Effect of zinc on high glucose-induced epithelial-to-mesenchymal transition in renal tubular epithelial cells

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Received November 24, 2014; Accepted March 23, 2015

DOI: 10.3892/ijmm.2015.2170

Abstract. Zinc (Zn) as an essential dietary element has been indicated in a number of protein functions in the prevention of numerous types of epithelial-to-mesenchymal transition (EMT)-driven fibrosis *in vivo*. However, relatively little is known regarding its effect in the EMT of the renal tubular epithelial cells, which play an important role in renal tubulointerstitial fibrosis and is an important component of the renal injury that is associated with diabetic nephropathy. The present study investigated the effect of Zn on the high glucose (HG)-induced EMT in a normal rat kidney tubular epithelial cell line (NRK-52E cells) and the underlying molecular mechanisms by immunofluorescence staining and western blot analysis. The present study identified that 10 μM of Zn supplementation prevented EMT changes, such as the loss of E-cadherin and the increase in α-smooth muscle actin and vimentin expression. Conversely, depletion of Zn with N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine in these cells aggravated changes in HG-induced EMT markers. Additionally, 10 μM Zn supplementation inhibited HG-induced transforming growth factor-β1 overexpression and reactive oxygen species production. Of note, HG increased phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways activation and Zn reversed HG-induced expression of PI3K/Akt, extracellular-signal-regulated kinase (ERK) and p38 MAPK, as well as EMT proteins. Finally, inhibitors of PI3K/Akt, ERK and p38 MAPK, and Zn supplementation blocked the HG-induced EMT in NRK-52E cells. These results indicate that physiologically optimal levels of Zn can inhibit HG-induced EMT of the NRK-52E cells possibly through several mechanisms, including abrogation of HG-induced oxidative stress, and PI3K/Akt, p38 MAPK and ERK activation in NRK-52E cells.

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Abbreviations: EMT, epithelial-to-mesenchymal transition; HG, high glucose; MAPK, mitogen-activated protein kinase; JNK, jun N-terminal kinase; ERK, extracellular-signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; SMA, smooth muscle cell actin; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; FITC, fluorescein isothiocyanate; TGF, transforming growth factor; PBS, phosphate-buffered saline; DCF-DA, 2,7-dichlorofluorescein diacetate; TBS, tris-buffered saline

Key words: renal tubular epithelial cells, epithelial-to-mesenchymal transition, zinc, high glucose

Introduction

Diabetic nephropathy (DN) is the leading cause of chronic kidney failure and end-stage renal disease worldwide, and the prevalence has progressively increased in recent years (1,2). DN is characterized by a decreased glomerular filtration rate, proteinuria, mesangial expansion, tubulointerstitial fibrosis and glomerulosclerosi (3). Hyperglycemia is the initiating factor in the development and progression of diabetic renal injury observed clinically in the DN (4). In certain patients, tubulointerstitial fibrosis also appears early in diabetic kidney injury, but it is more prominent in the later stages of the disease and correlates closely with the decline in renal function (5,6). Previous studies have suggested that hyperglycemia induced epithelial-to-mesenchymal transition (EMT) of tubular cells, and is an important mechanism of renal tubulointerstitial fibrosis in DN (7-9). The specific therapeutic options to inhibit the progression of chronic renal disease are not available in the clinic. Modulation of EMT may offer a novel therapeutic target to potentially inhibit renal fibrogenesis in the diabetic kidney.

EMT is a highly regulated process that may require the participation of growth factors or cytokines and integration of multiple signal pathways, involving loss of epithelial cell adhesion, *de novo* α-smooth muscle actin (α-SMA) expression and actin reorganization, disruption of tubular basement membrane and enhanced cell migration and invasion into the interstitium (8,9). Previous studies have indicated that high glucose (HG) levels induce a complex mixture of proinflam-

matory and profibrotic stimuli during renal tubular epithelial cells EMT *in vivo* (7), and HG can upregulate the expression of transforming growth factor-β1 (TGF-β1), a strong inducer of the EMT in the renal tubular epithelial cells (9,10). In addition, HG-induced damage in DN is primarily from mitochondrial superoxide overproduction, whose damage to proteins is one of the major pathogenic mechanisms in numerous chronic diseases including diabetes (11-13). HG, advanced glycation end products, angiotensin II and TGF-β1 all increase intracellular reactive oxygen species (ROS) and contribute to the development and progression of diabetic renal injury (7,14). ROS is associated with MAPK-mediated Smad activation during HG-induced EMT in proximal tubular epithelial cells and antioxidants effectively reversed HG-induced EMT in the renal tubular epithelial cells and DN (6,9,15).

Zinc (Zn) is an essential element that mediates a wide variety of physiological processes, including the enzymes involved in cellular signaling pathways and transcription factors (16,17). Evidence indicates that a low Zn concentration has important implications for patients with DN (10,11). The mechanisms of the protective functions or function of Zn in the pathogenesis of DN, including EMT in proximal tubular epithelial cells, vascular cell injury or dysfunction, are not clear. Numerous studies have indicated that Zn supplementation inhibits fibrosis, such as in myocardial, liver, perivascular and cystic fibrosis (18-22). However, it is not known whether Zn is involved in HG-induced EMT of the normal rat tubular epithelial cell line NRK-52E. For this purpose, the effect of Zn was measured on HG-induced EMT, cellular TGF-β1 and ROS production, as well as PI-3K and MAPK activation in NRK-52E cells.

Materials and methods

Cell culture. NRK-52E cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (low glucose) (HyClone, Logan, UT, USA), supplemented with 10% fetal calf serum (HyClone), glutamine (2 mM), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air and passaged twice a week. Cells were cultured at a density of $5x10^3$ cells/well in 6-well culture plates. Near confluent NRK-52E cells were subsequently transferred to serum-free DMEM medium for overnight starvation prior to each experiment. In the control groups, the NRK-52E cells were treated with serum-free DMEM medium only. In certain other groups, the cells were pretreated with 10 μM $ZnSO_4$ for 24 h followed by incubation of 30 mM HG for an addition 48 h. To deplete the intracellular Zn stores, the Zn chelator N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (1 μM) was added 12 h before the end of the 48-h incubation period with/without HG.

Assessment of cell viability. Cell viability was measured by the quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mossman (23) at 10^5 cells/ml in 96-well plates. 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 570 nm of each aliquot was measured using a Sunrise RC microplate reader (Tecan Schweiz AG, Männedorf, Switzerland). Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

Enzyme-linked immunosorbent assay (ELISA). The protein level of TGF-β1 was measured by a TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, the NRK-52E cells were seeded at a density of $3x10^5$ cells/well in a 12-well plate and cultured for 24 h. The cells were subsequently treated as previously described. The supernatants were collected from cultures of NRK-52E cells for ELISA testing. Secreted TGF-β1 protein concentration per 10^5 cells was measured and calculated from the standard curve by an ELISA kit. Briefly, 100 μl samples were added and incubated for 1 h with a plate shaker following washing with the washing buffer. An enzyme-conjugated secondary antibody was added to the wells and was incubated for 2 h before the substrate was added, and the reading was assessed with an absorbance ELISA reader at the 450 nm wavelength. All the procedures were performed at room temperature.

Detection of intracellular ROS level. The ROS assay experiments were performed using the reactive oxygen species assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Briefly, NRK-52E cells were treated as previously described in the section of cells culture. Subsequently, cells ($5x10^6$) were incubated with 10 μmol/l 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probes at 37˚C for 30 min and washed with phosphate-buffered saline (PBS) 3 times in order to remove the residual probes. DCFH-DA was deacetylated intracellularly by non-specific esterase, which was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). DCF fluorescence was detected by flow cytometer (Becton-Dickinson, San Jose, CA, USA). The results were analyzed by the CellQuest software (Becton-Dickinson).

Western blot analysis. Cells were pelleted by centrifugation at 125 g at 4˚C for 10 min and subsequently washed with ice-cold PBS. Cells were lysed using the radioimmunoprecipitation assay buffer and phenylmethanesulfonylfluoride mixture (1:100), on ice for 30 min with occasional vortexing. Lysed cells were sonicated and centrifuged at 8,000 x g at 4˚C for 5 min. The total protein concentration measurement was performed with the Bradford method (12). Protein samples were boiled for 5 min and 50 μg of total protein was loaded on the appropriate SDS-PAGE gel. The proteins on the gel were subsequently transferred to a polyvinylidene fluoride membrane using a Bio-Rad apparatus (Bio-Rad Laboratories, Hercules, CA, USA) for 2 h at 4˚C using 100 V. The protein-bound membrane was blocked in 5% milk in tris-buffered saline (TBS) (containing 0.5% Tween-20) at room temperature for 1 h and subsequently incubated with primary antibodies. The primary antibodies used included rabbit polyclonal anti-vimentin (1:400, sc-5565; Santa Cruz Biotechnology, Dallas, TX, USA), mouse monoclonal anti-α-SMA (1:1,000, sc-324317; Sigma, St. Louis, MO, USA), mouse monoclonal anti-β-actin (1:4,000, sc-8432; Sigma), rabbit monoclonal anti-E-cadherin (1:1,000, sc-7870; BD

Biosciences, San Jose, CA, USA), rabbit monoclonal anti-Akt (1:400, SAB4500797; Sigma), anti-phospho-Akt (1:400, SAB4503853; Sigma), rabbit polyclonal anti-c-Jun N-terminal kinase (JNK) (1:1,000, SAB4502398; Sigma), rabbit polyclonal anti-phospho JNK (1:1,000, SAB4504449; Sigma) rabbit polyclonal anti-p38 (1:1,000, M0800; Zymed Laboratories, San Francisco, CA, USA), rabbit polyclonal anti-phospho p38 (1:1,000, SAB4301534; Zymed Laboratories), rabbit polyclonal anti-extracellular-signal-regulated kinase (ERK)1/2 (1:800, M5670; Sigma) and rabbit polyclonal anti-phospho ERK1/2 (1:800, E7028; Sigma). Following completion of the primary antibody staining, the membranes were washed several times with TBS/0.1% Tween-20, which was followed by incubation with horseradish peroxidase-conjugated secondary antibodies overnight at 4˚C. The membrane was subsequently developed with an enhanced chemiluminescence kit (Walterson Biotechnology, Inc., Beijing, China) and the images were captured with UVP (G:BOX EF, Chemi HR16; Syngene, Frederick, MD, USA). The protein bands were quantified using the NIH ImageJ version 1.44 densitometry software.

Statistical analyses. Data are expressed as the means ± standard error of the mean. Variance was homogenous for use of standard analysis of variance (ANOVA) methodology. Subsequent to establishing the statistical significance by ANOVA, individual comparisons were performed using the Tukey's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Zn on the expression of HG-induced EMT in NRK-52E cells. First, exposure of the NRK-52E cells to HG (30 mM D-glucose) for 24-72 h decreased protein expression of E-cadherin and increased the expression of α-SMA and vimentin (Fig. 1). Mannitol or L-glucose (30 mM) did not change the expression of any of these markers, which suggested that it was not the high osmolarity, but HG that induced EMT in the NRK-52E cells (data not shown). Subsequently, the effects of Zn on HG-induced EMT of NRK-52E cells were assessed by western blotting. The HG-induced EMT can be attenuated by pre-treating the NRK-52E cells with 10 μM $ZnSO_4$, which was evidenced by the reduced upregulation of α-SMA and vimentin, and the ameliorated expression of E-cadherin (Fig. 2). These results showed that the physiologically optimal levels of Zn supplementation can reverse HG-induced EMT in NRK-52E cells.

Effect of Zn on TGF-β1 expression in the HG-treated NRK-52E cells. To assess the effect of Zn on the TGF-β1 expression in the HG-treated NRK-52E cells, TGF-β1 protein was measured by the ELISA assay (Fig. 3A). In contrast to the control group, the TGF-β1 expression was significantly higher in the HG-treated group, and the TGF-β1 expression was further enhanced in the TPEN/HG group. Conversely, $ZnSO_4$ treatment reduced the HG-induced TGF-β1 expression in the NRK-52E cells. Furthermore, Zn or TPEN alone did not significantly alter the TGF-β1 expression. Considering the above findings, the present study indicates that Zn can attenuate HG-induced TGF-β1 expression in the NRK-52E cells.

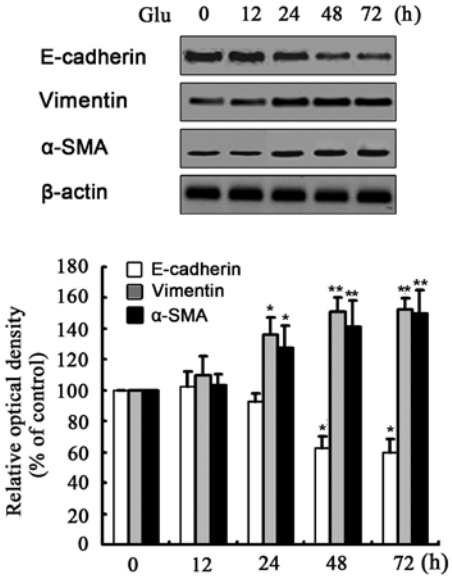

Figure 1. Effects of high glucose (HG) on epithelial-to-mesenchymal transition (EMT) in the NRK-52E cells. Representative western blot analysis shows that HG-induced E-cadherin downregulation, as well as vimentin and α-smooth muscle actin (α-SMA) upregulation at 24-72 h (n=6). All the experiments were repeated three times, and the results are depicted as mean ± standard error of the mean. ($^{**}P<0.01$, $^{*}P<0.05$ vs. control).

Effect of Zn on ROS production in the HG-treated NRK-52E cells. ROS is the initial and primary event that subsequently activates a number of other pathways implicated in the development of EMT in renal tubular epithelial cells (24-26). Therefore, the effect of Zn was examined on the HG-induced ROS induction in the NRK-52E cells by measuring the intracellular ROS with DCF-DA staining. The result indicated that depletion of Zn with TPEN, in conjunction with HG treatment, resulted in a substantial increase of ROS production in the NRK-52E cells (Fig. 3B). By contrast, Zn pre-treatment significantly attenuated HG-induced ROS production in the NRK-52E cells.

Effect of Zn supplementation on the HG-induced PI3K/Akt signaling pathway. The PI3K signaling pathway is involved in EMT in the NRK-52E cells (27,28). Having shown that Zn inhibited EMT, whether Zn mediated its effects on EMT in the NRK-52E cells through this pathway was determined under HG conditions by western blotting. When the cells were exposed to HG for 48 h, Akt phosphorylation increased compared to the control, whereas 10 μM $ZnSO_4$ treatment significantly decreased the expression of Akt phosphorylation (Fig. 4A and B). To further examine the effect of Zn on HG-induced EMT, the NRK-52E cells were incubated with or without 10 μM LY294002 [an inhibitor of upstream enzyme PI3K, the concentration of LY294002 is from reference (22)] for 1 h and were subsequently exposed to 30 mM HG in the presence or absence of 10 μM $ZnSO_4$ pretreatment for 24 h. The expected results showed that the HG/Zn or HG/LY294002 group decreased the expression of Akt phosphorylation and HG-induced EMT in the NRK-52E cells (Fig. 5A). There was no significant difference in the HG/Zn versus HG/LY294002 group. Taken together, these results indicated that the regulation mechanism of HG-induced EMT by Zn may be through

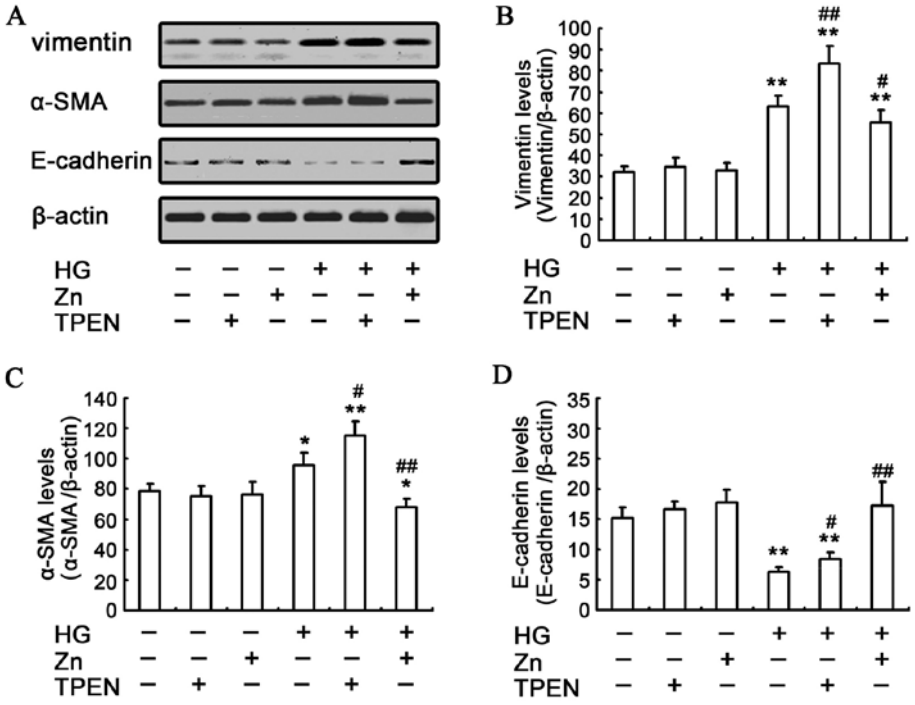

Figure 2. Effects of zinc (Zn) on epithelial-to-mesenchymal transition (EMT) in the NRK-52E cells. (A) Representative western blot analysis shows that $ZnSO_4$ treatment prevents high glucose (HG)-induced (D) E-cadherin downregulation, as well as (B) vimentin and (C) α-smooth muscle actin (α-SMA) upregulation at 48 h (n=6). All the experiments were repeated three times, and the results are depicted as mean ± standard error of the mean. (**P<0.01, *P<0.05 vs. control; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. HG). The Zn and N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) groups were not different compared to the control group, respectively (P>0.05).

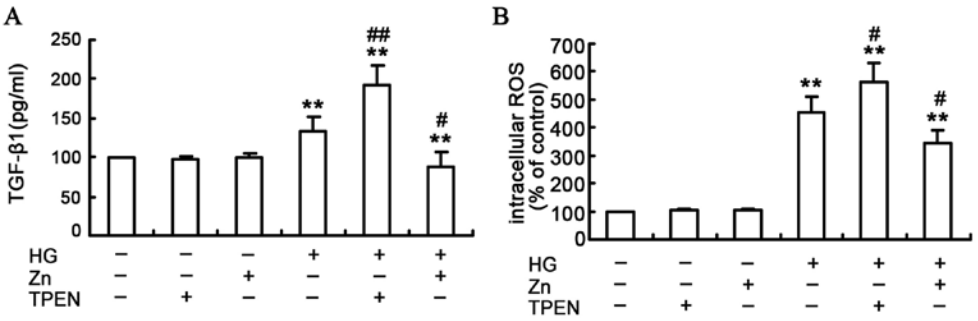

Figure 3. Effects of zinc (Zn) on transforming growth factor (TGF)-β1 production and intracellular reactive oxygen species (ROS) in the NRK-52E cells. (A) The level of TGF-β1 was measured by ELISA. All the results were obtained from three independent experiments. (B) The results of intracellular ROS from three independent experiments were analyzed. (**P<0.01, *P<0.05 vs. control; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. HG). HG, high glucose.

abrogation of HG-induced PI3K/Akt activation in the renal tubular epithelial cells.

Effect of Zn on HG-induced MAPK signaling pathway. The MAPK signaling pathway has been reported to be involved in the EMT (24,29-31), but whether Zn executes its effect on the EMT in the renal tubular epithelial cells through this pathway remains unknown. Therefore, the effect of Zn supplementation on the MAPK pathway, including JNK, p38 MAPK and ERK, was examined in the NRK-52E cells. The activation of the JNK, p38 MAPK and ERK pathways was analyzed by western blot analysis with phospho-p38, phospho-JNK and phospho-ERK antibodies. Compared to the control group, the phospho-p38, phospho-JNK and phospho-ERK in the HG group increased to varying degrees (Fig. 4A and C-E). The data are consistent with these earlier observations and provide a novel molecular signaling mechanism in which the MAPK pathway mediates HG-induced EMT in renal tubular epithelial cells (24). Of note, the TPEN/HG group, which depleted Zn with TPEN in the HG-treated NRK-52E cells, showed a robust increase of the phospho-p38, and phospho-ERK in comparison with the HG group. Conversely, preincubation of the NRK-52E cells with 10 μM $ZnSO_4$ significantly inhibited HG-induced expression of phospho-p38 MAPK and phospho-ERK. There was no significant difference of the expression of phospho-JNK in the HG/Zn versus HG group (Fig. 4A and D). All these results suggested that Zn may be involved in HG-induced EMT through regulation of the p38 MAPK and ERK pathways. To further examine the involvement of the p38 MAPK and ERK pathways in HG-induced EMT, the cells were incubated with

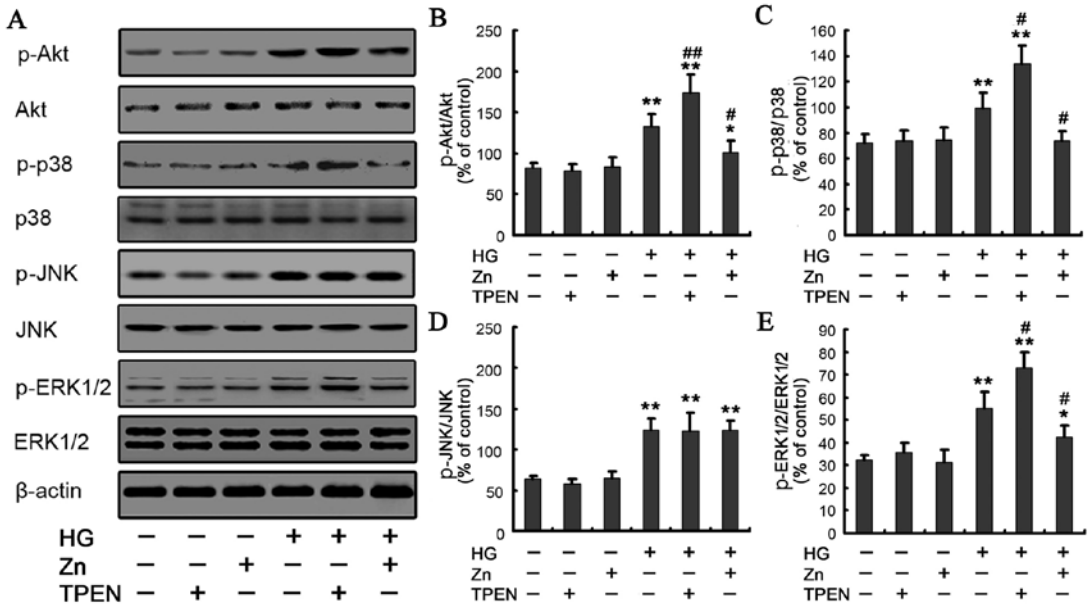

Figure 4. Effect of zinc (Zn) supplementation on high glucose (HG)-induced phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) signaling pathways. (A) Western blot analysis was performed with antibodies as indicated. Relative Akt, c-Jun N-terminal kinase (JNK), p38 MAPK, extracellular-signal-regulated kinase (ERK)1/2 and phospho (p)-Akt, p-JNK, p38 MAPK and p-ERK1/2 expression was calculated and normalized to the loading control. (B-E) The corresponding protein levels were assessed using densitometry and were expressed in relative intensities. All the results were obtained from three independent experiments. (**P<0.01, *P<0.05 vs. control; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. HG).

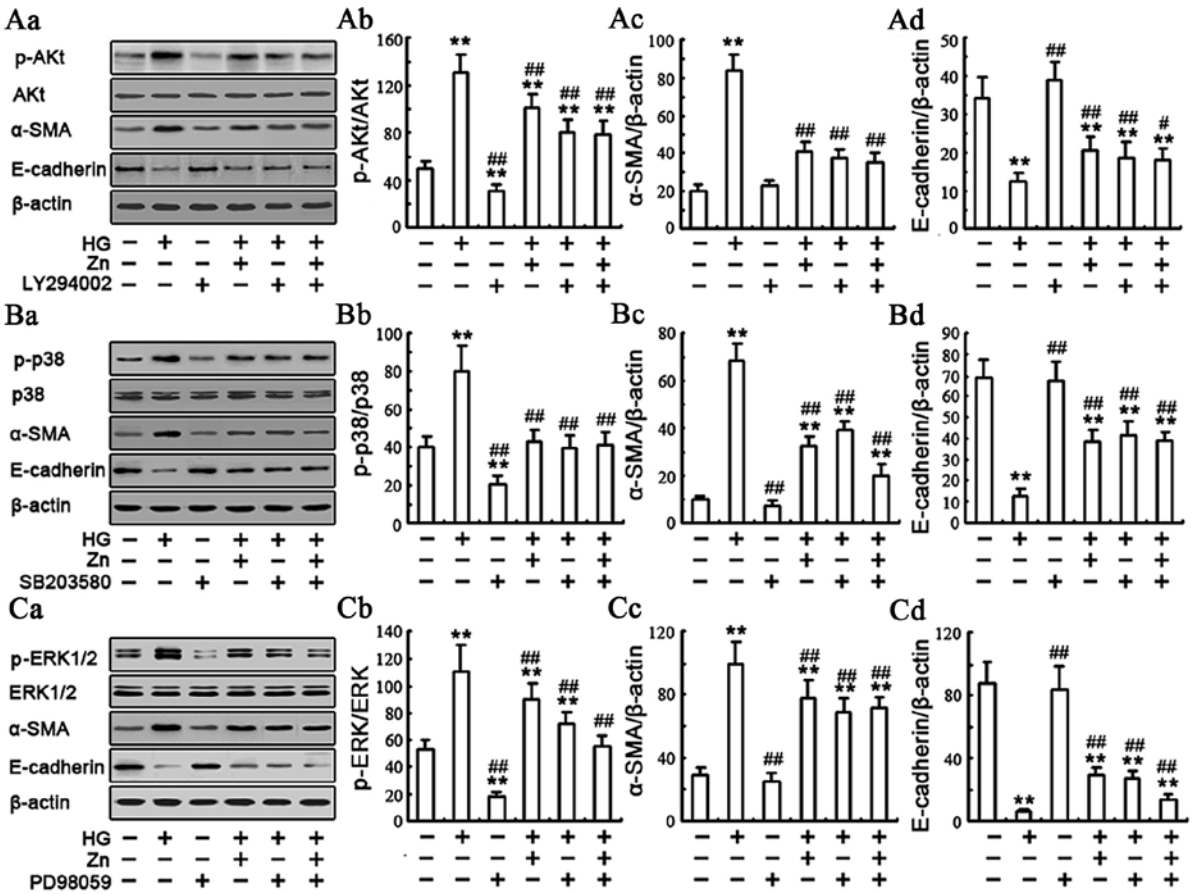

Figure 5. Zinc (Zn) inhibits the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway and mitogen-activated protein kinase (MAPK) protecting cells from epithelial-to-mesenchymal transition (EMT). Cells were incubated with or without 10 μM LY294002 for 1 h, 2 μM SB203580 for 1 h, 10 μM PD98059 for 1 h, respectively, and were subsequently exposed to 30 mM high glucose (HG) for 48 h in the presence or absence of 30 μM $ZnSO_4$ pretreatment for 24 h. (Aa-Ca) The expression of EMT proteins, total-Akt and phospho (p)-Akt, total-p38 and p-p38, total-ERK and p-ERK were assessed by western blot analysis. β-actin served as the loading control. (Ab-d, Bb-d and Cb-d) Quantitative analysis was performed by measuring the fluorescence intensity relative to the control. Each value represents the mean ± standard error of the mean (n=10). All the results were obtained from three independent experiments. (**P<0.01, *P<0.05 vs. control; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. HG.)

or without 2 μM of p38 MAPK inhibitor SB203580 [concentration is from (30)] or 10 μM of the ERK inhibitor PD98059 for 1 h [concentration is from (22)], respectively, and subsequently exposed to 30 mM of HG in the presence or absence of 10 μM $ZnSO_4$ pretreatment for 24 h. As shown in Fig. 5, HG evidently upregulated the expression of α-SMA and downregulated the expression of E-cadherin. As expected, when compared to the cells treated with HG alone, co-treatment of 10 μM $ZnSO_4$, SB203580 or PD98059 with HG significantly decreased the expression of α-SMA and ameliorated the expression of epithelial protein E-cadherin to varying degrees (Fig. 5B and C). Furthermore, similar to SB203580 or PD98059, Zn treatment decreased the HG-induced EMT and effectively inhibited p38 and ERK phosphorylation (Fig. 5B and C). Collectively, these results suggested that Zn protected the cells from HG-induced EMT possibly through abrogation of HG-induced p38 MAPK and ERK activation.

Discussion

Several studies in animal models and few clinical studies have demonstrated that Zn supplementation has a positive effect of inhibiting fibrosis in chronic inflammatory diseases, such as in liver, myocardial and cystic fibrosis (18,21,32). Conversely, previous studies have indicated that Zn deficiency can accelerate the degradation of E-cadherin and β-catenin proteins in lung and endothelial epithelial cells and lead to damage of membrane barrier integrity (33,34). The results in the present study demonstrate that Zn pre-treatment provides effective protection against HG-induced EMT in the renal tubular epithelial cells, as evidenced by a decrease in upregulation of vimentin and α-SMA and amelioration of E-cadherin associated with a transition in the epithelial phenotype of the NRK-52E cells to a myofibroblastic phenotype. The mechanism may be through abrogation of HG-induced oxidative stress and PI3K/Akt, and MAPK (p38 MAPK and ERK) activation in the NRK-52E cells. These results are the first to demonstrate that the physiologically optimal levels of Zn inhibit HG-induced EMT in the renal tubular epithelial cells.

TGF-β1, a strong profibrotic cytokine, as well as the TGF-β/Smad pathway were extensively studied for the EMT in previous years (14,15,35). TGF-β1 plays an important role in changing the phenotype of renal epithelial cells, actions that significantly contribute to the profibrotic actions of this growth factor (35,36). In addition, there is sufficient evidence that TGF-β1 signals through MAPKs and the activation of p38 MAPK is required in TGF-β1-induced EMT in human proximal tubular epithelial cells (7). A previous study indicated that a Zn deficiency resulted in the TGF-β1 induction in neurogenesis to regulate neuronal precursor cell proliferation and survival by regulating the p53-dependent molecular mechanism (37). Another study demonstrated that TGF-β1 has stimulating and inhibiting effects on osteoclast-like cell formation in mouse marrow culture, and that Zn can inhibit the stimulatory effect of TGF-β1 (38). Zn supplementation decreases ethanol- and acetaldehyde-induced liver stellate cell activation partly by inhibiting Smad signaling (39). In the present study, the physiologically optimal levels of Zn supplementation were confirmed to reduce the HG-induced TGF-β1 production from the NRK-52E cells.

The role of Zn in modulating oxidative stress has previously been recognized and Zn deficiency enhanced diabetic renal damage, which is associated with oxidative stress (40). Previous evidence has demonstrated that Zn deficiency can trigger oxidative stress and oxidant-mediated damage to cell components, alterations of cell functions and cell proliferation (41,42). HG, advanced glycation end products, angiotensin II and TGF-β1 all increase ROS and contribute to the development and progression of diabetic renal injury (7,14). Numerous studies have confirmed that EMT of tubular epithelial cells in DN patients is generally regarded to be the result of hyperglycemia-induced oxidative stress, as antioxidants effectively reverse the EMT in all tubular epithelial cells (6,9,13,43). In the present study, Zn treatment attenuated HG-induced ROS generation, whereas Zn depletion increased HG-induced ROS generation, suggesting that physiologically optimal levels of Zn inhibit the HG-induced EMT possibly through abrogation of HG-induced oxidative stress in NRK-52E cells.

The mechanisms by which HG and its metabolite regulate E-cadherin, vimentin and *α-SMA* gene expression as markers of EMT in the NRK-52E cells have not been completely elucidated. Several studies have reported that the MAPK and PI-3K pathways are involved in the pathology of various forms of kidney injury, including renal fibrosis (8,24,27,28). Phosphorylation of Akt is associated with a loss of cell-cell adhesion, a decrease in cell-matrix adhesion, and induction of cell motility and other characteristics of myofibroblasts (27,44). Furthermore, inhibition of PI3K/Akt activity causes a decrease in GSK-3β phosphorylation attenuated TGF-β1-mediated EMT in rat kidney epithelial cells (9,45). A previous study demonstrated that cyclosporin A activated JNK signaling in human renal epithelial cells and that JNK inhibition reduced the cyclosporin A-induced E-cadherin downregulation, cell migration and Snail-1 expression (46). The p38 MAPK activation is a key modulator in the progression of renal diseases and is thought to occur in HG-induced cell damage in renal tubular epithelial cells (8). Elevated ERK activity can enhance TGF-β1-mediated EMT in rat kidney epithelial cells, and ERK inhibition reduces the induced EMT (24). In PI3K-inhibited NRK-52E cells, the direct association between Akt and EMT was further confirmed. Phosphorylation of Akt increased in HG-treated NRK-52E cells and Zn supplementation decreased its level. The HG-mediated Akt activation, the reduction in E-cadherin and the upregulation of vimentin and α-SMA were reversed by a PI3K inhibitor, with no significance between with the effect of Zn, which is consistent with the results of the effect of Zn on HG-induced phosphorylation of ERK and p38 MAPK in NRK-52E cells. The results provide a novel molecular signaling mechanism in which Zn mediates HG-induced EMT possibly through abrogation of HG-induced PI3K/Akt, ERK and p38 MAPK activation in the renal tubular epithelial cells.

In conclusion, the present study provides new evidence regarding the association between Zn and EMT in NRK-52E cells. The results reveal that the physiologically optimal levels of Zn inhibit HG-induced EMT, most likely through inhibition of ROS, TGF-β1 production, and PI3K/Akt, ERK and p38 MAPK signaling pathways in NRK-52E cells. Given

the important role that EMT plays in the development and progression of interstitial fibrosis, the identification of Zn as a key regulator of HG-induced EMT represents an important finding. Further studies may confirm it as a potentially important target for therapeutic intervention in an attempt to limit EMT and with it the decline in renal function observed in patients with DN.

Acknowledgements

The present study was supported by the National Grand Fundamental Research 973 Program of China (grant no. 2012CB722405), the Natural Science Foundation of China (grant nos. 81170561, 81370517, 31171259 and 31271364), and the Shen Yang City Science and Technology Program (grant no. F11-264-1-21).

References

1. Schena FP and Gesualdo L: Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol 16 (Suppl 1): S30-S33, 2005.
2. Lapice E, Pinelli M, Riccardi G, *et al*: Pro12Ala polymorphism in the PPARG gene contributes to the development of diabetic nephropathy in Chinese type 2 diabetic patients: comment on the study by Liu et al. Diabetes Care 33: e114, 2010.
3. Ayodele OE, Alebiosu CO and Salako BL: Diabetic nephropathy - a review of the natural history, burden, risk factors and treatment. J Natl Med Assoc 96: 1445-1454, 2004.
4. Yeh CH, Chang CK, Cheng KC, *et al*: Role of bone morphogenetic proteins-7 (BMP-7) in the renal improvement effect of DangGui (*Angelica sinensis*) in type-1 diabetic rats. Evid Based Complement Alternat Med 2011: 796723, 2011.
5. Gilbert RE and Cooper ME: The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury? Kidney Int 56: 1627-1637, 1999.
6. Simonson MS: Phenotypic transitions and fibrosis in diabetic nephropathy. Kidney Int 71: 846-854, 2007.
7. Burns WC, Twigg SM, Forbes JM, *et al*: Connective tissue growth factor plays an important role in advanced glycation end product-induced tubular epithelial-to-mesenchymal transition: implications for diabetic renal disease. J Am Soc Nephrol 17: 2484-2494, 2006.
8. Lv ZM, Wang Q, Wan Q, *et al*: The role of the p38 MAPK signaling pathway in high glucose-induced epithelial-mesenchymal transition of cultured human renal tubular epithelial cells. PLoS One 6: e22806, 2011.
9. Lee YJ and Han HJ: Troglitazone ameliorates high glucose-induced EMT and dysfunction of SGLTs through PI3K/Akt, GSK-3beta, Snail1, and beta-catenin in renal proximal tubule cells. Am J Physiol Renal Physiol 298: F1263-F1275, 2009.
10. Karatug A, Kaptan E, Bolkent S, *et al*: Alterations in kidney tissue following zinc supplementation to STZ-induced diabetic rats. J Trace Elem Med Biol 27: 52-57, 2012.
11. Dogukan A, Sahin N, Tuzcu M, *et al*: The effects of chromium histidinate on mineral status of serum and tissue in fat-fed and streptozotocin-treated type II diabetic rats. Biol Trace Elem Res 131: 124-132, 2009.
12. Simonian MH and Smith JA: Spectrophotometric and colorimetric determination of protein concentration. Curr Protoc Mol Biol Chapter 10: Unit 10.1A, 2006.
13. Kalluri R and Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 112: 1776-1784, 2003.
14. Zeisberg M and Kalluri R: The role of epithelial-to-mesenchymal transition in renal fibrosis. J Mol Med (Berl) 82: 175-181, 2004.
15. Sato M, Muragaki Y, Saika S, *et al*: Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest 112: 1486-1494, 2003.
16. Hills CE and Squires PE: TGF-beta1-induced epithelial-to-mesenchymal transition and therapeutic intervention in diabetic nephropathy. Am J Nephrol 31: 68-74, 2010.
17. Hills CE and Brunskill NJ: Intracellular signalling by C-peptide. Exp Diabetes Res 2008: 635158, 2008.
18. Takahashi M, Saito H, Higashimoto M, *et al*: Possible inhibitory effect of oral zinc supplementation on hepatic fibrosis through downregulation of TIMP-1: a pilot study. Hepatol Res 37: 405-409, 2007.
19. Wang L, Zhou Z, Saari JT, *et al*: Alcohol-induced myocardial fibrosis in metallothionein-null mice: prevention by zinc supplementation. Am J Pathol 167: 337-344, 2005.
20. Gandhi MS, Deshmukh PA, Kamalov G, *et al*: Causes and consequences of zinc dyshomeostasis in rats with chronic aldosteronism. J Cardiovasc Pharmacol 52: 245-252, 2008.
21. Van Biervliet S, Vande Velde S, Van Biervliet JP, *et al*: The effect of zinc supplements in cystic fibrosis patients. Ann Nutr Metab 52: 152-156, 2008.
22. Zhang X, Liang D, Guo B, *et al*: Zinc inhibits high glucose-induced apoptosis in peritoneal mesothelial cells. Biol Trace Elem Res 150: 424-432, 2012.
23. Mossman BT: In vitro approaches for determining mechanisms of toxicity and carcinogenicity by asbestos in the gastrointestinal and respiratory tracts. Environ Health Perspect 53: 155-161, 1983.
24. Rhyu DY, Yang Y, Ha H, *et al*: Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. J Am Soc Nephrol 16: 667-675, 2005.
25. Yang J and Liu Y: Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. Am J Pathol 159: 1465-1475, 2001.
26. Ha H and Lee HB: Reactive oxygen species and matrix remodeling in diabetic kidney. J Am Soc Nephrol 14: S246-S249, 2003.
27. Zeng R, Yao Y, Han M, *et al*: Biliverdin reductase mediates hypoxia-induced EMT via PI3-kinase and Akt. J Am Soc Nephrol 19: 380-387, 2008.
28. Boca M, D'Amato L, Distefano G, *et al*: Polycystin-1 induces cell migration by regulating phosphatidylinositol 3-kinase-dependent cytoskeletal rearrangements and GSK3beta-dependent cell cell mechanical adhesion. Mol Biol Cell 18: 4050-4061, 2007.
29. Liu Q, Mao H, Nie J, *et al*: Transforming growth factor {beta}1 induces epithelial-mesenchymal transition by activating the JNK-Smad3 pathway in rat peritoneal mesothelial cells. Perit Dial Int 28 (Suppl 3): S88-S95, 2008.
30. Yang F, Chung AC, Huang XR, *et al*: Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3. Hypertension 54: 877-884, 2009.
31. Yamashita M, Fatyol K, Jin C, *et al*: TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. Mol Cell 31: 918-924, 2008.
32. von Bulow V, Dubben S, Engelhardt G, *et al*: Zinc-dependent suppression of TNF-alpha production is mediated by protein kinase A-induced inhibition of Raf-1, I kappa B kinase beta, and NF-kappa B. J Immunol 179: 4180-4186, 2007.
33. Bao S and Knoell DL: Zinc modulates cytokine-induced lung epithelial cell barrier permeability. Am J Physiol Lung Cell Mol Physiol 291: L1132-L1141, 2006.
34. Mengheri E, Nobili F, Vignolini F, *et al*: *Bifidobacterium animalis* protects intestine from damage induced by zinc deficiency in rats. J Nutr 129: 2251-2257, 1999.
35. Wang X, Pan X and Song J: AMP-activated protein kinase is required for induction of apoptosis and epithelial-to-mesenchymal transition. Cell Signal 22: 1790-1797, 2010.
36. Lan HY: Tubular epithelial-myofibroblast transdifferentiation mechanisms in proximal tubule cells. Curr Opin Nephrol Hypertens 12: 25-29, 2003.
37. Corniola RS, Tassabehji NM, Hare J, *et al*: Zinc deficiency impairs neuronal precursor cell proliferation and induces apoptosis via p53-mediated mechanisms. Brain Res 1237: 52-61, 2008.
38. Yamaguchi M and Kishi S: Differential effects of transforming growth factor-beta on osteoclast-like cell formation in mouse marrow culture: relation to the effect of zinc-chelating dipeptides. Peptides 16: 1483-1488, 1995.
39. Szuster-Ciesielska A, Plewka K, Daniluk J, *et al*: Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling. Biochem Pharmacol 78: 301-314, 2009.
40. Prasad AS: Clinical, immunological, anti-inflammatory and antioxidant roles of zinc. Exp Gerontol 43: 370-377, 2008.

41. Ho E and Ames BN: Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proc Natl Acad Sci USA 99: 16770-16775, 2002.
42. Zhang X, Liang D, Guo B, *et al*: Zinc transporter 5 and zinc transporter 7 induced by high glucose protects peritoneal mesothelial cells from undergoing apoptosis. Cell Signal 25: 999-1010, 2013.
43. Kosugi T and Sato W: Midkine and the kidney: health and diseases. Nephrol Dial Transplant 27: 16-21, 2012.
44. Agarwal E, Brattain MG and Chowdhury S: Cell survival and metastasis regulation by Akt signaling in colorectal cancer. Cell Signal 25: 1711-1719, 2013.
45. Kattla JJ, Carew RM, Heljic M, *et al*: Protein kinase B/Akt activity is involved in renal TGF-beta1-driven epithelial-mesenchymal transition in vitro and in vivo. Am J Physiol Renal Physiol 295: 215-225, 2008.
46. Pallet N, Thervet E and Anglicheau D: c-Jun-N-terminal kinase signaling is involved in cyclosporine-induced epithelial phenotypic changes. J Transplant 2012: 348604, 2012.