TNFα incubation was stopped at different time points, and cells were washed and transferred to complete culture medium (containing 10% FBS) for 24 h. Transepithelial electrical resistance (TEER) of cell monolayers was measured at 37°C under sterile conditions using a commercial apparatus (Millicell ERS; Millipore Co., USA) and calculated as $\Omega \cdot cm^2$ after subtracting the resistance value of the supporting filter. TEER of treated cells was expressed as percentage of the values recorded in untreated control cells.

2.3. Fluorescence staining

Immunofluorescence experiments were performed by standard protocols on cells fixed either in methanol at −20°C or in 2% paraformaldehyde with permeabilization in 0.1% TRITON X-100 [24], followed by staining with primary antibodies and corresponding secondary antibodies [27]. The specific antibodies employed were raised against ZO1, occludin, E-cadherin, β-catenin (Zymed, Invitrogen, Italy) and cytokeratin 18 fragments (M30-CytoDeath, Roche Diagnostics). Secondary conjugated antibodies were goat anti-mouse IgG FITC and goat anti-rabbit IgG TRITC (Jackson Immunoresearch Laboratories). F-actin was stained with TRITC conjugated phalloidin (Sigma-Aldrich, Italy) [24]. Intracellular zinc was imaged with 1 μM FluoZin-3-AM (Molecular Probes, Invitrogen), a cell-permeant dye with high affinity to zinc ions (KD=15 nM), and with 25 μM Zinquin ethyl ester (gift from Dr. A. D. Ward, Department of Chemistry, University of Adelaide, South Australia) in samples fixed in paraformaldehyde without permeabilization. Preparations were mounted using ProLong Gold antifade Reagent (Molecular Probes, Invitrogen). For nuclear staining, 300 nM 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Italy) was added directly to the mounting medium. Specimens were analyzed using an inverted laser-scanning confocal microscope with 40× oil immersion objective (LSM 700; Carl Zeiss, Jena, Germany). Serial optical sections were processed with ZEN 2009 software (Carl Zeiss, Jena, Germany). Section series were rendered in maximum intensity projections. Apoptotic cells (M30 positive) were counted using the fluorescence inverted microscope EVOS fl (AMG) with 20× objective and plotted as percent of total nuclei in the same field.

2.4. Protein analysis

Total proteins were extracted in sodium dodecyl sulfate (SDS) buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 2% SDS]. For purification of nuclear proteins, cells were lysed for 20 min in hypotonic buffer on ice [10 mM HEPES (pH 7.8), 1 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid and 5% glycerol] containing a cocktail of protease inhibitors (Roche Diagnostics). Nuclei were treated with 0.625% Nonidet P-40 for 5 min and centrifuged at 20,000*g* for 30 s. Nuclear proteins were extracted by incubation in hypertonic buffer [50 mM HEPES (pH 7.8), 400 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ethylene glycol tetraacetic acid and 10% glycerol] containing a cocktail of protease inhibitors. After treatment, nuclei were centrifuged at 20,000*g* for 5 min, and the supernatant was retained for Western blot analysis. Proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE), analyzed by Western blotting with the indicated antibodies and visualized by chemiluminescence (Amersham ECL Plus Western Blotting Detection Reagents, GE Healthcare). The specific antibodies employed were raised against cellular inhibitor of apoptosis protein (cIAP)1 (Cell Signaling Technology, Danvers, MA, USA), cIAP2 and X-linked inhibitor of apoptosis (XIAP) (R&D Systems, Inc., Minneapolis, MN, USA), NF-κB p65 (sc-109 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), β-actin (Abcam Cambridge, MA, USA) and tubulin (MP Biomedicals, Illkirch, France). Proteins were detected with horseradish-peroxidase-conjugated secondary antibodies (GE Healthcare, Italy) and enhanced chemiluminescence reagent (ECL, GE Healthcare Italy, followed by analysis and quantification of the chemiluminescence with the CCD camera detection system Las4000 Image Quant (GE Healthcare, Italy).

2.5. Gene expression analysis

Caco-2/TC7 cells were exposed to the different experimental conditions described above, and RNA was extracted after 4 h of TNFα treatment using TRIzol reagent (Invitrogen). As a control, RNA was also extracted from differentiated Caco-2/TC7 cells maintained in complete culture medium. Samples were treated with DNAse to avoid