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Zinc transporter 5 and zinc transporter 7 induced by high glucose protects peritoneal mesothelial cells from undergoing apoptosis

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ABSTRACT

Zinc is an essential micronutrient and cytoprotectant involved in many types of apoptosis. The zinc transporter family *SLC30A* (ZnTs) is an important factor in the regulation of zinc homeostasis; however, its function in apoptosis in peritoneal mesothelial cells (PMCs) remains unknown. This study explores the regulation of zinc transporters and how they play a role in cell survival, particularly in rat peritoneal mesothelial cells (RPMCs), surrounding glucose concentrations, and the molecular mechanism involved. The messenger RNA (mRNA) transcripts were quantitatively measured by real-time polymerase chain reaction for all known nine zinc transport exporters (*SLC30A1–8,10*), as well as in primary RPMCs and the cells cultured under nonstimulated and HG-stimulated conditions. While many zinc transporters were constitutively expressed, ZnT5 mRNA and ZnT7 mRNA were strongly induced by HG. Overexpression of ZnT5 and ZnT7 respectively resulted in a decrease in the expression of caspace 3, caspace 8, *BAX*, and AIF and coincided with cell survival in the presence of HG. Inhibition of ZnT5 and ZnT7 expression using considerable siRNA-mediated knockdown of RPMCs was examined and, afterwards, the impact on cell apoptosis was investigated. Increased levels of apoptosis were observed after knockdown of ZnT5 and ZnT7. Furthermore, overexpression of ZnT5 and ZnT7 is accompanied by activation of PI3K/Akt pathway and inhibiting HG-induced apoptosis. This study suggests that the zinc transporting system in RPMCs is influenced by exposure to HG, particularly ZnT5 and ZnT7. This may account for the inhibition of HG-induced RPMC apoptosis and peritoneum injury, likely through targeting PI3K/Akt pathway-mediated cell survival.

1. Introduction

Continuous ambulatory peritoneal dialysis (CAPD) is a form of renal replacement therapy for end-stage renal disease (ESRD) [1]. The peritoneal membrane surface is covered by a monolayer of peritoneal mesothelial cells (PMCs) that constitute the major surface area for molecular exchange and they produce a variety of cytokines. These cytokines are involved in the regulation of peritoneal permeability and local host defense during peritoneal dialysis (PD) [1,2]. Glucose is employed as an osmotic agent in peritoneal dialysis solutions (PDS) because it is inexpensive, safe, and readily metabolized. However, the use of glucose-based solutions has some disadvantages with known non-physiological features, such as low pH, high lactate, hyperosmolarity, and glucose-degradation products [3,4]. All of these may disrupt cell homeostasis and damage peritoneal membrane integrity, contributing to fibrosis of the peritoneal membrane and the failure of peritoneal function. Previous studies have shown that if the rate of apoptosis outweighs that of proliferation, the loss of homeostasis can occur, favoring cellular deletion of peritoneal cells, which as a potential mechanism for the development and progression of peritoneal fibrosis during long-term PD leads to the failure of peritoneal membrane function [5,6].

Zinc (Zn) is critical for the activities of various enzymes, including a number of Zn-dependent enzymes, such as Cu–Zn-superoxide dismutase (Cu–Zn-SOD) and the metalloproteinase, that contribute to cellular signaling pathways as well as transcription factors [7,8]. Changes

Abbreviations: RPMCs, rat peritoneal mesothelial cells; HPMCs, human peritoneal mesothelial cells; HG, high glucose; ZnT, zinc transporter; CAPD, continuous ambulatory peritoneal dialysis; ESRD, end-stage renal disease; CKD, chronic kidney disease; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine; RIPA, radioimmune precipitation assay; TGF, transforming growth factor; PI3K, Phosphoinositide 3-kinase; AIF, apoptosis-inducing factor; PBS, phosphate-buffered saline; DCF-DA, 2,7-dichlorofluorescein-diacetate; TBS, tris-buffered saline; PDF, peritoneal dialysis fluid; PI, propidium iodide.

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in intracellular Zn concentration have a strong influence on apoptotic processes induced by diverse physical, chemical, and immunological stimuli [7–10]. Regulation of zinc homeostasis is ensured by two different zinc-transporter families. The Irt-like protein (ZIP) family facilitates the influx of zinc into the cytosol from the outside of cells and from the lumen of intracellular compartments. Conversely, most ZnT family members facilitate removal of zinc from the cytosol, either out of the cell or into the lumen of vesicles and organelles [11,12]. The mammalian ZIP family consists of 14 different proteins, whereas the ZnT family comprises 10 proteins [13]. ZnT1 is a plasma membrane efflux transporter that was shown to confer Zn resistance to cells [11]. ZnT2 and ZnT4 are localized in cell vesicles [11]. ZnT3, which is expressed mainly by neuronal cells, incorporates Zn into vesicles at neuronal presynaptic terminals [14]. ZnT5 is thought to transport Zn into insulin-containing secretory granules of pancreatic β cells [15]. ZnT7 expression is highest in the liver and small intestine with moderate expression in the kidney, spleen, heart, brain, and lungs [9,16]. In addition, ZnT5 and ZnT7 are both located in the Golgi apparatus and implicated in the translocation of cytoplasmic zinc into the Golgi network [9,10,17].

These zinc transporters play a crucial role in maintaining the cellular balance between cell growth and cell death, and a model has recently been proposed that demonstrates how zinc is buffered and stored within cells [18]. Recently, some studies reported that a role for zinc transporter gene expression in zinc-mediated apoptosis and cytoprotection is vital following airway inflammation in mouse-lung or cultured-sheep pulmonary artery endothelial cells [19,20]. In addition to ZnT3 expression, it has been reported that glucose metabolism can be affected by alterations in the zinc transporter system in vivo using a knockout mouse model [21]. Most recently, we have been able to inhibit HG-mediated apoptosis by exposing RPMCs to supplemental zinc in the medium, but the mechanism remains unclear [22]. In light of these investigations, here aimed to explore whether HG influences zinc transporter expression, we investigated zinc transporters, the primary regulators of zinc homeostasis in mammals, and to what extent zinc transporters contribute to HG-induced apoptosis in RPMCs. Our investigation showed that the induction of ZnT5 and ZnT7 expression in RPMCs is essential for the mobilization of zinc into the Golgi under high-glucose conditions, thereby protecting cells from apoptosis.

2. Materials and methods

2.1. Reagents

Penicillin–streptomycin (5000 U/mL penicillin; 5000 U/mL streptomycin) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (NY, USA). Triton X-100 and DMSO were purchased from Sigma (St. Louis, USA). Zn sulfate was obtained from Xinhua Pure Chemical Industries (Shenyang, China). Anti-caspase3 and anti-caspase8 were obtained (Cell Signaling Technology, USA). Anti-Bax, anti-AIF, anti-p-Akt, 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) and flow cytometer (FACSCalibur, Becton-Dickinson, USA) were purchased. All reagents used were of trace element analysis grade. All water used was glass distilled.

2.2. Isolation and culture of RPMCs

The RPMCs were isolated and cultured as described before [23]. Briefly, the surgically resected omenta from Sprague–Dawley rats (150 g–180 g) were digested with 0.125% trypsin/0.01% ethylenediamine tetraacetic acid (EDTA) for 25 min at 37 °C, followed by neutralization with DMEM/F12 medium supplemented with 10% fetal calf serum (FCS, Sigma). The tissue-free cell suspension was then centrifuged at 60 *g* at 4 °C for 5 min before the supernatant was removed. The cell pellet was resuspended to a final volume of 4 mL culturing medium and then seeded into 25 cm^2 tissue culture flasks. Culturing medium is consisted of DMEM/F12 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin (all from Life Technologies Inc.). After 1–3 days incubation at 37 °C, the media were changed for the first time. The cells were passaged when they grew to 80% confluence. Cells were then transferred to serum free DMEM/F12 medium for overnight starvation prior to each experiment. In the control groups, the RPMCs cells were treated with serum free DMEM/F12 medium only.

2.3. Assessment of cell viability

Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 96-well plates, which is an indicator of the mitochondrial activity of living cells. Briefly, at the indicated time after treatment, 10 μL MTT (final concentration, 500 μg/mL) was added to the medium and incubated at 37 °C for 3 h. The MTT solution was removed and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the colored formazan crystals for 15 min. The absorbance at 490 nm of each aliquot was measured using a Sunrise RC microplate reader (TECAN, Switzerland). Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

2.4. Cell cycle

Cells were collected, and added precooled 70% ethanol fixed overnight. The next morning, the cells were collected and washed and centrifuged in PBS solution at 800 rpm for 5 min. Then the cells were resuspended in the solution of 1% R-Nase 0.5 mL in 37 °C water for 30 min. Lastly, cells were stained with propidium iodide (PI). Cell cycle analysis was performed to determine G1 stage and cell cycle distributions. DNA contents and cells were analyzed by flow cytometer (FACSCalibur, Becton-Dickinson, USA).

2.5. Analysis of apoptosis

RPMCs 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 then resuspended in 300 μL 1× binding buffer, centrifuged again at 1000 rpm for 5 min and then supernatant was removed. Cells were resuspended in 300 μL 1× binding buffer and transferred to a sterile flow cytometry glass tube. 10 μL Annexin V/FITC Annexin was added and incubated in the dark for 30 min at room temperature. Then cells were incubated in the dark with 5 μL propidium iodide. Lastly, cells were analyzed by flow cytometer. Cellular apoptosis was determined using the Annexin V/FITC Apoptosis Detection Kit I.

2.6. Terminal uridine nick 3′end labeling (TUNEL) method

After incubation with 1% paraformaldehyde for 5 min, cells were fixed in 2:1 v/v ethanoluacetic acid for 10 min at room temperature. After three washings with PBS, cells were incubated with 100 U/mL terminal deoxyuridine transferase, 0.5 μg/mL biotinylated uridine in 1 M potassium cacodylate and 125 mM Tris–HC1, 2.5 mM cobalt chloride (Boeringher, Mannheim, Germany), at pH 6.6 for 1 h at 37 °C in a humidified chamber. After washes, a 1:40 solution of fluoresceinated streptavidin (Boeringher) was incubated for 30 min at room temperature. Slides were counter-stained with 0.3 μg/mL propidium iodine (Sigma) in PBS for 1 min at room temperature. An epi-fluorescent microscope (Ernst Leitz, Inc., Rockleigh, NJ, USA) was used to detect apoptotic cells, which were quantified by counting the number of fluorescein-positive cells relative to the total number of cells in at least 10 microscopic fields. For each experiment 200 cells were counted.