Abstract. Zinc (Zn) deficiency is important for inducing nucleotide-binding domain and leucine-rich-repeat-containing family, pyrin domain-containing-3 (NLRP3) inflammasome activation in macrophages. However, its function in the NLRP3 inflammasome activation of peritoneal mesothelial cells (PMCs) remains to be elucidated. In the present study, the human PMC (HPMC) line HMrSV5 was co‑treated with high glucose and either ZnSO4 or a Zn chelator. The activity of the NLRP3/caspase‑1 inflammasome was assessed via western blot analysis, immunofluorescence, reverse transcription‑quantitative polymerase chain reaction and ELISA. In addition, the activity of the nuclear factor erythroid 2‑related factor 2 (Nrf2) pathway was detected using western blotting, and the level of reactive oxygen species (ROS) was assessed by 2,7‑dichlorofluorescein fluorescence and flow cytometry. It was found that Zn supplementation inhibited HG‑induced NLRP3 inflammasome activation in the HPMCs by attenuating ROS production. Further experiments revealed that Zn supplementation inhibited the HG‑induced production of ROS through activation of the Nrf2 antioxidant pathway. These results indicated that Zn inhibited NLRP3 inflammasome activation in the HG‑treated HPMCs by activating the Nrf2 antioxidant pathway and reducing the production of ROS.

Introduction

Peritoneal dialysis (PD) is a key model of renal replacement therapy for end-stage renal disease (1). The peritoneal membrane is covered with a monolayer of mesothelial cells, which exhibit characteristics of epithelial cells, act as a permeability barrier, and produce a variety of cytokines involved in the regulation of peritoneal permeability and local host defense (1,2). Conventional PD solutions contain glucose as an osmotic agent for peritoneal dialysis fluids, which include commercially available 1.5, 2.5 and 4.25% glucose solutions (3). However, long‑term exposure to the conventional PD solutions with high concentrations of glucose and glucose degradation products may activate various inflammatory cytokines and growth factors, which can result in damage to human peritoneal mesothelial cells (HPMCs), including high glucose (HG)-induced profibrotic and proinflammatory reactions (4,5).

The nucleotide‑binding domain and leucine‑rich repeat-containing family, pyrin domain-containing-3 (NLRP3) inflammasome is an important component of the innate immune system and is composed of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase‑1. The NLRP3 inflammasome senses endogenous and exogenous danger signals, including lipopolysaccharide (LPS) and HG, resulting in the activation of caspase‑1 and subsequent activation of cytokines interleukin (IL)-1β, IL‑18 and IL‑33 (6). This trigger sustained inflammation and has been associated with the HG-containing peritoneal dialysis-associated proinflammatory reaction, and may be an effective therapeutic target for preventing HG-induced profibrotic and proinflammatory reactions to PMCs (7).

Zinc (Zn) is an essential trace element, which is important for various cellular functions, including apoptosis, signal transduction, transcription, differentiation and replication, in all organ systems and during embryonic development (8‑12). Several studies have demonstrated that Zn supplementation inhibits fibrosis in animal models and human chronic inflammatory diseases, including liver fibrosis, myocardial fibrosis, vasculitis, perivascular fibrosis and cystic fibrosis (13‑16). Zn deficiency has been reported to be associated with NLRP3 inflammasome activation and the production of IL‑1β in macrophages (17). However, the effect of Zn on HG-induced NLRP3 inflammasome activation in HPMCs remains to be fully elucidated. To address these questions, the present study investigated the effect of Zn on HG‑induced NLRP3 inflammasome activation in HPMCs in vitro. To examine the mechanism, the effects of Zn on the production of reactive oxygen species (ROS) and activity of the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway were measured.
Materials and methods

Reagents. Penicillin-streptomycin (5,000 U/ml penicillin; 5,000 U/ml streptomycin) and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). ZnSO₄ was obtained from Xinhua Pure Chemical Industries (Shenyang, China). Zn chelator N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was obtained from Sigma; Merck Millipore (Darmstadt, Germany). The ECL kit was purchased from Pierce; Thermo Fisher Scientific, Inc.). All reagents used were trace element analysis grade.

Cell culture. The HPMC line HMrSV5 was provided by Professor Huimian Xu of The First Affiliated Hospital of China Medical University (Shenyang, China) and cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS. The cells were incubated at 37°C in a 5% CO₂ atmosphere and the culture medium was replaced every 2-4 days. The cells were liberated with trypsin-EDTA for subculture in new dishes with a subcultivation ratio of 1:2-1:3. Cells at passages 5-10 were used in all experiments.

MTT assay. The cells were seeded into 96-well plates (4,000 cells/well) and cultured to 70-80% confluence. Following incubation in DMEM with 0.01% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h, the cells were divided into different treatment groups, with six replicate wells for each treatment group. Following incubation at 37°C for 6, 12, 24 or 48 h, 20 µl of MTT (5 mg/ml) was added to each well and the plates were incubated for an additional 4 h. Subsequently, the medium was discarded, and 100 µl of DMSO was added to each well and mixed thoroughly. The absorbance value of the wells was read at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transfection and Zn treatment. For transfection, 150 nM of Nrf2 siRNA (sense, 5'-GAAGCCAGAUGUUAGAAAUU-3'; antisense, 3'-UUUCUUCGUGUACCAUUUCUUU-5') or scrambled siRNA (sense, 5'-UAGCGACUAAACACAUCAAUU-3'; antisense, 3'-UUUAUCGCUGAUUUGUGAUU-5'; both GenePharma, Shanghai, China) was transfected into the HPMCs respectively, with Lipofectamine 2000. Following incubation for 24 h, the cells were treated with 10 µM ZnSO₄ at 37°C for 24 h, followed by the incubation with 126 mM HG for an additional 24 h. To deplete intracellular Zn stores, the Zn chelator (TPEN; 1 µM) was added 4 h prior to the 24 h incubation with 126 mMol/l HG.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. The mRNA levels of NLRP3, caspase-1, heme oxygenase-1 (HO-1), NQO1, IL-1β and IL-18 were evaluated using RT-qPCR analysis. Total RNAs were isolated from the cells using TRIzol reagent and reverse transcription was performed using a Takara RNA PCR kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The qPCR was performed using SYBR Green Premix Ex Taq (Takara Bio, Inc.) on a Light Cycler 480 (Roche Diagnostics, Basel, Switzerland). β-actin mRNA was used as an internal control. The forward and reverse primers were as follows: β-actin, forward 5'-CTGTCCTCCTGTAGCCCTCG-3' and reverse 5'-ATGTCACGCACAGATTTC-3'; Nr2f2, forward 5'-CAGTGCCTCTATGCGTGA-3' and reverse 5'-GCGCTTGAATGTTCGTC-3'; NQO1, forward 5'-CCTTAAGGTT CGTCTTTGCG-3' and reverse 5'-CAATCGAGGCCTCTTC TG-3'; NLRP3, forward 5'-CAAGGCAGCTCGTATTG-3' and reverse 5'-CTTCAATCTCAACAGCGCAT-3'; caspase-1, forward 5'-AGACC CGACGTGTAGTGACT-5' and reverse 5'-AAATCTCTCG CGCATTCTTGCTTCC-3'; IL-1β, forward 5'-ATAGGCCAGC CTAGAGTA-3' and reverse 5'-ATCAAGGAGTATCAT TCT-3'; HO-1, forward 5'-GTCCAAATCTCAACAGCGCAT-3' and reverse 5'-GGC-3' and reverse 5'-GACAAGTTGCTGCCCTGGGA-3'. All reactions were run in triplicate, from which an average quantification cycle (Cq) was calculated. The gene expression levels were analyzed by comparing the values of ΔCq (ΔCq=Cq of target gene-Cq of housekeeper gene) transformed into a linear scale (2^ΔCq) (18).

Western blot analysis. The HPMCs were treated with medium alone or with 126 mM glucose in the presence or absence of ZnSO₄ or TPEN for 24 h. Proteins were extracted using a nuclear-cytosol extraction kit (Applygen Technologies, Inc., Beijing, China) according to the manufacturer's protocol. Protein concentrations were quantified using a BCA protein assay kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). For the western blot analysis, 30 or 50 mg/lane of total protein were boiled and separated by SDS-PAGE (6 or 12%). Following electrophoresis, proteins were blotted onto a PVDF membrane and blocked for 1 h at room temperature. Each membrane was incubated at 4°C overnight with primary antibodies against NLRP3 (cat. no. ab214185; Abcam, Cambridge, MA, USA; 1:1,000 dilution), Pro-caspase-1 (cat. no. ab14367; Abcam; 1:1,000 dilution), Caspase-1 (cat. no. #4199; Cell Signaling Technology, Inc.; 1:1,000 dilution), NQO1 (cat. no. #3700; Cell Signaling Technology, Inc.; 1:1,000 dilution), IL-1β (cat. no. D043-3; R&D Systems, Inc.; 1:500 dilution), IL-18 (cat. no. #3187; Cell Signaling Technology, Inc.; 1:1,000 dilution), α-tubulin (cat. no. #8004S; Cell Signaling Technology, Inc.; 1:1,000 dilution) and b-actin (cat. no. #3700; Cell Signaling Technology, Inc.; 1:1,000 dilution) as a control. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies [goat anti-mouse IgG(H+L); cat. no. E030120-02; EarthOx Life Sciences, Millbrae, CA, USA, 1:20,000 dilution] for 1 h at room temperature. Specific signals were detected using an enhanced chemiluminescence detection system. The experiments were repeated at least three times and densitometry was performed using Image J v1.48 software (National Institutes of Health, Bethesda, MD, USA).

ELISA. The HPMCs were treated with medium alone or with 126 mM glucose in the presence or absence of ZnSO₄ or TPEN for 24 h. The cell culture media were centrifuged at 1,500 x g for 10 min at 4°C and the supernatants were collected and stored at -20°C prior to analysis. The levels of IL-1β and IL-18 in the supernatants were determined using a human
IL-1β and IL-18 ELISA kit (R&D Systems, Inc.) according to the manufacturer's protocol.

Detection of intracellular ROS levels. The HPMCs were treated with medium alone or with 126 mM glucose in the presence or absence of ZnSO$_4$ or TPEN for 24 h. The levels of ROS were determined using a ROS assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, the HPMCs (5x10$^6$) were incubated with 10 µmol/l DCFH-DA probes at 37˚C for 30 min and washed with phosphate-buffered saline (PBS) three times in order to remove residual probes. DCFH-DA was deacetylated intracellularly using nonspecific esterase, which was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). DCF fluorescence was detected using a flow cytometer (Miltenyi Biotec, Inc., Auburn, CA, USA). The results were analyzed using MACSQuantify™ Software version 5.2 (Miltenyi Biotec, Inc.).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons among groups were performed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of HG on the production of IL-1β and IL-18. The HPMCs were treated with 76, 126 and 214 mM glucose for 6, 12, 24 and 48 h, and the levels of IL-1β and IL-18 in the supernatant were detected by ELISA. As shown in Fig. 1, at 24 and 48 h, the levels of the two cytokines increased significantly in a concentration-dependent manner following glucose treatment. However, the effect plateaued at the concentration of 126 mM for 24 h. Therefore, the HPMCs were treated with 126 mM glucose for 24 h in subsequent experiments.

Zn inhibits HG-induced production of IL-1β and IL-18 in HPMCs. The HPMCs were pretreated with 10 µM ZnSO$_4$ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h, following which the secretion of IL-1β and IL-18 in the supernatants of the HPMCs were examined by ELISA (Fig. 3A and B). The mRNA expression levels of IL-1β and IL-18 in the HPMCs were detected using RT-qPCR analysis (Fig. 3C and D). The protein expression levels of pro-IL-1β, pro-IL-18, IL-1β, IL-18 in the HPMCs were detected using western blot analysis (Fig. 3E-G). As shown in Fig. 3,
Zinc inhibits high glucose-induced NLRP3 inflammasome activation in the HPMCs. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. The levels of IL-1β and IL-18 were measured using enzyme-linked immunosorbent assays (ELISA). The mRNA expression levels of caspase-1 and NLRP3 in the HPMCs were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Immunofluorescence was used to analyze the coexpression of ASC (green) and NLRP3 (red) in the HPMCs. The HG-induced NLRP3 inflammasome activation was attenuated by pretreating the HPMCs with 10 µM ZnSO₄, which was shown by the reduced expression of NLRP3 and caspase-1, and the reduced coexpression of NLRP3 and ASC. Pretreating the HPMCs with 1 µM TPEN further increased the expression of NLRP3 and caspase-1, and the coexpression of NLRP3 and ASC.

Zinc inhibits high glucose-induced intracellular generation of ROS in HPMCs. The HPMCs were treated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h, and then incubated with 126 mM glucose for 24 h. The levels of intracellular ROS were detected using a DCFH-DA assay. As shown in Fig. 5A and B, the levels of ROS were reduced in the ZnSO₄-treated group compared to the HG group. The ROS levels were further increased in the TPEN-treated group. These results suggest that zinc can inhibit the high glucose-induced generation of ROS in the HPMCs.
intracellular ROS were increased in the HG-induced cells, compared with those of the control group. However, pretreatment with 10 µM ZnSO₄ significantly reversed the increased intracellular production of ROS induced by HG, whereas pretreating the HPMCs with 1 µM TPEN potentiated the HG-induced intracellular production of ROS.

Zn activates the Nrf2 antioxidant pathway in HPMCs. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h, and then incubated with 126 mM glucose for 24 h. The mRNA and protein expression levels for Nrf2 and Nrf2-regulated genes, HO-1 and NQO1, were analyzed to determine whether the Nrf2-antioxidant system was affected by Zn supplementation or inhibition. The results indicated that the mRNA expression of Nrf2, nuclear protein expression of Nrf2 and the expression of target genes of the Nrf2 antioxidant pathway were all increased in the HG-induced cells, compared with those in the control group. Pretreatment with 10 µM ZnSO₄ significantly reversed the increased intracellular production of ROS induced by HG, whereas pretreating the HPMCs with 1 µM TPEN potentiated the HG-induced intracellular production of ROS.

Figure 4. Effects of Zn intervention on HG-induced NLRP3 inflammasome activation in HPMCs. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. Protein expression levels of (A and B) pro-caspase-1, caspase-1 and (C and D) NLRP3 in HPMCs were detected using western blot analysis. mRNA expression levels of (E) NLRP3 and (F) caspase-1 in HPMCs were detected using reverse transcription-quantitative polymerase chain reaction analysis. (G) Immunofluorescence was used to analyze the coexpression of apoptosis-associated speck-like protein containing a CARD (green) and NLRP3 (red) in HPMCs. Counterstaining with 4',6-Diamidino-2-phenylindole dihydrochloride (blue). Scale bar=50 µm. Values are presented as the mean ± standard deviation of three independent experiments. Student's two-tailed t-test was used to analyze significant differences. *P<0.05, compared with the Con group, #P<0.05, compared with the HG group. HPMCs, human peritoneal mesothelial cells; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3; Zn, zinc; HG, high glucose; TPEN, N, N, N', N'-tetrakis(2-pyridylmethyl) ethylenediamine; Con, control.
Figure 5. Effects of Zn intervention on HG-induced intracellular generation of ROS in HPMCs. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. (A) Levels of intracellular ROS were detected using a DCFH-DA assay and (B) quantified. Values are presented as the mean ± standard deviation of three independent experiments. Student's two-tailed t-test was used to analyze significant differences. *P<0.05, compared with the Con group; †P<0.05, compared with the HG group. HPMCs, human peritoneal mesothelial cells; ROS, reactive oxygen species; Zn, zinc; HG, high glucose; TPEN, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine; Con, control.

Figure 6. Effects of Zn intervention on Nrf2 antioxidant pathway in HPMCs. The HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. mRNA expression levels of (A) HO-1, (B) NQO1 and (C) Nrf2 in the HPMCs were detected using reverse transcription-quantitative polymerase chain reaction analysis. (D) Protein expression levels of cytosolic and nuclear (E) HO-1, (F) NQO1 and (G and H) Nrf2 in the HPMCs were detected using western blot analysis. Values are presented as the mean ± standard deviation of three independent experiments. Student's two-tailed t-test was used to analyze significant differences. *P<0.05, compared with the Con group; †P<0.05, compared with the HG group. HPMCs, human peritoneal mesothelial cells; Zn, zinc; HG, high glucose; TPEN, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; Con, control.
ZnSO₄ promoted activation of the Nrf2-antioxidant system, whereas pretreatment with 1 µM TPEN reversed the activation of the Nrf2 antioxidant system induced by HG, as shown in Fig. 6.

Zn inhibits HG-induced NLRP3 inflammasome activation by activating the Nrf2 antioxidant pathway in HPMCs. Nrf2 siRNA or scrambled siRNA (150 nM) were transfected into the HPMCs. Following transfection for 24 h, HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. (A) Intracellular generation of ROS was detected using a DCFH-DA assay. The levels of (B) IL-1β and (C) IL-18 in the supernatants were determined by an ELISA assay. (D-F) NLRP3 inflammasome activity was examined using western blot analysis. (G and H) Activity of the Nrf2 antioxidant pathway was detected using western blot analysis. Values are presented as the mean ± standard deviation of three independent experiments. Student's two-tailed t-test was used to analyze significant differences. *P<0.05. HPMCs, human peritoneal mesothelial cells; Zn, zinc; HG, high glucose; TPEN, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine; siRNA, small interfering RNA; ROS, reactive oxygen species; IL, interleukin; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; Con, control.

Figure 7. Role of the Nrf2 antioxidant pathway in the inhibitory effect of Zn on HG-induced NLRP3 inflammasome activation in HPMCs. Nrf2 siRNA or scrambled siRNA (150 nM) were transfected into the HPMCs. Following transfection for 24 h, HPMCs were pretreated with 10 µM ZnSO₄ or 1 µM TPEN for 24 h and then incubated with 126 mM glucose for 24 h. (A) Intracellular generation of ROS was detected using a DCFH-DA assay. The levels of (B) IL-1β and (C) IL-18 in the supernatants were determined by an ELISA assay. (D-F) NLRP3 inflammasome activity was examined using western blot analysis. (G and H) Activity of the Nrf2 antioxidant pathway was detected using western blot analysis. Values are presented as the mean ± standard deviation of three independent experiments. Student’s two-tailed t-test was used to analyze significant differences. *P<0.05. HPMCs, human peritoneal mesothelial cells; Zn, zinc; HG, high glucose; TPEN, N, N, N’, N’-tetrakis (2-pyridylmethyl) ethylenediamine; siRNA, small interfering RNA; ROS, reactive oxygen species; IL, interleukin; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; Con, control.

Discussion

Zn has been shown to effectively inhibit fibrosis, exhibiting antioxidant, anti-apoptotic and anti-inflammatory properties (14,16,19). Zn deficiency has been reported to be associated with NLRP3 inflammasome activation and the production of IL-1β in macrophages, however, the effect of Zn on HG-induced NLRP3 inflammasome activation in HPMCs remains to be fully elucidated. In the present study, it was found that HG induced NLRP3 inflammasome activation and the production of IL-1β and IL-18, consistent with the previous report (7). Furthermore, the present study demonstrated for the first time, to the best of our knowledge, that Zn was important in protecting against NLRP3 inflammasome activation under HG conditions in HPMCs.

Canonical NLRP3 inflammasome activation has been reported to depend upon one, or a combination of signals, which include K⁺ ion efflux, ROS generation and destabilization of lysosomal membranes. In the present study, data showed that HG caused ROS generation, which indicated that HG induced NLRP3 inflammasome activation by inducing the generation of ROS. The ability of Zn to retard oxidative processes has long been recognized. Accumulating evidence has demonstrated that Zn deficiency can trigger oxidative stress and oxidant-mediated damage to cell components (20). By contrast, Zn supplementation is known to decrease oxidative stress by reducing ROS levels (21). The data obtained in the present study suggested that Zn treatment attenuated HG-induced ROS generation, suggesting that Zn may inhibit HG-induced NLRP3 inflammasome activation by decreasing oxidative stress.
The Nrf2 pathway is central in the antioxidant system. Under normal conditions, Nrf2 is captured by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, and is constantly degraded via the ubiquitin-proteasome pathway (22). On exposure to excess ROS and Zn\(^{2+}\), Nrf2 dissociates from Keap1, accumulates in the nucleus, binds to antioxidant responsive element sequences (AREs) and activates antioxidant genes, including glutamate-cysteine ligase catalytic subunit and NQO1 (23,24). In the present study, the results indicated that HG induced the upregulation of nuclear Nrf2 and the target gene of the Nrf2 pathway. Zn treatment activated the Nrf2 pathway, which inhibited the HG-induced generation of ROS and NLRP3 inflammasome activation. The effects of Zn treatment on ROS generation and NLRP3 inflammasome activation were reduced by the knock down of Nrf2.

In conclusion, the present study demonstrated that HG induced NLRP3 inflammasome activation in HPVCs, whereas Zn treatment inhibited NLRP3 inflammasome activation by attenuating oxidative stress. Zn also inhibited oxidative stress by activating the Nrf2 pathway. These results indicated that Zn inhibited HG-induced NLRP3 inflammasome activation in HPVCs through activation of the Nrf2 antioxidant pathway. A more detailed understanding of the critical role of Zn is likely to provide insight at the molecular level into innovative therapeutic strategies for used in PD.

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