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ScienceDirect

Journal of Nutritional Biochemistry 43 (2017) 18–26

Journal of Nutritional Biochemistry

Zinc enhances intestinal epithelial barrier function through the PI3K/AKT/mTOR signaling pathway in Caco-2 cells☆

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Received 30 September 2016; received in revised form 17 January 2017; accepted 19 January 2017

Abstract

Zinc plays an important role in maintaining intestinal barrier function as well as modulating cellular signaling recognition and protein kinase activities. The phosphatidylinositol 3-kinase (PI3K) cascade has been demonstrated to affect intercellular integrity and tight junction (TJ) proteins. The current study investigated the hypothesis that zinc regulates intestinal intercellular junction integrity through the PI3K/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway. A transwell model of Caco-2 cell was incubated with 0, 50 and 100 μM of zinc at various time points. Transepithelial electrical resistance (TEER), paracellular permeability, TJ proteins, cell proliferation, differentiation and cell damage were measured. Compared with controls, 50 and 100 μM of zinc increased cell growth at 6, 12 and 24 h and the expression of proliferating cell nuclear antigen at 24 h. Zinc (100 μM) significantly elevated TEER at 6–24 h and reduced TJ permeability at 24 h, accompanied by the up-regulation of alkaline phosphatase (AP) activity and zonula occludens (ZO)-1 expression. In addition, zinc (100 μM) affected the PI3K/AKT/mTOR pathway by stimulating phosphorylation of AKT and the downstream target mTOR. Inhibition of PI3K signaling by LY294002 counteracted zinc promotion, as shown by a decrease in AP activity, TEER, the abundance of ZO-1 and phosphorylation of AKT and mTOR. Additionally, TJ permeability and the expression of caspase-3 and LC3II (markers of cell damage) were increased by addition of PI3K inhibitor. In conclusion, the activation of PI3K/AKT/mTOR signaling by zinc is involved in improving intestinal barrier function by enhancing cell differentiation and expression of TJ protein ZO-1.

Keyword: Zinc; Barrier function; Tight junction protein; PI3K/AKT/mTOR; Caco-2 cells

1. Introduction

The intestinal epithelium serves as both a selective barrier to permit the absorption of nutrients, salts and water from the intestinal lumen into circulation [1,2] and also a protective barrier to prevent luminal antigens, microorganisms and toxins from entering the internal environment [3–5]. Intestinal barrier function is composed of epithelial cells and the paracellular apical junction complex. The homeostatic state of epithelial cells, which includes cycles of cell proliferation, differentiation and apoptosis every 3–4 days, is required for physiological intestinal barrier function [5,6]. The paracellular apical junction complex is maintained through adherens junction (AJ) and tight junction (TJ) expression, including cadherins, claudins, occludin, tricellulin and junctional adhesion molecules, which form a seal between adjacent cells and provide cytoskeletal anchoring [7,8]. Additionally, the scaffolding proteins zonula occludens (ZO-1, -2 and -3) play a central role in TJ organization and regulation since they are responsible for cytoskeleton reorganization [9]. The disruption of intestinal barrier integrity is associated with intestinal inflammation [10,11] and a number of gastrointestinal diseases, including irritable bowel syndrome, inflammatory bowel disease and celiac disease [12,13]. This process often results from the imbalance between cell growth and apoptosis, as well as alterations in TJ proteins. Therefore, it is essential to advance our knowledge in improving intestinal barrier function by modulating the underlying molecular mechanisms in healthy and diseased states.

Zinc has several essential functions in cellular physiology, such as the regulation of enzymatic reactions, DNA synthesis and signal recognition [14,15]. In particular, zinc plays a vital role in the development and function of the intestine by increasing epithelial

Abbreviations: AJ, adherens junction; AKT, protein kinase B; AP, alkaline phosphatase; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; mTOR, mammalian target of rapamycin; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; TEER, transepithelial electrical resistance; TJ, tight junction; ZO, zonula occludens.

☆ This work was supported by the Beijing Higher Education Young Elite Teacher Project, the National Natural Science Foundation of China (no. 30901031) and the Chinese Universities Scientific Fund (no. 2015DK005).

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http://dx.doi.org/10.1016/j.jnutbio.2017.01.013
0955-2863/

cell proliferation [17,18], improving cell turnover and repair [9,20], and maintaining structure and barrier function [18,21–23]. In animal studies, pharmacological doses of zinc are widely used to improve performance and alleviate intestinal permeability and diarrhea [22,24]. In studies using human epithelial cell lines, zinc deficiency is causing release of zinc bound to protein in order to elevate cytosolic free zinc [25–27], which may directly inhibit cell growth and disassemble TJ proteins, leading to intestinal barrier dysfunction [26,28]. Additionally, zinc supplementation attenuated chloride secretion and diarrhea, as well as improved the membrane barrier permeability and the arrangement of TJ [6,29,30]. However, the molecular pathways mediating the regulation of intestinal barrier function by supplemental zinc remain unclear. Several papers report that the phosphatidylinositol 3-kinase (PI3K) cascade plays critical roles in controlling proliferation, differentiation and apoptosis [31–34] and regulating intercellular junction integrity, as well as TJ proteins [34–36]. Zinc has been shown to have insulin-mimetic effects in activating PI3K/protein kinase B (AKT) signaling cascade *via* the regulation of gene expression [37]. Zinc induces the phosphorylation of PI3K/AKT, as a part of the signaling cascade, promoting activation and proliferation of lung epithelial cells [38] and myogenic cells [39,40]. However, whether PI3K/AKT is involved in zinc-enhanced intestinal barrier function has not been investigated.

In the current study, we tested the hypothesis that the PI3K-dependent signaling pathway might play an important role in the modulation of zinc-induced intestinal epithelial junction integrity. Human intestinal epithelial Caco-2 cells were subjected to zinc supplementation with and without the inhibition of PI3K pathway by LY294002. The transepithelial electrical resistance (TEER), paracellular permeability, TJ expression, cell proliferation, differentiation and cell damage were examined to explore the cellular response.

2. Methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum and antibiotics were from GIBCO. LY294002 was from Calbiochem. Cell counting kit-8 (CCK-8) was from Dojindo. Alkaline phosphatase (AP) colorimetric assay kit was from Abcam. TRIzol and PrimeScript RT Master Mix were from TaKaRa. Fluorescein isothiocyanate (FITC)-dextran, lactic dehydrogenase (LDH)-based kit, zinc sulfate heptahydrate and antibody against β-actin were from Sigma-Aldrich. Primary antibodies for ZO-1 and occludin were obtained from Invitrogen; E-cadherin, LC3I, LC3II, phosphorylated AKT (p-AKT), AKT, phosphorylated-mTOR (p-mTOR) and mTOR were purchased from Cell Signaling Technology; and caspase-3 and proliferating cell nuclear antigen (PCNA) were from Bioworld Technology.

2.2. Cell culture

The human colon carcinoma Caco-2 cells were obtained from ATCC (HTB-37) and grown in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. For all experiments, unless indicated, the cells between passage 20 and 30 were serum deprived overnight to synchronize the cell cycle and then were incubated with 0, 50 and 100 μM zinc-supplemented DMEM for the indicated time periods. Supplemental zinc ($ZnSO_4 \cdot 7H_2O$, Sigma) was dissolved in deionized distilled water and filter-sterilized with a 0.2-mm filter (Corning) prior to culture media supplementation. Zinc concentrations used in this study are based on previous studies [6,19] and our preliminary observations that low levels (at or below 30 μM) did not enhance cell growth and proliferation and that high levels (at or above 150 μM) were cytotoxic and induced cell rounding and loss of adherence. All samples were pretreated in the absence or presence of the PI3K inhibitor LY249002 (20 μM) for 2 h prior to treatment with or without zinc.

2.3. Cell viability assay

Cell viability was determined using the CCK-8 assay according to manufacturer protocol. Briefly, Caco-2 cells were plated in 96-well culture plates at a density of 6000 cells/well 24 h before treatment. Then, the cells were treated with 0, 50 and 100 μM zinc in serum-free DMEM for the indicated time point. Each value was normalized to the control and then calculated as previously described [41].

2.4. Measurement of TEER and permeability

For measurement of TEER, Caco-2 cells were grown to confluence on transwell inserts (membrane area 0.33 cm^2, pore size 0.4 μm, Coring) and achieved a polarized and differentiated state within 21 days after seeding. TEER was monitored daily prior to differentiation to test the effect of treatment using Millicell Electrical Resistance System-2 (Millipore). TEER was recorded with three consecutive measurements and expressed as Ω (resistance) × cm^2 (surface area of the monolayer) after subtracting the filter resistance value. For evaluating intestinal paracellular permeability of the Caco-2 cell monolayer, FITC-dextran (4 kDa) was widely used as an established paracellular marker in recent years [5,26]. In brief, 5 μl of 10 mg/ml FITC-dextran was added to the apical side of the transwell chamber. Thirty minutes after the addition of FITC-dextran, medium from the basolateral side of the transwell chamber was collected, and fluorescence intensity was measured using a Multimode Reader (SpectraMaz M3, Molecular Devices) at 485-nm excitation and 520-nm emission wavelengths.

2.5. Cell cytotoxicity and differentiation

Caco-2 cells were grown on transwell inserts to achieve a polarized and differentiated state, and then pretreated with or without PI3K inhibitor for 2 h followed by treatment with and without zinc (100 μM) for 24 h. Cell cytotoxicity was analyzed by an LDH-based kit according to a protocol described by Wang et al. [6]. Cell differentiation was determined by AP colorimetric assay kit as previously described [42].

2.6. Quantification of TJ protein mRNA levels

Total RNA was isolated using TRIzol reagent and reverse transcribed using PrimeScript RT Master Mix according to the manufacturer's instructions. cDNA was used as template for polymerase chain reaction (PCR) amplification using SYBR Green PCR technology (TaKaRa) in an ABI 7500 Real-Time PCR machine (Applied Biosystems, USA). The gene-specific primers for *PI3K110α* (NM_006218, forward, CCACGACCATCATCAGGTGAA; reverse, CCTCACGGAGGCATTC TAAAGT), *RHOA* (NM_001664, forward, AGTTTCTTCCGGATGGCAG; reverse, CGGTCTGGTCTTCAGCTACC), *MLCK* (NM_001256799, forward, CACCGTCCATGAAAAGAAGAGTAG; reverse, GAGAGGCCCTG CAGGAAGATGG) and *GAPDH* (NM_001256799, forward, GGAGCGA GATCCCTCCAAAAT; reverse, GGCTGTTGTCATACTTCTCATGG). Relative gene expression was calculated using the ΔΔCt method and expressed as the ratio of target genes to *GAPDH*.

2.7. Assessment of protein expression by Western blot analysis.

Cells were collected and lysed as previously described [39], and the protein concentration was quantified using the BCA Assay (CWBiotech). Proteins (30 μg) were separated on a 4%–20% Mini-PROTEAN TGX Gel (Bio-Rad) and then transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked with 5% nonfat