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Zinc supplementation partially prevents renal pathological changes in diabetic rats☆

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Abstract

We have demonstrated that Zn supplementation mediated up-regulation of cardiac metallothionein (MT) as a potent antioxidant prevented the development of diabetic cardiomyopathy. The present study was undertaken to test whether induction of renal MT synthesis by Zn supplementation protects the kidney from diabetes-induced damage. Streptozotocin-induced diabetic rats were treated with and without Zn supplementation at 5 mg/kg in drinking water for 3 months. Diabetic renal damage was detected by examining renal pathological alterations and 24-h urinary protein levels. Three-month Zn supplementation immediately after the onset of diabetes, partially but significantly, prevented the kidney from diabetes-induced increases in 24-h urinary proteins and pathological alterations. Diabetes-induced renal oxidative damage, inflammation and up-regulated expression of profibrosis mediator connective tissue growth factor (CTGF) were also markedly attenuated by Zn supplementation, along with significant increases in Zn levels concomitant with MT expression in renal tubular cells. Direct exposure of renal tubular (HK11) cells to high levels of glucose (HG) induced CTGF up-regulation predominantly through ERK (extracellular signal-regulated kinase)1/2-dependent, and partially through p38 mitogen-activated protein kinase (MAPK)-dependent pathways. Pretreatment of HK11 cells with Zn or cadmium induced MT expression and also significantly suppressed HG-induced CTGF expression. These results provide the first evidence for Zn supplementation to attenuate diabetes-induced renal pathological changes, likely through prevention of hyperglycemia-induced CTGF expression by Zn-induced MT in renal tubular cells.

Keywords: Diabetic nephropathy; Metallothionein; Zinc; CTGF; Renal tubular cells

1. Introduction

Diabetes is a major risk factor for chronic cardiovascular diseases [1]. Diabetic nephropathy (DN) has become one of the leading causes for the high mortality of diabetic patients [1]. It is of utmost importance to develop novel therapeutic strategies that efficiently prevent DN for diabetic patients. Several mechanisms for the pathogenesis of DN have been proposed [2,3], one of which is abnormal homeostasis of trace elements [4–6]. For example, Zn is one of the essential trace elements for eukaryotes and has numerous physiological functions [7]. Zn deficiency was found to increase the risks of diabetes and diabetic complications [4–6].

Zn is also an effective inducer for gene and protein expression of metallothionein (MT), a potent antioxidant [8]. We have demonstrated that Zn supplementation in diabetic mice significantly induced cardiac MT expression, along with a significant prevention of the development of diabetic cardiomyopathy [9], suggesting that induction of systemic MT synthesis by Zn supplementation may be a potential approach to preventing diabetes-induced complications in multiple organs.

However, there was lack of direct evidence for the role of Zn-induced MT in preventing other diabetic complications such as renal damage. The information from literature regarding the role of MT in the kidney of diabetic and nondiabetic subjects is controversial. Several studies have shown a high susceptibility of the kidney with Cd-induced MT to subsequent oxidative damage [10–12], suggesting that the induction of renal MT might not protect the kidney from oxidative stress-induced damage. However, these nonprotective

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results may be due to the use of Cd as MT inducer in these studies [10–12]. In fact, renal MT synthesis was enhanced in diabetic animal models, implying a possible protective role of MT against diabetic kidneys [13]. Zn and Zn-induced MT in the kidney were found to provide a significant protection against renal damage caused by various oxidative stress conditions [8,14–16]. Given that one of the major causes for various diabetic complications is oxidative stress [2,3] and both MT and Zn act as potent antioxidants [7,8], it is urgent to clarify whether Zn-induced renal MT can protect the kidney from diabetic damage.

The present study was thus undertaken to determine the effects of Zn supplementation on diabetes-induced pathological alterations of the kidney. Rats were used to produce Type 1 diabetes by streptozotocin (STZ) and the hyperglycemic rats were treated with and without Zn supplementation for three months. Pathological changes of the kidney were examined at the time of experimental termination. Zn supplementation was found to partially but significantly protect the kidney against diabetes-induced urinary protein increase and pathological alterations, along with a significant induction of renal MT synthesis predominantly in tubular cells. Furthermore, in vitro cultured renal tubular cells were used to investigate the signaling mechanisms responsible for the fibrotic effect of high levels of glucose (HG) and the possible role of MT in the protection of profibrotic effects by Zn supplementation.

2. Materials and methods

2.1. Diabetic rat model

Male Wistar rats (180–200 g) were purchased from Shanghai Experimental Animal Center and housed in Guiyang Medical College Research Resources Center at 22°C with a 12-h light/dark cycle. Rats had free access to rodent diet and tap water. The College Animal Care and Use Committee approved all animal procedures. Rats were divided into nondiabetic and diabetic groups (n=20 in each nondiabetic group, n=30 in each diabetic group). Diabetes was induced by tail intravenous injection of a single dose of STZ (55 mg/kg, Sigma Chemical, St. Louis, MO, USA) dissolved in a sodium citrate buffer (pH 4.5). Whole blood obtained from each rat's tail vein was used for glucose monitoring with a Glucometer 4 complete blood glucose monitor. STZ-treated rats with whole-blood glucose levels >12.0 mM, examined on Day 3 after STZ treatment, were considered diabetic. Rats serving as vehicle controls (nondiabetic rats) were given the same volume of sodium citrate. These rats were housed for 3 months with daily general checking and weekly body weight recording.

2.2. Zn supplementation

When diabetic rats were diagnosed on Day 3 after STZ treatment, both nondiabetic and diabetic rats were randomly divided into 2 groups with and without Zn supplementation (n=10 in each nondiabetic group, n=15 in each diabetic group). Zn supplementation was given by drinking water at 5 mg $ZnSO_4$/kg daily for 3 months.

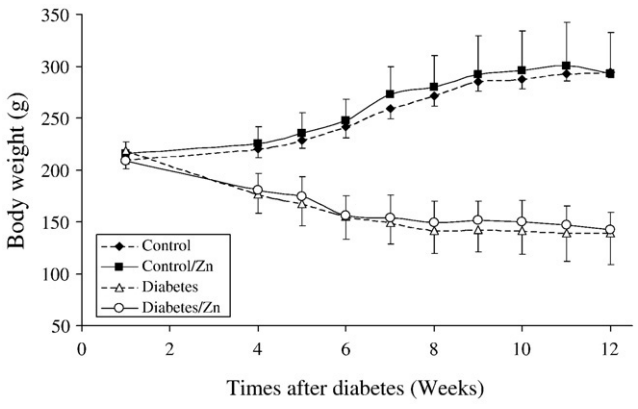

Fig. 1. Body weight of diabetic rats. Body weights of rats were monitored at the indicated times.

Table 1
The levels of blood glucose and 24-h urine proteins in the control and diabetic rats with or without Zn supplementation (mean±S.D.)

Groups	Blood glucose, mmol/L	24 h urine proteins, mg
Control	8.95±1.41	1.93±0.42
Control/Zn	7.77±1.68	1.48±0.38
Diabetes	18.03±1.72 *	59.25±11.31 *
Diabetes/Zn	15.84±1.38 *	42.28±15.15 *,†

* P<.01 vs control.
† P<.05 vs diabetes.

These rats were kept individually and daily in the morning they were first given 20–35 ml of tap water dissolved $ZnSO_4$ (50 μg/ml). Volume of $ZnSO_4$ solution was calculated based on individual rat body weight (0.1 ml $ZnSO_4$/g body weight). After completely $ZnSO_4$ solution, rats were provided free access tap water. The basal diet contained Zn (30 mg/kg) as the AIN-93G standard diet, which was purchased from Shanghai Experimental Animal Center.

2.3. Measurement of 24 h urinary proteins

To collect urine samples, animals were individually placed in metabolic cages for 24 h prior to the end of 12 weeks after STZ treatment. The total 24-h urinary protein (mg) concentrations were measured using a Coomassie Brilliant Blue assay following the instructions provided in the manufacturer's kits (Beijing Mike, China).

2.4. Measurements of Zn levels in the serum, kidney, liver and muscle

Zn levels in the serum, kidney, liver and skeletal muscle were measured by an atomic absorption spectrophotometer using air-acetylene flame after tissue digestion by nitric acid as described previously [13]. By this assay, total Zn in the tissues including free Zn and protein-bound Zn were measured and expressed as micrograms per gram of dry tissue.

2.5. Pathological examination

Kidneys were collected and immersion-fixed in 10% neutral formalin, embedded in paraffin and sectioned into 4-μm-thick sections onto glass slides. After deparaffinization, the tissue sections were rehydrated and stained by hematoxylin and eosin, periodic acid-Schiff (PAS) reagent and Sirius-red.

Pathological changes of glomeruli, renal tubules and interstitium were examined by light microscopy. All morphometric and semiquantitative investigations were performed in a blinded manner by two investigators without knowledge of the origin of the slides. Mesangial index was assessed based on at least 20 glomeruli per kidney using computer image analysis by measuring the proportion of glomerular tuft comprised of extracellular matrix (ECM). The ratio of measured PAS-positive area (ECM) in the full glomerular area (GA) (i.e., ECM/GA) was used to indicate the magnitude of ECM accumulation as previously described [17]. The tubulointerstitial damage index was assessed on PAS-stained sections at a magnification of ×100 using a semiquantitative scoring systems [18]. For determination of the tubulointerstitial damage score, 10 fields per kidney were randomly sampled, and the changes (0–4) were graded as follows: Grade 0, no change; Grade 1, lesions involving less than 25% of the area; Grade 2, lesions affecting 25% to 50%; Grade 3, lesions involving more than 50% and Grade 4, involving (almost) the entire area. The thickness of glomerular basement membrane (GBM) was also measured based on at least 5 glomeruli per section of the three sections from each rat using computer image analysis system. For Sirius red staining, the sections of 4 μm thickness were stained with 0.1% Sirius red F3BA and 0.25% Fast green FCF, and the Sirius red-stained sections were assessed for the proportion of fibrosis (collagen) in the kidney tissues using computer image analysis system, as described in our previous study [17].

2.6. Detection of MT-1 mRNA expression by reverse transcriptase-polymerase chain reaction

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, the tissue pellets were homogenized in TRIzol Reagent. Total RNA was extracted with phenol/chloroform, precipitated by isopropyl alcohol and subjected to further purification using an RNeasy mini kit (Qiagen, Valencia, CA, USA). MT-1 mRNA expression level was measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). A 50-μl reaction mixture containing 2 μg of total RNA was reversely transcribed to cDNA using oligo(dT) primers and SuperScript II RT-polymerase (Invitrogen). Real-time quantitative PCR was performed using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) in combination with SYBRgreen dye. The reaction was performed at 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min in triplicate and analyzed using the comparative C_t method according to the TaqMan manual. The following primers were used: for MT-1, 5′-GTGCCCAAGGCTGCATCT-3′ and 5′-

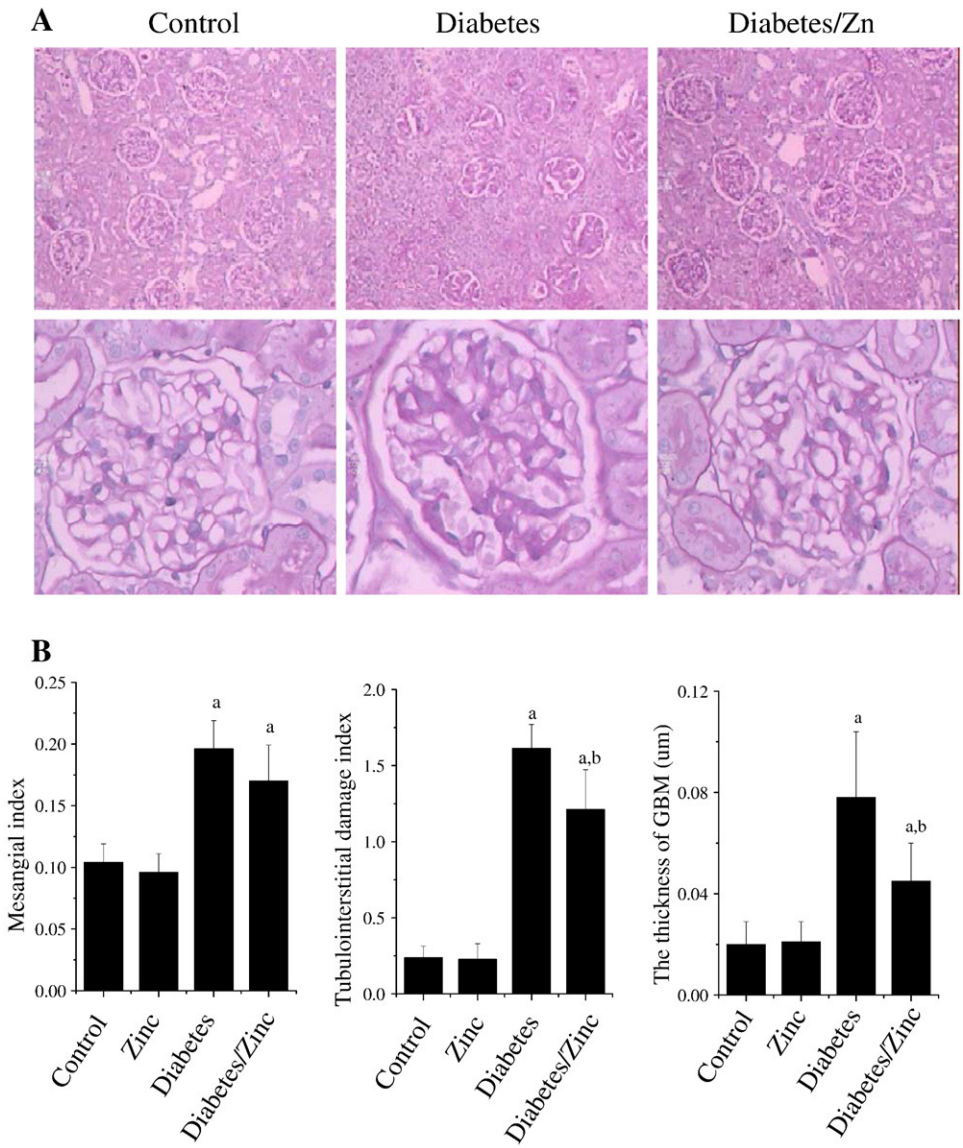

Fig. 2. Renal pathological changes. Renal tissues were stained by PAS (Upper panel, original magnification ×100; Lower panel, original magnification ×400), which showed that diabetes increased PAS-positive materials in renal tubule interstitial components and glomeruli (A). Renal histological changes were semiquantitatively evaluated under light microscopy based on PAS staining (B) for the mesangial damage index, tubular damage index and GBM thickness, as described in Materials and Methods. Statistical analysis: (a) $P<.05$ vs control. (b) $P<.05$ vs. diabetes alone.

GGTCACGGTCAGGGTTGTACA-3′; for β-actin, 5′-ACCACCATGTACCCAGGCAT-3′ and 5′-CCGGACTCATCGTACTCCTG-3′.

2.7. Immunohistochemical staining of MT, connective tissue growth factor and plasminogen activator inhibitor-1 expression

Renal tissue sections (4 μm) were placed into xylene to remove the paraffin wax and then hydrated in graded ethanol. The slides were subjected to immunohistochemical staining for MT, CTGF and plasminogen activator inhibitor-1 (PAI-1) based on our published methods [13] with the following specific antibodies: monoclonal mouse anti-MT antibody (Dako North America, Carpinteria, CA, USA), polyclonal goat anti-CTGF and rabbit anti-PAI-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To achieve color development, samples were incubated with diaminobenzidine and counter-staining with hematoxylin. Controls were obtained by replacing the primary antibody with phosphate-buffered saline. Semiquantitative analysis was performed using computer imaging system for the percentage of positive staining as described above.

2.8. Measurement of malondialdehyde and superoxide dismutase

Renal tissue (100 mg) was homogenized and malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured following the kit instructions provided from the company (Nanjing Jiancheng Biological, Nanjing, China).

2.9. Cell culture and treatments

Human renal proximal tubular (HK11) cells [19] were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA). When cell populations reached 40–50% confluence, the cells were exposed to $ZnSO_4$ (50 μM) or $CdCl_2$ (1.0 μM) in DMEM/F12 media with 5% FBS for 24 h. Then cells were exposed to D-glucose in a final concentration of 27.5 mM as HG or exposed to 5.5 mM D-glucose as control in serum free DMEM/F12 media for another 24 h. To exclude a hyperosmotic effect, we added identical concentrations of D-mannitol (5.5 mM Glucose+22 mM mannitol; Sigma) in control cultures. In addition, some cultured cells were exposed to p38 mitogen-activated protein kinase (MAPK) inhibitor–SB203580 at a final concentration of 10 μM or extracellular signal-regulated kinase (ERK)1/2 inhibitor–PD098059 at a final concentration of 50 μM for 30 min (both inhibitors from Calbiochem, San Diego, CA, USA). Then the cells were exposed to $ZnSO_4$ or $CdCl_2$ with one of the two inhibitors for 24 h and further exposed to HG with one of the two inhibitors for another 24 h.

2.10. Measurements of CTGF and MT expression by Western blotting in HK11 cells

After treatments, the monolayer cultures were collected with a gum rubber-scraping device and lysed by RIPA buffer (sc-24948, Santa Cruz Biotechnology).

Proteins were collected by centrifuging at 12 000 rpm for 10 min at 4°C and supernatants were aliquoted and saved for further experiments. The protein concentration was determined by Bio-Rad Protein Assay according to the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA, USA). Sample was then mixed with loading buffer (sc-24945, Santa Cruz Biotechnology), heated at 95°C for 5 min and then subjected to Western blotting assays for CTGF with goat anti-CTGF polyclonal antibody (sc-14939, Santa Cruz Biotechnology), as described before [9]. After detection of CTGF, the membranes were stripped with stripping buffer for 1 h at room temperature and reprobed with monoclonal anti-β-actin antibody (Sigma) at a concentration of 1:10,000 for 1 h, as a loading control [9].

MT expression was detected by a modified Western blotting protocol [9]. Briefly, protein samples were treated with DTT at a final concentration of 20 mM at 56°C for 30 min, added with iodoacetamide (Sigma) at a final concentration of 50 mM at room temperature for 1 h in the dark and centrifuged at 800×*g* for 5 min to collect the supernatant. Supernatant was mixed with loading buffer and heated at 95°C for 5 min and then subjected to electrophoresis on 18% SDS-PAGE and to Western blotting with anti-MT monoclonal antibody (Dako North America). Antigen-antibody complexes were visualized with enhanced chemiluminescence (ECL) system. However, since in the transfer conditions $CaCl_2$ was utilized for MT protein analysis, these blots could not be stripped and reprobed for actin. Thus, 2 parallel gels were run under the same conditions. One gel (MT gel) was transferred to a membrane in transfer buffer containing $CaCl_2$, while another (actin gel) was transferred to membrane in buffer without $CaCl_2$. Western blotting was performed on two membranes separately, with actin blot only to confirm equal loading of proteins. Therefore, data analysis of MT blots was based on the fold difference relative to control (in the same membrane) rather than on the fold difference relative to actin (in a separate membrane) [9].

2.11. Statistical analysis

All results are presented as mean±S.D. The data were analyzed by analysis of variance and post-multiple comparison using Scheffe' Test. Differences between groups were considered significant at $P<.05$.

3. Results

3.1. Zn supplementation partially prevented diabetes-induced renal functional and pathological changes

To determine whether Zn supplementation prevents diabetes-induced renal damage, STZ-induced diabetic and age-matched, non-diabetic rats were supplemented with Zn in drinking water at 5mg $ZnSO_4$/kg for 3 months. Monitoring the body weight (Fig. 1) showed that body weight gain in diabetic rats was significantly decreased compared with control group ($P<.05$). Zn supplementation did not affect body weight gain either in nondiabetic or diabetic mice. At the end of the experiment, 24 h urinary protein and fasting whole blood glucose were all significantly higher in the diabetic rats than those in nondiabetic groups ($P<.01$). Diabetes-induced increase of 24 h urinary protein was partially prevented by Zn supplementation ($P<.05$, Table 1).

Functional improvement of the kidney by Zn supplementation in the diabetic rats was accompanied by a significant prevention of diabetes-induced renal pathological changes. PAS staining indicated that diabetes induced renal fibrosis, characterized by mesangial expansion and ECM accumulation, and increased tubule interstitial accumulation of PAS-positive materials (Fig. 2A). Semiquantitative analysis of mesangial index, tubulointerstitial damage index and GBM thickness were performed based on PAS staining (Fig. 2B), using the criteria described in Materials and Methods. Diabetes-induced significant increases in mesangial index, tubulointerstitial damage index and GBM thickness. Zn supplementation partially prevented diabetes-induced tubulointerstitial damage and GBM thickness (Fig. 2B, $P<.05$) and slightly (no statistical difference) prevented diabetes-induced mesangial damage.

Sirius-red staining of collagen further confirmed that interstitial collagen accumulation is predominant in proximal tubules (Fig. 3A). Glomerular deposition (Fig. 3B) was also observed in the kidneys of diabetic rats, which was significantly inhibited with Zn supplementation (Fig. 3C).

To further examine the protective effects of Zn supplementation on diabetes-induced renal inflammation, we performed PAI-1 immunohistochemical staining. These results demonstrated that diabetes induced a significant increase in PAI-1 expression predominantly in tubules, and Zn supplementation partially prevented its expression (Fig. 4A, C). Since PAI-1 also plays a critical role in renal fibrosis [1,2], the critical fibrosis mediator CTGF was examined by immunohistochemical staining (Fig. 4B). Expression of CTGF was significantly increased in the kidneys of diabetic rats, which was partially attenuated after Zn supplementation diabetic rats (Fig. 4B, D). Similar to PAI-1 expression, CTGF expression was predominantly increased in the proximal renal tubules of diabetic rats (Fig. 4B).

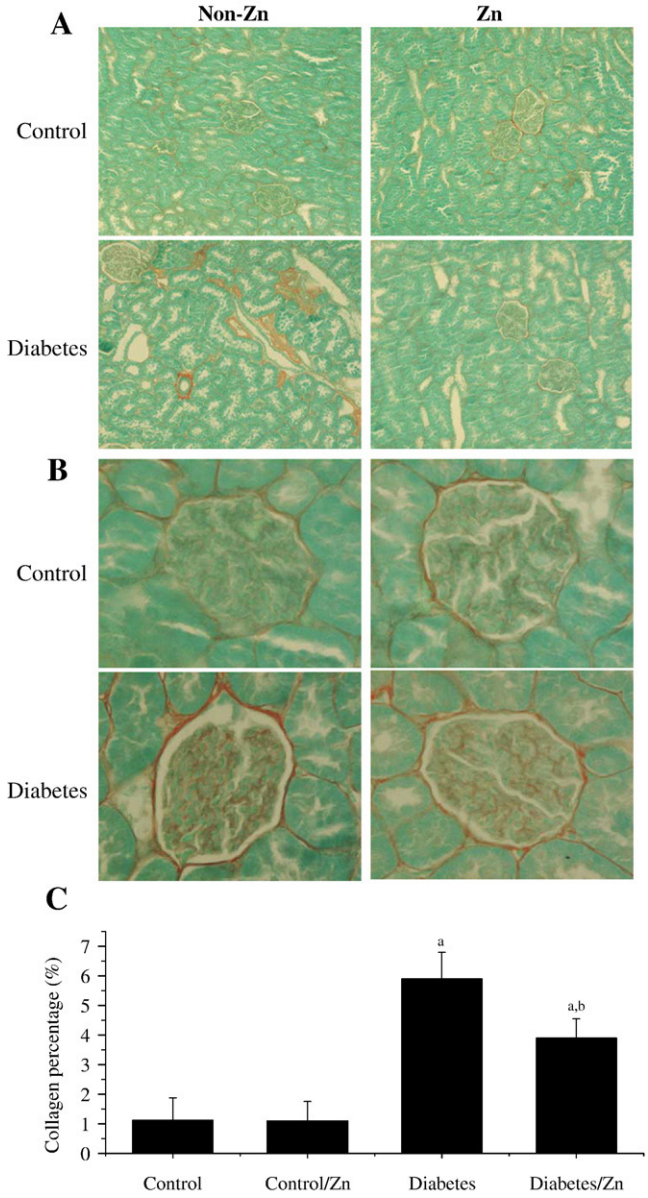

Fig. 3. Sirius-red staining of collagen. Renal fibrosis (collagen deposition) was observed by Sirius-red staining, and images of this staining are representatively provided with low (A, original magnification ×100) and high (B, original magnification ×400) powers. Semiquantitative analysis was performed using Biomias99 computer image analysis system for the percentage of positive staining (C). Statistical analysis: (a) $P<.05$ vs control. (b) $P<.05$ vs. diabetes alone.

3.2. Zn supplementation increased tissue Zn levels and renal MT synthesis and also ameliorated diabetes-induced renal oxidative damage

To ensure that there was an increase in systemic Zn levels after Zn supplementation, serum and tissue Zn levels were measured. Serum

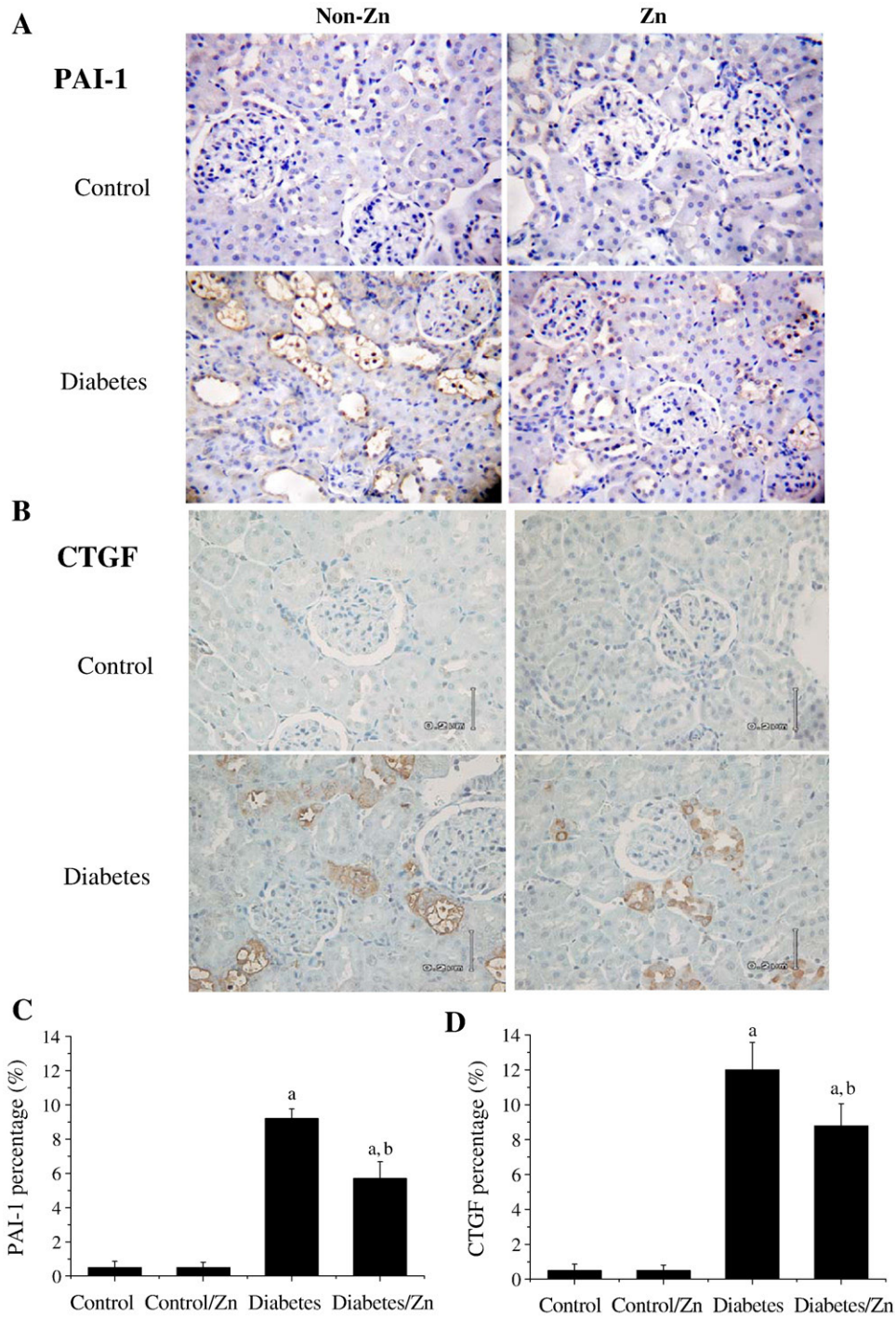

Fig. 4. Immunohistochemical examination for PAI-1 and CTGF. Examination using immunohistochemical staining and representative images of the staining for PAI-1 (A) and CTGF (B) are presented. Semiquantitative analysis for PAI-1 (C) and CTGF expression (D) was performed using Biomias99 computer image analysis system to calculate the percentage of positive staining. Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. diabetes alone.

and renal Zn levels were significantly increased in the Zn-supplemented control and diabetic rats (Fig. 5A, B, $P<.05$). Hepatic Zn levels (Fig. 5C, $P<.05$), but not muscular Zn levels (Fig. 5D), were also significantly increased in Zn-supplemented groups compared to control groups.

To determine whether Zn supplementation induces renal MT induction, renal MT-1 mRNA expression was measured by real-time RT-PCR. Results demonstrated a significant increase in MT-1 mRNA in the kidneys of Zn-supplemented nondiabetic and diabetic rats (Fig. 6A). Although diabetes also increased MT expression, which is consistent with our previous studies [13], Zn supplementation with diabetes further enhanced renal MT-1 mRNA expression. Consistent with mRNA expression (Fig. 6A), immunohistochemical staining using anti-MT antibody confirmed slight MT induction in the kidneys of nondiabetic rats, which was further enhanced in the kidneys of diabetic rats that were on Zn supplementation (Fig. 6B). Immunohistochemical staining also suggested that Zn-induced renal MT induction was predominantly in renal tubular cells (Fig. 6B).

To define whether Zn supplementation also induces other antioxidants that may contribute to renal protection from diabetes, total activity of SOD in the kidney tissues was measured. Zn supplementation did not affect nondiabetic renal SOD activity (Fig. 6C). Diabetes caused a slight decrease in renal SOD activity ($P<.05$), which was prevented by Zn supplementation.

It is well recognized that diabetes causes oxidative damage in organs, leading to dysfunction [2,3]; therefore, renal oxidative damage was also examined by measuring lipid peroxides (MDA). Diabetes induced a significant increase in renal lipid peroxides (Fig. 6D), and Zn supplementation did not affect the lipid peroxide level in non-diabetic rats but partially attenuated diabetes-induced renal lipid peroxide levels ($P<.05$).

3.3. HG induced CTGF expression in vitro and Zn-pretreatment presented HG-induced CTGF expression

To explore the possibly possibility of a direct role of Zn-induced MT in preventing diabetes-induced renal fibrosis, we examined CTGF expression in cultured HK11 renal tubular cells directly exposed to HG at 27.5 mM to mimic diabetes in vitro in the presence and absence of Zn [20]. Selection of renal proximal tubular cells for in vitro experiments is based on the findings that diabetes-induced collagen deposition (Fig. 3A), up-regulation of fibrosis mediator CTGF (Fig. 4B) and MT induction (Fig. 6B) are all predominant in proximal tubules and interstitial tissues. Direct exposure of HK11 cells to HG for 24 and 48 h significantly increased the CTGF expression. There was no significant difference between 24 and 48 h (data not shown). Therefore, exposure of HK11 cells to HG for 24 h was used for the consequent experiments.

For in vitro study, HK11 cells were pre-treated with 50 μM Zn for 24 h and then exposed to HG for another 24 h in fresh medium without Zn supplementation. Western blotting demonstrated HG-induced CTGF expression, which was significantly prevented by Zn-pretreatment. A concomitant increase in MT expression was evidenced in HG-treated HK-11 cells that were pre-treated with Zn for 24 hr (Fig. 7A and B). In the absence of Zn, HG alone only induced a slight increase in MT expression (no statistical difference). Combination of this in vitro finding with the above in vivo findings suggests that the significant prevention of diabetes- or HG-induced renal fibrosis by Zn supplementation may be attributed to its induction of renal MT expression. To negate a direct role of Zn and to demonstrate a critical role of MT expression in protection of HG-induced CTGF expression, HK11 cells were pre-exposed to low-dose Cd, a nonessential trace metal that is also well-known MT inducer, under the same conditions as Zn pretreatment. Like Zn pretreatment, treatment of HK11 cells with low-dose Cd that induced MT synthesis (Fig. 7B) also provided a significant protection against HG-increased CTGF expression and (Fig. 7A). Therefore, both Zn and Cd pretreatments caused MT protein induction concomitant with a decrease in CTGF expression, indirectly suggesting a possible role for MT in the prevention of HG-induced CTGF up-regulation.

3.4. Contributions of p38 and MEK/ERK MAPK pathways to HG-mediated CTGF expression and Zn-induced MT induction in HK11 cells

Previous studies have extensively demonstrated that activation of both p38 MAPK and MEK/ERK1/2 was involved in the pathogenesis of DN [21–23]. Therefore, we used the specific inhibitors of p38 and MEK/ERK1/2 MAPK to determine their roles in HG-induced CTGF expression and Zn-induced MT induction in renal tubular cells. HK11 cells were preincubated separately with or without p38 MAPK inhibitor (SB203580 at 10 μM) or ERK1/2 inhibitor (PD098059 at 50 μM) for 30 min, followed by treatment with or without Zn (50 μM)

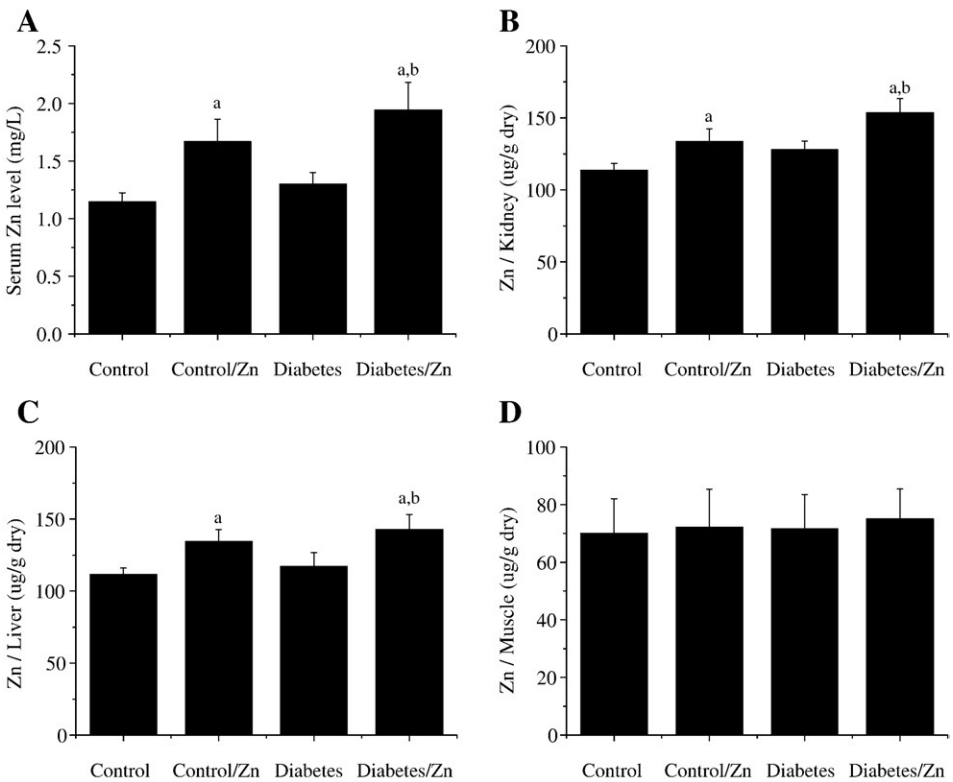

Fig. 5. Zn levels in the serum and tissues. Zn levels in the serum (A), kidney (B), liver (C) and muscle (D) of six rats at lease for each group were measured using atomic absorption spectrometer as described in Materials and Methods. Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. diabetes alone.

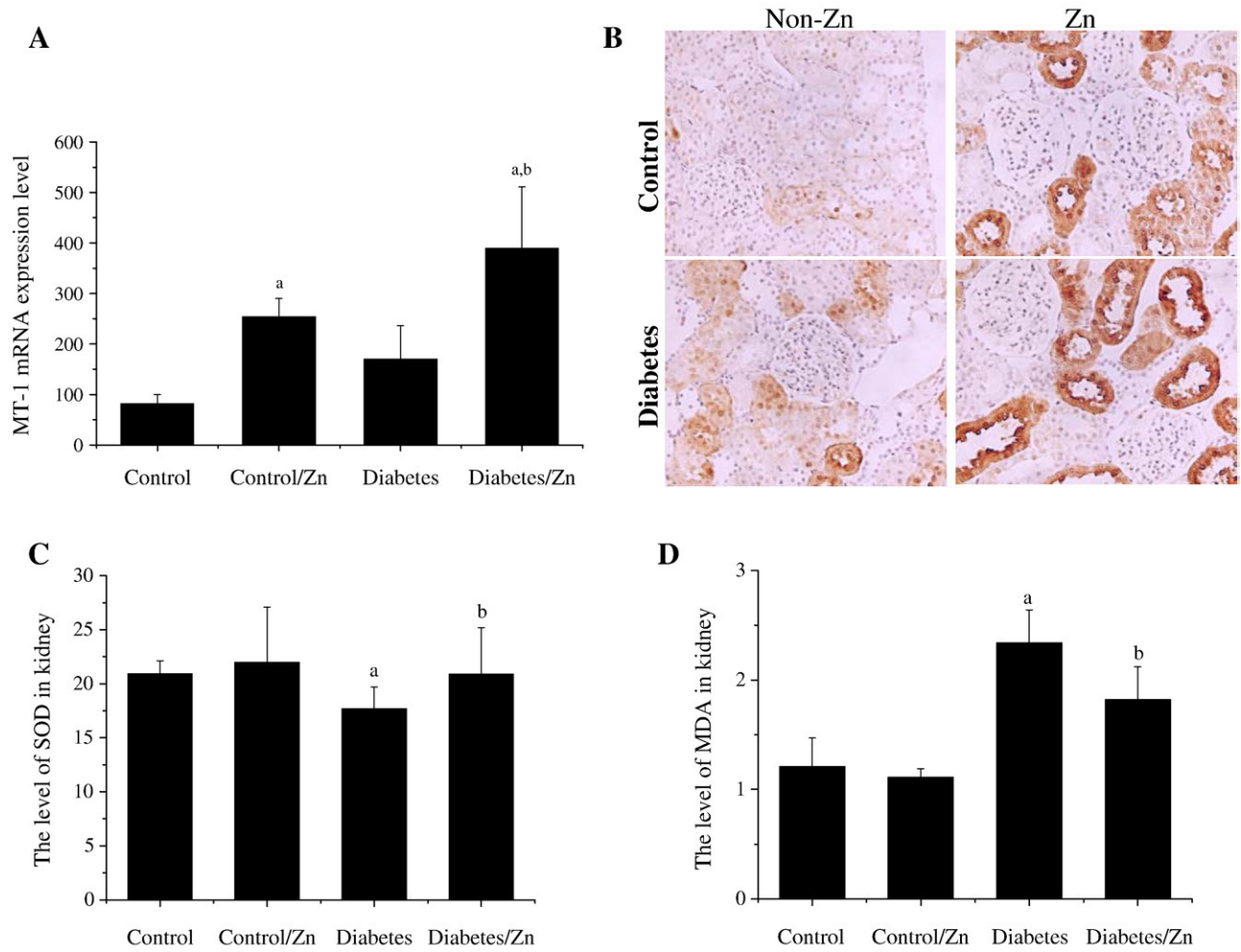

Fig. 6. Effects of Zn supplementation on renal MT expression and synthesis, renal SOD activity and lipid oxidation. Renal MT-1 mRNA expression was measured by RT-PCR method (A) and immunohistochemical staining (B). Total activity of SOD in the kidney was measured by a biochemical assay (C), and renal lipid peroxidation was also measured by MDA contents (D). Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. diabetes.

in continual presence of the inhibitors for 24 h. Cells were then subjected to HG or low glucose (LG) medium for an additional 24 h in the presence or absence of inhibitors. Cell lysates were subjected to anti-CTGF and anti-MT immunoblotting. Results showed that HG-induced CTGF up-regulation was prevented partially by p38 MAPK inhibition (Fig. 8, the fourth column vs. the second column in the middle panel), while MEK/ERK inhibition completely abrogated HG-induced CTGF expression (Fig. 9, the fourth column vs. the second column in the middle panel). Similar to MEK/ERK MAPK inhibition, pretreatment of Zn inhibited HG-induced CTGF expression in HK11 cells. However, Zn pretreatment inhibited HG-induced CTGF expression to a higher extent than was seen in the presence of SB203580 alone (the fifth and sixth columns vs. the third column in the middle panels of Figs. 8 and 9). These results demonstrate that hyperglycemia- or HG-induced CTGF up-regulation is mediated by both ERK1/2 and p38 MAPK signaling pathways in human renal proximal tubular cells.

Next we explored the contributions of these two MAPK signaling pathways to Zn-induced MT expression in HG-treated HK11 cells. Inhibition of p38 MAPK did not affect Zn-induced MT expression with or without HG (Fig. 8, the fifth and sixth columns vs. the third column in the low panel). In contrast, inhibition of MEK/ERK1/2 partially inhibited Zn-induced MT expression with or without HG (Fig. 9, the fifth and sixth columns vs. the third column in the low panel).

4. Discussion

The present study provides the first evidence that Zn supplementation can partially prevent the kidney from diabetes-induced pathological changes, likely through renal MT induction.

DN is characterized by excessive ECM accumulation in the kidney. Decreased degradation and increased synthesis of ECM both play important roles in ECM remodeling and eventual fibrosis. PAI-1 plays a critical role in ECM remodeling in the kidney [24–27]. Normal human kidneys do not express PAI-1. However, PAI-1 is overexpressed in pathologic conditions associated with renal fibrosis, including DN. To support these previous studies, we also found the significant up-regulation of PAI-1in the diabetic kidney, which was partially down-regulated by Zn supplementation (Fig. 4A). Up-regulation of transforming growth factor (TGF)-β1 has been considered as one of the key factors responsible for the increased ECM synthesis observed in the diabetic kidney; however, we found that renal fibrotic effect in diabetic and nondiabetic conditions may not be mediated only by TGF-β1 under certain conditions [28]. In fact, CTGF plays a pivotal role in renal fibrosis in the most conditions [28–30]. Treatment with CTGF antisense oligonucleotide (ASOs) of STZ-induced Type 1 diabetic mice for 4 month or of db/db Type 2 db/db diabetic mice for 8 months

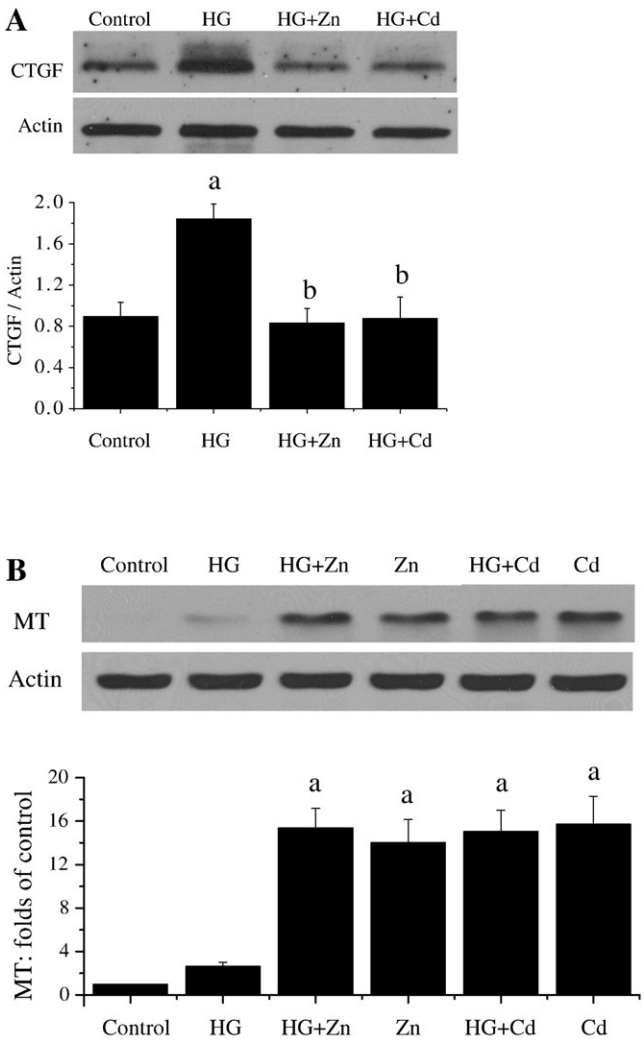

Fig. 7. Zn-induced MT and its effect on HG-induced CTGF. Western blotting was used to detect CTGF expression (A) and MT expression (B) in the cultured HK11 renal tubular cells exposed to HG at 27.5 mM for 24 h with and without pretreatment with 50 μM Zn or 1.0 μM Cd for 24 h. Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. HG.

significantly reduced renal CTGF expression with a concomitant decrease in proteinuria and albuminuria [30]. In TGF-β1 transgenic mice, intravenous administration of CTGF ASO also significantly blocked CTGF expression in proximal tubular epithelial cells along with a concomitant decrease in renal interstitial fibrosis [29]. These studies clearly indicated the pivotal role of CTGF pathway in the development of diabetic or nondiabetic interstitial fibrosis.

The first novel finding of the present study is that Zn supplementation did not affect the animal body weight either in nondiabetic or diabetic rats (Fig. 1) but partially prevents diabetes-induced pathological changes, fibrosis and 24 h urinary protein increase (Figs. 2, 3 and Table 1) due to its partial suppression of diabetes-induced CTGF up-regulation (Fig. 4). In vitro studies suggest that HG significantly induced CTGF up-regulation that was also completely inhibited by Zn pretreatment. It should be mentioned that the in vivo study showed only partial protection from diabetes-induced CTGF expression (Fig. 4D). This implies that although MT can efficiently prevent HG-induced CTGF expression, other mechanisms, rather than hyperglycemia alone, may be also responsible for the CTGF up-regulation under the in vivo diabetic condition. We have previously shown that renal CTGF expression and fibrosis could be induced by chronic treatment with advanced glycation end-products [28] and also by renal ablation [17].

Emerging evidence has indicated an important role of oxidative stress for hyperglycemia-induced renal damage and fibrosis in vitro and in vivo [3,31]. MT, as a potent antioxidant, protects against oxidative damage more efficiently than other known antioxidants [32–35]. Our group and others have demonstrated that MT significantly prevented diabetes-derived oxidative damage, leading to a significant protection against diabetic cardiomyopathy [32,36–40]. Whether the protective effect of Zn supplementation-induced MT on diabetes-mediated renal pathological changes and fibrosis is also mediated by antioxidant action remains unclear; however, several pieces of indirect evidence support the importance of antioxidant action against DN. Patients with defective SOD accelerated the development of DN [41,42]. Mice overexpressing SOD or catalase showed complete or partial prevention of the development of DN [43,44]. Antioxidants administered to diabetic rats or mice significantly prevented diabetes-induced renal dysfunction [45,46]. Previous studies [8,14–16] and our current study (Fig. 6D) showed that induction of renal tubular MT synthesis by Zn showed a significant protection against oxidative damage.

It should be noted that the protective effects of Zn supplementation on renal pathological changes (Fig. 2), fibrosis (Figs. 3, 4) and oxidative damage (Fig. 6D) were more significant than that on 24 h urinary protein increase (Table 1). This is probably because urinary protein level is a reflection of both tubular and glomerular

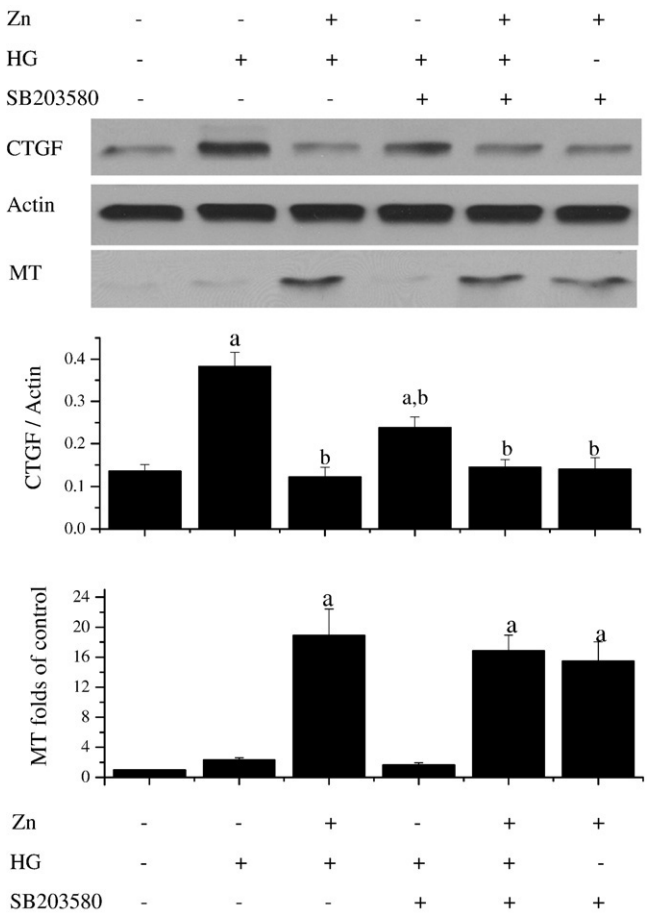

Fig. 8. Effects of p38 MAPK inhibition on HG-induced CTGF and Zn-induced MT. CTGF (middle panel) and MT (low panel) expressions were detected with Western blotting in the HK11 cells exposed to the indicated conditions. SB203580, p38 MAPK inhibitor at 10 μM. Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. HG.

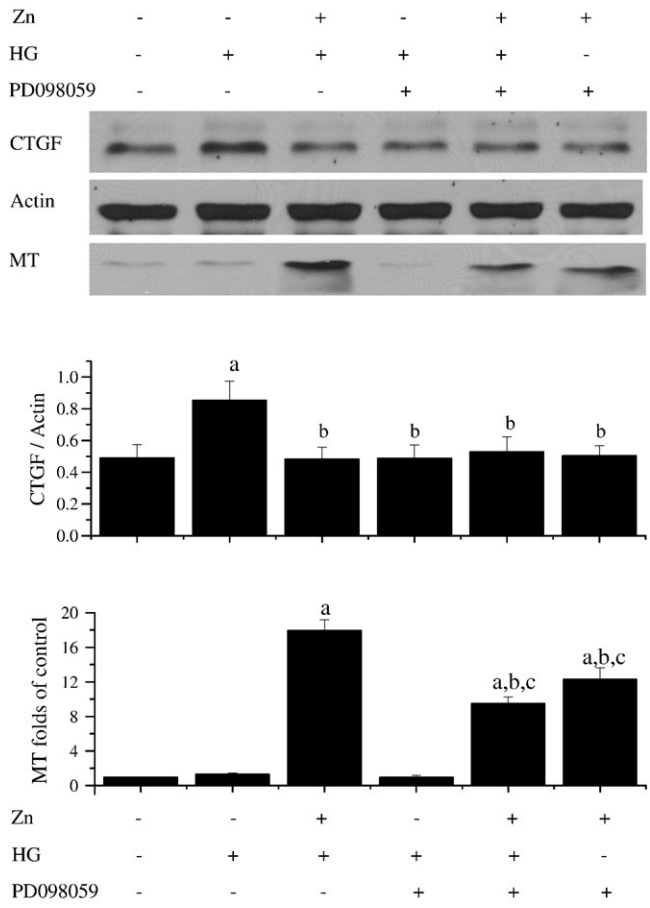

Fig. 9. Effects of MEK/ERK1/2 inhibition on HG-induced CTGF and Zn-induced MT. CTGF (middle panel) and MT (low panel) expressions were detected with Western blotting in the HK11 cells exposed to the indicated conditions. PD098059: MEK/ERK1/2 inhibitor at 50 μM. Statistical analysis: (a) $P<.05$ vs. control. (b) $P<.05$ vs. HG. (c) $P<.05$ vs. HG+Zn.

dysfunction, while Zn supplementation-induced MT is mainly expressed in tubular cells. Therefore, Zn supplementation may predominantly protect from tubular pathogeneses but not significantly from glomerular pathogenesis (Fig. 2), which results in a less protective effect on 24-h urinary protein increase (Table 1).

Since Zn also functions as a potent antioxidant [7,47] and Zn deficiency enhanced diabetic renal damage [48], we further determine whether the protective effect of Zn supplementation against DN is mediated by Zn in an MT dependent or independent manner. We demonstrated that a pre-exposure of HK11 cells to low-dose Cd offered a protection similar to Zn, indirectly indicating that the protective effect of Zn-pretreatment on HG-induced CTGF up-regulation is attributed to MT rather than Zn. We have previously shown that pretreatment with low-dose Cd that did not have cytotoxic effect on the cultured neuronal and cardiac cells significantly induced MT expression and protected these cells from radiation- and HG-induced damage [7,49,50]. Furthermore, when MT expression was silenced with MT siRNA, the protective effect of pretreatment with Zn or low-dose Cd against HG-induced toxicity was abolished in cardiac cells [9]. Consistent with the above notion, mice with podocyte-specific overexpression of MT are significantly resistant to the development of renal pathogenic alterations and dysfunction, which were evident in their wild-type diabetic mice [51]. Collectively, these results imply that the significant protection of renal tubular cells from HG by pretreatment with Zn or low-dose Cd is most likely mediated by Zn- or Cd-induced MT. However, it should be noted that we used a low-dose of Cd in the current study only to demonstrate a critical role for MT-induction in protection against diabetes-mediated renal damage. Unlike Zn, we do not believe that Cd supplementation will have any translational benefits in a clinical setting, as Cd-induced MT may enhance the renal susceptibility to oxidative damage under in vivo conditions, as aforementioned [10–12].

While it is likely that MTs are the key players in the protective mechanism triggered by Zn supplementation, whether elevation of intracellular Zn also affects other players of Zn homeostasis, such as Zn transporters (ZnTs), contributing to the renal protection from diabetes remains unclear. Zn ions cross biological membranes with the aid of specialized membrane proteins, belonging to the ZRT/IRT-related proteins (ZIP) and ZnT families. The ZIPs are encoded by the Slc39A gene family and are responsible for uptake of the Zn, while ZnTs are encoded by the Slc30A genes and are involved in intracellular traffic and/or excretion [52–54]. Intracellular Zn concentration is buffered by MT. Although ZnTs have been implicated in the onset of Types 1 and 2 diabetes [52–54], whether changes of ZIPs and ZnTs are also involved in the development of or protection remains to be determined.

A limitation of the present study was that we did not perform detail dose effects of Zn supplementation against diabetic nephropathy. We selected one dose of Zn supplementation in the present study as we have previously shown that this dose of Zn supplementation has afforded complete protection against diabetes-induced cardiac dysfunction [9]. The present study was originally intended to determine whether Zn supplementation that significantly protected the heart from diabetes also significantly protected the kidney from diabetes. As a consequence, only partial protection of such dose Zn supplementation against diabetes-induced renal dysfunction was found in the present study. Interestingly, clinical studies also showed a partial renal protection by Zn supplementation from diabetic patients [55–57]. Therefore, whether increasing doses of Zn supplementation can enhance the protective effect will be addressed in the future studies.

Clinical studies have shown that diabetic patients with low serum Zn levels have a high risk for developing cardiovascular disease compared to those with normal serum Zn levels [6], and Zn supplementation with and without other medications for diabetic patients provided several benefits, including correcting diabetic Zn deficiency, better glycemic control [56,58], modifying the lipid profile [56], improvement in peripheral neuropathy [59] and partial improvement of renal function [55,56]. Combined, these clinical observation as well as experimental studies suggest that although protective effects are not equal in the heart (completely) and kidney (partially), Zn supplementation will provide a greatly potential for clinical application to preventing diabetic cardiovascular diseases, at least via induction of MT as one of the protective mechanisms.

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