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Original Article

Darbepoetin-α ameliorates diabetes-induced mesangial cell damage *In Vitro* and *In Vivo*

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Abstract: The present research investigated the long-term effects of darbepoetin-α (DPO) on streptozotocin (STZ)-induced diabetic nephropathy and on mesangial cells (MCs) under high glucose (HG) conditions. Sprague-Dawley rats were assigned as follows: control group, diabetic group, and DPO group. Rats in the diabetic and DPO groups were treated with STZ to induce diabetes. Rats in the DPO group were administered DPO (0.75 μg/kg BW) after the onset of diabetes. MCs were cultured in NG or HG with or without DPO. DPO did not increase hematocrit levels, but significantly reversed diabetes-induced changes in glucose level, HbA_{1c}, serum creatinine levels, urinary microalbumin levels and creatinine clearance rate. DPO attenuated apoptosis as well as fibrosis and morphological alterations in glomerular of STZ. DPO significantly reduced ROS production and inhibited apoptosis and fibrosis of MCs in HG. DPO inhibited Nox4 expression and activation of ERK1/2 in renal cortex of STZ and HG-induced MCs. Moreover, DPO suppressed the diabetes-induced up-regulation of α-SMA and collagen IV by inhibiting Nox4/ERK1/2 signaling pathways. Long-term treatment with low-dose DPO did not stimulate hematopoiesis but ameliorated hyperglycemia in rats treated with STZ. DPO may attenuate diabetes-induced MC damage *In Vitro* and *In Vivo* by anti-apoptosis and anti-fibrosis. NADPH regulated the ERK1/2 signaling pathway to mediate phenotypic transition and collagen synthesis in HG-treated MCs. DPO is a feasible therapeutic agent against renal fibrosis in DN by inhibition of Nox4-mediated activation of ERK1/2.

Keywords: Darbepoetin-α, streptozotocin, mesangial cell, hyperglycemia, fibrosis

Introduction

As a serious complication of diabetes, diabetic nephropathy (DN) usually leads to end-stage renal disease and contributes to nearly half of cases of this condition worldwide [1]. Morbidity and mortality of individuals with DN would increase without early detection of the condition. DN consists of morphological, ultrastructural, and functional changes in the diabetic kidney [2]. Glomerular changes in DN include abnormal deposition of extracellular matrix (ECM) proteins, proliferation and hypertrophy of mesangial cells (MCs) as well as incrassation of the glomerular basement membrane (GBM) [3]. In contrast, tubulointerstitial fibrosis, hypertrophy of tubular cells, and incrassation of the tubular basement membrane (TBM) are the characteristic changes of the tubulointerstitial areas in DN [4, 5].

Erythropoietin (EPO) is an erythropoiesis-stimulating hormone. It regulates the generation of erythrocytes and assists in oxygen delivery. EPO is normally used in the treatment of anemia. However, EPO also has inhibitory effects on apoptosis, oxidative stress, and inflammation [6]. Renal cells are found to express functional EPO receptors [7]. There is a growing number of research on renal-protective effects of EPO in animal models of acute kidney damage [8] or chronic kidney disease [9, 10]. Analysis of the pharmacological mechanisms of EPO in these studies indicates that treatment with erythropoiesis-stimulating agents (ESAs) may exert renal protection by pleiotropic actions on several targets directly or indirectly.

Darbepoetin-α (DPO), an ESA, has a serum half-life nearly three times as long as that of EPO [11]. Low-dose DPO significantly attenuates

renal injury, preserves renal function, and improves the survival rate in rats that have undergone 5/6 nephrectomy without changing hematocrit (Hct) levels [12]. Administration of DPO also protects the kidney against ischemic acute renal injury [13]. DPO has been proven to ameliorate apoptosis of murine MCs exposed to toxic or hypoxic stimuli [14]. DPO has also been found to inhibit apoptosis of podocytes in both *In Vitro* and *In Vivo* experiments [15]. However, the effects of DPO on maintaining renal function and structure in type 1 experimental diabetic rat have been seldom investigated. EPO has been recorded to prevent reactive oxygen species (ROS) production and attenuate apoptosis in HG-treated renal tubular cells [16]. However, there is little data available regarding the effects of DPO on HG-treated MCs. Therefore, the aim of our research was to determinewhether DPO administration exhibited renal-protective effects in type 1 experimental diabetic rats, as well as to investigate the effects of DPO on HG-treated MCs.

Materials and methods

Animals, induction of diabetic model, and drug administration

Male Sprague-Dawley (SD) rats weighed about 200-250 g were purchased from Orient Bio Inc. (Seongnam, Korea). Rats were provided with standard rat chow and water. Seoul National University Bundang Hospital Committee of Animal Experiments approved our research, and institutional guidelines were followed.

SD rats were then randomly assigned to 3 groups: control (n = 5), diabetic rats (DM) (n = 8), and diabetic rats treated with DPO (DPO) (n = 8). The rats were raised in a temperature- and light-controlled setting for 2 weeks before the induction of diabetes. Rats from the DM and DPO groups were treated intraperitoneally with a dose of 65 mg/kg BW streptozotocin (STZ) to induce diabetes. Fasting glucose levels over 300 mg/dL were indicated as diabetes. Two weeks after the confirmation of diabetic status, rats from the DPO group received weekly subcutaneous injections of 0.75 μg DPO/kg BW. Control and DM groups were addressed with an equal volume of 0.9% NaCl as the vehicle. STZ was supplied by Sigma-Aldrich Co. LLC. (St. Louis,MO, USA). DPO was provided by Kyowa Hakko Kirin Co., Ltd. (NESP® Injection, Tokyo, Japan). The animal experiment was terminated 12 weeks after the onset of diabetes. Urine and blood were sampled, and kidney tissues were collected.

Cell culture

Mesangial cells (MCs) were collected from C57B6 mice [17]. We cultured MCs in DMEM (Gibco, Life Technologies, USA) with normal glucose (NG; 5.5 mM) or high glucose (HG; 25.5 mM), supplemented with 10% fetal bovine serum, 10 mg/ml streptomycin and 10,000 IU/mL penicillin G in a humidified incubator with 5% CO_2 at 37°C. The cells were incubated in a 6-well cell culture plates (2×10^5 MC cells/well) until they reached 60%-80% confluence for grouping.

Detection of ROS

We measured intracellular generation of ROS by 2',7'-dicholorofluoresce in diacetate (H2DCF-DA). 10 μM H2DCF-DA were added to the cell medium. The cells were then incubated at 37°C for 30 min. We then used a fluorescence spectrophotometer to measure the fluorescence intensity. The excitation and emission wavelengths were 490 nm and 526 nm, respectively. H2D-CF-DA was provided by Sigma-Aldrich Co. LLC. (St. Louis, MO, USA).

Laboratory analyses and regents

Glucose levels were collected from rat tail veins and evaluated using a HemoCue B-glucose analyzer (HemoCue AB, Angelholm, Sweden). Urinary albumin concentration (Exocell Inc., Philadelphia, PA, USA), urinary creatinine level (Exocell Inc.), and serum creatinine level (Bioassay Systems, Hayward, CA, USA) were measured with commercial kits. Serum insulin levels (Thermo Fisher Scientific, Waltham, MA, USA) were detected using enzyme-linked immunosorbent assay (ELISA) kits. ERK1/2 inhibitor (UO126) was provided by Cell Signaling Technology. Inc. (#9903).

Morphological analyses

We fixed the tissue samples in formalin and embedded them in paraffin. We then stained the renal sections with periodic acid-Schiff (PAS) or Masson's trichrome (MT). Within each section, 10-15 renal glomerular areas were identified at 400× magnification. All analyses

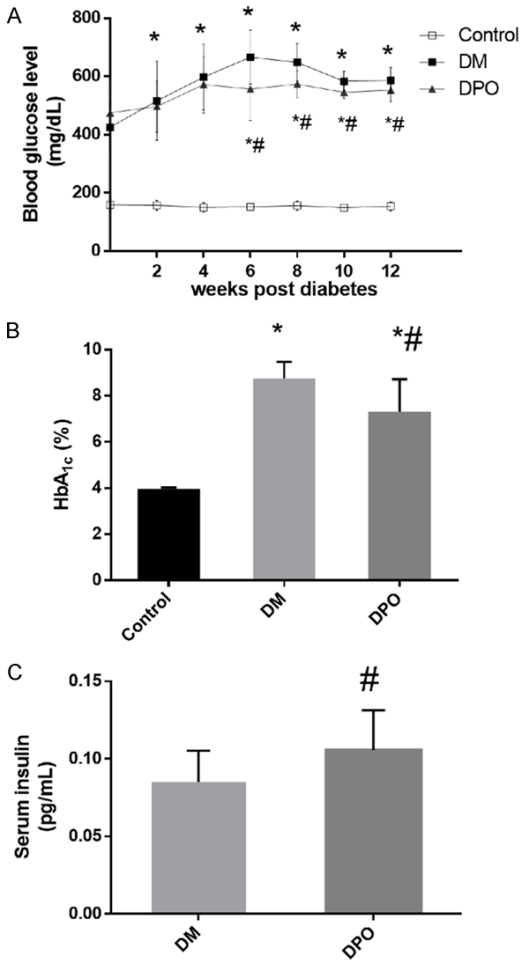

Figure 1. *DPO ameliorated hyperglycemia in STZ rats.* A. Sequential changes in fasting blood glucose levels after STZ injection at weeks 2, 4, 6, 8, 10, and 12 in control, diabetic rats (DM), and diabetic rats treated with DPO (DPO). Each value of blood glucose level was collected from 2 weeks record of fasting blood glucose levels. B. Blood glycated hemoglobin (HbA_{1c}) at week 12 in each group. C. Serum insulin levels at week 12 in diabetic rats (DM), and diabetic rats treated with DPO (DPO). Results represent mean ± SD. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

were performed blindly. Image J software (NIH, Bethesda, MD, USA) was utilized to analyze images.

Apoptosis

Apoptosis in the renal cortex and cultured MCs was evaluated with an *In Situ* Cell Death Detection Fluorescein Kit (Roche Applied Science, Mannheim, Germany). The instructions of the kit were followed. We detected apoptotic cells by using terminal deoxynucleotidyltransferase-mediated uridine triphosphate nick-end labeling (TUNEL) and stained the total nuclei by using 4',6-diamidine-2'-phenylindole (DAPI) staining. TUNEL-positive cells were detected by fluorescence microscopy (Carl Zeiss, Jena, Germany) in 10 consecutive fields. Apoptosis was evaluated by a ratio of TUNEL (+) cells to total cells.

Transient transfection

Small interfering (si) RNA against mouse Nox4 or control siRNA was transfected into MCs using Lipofectamine Plus reagent. The instructions from the manufacturer were followed. The siRNA was supplied by Santa Cruz Biotechnology. Inc. (Santa Cruz, CA, USA), whereas, the Lipofectamine Plus reagent was provided by Invitrogen-BRL (Carlsbad, CA, USA).

Western blot analysis

Proteins from tissues and MCs were extracted and measured using a bicinchoninic acid assay (BCA) method, and then loaded equivalently onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel. After that, we transferred the gel to a nitrocellulose membrane after electrophoresis, and then incubated the membrane all-night with the primary antibodies against β-actin, Nox4, $p22^{phox}$ collagen I and IV (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3, phospho-ERK1/2, ERK1/2, fibronectin (Cell Signaling Technology, Danvers, MA, USA), α-SMA (Abcam Inc, Cambridge, MA, USA) then incubated it with secondary antibodies (Santa Cruz). We measured the chemiluminescence by using an enhanced chemiluminescence substrate (Thermo Fisher Scientific) and analyzed the blots by using Image J software (NIH, Bethesda, MD, USA).

Data analysis

The results were expressed as the mean ± standard deviation (SD). Results were analyzed using Student's t-test or one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

The effects of DPO on blood glucose of STZ rats

SD rats developed hyperglycemia after STZ injection. Blood glucose levels were elevated

Table 1. Parameters of RBC counts, Hemoglobin, Hematocrit, Renal Weight, Body Weight, Renal/Body Weight at week 12 in control rats, diabetic rats (DM), and diabetic rats treated with DPO (DPO)

Parameter	Control	DM	DPO	Overall *P*	Specific *P*	
					*	#
Red blood cell counts (×10^6/μL)	6.11 ± 1.07	6.7 ± 0.82	4.96 ± 2.10	0.105	0.833	0.097
Hemoglobin (g/dL)	11.93 ± 1.38	13.16 ± 1.39	10.01 ± 4.00	0.122	0.798	0.110
Hematocrit (%)	34.13 ± 5.51	38.27 ± 4.65	29.46 ± 12.13	0.178	0.766	0.158
Renal Weight (mg)	1.85 ± 0.16	2.33 ± 0.32	2.39 ± 0.21	0.003	0.013	0.869
Body Weight (mg)	660.8 ± 61.39	357.1 ± 48.58	367.5 ± 49.80	< 0.001	< 0.001	0.909
Renal/Body Weight (mg/mg)	0.0028 ± 0.00013	0.0065 ± 0.00054	0.0066 ± 0.00088	< 0.001	< 0.001	0.990

Values are expressed as mean ± SD. Overall *P* values are estimated for the 3 groups by using an ANOVA tests; **P* and *#P* values are for the comparisons between DM and Control, and between DM and DPO groups by using ANOVA and post-hoc Tukey analysis, respectively. The values of renal weight, body weight, and renal/body weight were significantly different between the control and DM group. Red blood cell counts, hemoglobin levels, and hematocrit levels did not show any significant differences among, and between the three groups, as determined using ANOVA and Tukey post-hoc analyses.

substantially and remained consistently higher in STZ rats compared to levels in controls over the next several weeks ($P < 0.05$). However, levels of blood glucose were lower in STZ rats treated with DPO when compared with the STZ-only rats at weeks 6, 8, 10, and 12 ($P < 0.05$) (**Figure 1A**). Moreover, at weeks 12, the serum insulin level was higher in STZ rats treated with versus without DPO (DPO:DM = 0.106 ± 0.026:0.085 ± 0.020 pg/mL, $P < 0.05$) (**Figure 1C**), whereas blood HbA_{1c} was lower in the DPO group compared with the DM group (DPO:DM = 7.31 ± 1.40:8.74 ± 0.73%, $P < 0.05$) (**Figure 1B**).

The effects of DPO on physical data, renal function and structures in STZ rats

We did not observe a statistical difference between the DM and DPO groups in red blood cell (RBC) count, hemoglobin level, Hematocrit (Hct), renal weight, body weight, and the ratio of kidney weight to body weight (**Table 1**). Renal functions were evaluated by microalbuminuria and creatinine clearance rate (Ccr). 24-hour urinary microalbumin excretion was considerably higher in the DM group compared with the control group, whereas a lower excretion was observed in STZ rats treated with versus without DPO (DPO:DM = 20.80 ± 10.47:59.94 ± 28.98 mg/day, $P < 0.05$). A higher serum creatinine level was found in STZ rats than that in control ones (DM:control = 0.37 ± 0.050:0.23 ± 0.031 mg/dL, $P < 0.05$), whereas a markedly reduced serum creatinine level was observed in the DM group with DPO treatment (0.31 ± 0.027 mg/dL, $P < 0.05$). The creatinine clearance rate (Ccr) was elevated in diabetic rats treated with versus without DPO (DPO: DM = 3.41 ± 0.79:1.80 ± 0.56 mL/min, $P < 0.05$). (**Figure 2A**).

The glomerular area of STZ rat kidneys displayed glomerular hypertrophy, mesangial expansion, indicated by PAS staining. The glomerular tuft area was larger in diabetic versus control rats; however, DPO treatment ameliorated these changes (DM:DPO = 3866 ± 803.3:3088 ± 602.0 μm^2, $P < 0.05$). In STZ rats, the mesangial matrix fraction increased significantly; however, it was reduced by DPO treatment (DM:DPO = 0.34 ± 0.071:0.26 ± 0.054, $P < 0.05$). STZ rats exhibited more severe glomerular fibrosis by MT staining when compared with controls. DPO treatment markedly reversed diabetes-induced glomerular fibrosis (volume fraction, DM:DPO = 0.26 ± 0.12:0.16 ± 0.07, P < 0.05) (**Figure 2B**, **2C**).

To measure the STZ-induced apoptosis in the glomerular area of rat kidneys, we detected apoptotic cells in kidney sections by TUNEL staining. In STZ rats, the numbers of apoptotic cells were remarkably elevated in the glomerular of rat kidneys; however, we observed a notably less apoptotic cells in the STZ rats treated with DPO (ratio of apoptotic cells to total cells, DM:DPO = 0.27 ± 0.10:0.17 ± 0.07, $P < 0.05$) (**Figure 2B**, **2C**).

The above demonstrated that DPO protected against glomerular damage in a type 1 model of diabetes.

The effects of DPO on ROS generation, NADPH oxidase 4 (Nox4) expressions in HG-treated MCs

To reveal the effects of DPO on oxidative stress, we examined NADPH oxidase activity and ROS

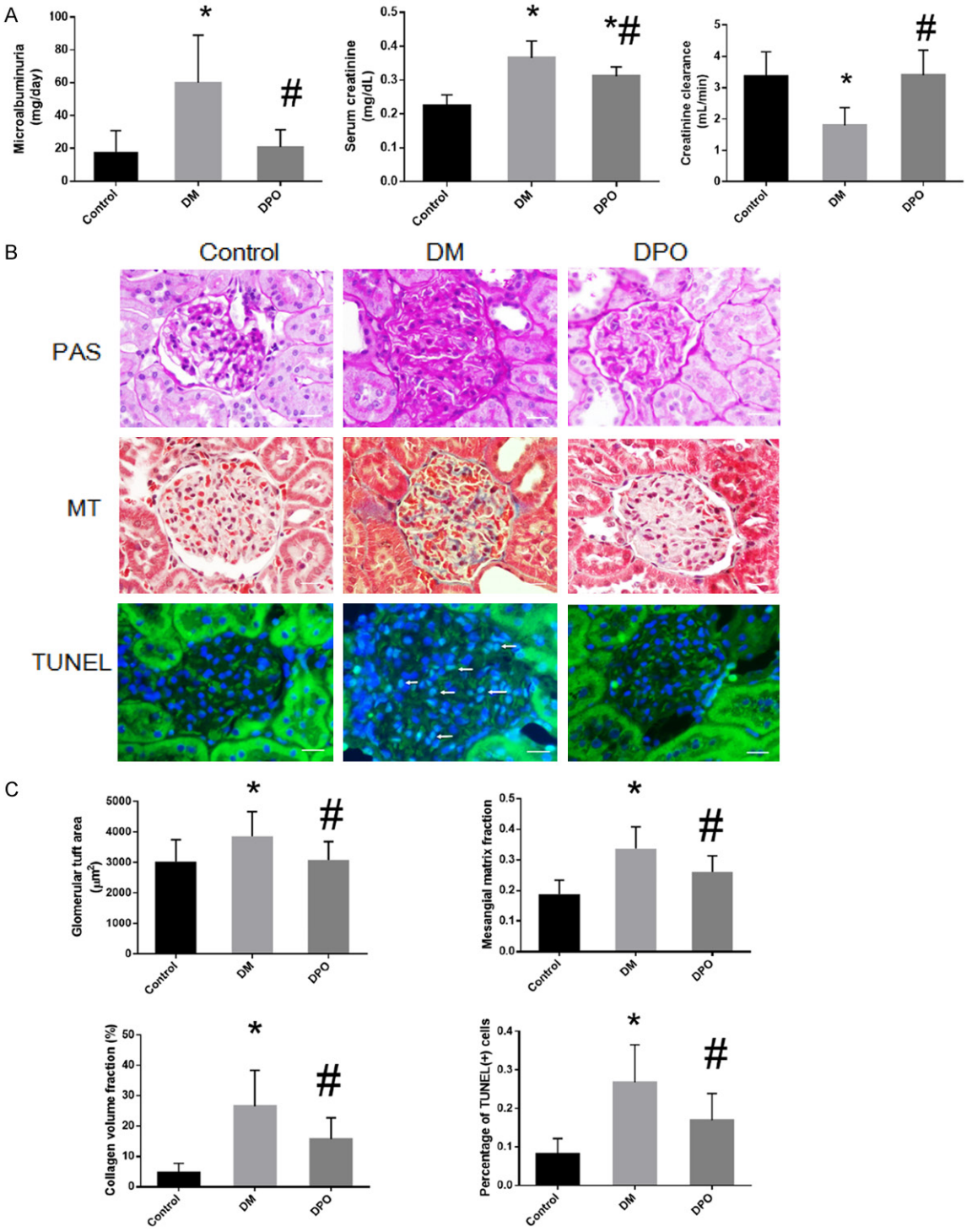

Figure 2. *The effects of DPO on renal function and renal structures of STZ rats*. Sprague-Dawley (SD) rats were administered with STZ to induce diabetes. They were treated with DPO (0.75 μg/kg/week) or vehicle (0.9% NaCl) for 10 weeks. A. 24-hour urinary microalbumin excretion, serum creatinine level, and creatinine clearance rate at week 12 were measured. B. Representative photomicrographs of the kidneys stained with periodic acid-Schiff (PAS), Masson's trichrome (MT) and terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling (TUNEL) (original magnification 400×). Scale bars: 40 μm. Arrows indicate apoptotic cell with condensed nuclear DNA. C. Statistical analysis of glomerular tuft area, glomerular mesangial expansion, glomerular fibrosis and glomerular TUNEL-positive cells percentages. Data are presented as mean ± SEM. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

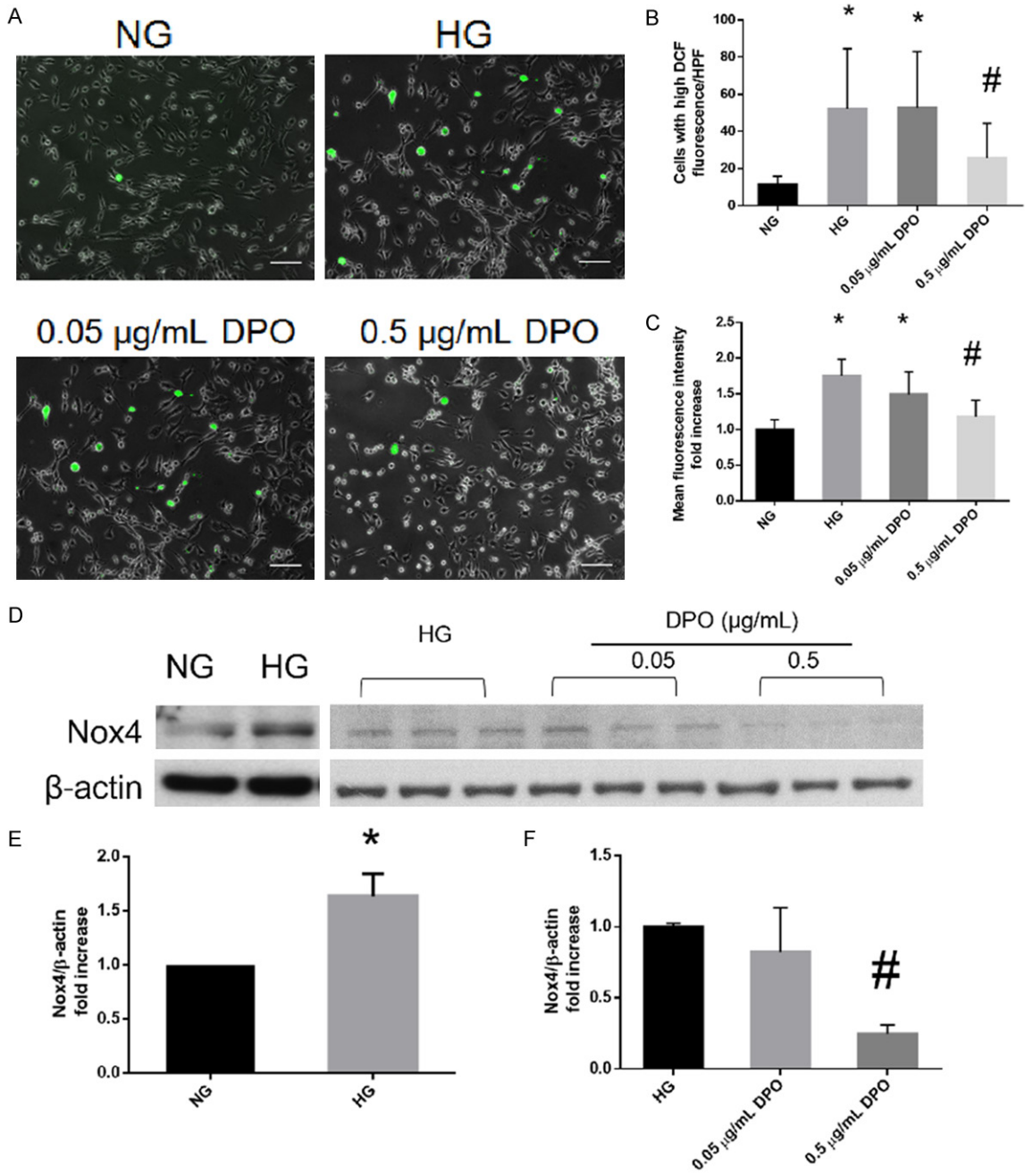

Figure 3. *The effects of DPO on ROS generation, NADPH oxidase 4 expressions in HG-treated MCs*. Intracellular ROS production. MCs were treated with normal glucose (NG) or high glucose (HG) for 24 h. After treatment of DPO at different concentrations (0.05 and 0.5 μg/mL DPO) for 24 h, cells were stained with ROS-sensitive dye DCF-DA for 30 min at 37°C, then observed under a fluorescence microscope (magnification 400×). Bars indicate 40 μm (A). The number of cells with intracellular ROS obtained in at least 10 fields (B). ROS generation was also detected by using a florescence-quantifying instrument and the data was normalized to NG group (C). Representative expression of NADPH 4 protein in the MCs treated with normal glucose (NG) or high glucose (HG) plus DPO at different concentrations (0.05 and 0.5 μg/mL DPO), assayed by Western blotting (D). Quantitative analysis of the expression of NADPH 4 protein, normalized with β-actin (E, F). Results represent mean ± SD. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

generation. HG increased intracellular ROS detected by DCF-DA probe in MCs; however, 0.5 μg/mL DPO significantly reduced the intracellular ROS ($P < 0.05$) (**Figure 3A**). The numbers

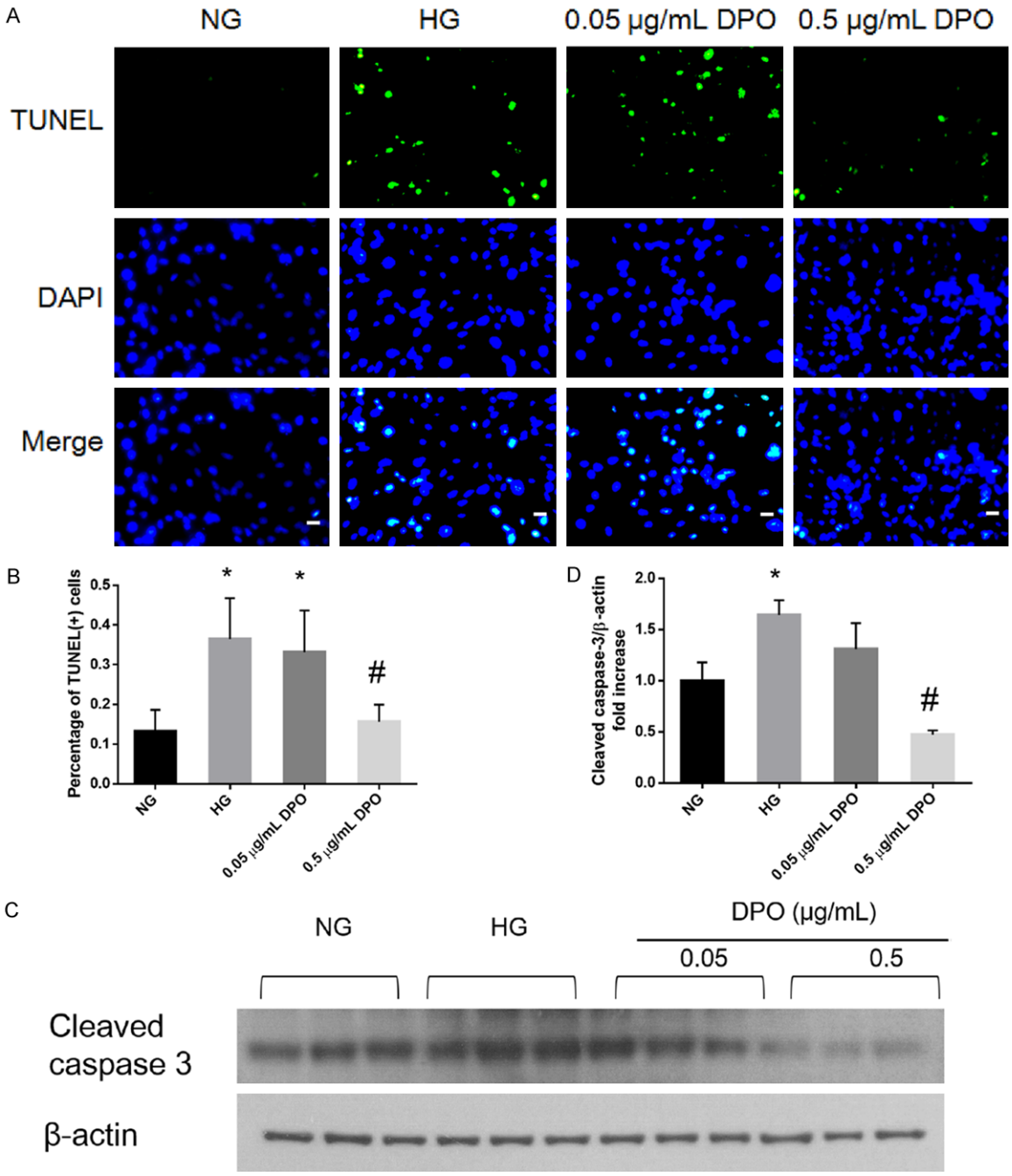

Figure 4. *The effects of DPO on apoptosis in HG-treated MCs*. Representative TUNEL staining (magnification ×400) in MCs treated with normal glucose (NG) or high glucose (HG) plus DPO at different concentrations (0.05 and 0.5 μg/mL DPO). Bars indicate 20 μm (A). Apoptosis was assessed by the ratio of TUNEL-positive cells to total cells (B). Representative expression of cleaved caspase 3 in MCs treated with normal glucose (NG) or high glucose (HG) plus DPO at different concentrations (0.05 and 0.5 μg/mL DPO), assayed by Western blotting (C). Quantitative analysis of the expression of cleaved caspase 3 protein was normalized with β-actin (D). Results represent mean ± SD. $^{*}P < 0.05$ compared to NG group, $^{\#}P < 0.05$ compared to HG group.

of cells with DCF-DA staining were 11.46 ± 4.41 cells/HPF, 52.14 ± 32.39 cells/HPF, and 25.6 ± 18.69 cells/HPF (**Figure 3B**). Similar findings were observed by using a fluorometric assay (**Figure 3C**). HG also up-regulated the protein expression of Nox4 by 63.7% (**Figure 3D**, **3E**),

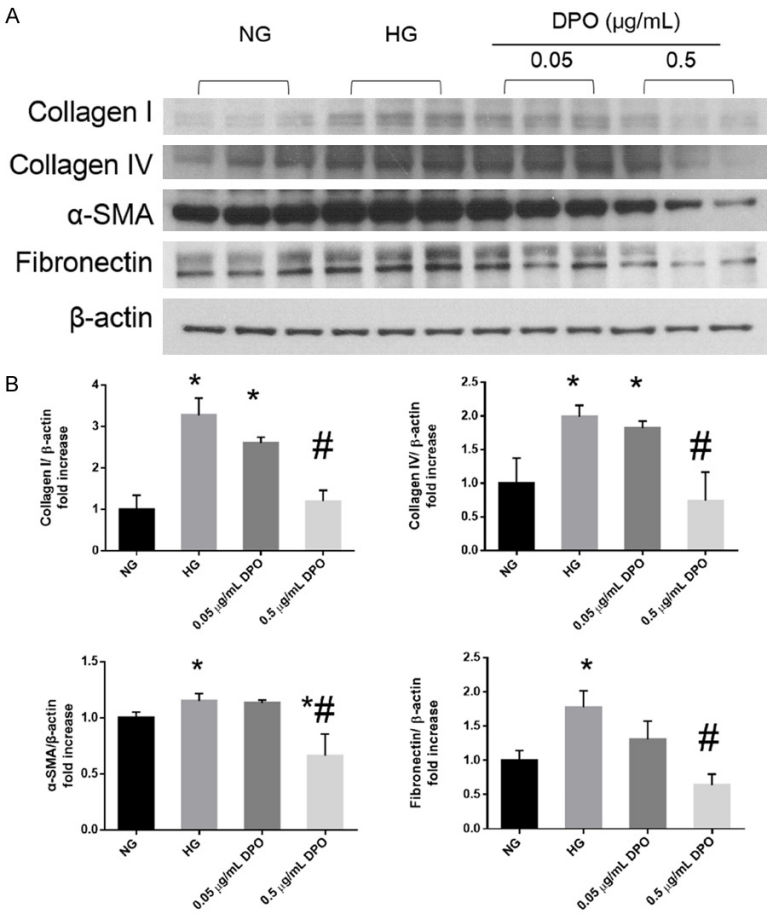

Figure 5. *The effect of DPO on the expression of α-SMA and ECM component in HG-treated MCs*. A. Representative expression of fibronectin, α-SMA, collagen I and IV protein in the MCs treated with normal glucose (NG) or high glucose (HG) plus DPO at different concentrations (0.05 and 0.5 μg/mL DPO), assayed by Western blotting. B. Quantitative analysis of the expression of fibronectin, α-SMA, collagen I and IV protein, normalized with β-actin. Results represent mean ± SD. $^{*}P < 0.05$ compared to NG group, $^{\#}P < 0.05$ compared to HG group.

assayed by Western blotting, however, DPO, at 0.5 μg/mL, remarkably reduced the expression by 75.2% in HG-treated MCs (**Figure 3D**, **3F**).

The effects of DPO on apoptosis in HG-treated MCs

The apoptosis in HG-treated MCs was also evaluated by TUNEL-staining and protein expression of caspase 3. A reduced TUNEL-positive cells was observed in HG-treated MCs by DPO treatment (0.5 μg/mL) (ratio of apoptotic cells to total cells, DM:DPO = 0.37 ± 0.10:0.16 ± 0.04, P < 0.05) (**Figure 4A**, **4B**), and DPO treatment (0.5 μg/mL) also reduced the protein expression of cleaved caspase 3 by 71.2% in HG-treated MCs (**Figure 4C**, **4D**).

The effect of DPO on the expression of α-SMA and ECM components in HG- treated MCs

The overexpression of α-SMA in MC is considered as a shift to a fibrogenic phenotype. Phenotype-transited MCs can promote an increased matrix turnover. Thus, the effects of DPO on the MC phenotype transition were investigated. The present research showed that the expression of α-SMA was up-regulated in MCs under HG conditions. While after treatment with 0.5 μg/mL DPO, protein expression of α-SMA was significantly decreased by 42.5% in HG-treated MCs. We also investigated that HG increased the protein expression of fibronectin, collagen I and IV by Western blotting, however, DPO treatment (0.5 μg/mL) caused 63.5%, 62.8%, and 63.9% reduction of collagen I and IV, fibronectin relative to HG counterparts (**Figure 5A**, **5B**).

DPO inhibits the expression of α-SMA and collagen IV in HG-treated MCs through Nox4/ERK1/2

We then evaluated the underlying mechanism of DPO's inhibitory effects on HG-induced overexpression of ECM proteins in MCs. Protein expression of Nox4 was up-regulated in HG-treated MCs when compared with the NG group as mentioned above (**Figure 3E**). The cells were transfected with Nox4 siRNA before transferred into HG. As showed in **Figure 6A**, transfection with Nox4 siRNA decreased Nox4 expression induced by HG in MCs. This was similar to the effects of DPO in HG-treated MCs. Meanwhile, a significant decline was observed in the phosphorylation of ERK1/2 and in the expression of α-SMA and collagen IV in MC cells after expo-

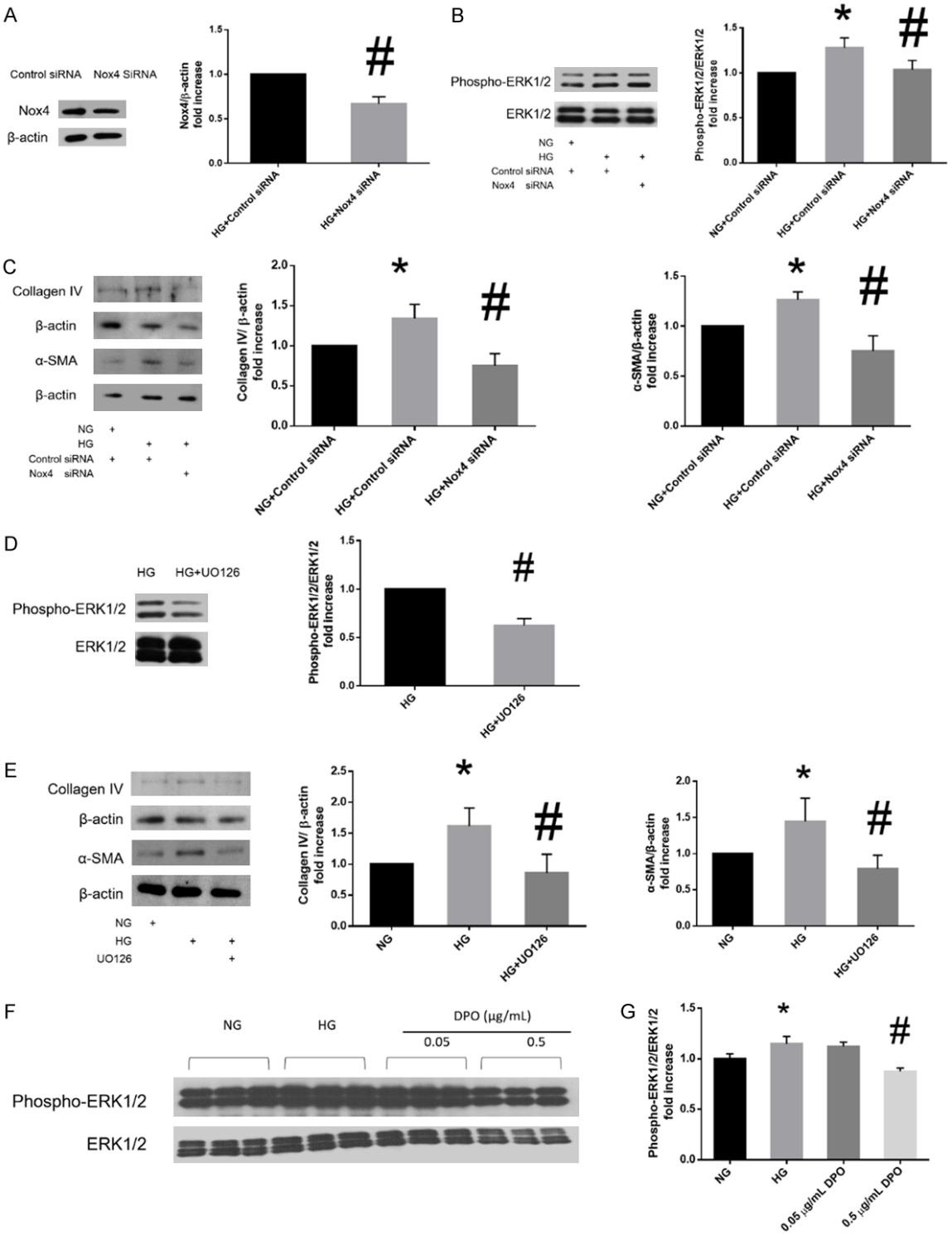

Figure 6. *HG up-regulated the expression of α-SMA and collagen IV in MCs through Nox4/ERK1/2 and DPO suppressed the activation of ERK1/2 in HG-treated MCs*. MCs serum-starved for 24 h were transiently transfected with Nox4 or control siRNA; 48 h later, the cells were incubation with normal glucose (NG) or high glucose (HG) for 48 h. After that, cells were collected for Western blot analysis. Representative Western blots and statistical analysis are shown in (A-C). Depletion Nox4 by Nox4 siRNA significantly reduced Nox4 protein expression compared with scrambled control in HG-treated MCs (A). HG markedly up-regulated the expression of phospho-ERK1/2, however, transfection with Nox4 siRNA suppressed HG-induced up-regulation of phospho-ERK1/2 (B). HG significantly up-regulated the expression of α-SMA and collagen IV, however, transfection of MCs with Nox4 siRNA suppressed

HG-induced up-regulation of α-SMA and collagen IV (C). Therefore, HG activated ERK1/2 and up-regulated the expression of α-SMA and collagen IV via NADPH. MCs were pretreated with U0126 (10 μM) for 30 min, followed by treatment with NG or HG for 48 h. MCs were then collected for Western blot analysis. Representative Western blots and statistical analysis are present in (D, E). Pre-treatment with U0126 significantly inhibited the expression of phospho-ERK1/2 in HG-treated MCs (D). HG significantly increased the expression of α-SMA and collagen IV, U0126 abated the increased expression of α-SMA and collagen IV induced by HG in MCs (E). Hence, HG upregulated the expression of α-SMA and collagen IV via ERK1/2. In general, HG up-regulated the expression of α-SMA and collagen IV in MCs through Nox4/ERK1/2. MCs were treated with NG or HG for 24 h. After treatment of DPO at different concentrations (0.05 and 0.5 μg/mL DPO) for another 24 h, cells were harvested for Western blot analysis. Representative expression of the ERK1/2 protein in the MCs treated with NG or HG plus DPO at different concentrations (0.05 and 0.5 μg/mL DPO), assayed by Western blotting (F). Quantitative analysis of the expression of the phospho-ERK1/2 protein (G). DPO inhibited the activation of ERK1/2 in HG-treated MCs. The expression of Nox4, α-SMA and collagen IV protein was normalized with β-actin. The expression of the phospho-ERK1/2 protein was normalized with total ERK1/2 protein. Results represent mean ± SD. $^{*}P < 0.05$ compared to NG or NG + Control siRNA group, $^{\#}P < 0.05$ compared to HG or HG + Control siRNA group.

sure to HG (**Figure 6B**, **6C**).Moreover, like siNOX4 treatment, pretreatment with U0126, an ERK1/2 inhibitor, could markedly suppress the expression of phospho-ERK1/2 and subsequently reduced the expression of α-SMA and collagen IV in HG-treated MCs (**Figure 6D**, **6E**). These data demonstrated that the NADPH pathway was upstream of ERK1/2 in regulating the expression of α-SMA and collagen IV in MCs under hyperglycemic conditions. Accordingly, DPO significantly inhibited HG-induced activation of ERK1/2 in MCs (**Figure 6F**, **6G**). Combined with those aforementioned inhibition effects on the expression of Nox4 (**Figure 3F**), α-SMA and collagen IV (**Figure 5**), DPO may reduce the expression of α-SMA and collagen IV in MCs through inhibiting Nox4/ERK1/2.

DPO inhibits ERK1/2 activation and suppresses the expression of Nox4, α-SMA and collagen IV in renal cortexes of STZ

To uncover the mechanism of DPO's impact on renal fibrosis in STZ rats, the effects of DPO on Nox4/ERK1/2 signaling pathway *In Vivo* were investigated. The expression of Nox4 was determined by Western blotting. The protein level of Nox4 was significantly increased 5.24 ± 1.53 fold in STZ rats compared with controls, but it was decreased 1.39 ± 0.35 fold by treatment with DPO ($P < 0.05$). Similarly, the expression of $p22^{phox}$ was also higher in STZ when compared with the control rats, while it was lower in STZ rats treated with versus without DPO (DM:DPO = 1.65 ± 0.16:0.93 ± 0.23 fold control, $P < 0.05$). The ratio of phospho-ERK1/2 to ERK1/2 protein expression was increased in HG-treated MCs compared with controls, as indicated by Western blot analysis. However, DPO treatment suppressed the ratio by 44.1%. DPO also reduced the protein expression of collagen IV and α-SMA by 30.4% and 48.7%, respectively, in STZ. These results demonstrated that the DPO may protect against diabetic renal fibrosis through Nox4/ERK1/2 signaling (**Figure 7**).

Discussion

In our research, Effects of DPO on STZ-induced nephropathy and HG-treated MCs were investigated. Long-term treatment with low-dose DPO ameliorated hyperglycemia and improved renal function without stimulating hematopoiesis. DPO reduced glomerular hypertrophy and mesangial expansion in STZ. DPO also attenuated apoptosis and fibrosis in the glomerular area of STZ. Furthermore, DPO significantly reduced ROS production and inhibited apoptosis and fibrosis in HG-cultured MCs.

It has been suggested that recombinant human (rh) EPO (150 U/kg) exerts renal-protective effects without stimulating hematopoiesis in STZ rats [10]. 150 U/kg rhEPO has been found to reverse the creatinine clearance rate, urinary albumin and serum creatinine in STZ rats, attenuate STZ-induced collagen accumulation in the glomerular area, exert antioxidant properties by inhibiting Nox4, and reduce apoptotic cell in diabetic rat kidneys [10]. DPO has a longer half-life than EPO [11]. The amount of DPO was pharmacologically equivalent to a dose of EPO divided by 200 [13]. Therefore, the effects of 0.75 μg/kg DPO in STZ rats were investigated in our research. We also observed higher levels of Ccr, lower levels of serum creatinine and urinary microalbumin in STZ rats treated with versus without DPO. DPO improved oxidative stress in STZ rat kidneys by inhibiting the expression of Nox4 and $p22^{phox}$. DPO inhib-

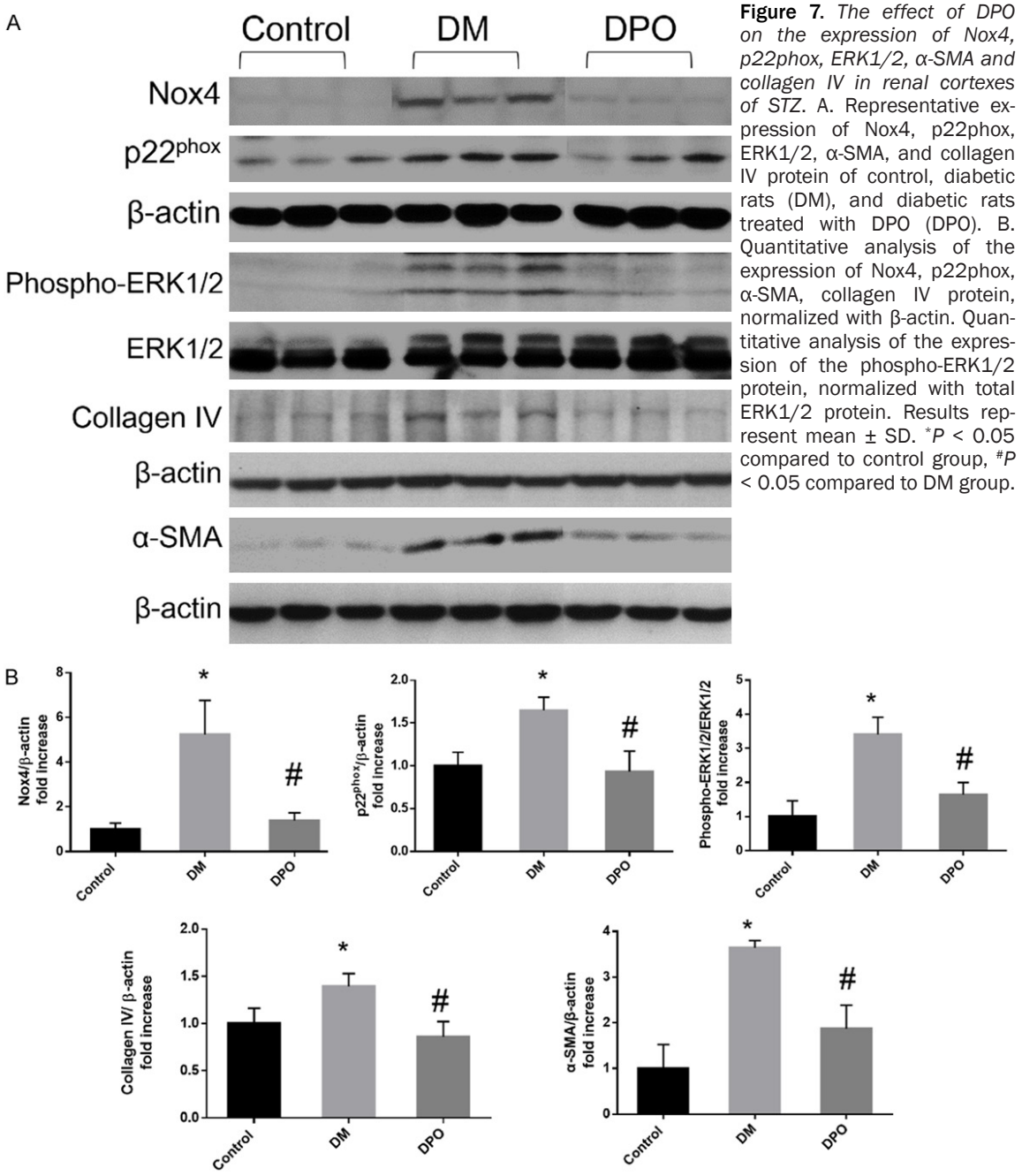

Figure 7. *The effect of DPO on the expression of Nox4, p22phox, ERK1/2, α-SMA and collagen IV in renal cortexes of STZ.* A. Representative expression of Nox4, p22phox, ERK1/2, α-SMA, and collagen IV protein of control, diabetic rats (DM), and diabetic rats treated with DPO (DPO). B. Quantitative analysis of the expression of Nox4, p22phox, α-SMA, collagen IV protein, normalized with β-actin. Quantitative analysis of the expression of the phospho-ERK1/2 protein, normalized with total ERK1/2 protein. Results represent mean ± SD. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

ited apoptosis and fibrosis in the glomerular area of STZ (**Figures 2**, S1 and S2), as rhEPO did in STZ rats.

RhEPO improves pancreatic β-cell damage and glucose metabolism in STZ rats [18], and epoetin beta ameliorates hyperglycemia in STZ rats [19]. However, some studies have not observed an improvement in glucose levels in STZ rats treated with rhEPO [10]. The reasons for these conflicting results might involve the dose of EPO, the treatment period, or others. 300 U/kg rhEPO (3 times/week for 4 weeks) was found to improve glucose metabolism in STZ (25 mg/kg) rat provided with a high fat diet [18], while 150 U/kg rhEPO (3 times/week for 4 weeks) did not in STZ (65 mg/kg) rat provided with standard rodent chow [10]. The present study showed that the glucose levels of STZ rats (65 mg/kg, and the rats were treated with standard chow) were decreased significantly by DPO (0.75 μg/kg, once a week for 10 weeks) treat-

ment. Therefore, we didn't know whether DPO exerted its renal-protection through its anti-oxidative properties or its indirect glucose-lowering effects in STZ rats, thus an *In Vitro* experiment was carried out in our research.

In the animal experiment, we found that Ccr was improved in DPO treated STZ rat. DPO reduced mesangial expansion and inhibited glomerular hypertrophy in STZ by histopathological analysis. Given that mesangial expansion leads to the loss of Ccr, and is present in DN patients before the onset of clinical manifestations [20, 21]. The mesangial expansion was inversely related to Ccr in type I diabetes mellitus [22]. Amelioration of mesangial expansion is one of the targets against DN [23]. Thus, DPO may maintain Ccr through amelioration of mesangial expansion in STZ. Our *In Vivo* experiment has confirmed that DPO protected against glomerular injury in STZ by anti-apoptosis and anti-fibrosis. In order to understand the cellular events that DPO mediates to inhibit mesangial expansion in DN. Therefore, we investigated the effects of DPO in HG-treated MCs. In the present study, we found that DPO also had anti-oxidative effects in HG-treated MCs by inhibiting Nox4 and reducing ROS production. Moreover, DPO attenuated HG-induced MC apoptosis by inhibiting caspase 3. We further found that DPO also inhibiting HG-induced MC fibrosis by inhibiting the expression of ECM proteins. The overexpression of α-SMA is a sign of the MC activation and trans-differentiation, indicating a myofibroblast phenotypic transition [24]. When in pathological conditions, MC may up-regulate the expression of α-SMA, transit into a myofibroblast phenotype and secrete extracellular matrix [25-27]. Our findings showed that HG can facilitate the transition of MC into myofibroblast phenotype by overexpression of α-SMA and promote the production of ECM by up-regulating the expression of fibronectin, collagen I and IV, which would contribute to mesangial expansion. However, DPO inhibited these changes. Beyond its lowering glucose effects in STZ rats *In Vivo*, DPO still protected against HG-induced MCs damage *In Vitro*.

Extracellular signal-regulated kinase (ERK) 1/2 is a crucial signaling molecule in the regulation of ECM protein synthesis [28]. It has been reported that ERK1/2 signaling is activated in the diabetic rat kidneys and in HG-treated MCs [29], which was also observed in our research. It has been suggested that NADPH regulates the ERK1/2 signaling pathway to mediate fibrosis [30, 31]. EPO attenuates TGF-β-induced myocardial fibrosis through the inhibitory effects on NADPH/ERK1/2 pathway [30]. Based on these investigations, we were prompted to examine the effects of DPO on NADPH/ERK1/2 pathway in renal cortex of STZ kidneys and HG-treated MCs. In the present study, DPO treatment significantly inhibited the expression of Nox4 and phosphorylation of ERK1/2 in HG-treated MCs and renal cortex of STZ kidney. Our *In Vitro* experiment further reveal that down-regulated Nox4 inhibited the phosphorylation of ERK1/2 and the expression of α-SMA and collagen IV, whereas, down-regulated phosphorylation of ERK1/2 suppressed the expression of α-SMA and collagen IV. These data indicate that NADPH regulates the ERK1/2 signaling pathway to mediate mesangial cell phenotypic transition and collagen production in HG-treated MCs. Moreover, it seems that DPO suppresses HG-induced ECM accumulation in MCs by inhibiting Nox4/ERK1/2, which would contribute to the amelioration of mesangial expansion in STZ kidney, and the improvement of renal function of STZ rats, eventually.

We did not employ osmotic control in *In Vitro* experiment; however, there seems little influence of osmotic stress on fibrosis, oxidation, and apoptosis in NG-treated MCs [32, 33]. But further research is needed to provide solid evidence of the effects of osmotic stress in MCs.

In conclusion, the present research showed that long-term low-dose DPO treatment ameliorated hyperglycemia and renal damage beyond hematopoiesis in STZ rats. DPO protected against diabetes-induced glomerular hypertrophy, mesangial expansion. DPO may attenuate diabetes-induced MC damage *In Vitro* and *In Vivo* by anti-apoptosis and anti-fibrosis. NADPH regulates the ERK1/2 signaling pathway to mediate phenotypic transition and collagen production in HG-treated MCs. DPO suppresses renal fibrosis in DN by inhibiting NADPH/ERK1/2. Low-dose DPO may have a therapeutic application in chronic DN.

Acknowledgements

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Disclosure of conflict of interest

None.

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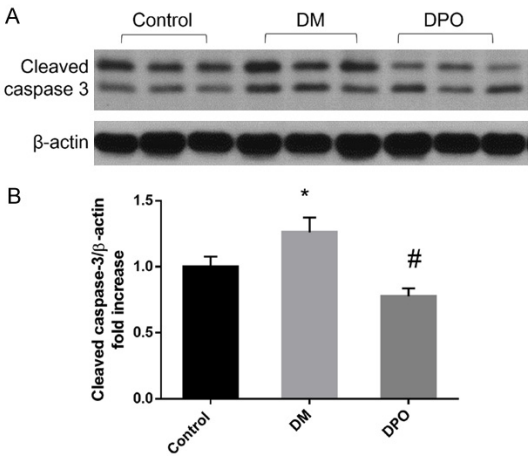

Figure S1. *The effect of DPO on the expression of cleaved caspase 3 in renal cortexes of STZ*. A. Representative expression of cleaved caspase 3 protein of control, diabetic rats (DM), and diabetic rats treated with DPO (DPO). B. Quantitative analysis of the expression of cleaved caspase 3 protein, normalized with β-actin. Expression of cleaved caspase 3 was higher in the DM group compared with the controls; however, the expression of cleaved caspase 3 was decreased by DPO treatment. Results represent mean ± SD. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

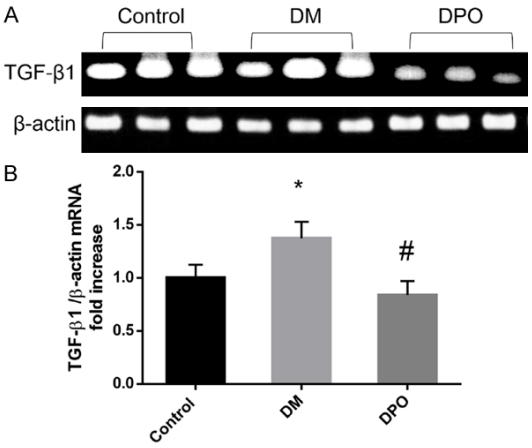

Figure S2. *TGF-β1 mRNA expression renal cortexes*. A. Representative expression of the mRNA for TGF-β1 in renal cortexes of control, diabetic rats (DM), and diabetic rats treated with DPO (DPO), assayed by RT-PCR. B. Quantitative analysis of expression of the mRNA for TGF-β1, normalized with β-actin. Total RNA was isolated from renal cortex using Tri-Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was then DNased. cDNA was synthesized from RNA (1 μg) using a commercial kit (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen Co., CA, USA). cDNA was amplified using Taq DNA polymerase (TaKaRa Ex Taq™, TAKARA, Kyoto, Japan). RT-PCR was performed as described previously [1]. The primers used in this study, including rat TGF-β1 and β-actin, were described previously [1, 2]. The ratio for TGF-β1 mRNA was normalized with β-actin. Expression of TGF-β1 mRNA was higher in the DM group compared with the controls; however, the expression was reduced by DPO treatment. Results represent mean ± SD. $^{*}P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to DM group.

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