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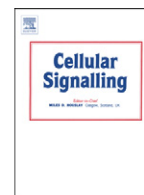

ZnT7 can protect MC3T3-E1 cells from oxidative stress-induced apoptosis via PI3K/Akt and MAPK/ERK signaling pathways

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ABSTRACT

The osteoblasts could be lead to the occurrence of apoptosis by oxidative stress. The zinc transporter family *SLC30A* (ZnTs) plays an important role in the regulation of zinc homeostasis, however, its function in apoptosis of MC3T3-E1 cells remains unknown. This study was aimed to investigate the role of zinc transporters in cell survival, particularly in MC3T3-E1 cells, during oxidative stress, and the molecular mechanism involved. Our study found that hydrogen peroxide can induce zinc-overloaded in the cells. While high concentration of zinc plays an important role in inducing apoptosis of the MC3T3-E1 cells, we demonstrated that ZnT7 can protect MC3T3-E1 cells and reduce the aggregation of intracellular free zinc ions as well as inhibit apoptosis induced by H_2O_2. Moreover, ZnT7 overexpression enhanced the anti-apoptotic effects. Interestingly, suppression of ZnT7 by siRNA could significantly exacerbate apoptosis in MC3T3-E1 cells. We also found that ZnT7 promotes cell survival via two distinct signaling pathways involving activation of the PI3K/Akt-mediated survival pathway and activation of MAPK/ERK pathway. Collectively, these results suggest that ZnT7 overexpression significantly protects osteoblasts cells from apoptosis induced by H_2O_2. This effect is mediated, at least in part, through activation of PI3K/Akt and MAPK/ERK pathways.

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1. Introduction

Zinc is an essential micronutrient and cytoprotectant involved in oxidative stress. Because zinc cannot freely pass the membrane of cells and intracellular organelles, zinc transporter proteins have been thought to play a critical role in regulating the zinc concentration in the cells. We tested whether zinc transporters, the critical regulators that maintain intracellular zinc concentrations, play a role in cell survival, particularly in mouse osteoblasts cells, during oxidative stress and apoptosis. Zinc transporters comprise a family of multiple transmembrane-spanning domain proteins that are encoded by two solute-linked carrier (SLC) gene families, the ZIP proteins (SLC39a) and the ZnT proteins (SLC30a). ZIPs facilitate influx of zinc to the cytosol from the outside of cells or from the lumen of intracellular compartments while ZnTs ensure zinc efflux from cytosol to the outside of cells or to intracellular organelles [1]. Ten genes encoding ZnT protein family members (ZnT1 through ZnT10) have been identified in the human genome [2]. Members of the ZnT family have similar membrane topology with six transmembrane domains and a histidine-rich loop between transmembrane domains IV and V, where zinc may be bound by histidines and subsequently transported across the membrane [3]. ZnT9 has been isolated from human embryonic lung cells where it is localized to the cytoplasm and nucleus. However, for ZnT9 no zinc-transporting role has been identified [4]. ZnT10 has a role in regulating Zn homeostasis in the brain so it may have relevance to the development of neurodegenerative disease. But, the ZnT10 protein has not yet been characterized, thus its role in zinc transport remains hypothetical [5, 6]. ZnTs are not expressed evenly in all tissues: in mammals the distribution of ZnTs is as follows: ZnT1 which expressed with a high expression in placenta is involved in zinc efflux across the plasma membrane [7]. ZnT2 expression is restricted to tissues with unique zinc requirements such as mammary and prostate glands [8]. Increasing evidence suggests that ZnT3 is present in various cell types like different cell types in the brain, cells from adipose tissue, beta-cells from pancreatic islets, epithelial cells, cells from testis, prostate cancer cells and cells from

Abbreviations: ZnT, zinc transporter; α-MEM, α-Minimal essential medium; FITC, fluorescein isothiocyanate; PI3K, Phosphoinositide 3-kinase; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; DCF-DA, 2,7-dichlorofluorescein-diacetate; TBS, tris-buffered saline; PI, propidium iodide; AIF, Apoptosis-inducing factor; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase.

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retina. The expression of ZnT3 is regulated by age, hormones, fatty acids, zinc chelation, and glucose [9]. ZnT4 is abundantly expressed in brain and mammary glands with low level expression in other tissues [10]. Znt5 is selectively required for the mast cell-mediated delayed-type allergic response, and it is a novel player in mast cell activation [11]. ZnT6 may function in transporting the cytoplasmic zinc into the Golgi apparatus, as evidenced by its overlapping intracellular localization with TGN38 and transferrin receptor in the normal rat kidney cells [12]. ZnT7 is abundantly expressed in the ganglion cells and pigment epithelial cells of the mouse retina [13]. ZnT8 is specifically expressed in the pancreatic β-cells and has been identified as a novel target autoantigen in patients with type 1 diabetes [14]. The expression of zinc transporter may have an impact on apoptosis in cells. Previous studies have proved that the expression of zinc transporter has an important implications on the occurrence of apoptosis [15, 16]. However, the relation between zinc transporter and osteoblast apoptosis has not been reported.

Here we describe the identification and functional characterization of ZnT7. Our studies demonstrate that the ZnT7 protein is abundantly expressed in mouse osteoblasts MC3T3-E1 cells. ZnT7 is involved in two transporting mechanisms. Firstly, it facilitates transportation of the cytoplasmic zinc into the Golgi apparatus of the cell for zinc storage. Secondly, it mediates incorporation of zinc into newly synthesized zinc transporter proteins [17]. Here, we have investigated whether this protein also controls H_2O_2-induced apoptosis in MC3T3-E1 cells. Our investigation showed that the induction of ZnT7 is essential for the mobilization of zinc into the Golgi under H_2O_2 conditions, thereby protecting cells from undergoing apoptosis. We then measured the mechanism of the cytoprotective effects of ZnT7. The results were related to the activation of ERK dephosphorylation and activation of PI3K/Akt signaling.

2. Material and methods

2.1. Reagents

α-Minimal essential medium (α-MEM), penicillin–streptomycin (5000 U/ penicillin; 5000 U/ml streptomycin), and fetal bovine serum (FBS) were obtained from Gibco Laboratories (NY, USA). H_2O_2 Triton X-100 and DMSO were purchased from Sigma (St. Louis, USA). Anti-caspase3, and anti-caspase9, were obtained (Cell Signaling Technology, USA). Anti-Bax, anti-AIF, anti-p-AKT, anti-p-ERK, and anti-β-actin were obtained (Santa Cruz Biotechnology, USA). Annexin V/FITC Apoptosis Detection Kit I was purchased (Clontech Laboratories Inc., USA). ECL kit was purchased (Pierce, thermo Co. Ltd, USA). Microplate spectrophotometer (Bio Tek, USA), flow cytometer (FACSCalibur, Becton-Dickinson, USA). All reagents used were trace element analysis grade. All waters used were glass distilled.

2.2. Cell culture

Mouse osteoblastic MC3T3-E1 cells (ATCC, CRL-2593, USA) were cultured in regular growth medium (α-MEM with 10% FBS, and 1% penicillin/streptomycin) at 37 °C in a 5% CO_2 incubator. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA for experiments, and cells were cultured for 24 h to obtain monolayers containing 3 ml of α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium containing either H_2O_2 or other disposal agents and the cells were cultured further.

2.3. MTT assay

Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 96-well plates. Briefly, cells were plated at seeding MC3T3-E1 cells (6×10^3 cells/well in 96-well plate) and maintained in growth media for 24 h at 5% CO_2 at 37 °C. At 60% confluence, the cells were treated with (0–1 mmol/l) H_2O_2 for 24, 48 and 72 h. Thereafter, 10 μl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 4 h at 37 °C. After formation of formazan crystals, MTT medium was then aspirated and replaced with 150 μl of solubilization solution dimethyl sulfoxide (DMSO) for dissolving the formazan crystals. And the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm. Relative cellular growth was determined by a ratio of average absorbance in treatment cells versus the average absorbance in control cells. The cell viability was calculated as the ratio of optical densities.

2.4. Analysis of apoptosis

Cellular apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit I (Clontech Laboratories Inc., USA). Briefly, MC3T3-E1 cells were cultured at 4×10^6 cells/ml and seeded in 6-well plates. Cells were harvested by trypsinization, then washed twice with cold PBS and centrifuged at 1000 rpm. About 1×10^5–1×10^6 cells were resuspended in 300 μl of 1× binding buffer, centrifuged again at 1000 rpm for 5 min and then supernatant was removed. Cells were resuspended in 300 μl of 1× binding buffer and transferred to a sterile flow cytometry glass tube. 10 μl of Annexin V-FITC was added and incubated in the dark for 30 min at room temperature. Then cells were incubated in the dark with 5 μl of propidium iodide and analyzed by a flow cytometer (FACSCalibur, Becton-Dickinson, USA).

2.5. Detection of intracellular ROS level

To determine ROS generation within H_2O_2-treated cells, FACS analysis was performed. Cells were exposed to H_2O_2 for different hours and then stained with 5 μg/ml of DCF-DA for 30 min and subjected to flow cytometry using a Becton-Dickinson FACScalibur and analyzed by CellQuest software (Becton-Dickinson, San Jose, CA).

2.6. Measurement of Zn in MC3T3-E1 cells

Harvested cells were rinsed three times in PBS, digested in 2% SDS and boiled for 10 min. Protein concentrations were measured with BCA protein assay (Pierce, Rockford, IL). Zn levels were measured by flame mode using a Shimadzu AA-6300 atomic absorption spectrophotometer.

2.7. Transfection and RNA interference

We cloned hZnT-7-EGFP and control EGFP vector, human ZnT7 sequence obtained from GenBank (ZnT7; GenBank accession no.NM_133496), then we cut hZnT-7 by restriction enzymes EcoR I and Xho I, and after that we inserted hZnT-7 into a pcDNA3.1/myc-hisA vector for sequencing. hZnT-7-pcDNA3.1/myc-hisA and pcDNA3.1/myc-hisA were transiently transfected into MC3T3-E1 cells and checked the presence of the hZnT-7 RNA in it by reverse transcription polymerase chain reaction ZnT7 siRNA (Stealth RNAi) prepared by Invitrogen. The sequence of the siRNA targeting a specific sequence in ZnT7 mRNA was 5′-GCCAUAGUCACGAAGCCAATT-3′. The scrambled siRNA sequence was 5′-UUGGCUUCGUGACUAUGGCTT-3′. The target sequences of the ZnT7 siRNA and control ZnT7 siRNA were BLAST searched against the GenBank database. All transfection experiments were accomplished in independent triplicates in accordance with the manufacture's protocol for Lipofectamine 2000. Cells were allowed to recover in α-MEM for 24 h after transfection.

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