Original Article

Over-production of nitric oxide by oxidative stress-induced activation of the TGF- β 1/PI3K/Akt pathway in mesangial cells cultured in high glucose

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Aim: To investigate whether NO over-production in rat mesangial cells cultured in high glucose (HG) is related to activation of the TGF- β 1/PI3K/Akt pathway.

Methods: Rat mesangial cells line (HBZY-1) was exposed to HG (24.44 mmol/L) or H₂O₂ (10 μ mol/L) for 16 h. NO release was quantified using the Griess assay. The TGF- β 1 level was measured using ELISA. The protein expression of p-Akt, t-Akt, Bim, and iNOS was examined by Western blotting. The mRNA levels of TGF- β 1 and Bim were measured using RT-PCR. The cell proliferation rate was estimated using a BrdU incorporation assay.

Results: Treatment of the cells with HG, H₂O₂, or TGF- β 1 (5 ng/mL) significantly increased the NO level that was substantially inhibited by co-treatment with the NADPH oxidase inhibitor diphenylene iodonium (DPI), TGF- β 1 inhibitor SB431542, or PI3K inhibitor LY294002. Both HG and H₂O₂ significantly increased the protein and mRNA levels of TGF- β 1 in the cells, and HG-induced increases of TGF- β 1 protein and mRNA were blocked by co-treatment with DPI. Furthermore, the treatment with HG or H₂O₂ significantly increased the expression of phosphorylated Akt and iNOS and cell proliferation rate, which was blocked by co-treatment with DPI, SB431542, or LY294002. Moreover, the treatment with HG or H₂O₂ significantly inhibited Bim protein and mRNA expression, which was reversed by co-treatment with DPI, SB431542, or LY294002.

Conclusion: The results demonstrate that high glucose causes oxidative stress and NO over-production in rat mesangial cells *in vitro* via decreasing Bim and increasing iNOS, which are at least partially mediated by the TGF- β 1/PI3K/Akt pathway.

Keywords: mesangial cells; high glucose; oxidative stress; NO; iNOS; Bim; TGF- β 1; PI3K/Akt pathway

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Introduction

Diabetic nephropathy (DN) is characterized by glomerular hypertrophy, excessive accumulation of extracellular matrix (ECM) and glomerulosclerosis, which ultimately lead to a progressive loss of renal function^[1]. Hyperglycemia is considered to be the first step in initiating DN, in which high glucose (HG) activates multiple intracellular signaling factors and causes target organ injury^[2]. Over-production of reactive oxygen species (ROS) and oxidative stress are the direct consequences of hyperglycemia^[3, 4], and excessive mitochondrial ROS serve as a common upstream mediator of diabetic complications^[5]. Our previous research demonstrated that extracts from herbal medicines could prevent DN progression by scavenging ROS^[6–8].

Nitric oxide (NO) plays an important role in DN progression. There have been reports suggesting that the physiological and morphological changes in DN are mediated by the NO level in the kidneys^[9–12]. However, there is still disagreement over the effect of hyperglycemia on NO production in renal cells^[13, 14]. Mesangial cells (MCs) provide structural support for capillary loops and modulate glomerular filtration, playing a crucial role in maintaining the structure and function of the renal glomerular tufts. Lipopolysaccharide increases the enzymatic activity and protein and mRNA levels of inducible nitric oxide synthase (iNOS) in MCs, suggesting that MCs are a potential source of NO production^[15, 16]. Reports from animal experiments show that increased renal NO production is one of the major pathogenic features of early DN^[17–19]. However, the molecular mechanism for the effects of hyperglycemia on renal NO production remains unclear.

Hyperglycemia can be mediated through the induction of various growth factors and cytokines. Data from other laboratories^[20–22] and our team^[6–8, 23–25] have demonstrated

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that transforming growth factor $\beta 1$ (TGF- $\beta 1$) is the core factor that contributes to DN pathogenesis. Most of the studies on the pathologic effects of TGF- $\beta 1$ on DN simply focus on its profibrotic effect, while other potential mechanisms remain unclear. Studies have shown that TGF- $\beta 1$ expression in MCs is increased under high glucose conditions^[20–22, 24, 25] and that TGF- $\beta 1$ participates in activation of the PI3K/Akt pathway^[26–29]. PI3K/Akt pathway activation by TGF- $\beta 1$ induces the phosphorylation and inactivation of FoxO3a and decreases expression of its downstream target genes, Bim and MnSOD^[30–36]. Consequently, both the cell proliferation rate and ROS production increase^[37]. Furthermore, Bim deficiency in Birt-Hogg-Dubé (–/–) cells can be attributed to reduced Bim transcription, which is associated with a loss of TGF- β -mediated transcription and chromatin modifications^[38]. Autocrine TGF- β protects breast cancer cells from apoptosis through Bim reduction^[39]. Additionally, iNOS is a major enzyme for NO over-production after stress or under pathological conditions, but whether increased TGF- $\beta 1$ expression can promote iNOS protein expression is not well understood. Therefore, investigating the relationships among high glucose-induced TGF- $\beta 1$, NO production and the downstream targets of the PI3K/Akt pathway in rat MCs under high glucose conditions is necessary.

Based on the above findings, we investigated whether NO over-production in rat mesangial cells cultured in HG is related to activation of the TGF- $\beta 1$ /PI3K/Akt pathway.

Materials and methods

Mesangial cell culture and treatment

A rat mesangial cell line (HBZT-1) was purchased from the China Center for Type Culture Collection (Wuhan University, Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C. The cells were cultured under normal glucose conditions (NS, 5.56 mmol/L) for 24 h and treated with mannitol (MA, 24.44 mmol/L), high glucose (HG, 24.44 mmol/L), high glucose+dimethyl sulfoxide (HG+DMSO, with 20 μ mol/L DMSO), high glucose+the TGF- $\beta 1$ inhibitor SB43154 (HG+SB431542, with 10 μ mol/L SB431542), high glucose+the PI3K inhibitor LY294002 (HG+LY294002, with 20 μ mol/L LY294002), high glucose+the NADPH oxidase inhibitor diphenylene iodonium (HG+DPI, with 10 μ mol/L DPI), hydrogen peroxide (H₂O₂, 10 μ mol/L), hydrogen peroxide+the TGF- $\beta 1$ inhibitor SB431542 (H₂O₂+SB431542, with 10 μ mol/L SB431542), H₂O₂+the PI3K inhibitor LY294002 (H₂O₂+LY294002, with 20 μ mol/L LY294002), or TGF- $\beta 1$ (5 ng/mL). All of the experiments were performed with cells between passages 5 and 8. After treatment with the different agents for 16 h, the cells were harvested for analysis.

Spectrophotometric determination of total nitric oxide level

The MCs were treated with different inhibitors and then cultured for 16 h before supernatant collection for the NO assay. The total NO level was estimated using the Griess method, which determined the levels of nitrate and nitrite to reflect the

total NO level. The Griess kit was purchased from the Beyotime Institute of Biotechnology (Haimen, China), and the measurements were performed according to the manufacturer's instructions.

ELISA assay for TGF- $\beta 1$ measurement

The MCs were seeded in 12-well plates at approximately 10⁵ cell/well with DMEM medium containing 10% FBS. After 24 h, the medium was replaced with serum-free DMEM containing penicillin/streptomycin. The conditioned medium was collected after 16 h of treatment and centrifuged at 10000×g for 15 min. The resulting supernatant was subjected to TGF- $\beta 1$ measurement using a rat enzyme-linked immunosorbent assay (ELISA) kit (Boster Bio-engineering Co, Ltd, Wuhan, China), according to the manufacturer's instructions. The colorimetric reaction was measured at 450 nm. Each experiment was repeated in triplicate.

Western blotting analysis for Akt, iNOS and Bim protein expression

The cell samples were separated by SDS-PAGE under reducing conditions and then transferred onto nitrocellulose membranes. The membranes were blocked with Western blocking buffer and then incubated with different antibodies, including rabbit polyclonal antibodies against t-Akt and p-Akt (Beyotime Institute of Biotechnology, Haimen, China, both 1:1000 dilution), rabbit monoclonal antibody against Bim (Cell Signaling Technology, Beverly, MA, USA, 1:1000 dilution), rabbit polyclonal antibodies against iNOS and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA, both 1:500 dilution), and goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000 dilution). Detection was performed by using Image J version 1.34s software (Wayne Rasband National Institutes of Health, Detroit, MI, USA) and normalized to β -actin expression. The statistical data for Bim protein expression were obtained by determining the average of band optical density of three different molecular masses.

Analysis of TGF- $\beta 1$ and Bim by RT-PCR

The MCs were cultured for 16 h in the normal and experimental groups. Total RNA was extracted using TRIzol reagent (Tiangen Biotech, Co, Ltd, Beijing, China) according to the manufacturer's instructions and was then subjected to reverse transcription using a Quant One Step RT-PCR kit (Applied Biosystems, Carlsbad, CA, USA). Complementary DNA (cDNA) was amplified using the appropriate primers. The primer sequences used were as follows: TGF- $\beta 1$, 5'-CCCGCATCCCAGGACCTCTCT-3' (forward) and 5'-CGGGGACTGGCGAGCCTTAG-3' (reverse); Bim, 5'-GCCAAGCAACCTTCTGATGTA-3' (forward) and 5'-CAGTGCCTTCTCCAGACCAG-3' (reverse); and β -actin, 5'-GCTGCGTGTGGCCCTGAG-3' (forward) and 5'-ACGCAGGATGGCATGAGGGA-3' (reverse). The resulting products were separated by 1.5% agarose electrophoresis, and the band densities were analyzed by using Image J version 1.34s software (Wayne Rasband National Institutes of Health, Detroit, MI, USA). The

data for Bim mRNA expression were obtained by determining the average band optical density of three different molecular masses.

Cell proliferation determination using a BrdU incorporation assay

Cells (10^4 /well) were seeded in Falcon 96-well plates for 24 h and then exposed to different agents. After incubation for 16 h, a cell proliferation assay was performed using a BrdU Cell Proliferation Assay kit (Roche, Berlin, Germany). The optical density was measured at 370 nm using an automatic microplate reader (ClinBio 128Ce, Asys Hitech GmbH, Austria).

Statistical analysis

All of the data in the different experimental groups were expressed as the mean $\pm$ SD. Differences between the groups were assessed using one-way ANOVA and the *t*-test. $P<0.05$ was considered to be statistically significant.

Results

Effects of osmotic pressure and solvent

Mannitol was used to mimic the osmotic pressure produced under high glucose conditions, and DMSO served as the solvent. We examined the effects of mannitol and DMSO on NO release from rat MCs; mRNA expression of TGF- β 1 and Bim; the protein expression of Akt, Bim, and iNOS; as well as cell proliferation. The results in the mannitol group were almost identical to the NS group, indicating that the osmotic pressure induced by high glucose did not affect the experimental data. The results in the HG+DMSO group were similar to the HG group, demonstrating that the presence of DMSO had no effect on the experimental data. Therefore, neither the osmotic pressure induced by high glucose nor the presence of DMSO significantly affected the experimental results.

NO level in rat mesangial cells

The amount of NO released from rat MCs was examined by visible spectrophotometry. Compared to the NS group, NO release from the rat MCs significantly increased ($P<0.01$) in the HG, HG+DMSO, and H_2O_2 groups and was decreased in the HG+DPI group ($P<0.01$) (Figure 1). This result indicates that excessive ROS generation can stimulate NO over-production in MCs cultured in high glucose. NO release from the rat MCs also increased ($P<0.01$) in the TGF- β 1 group compared to the NS group. Compared to the HG group, NO release was decreased ($P<0.01$) in the HG+SB431542 and HG+LY294002 groups. In conclusion, the TGF- β 1/PI3K pathway may participate in NO over-production in MCs with high glucose.

TGF- β 1 protein and mRNA expression in rat mesangial cells

Compared to the NS group, TGF- β 1 expression in the rat MCs was significantly increased ($P<0.05$) in the HG group, but decreased ($P<0.05$) in the HG+DPI group. Moreover, TGF- β 1 expression was also markedly elevated ($P<0.01$) in the rat MCs treated with H_2O_2 (Figure 2A). These results indicate that excessive ROS generation mediates increased TGF- β 1 expres-

Figure 1. Intracellular NO production in the rat mesangial cells treated with different agents for 16 h. NS, MA, HG, HG+DMSO, HG+DPI, H_2O_2 , TGF- β 1, HG+SB431542, and HG+LY294002 represent the normal glucose (5.56 mmol/L glucose), osmotic pressure (24.44 mmol/L mannitol), high glucose (24.44 mmol/L glucose), HG+solvent (24.44 mmol/L glucose and 20 μ mol/L DMSO), HG+NADPH oxidase inhibitor (24.44 mmol/L glucose and 10 μ mol/L DPI), hydrogen peroxide (10 μ mol/L H_2O_2), transforming growth factor β 1 (5 ng/mL), HG+TGF- β 1 inhibitor (24.44 mmol/L glucose and 10 μ mol/L SB431542) and HG+PI3K inhibitor (24.44 mmol/L glucose and 20 μ mol/L LY294002) conditions, respectively. The data in the different experimental groups are expressed as the mean $\pm$ SD. $n=3$. $^cP<0.01$, compared to the NS group. $^fP<0.01$, compared to the HG group.

sion in MCs with high glucose.

To further evaluate the role of TGF- β 1 in NO over-production in MCs under high glucose conditions, we examined the changes in TGF- β 1 mRNA expression (Figure 2B). The results showed that TGF- β 1 mRNA expression in the rat MCs was significantly increased in the HG group ($P<0.05$) but decreased in the HG+DPI group ($P<0.01$). Similarly, TGF- β 1 mRNA expression was markedly elevated ($P<0.01$) in the rat MCs treated with H_2O_2 . These results indicate that excessive ROS generation promotes TGF- β 1 mRNA expression in the MCs under high glucose conditions.

Akt phosphorylation in rat mesangial cells

Both high glucose and H_2O_2 significantly increased ($P<0.01$) the phosphorylated Akt (p-Akt) level in rat MCs, which was reduced ($P<0.01$) by treatment with the TGF- β 1 inhibitor SB431542 or the PI3K inhibitor LY294002 (Figure 3A). This finding indicates that high glucose activates the TGF- β 1/PI3K/Akt pathway in rat MCs. Furthermore, the level of p-Akt in the rat MCs decreased after adding DPI ($P<0.01$). Therefore, these findings demonstrate that excessive ROS generation leads to activation of the TGF- β 1/PI3K/Akt pathway.

iNOS protein expression in rat mesangial cells

Compared to the NS group, iNOS protein expression was significantly increased in the HG group ($P<0.01$) but decreased ($P<0.01$) in the HG+SB431542, HG+LY294002, and HG+DPI groups (Figure 3B). Moreover, iNOS protein expression was markedly elevated ($P<0.01$) in the rat MCs treated with H_2O_2 but reduced ($P<0.01$) in the H_2O_2 +SB431542 and HG+LY294002 groups. These results indicate that excessive ROS generation increases iNOS expression in MCs under high glucose condi-

Figure 2. TGF-β1 protein (A) and mRNA (B) expression in the rat mesangial cells treated with different agents for 16 h. NS, MA, HG, HG+DMSO, HG+DPI, and H₂O₂ represent the normal glucose (5.56 mmol/L glucose), osmotic pressure (24.44 mmol/L mannitol), high glucose (24.44 mmol/L glucose), HG+solvent (24.44 mmol/L glucose and 20 μmol/L DMSO), HG+NADPH oxidase inhibitor (24.44 mmol/L glucose and 10 μmol/L DPI) and hydrogen peroxide (10 μmol/L H₂O₂) conditions, respectively. The data in the different experimental groups are expressed as the mean±SD. *n*=3. ^b*P*<0.05, ^c*P*<0.01, compared to the NS group. ^e*P*<0.05, ^f*P*<0.01, compared to the HG group.

tions, which may be mediated by the TGF-β1/PI3K/Akt pathway.

Bim protein and mRNA expression in rat mesangial cells

Compared to the NS group, Bim protein expression in the rat MCs was significantly decreased (*P*<0.01) in the HG group but increased (*P*<0.01) in the HG+SB431542, HG+LY294002, and HG+DPI groups (Figure 4A). Moreover, Bim protein expression was remarkably (*P*<0.01) decreased in the rat MCs treated with H₂O₂ but increased (*P*<0.01) in the H₂O₂+SB431542 and H₂O₂+LY294002 groups. These results indicate that excessive ROS generation decreases Bim expression in MCs under high glucose conditions, which may be mediated by the TGF-β1/PI3K/Akt pathway. The same effect can be seen for Bim mRNA expression (Figure 4B).

Cell proliferation in the rat mesangial cells

Compared to the NS group, the cell proliferation rate was significantly increased (*P*<0.01) in the HG group but decreased (*P*<0.01) in the HG+SB431542, HG+LY294002, and HG+DPI groups (Figure 5). Moreover, increased cell proliferation rate was observed (*P*<0.05) in rat MCs treated with H₂O₂, but the cell proliferation rate was lower (*P*<0.05) in the rat MCs

Figure 3. P-Akt (A) and iNOS (B) protein levels in rat mesangial cells treated with different agents for 16 h. NS, MA, HG, HG+DMSO, HG+SB431542, HG+LY294002, HG+DPI, H₂O₂, H₂O₂+SB431542, and H₂O₂+LY294002 represent the normal glucose (5.56 mmol/L glucose), osmotic pressure (24.44 mmol/L mannitol), high glucose (24.44 mmol/L glucose), HG+solvent (24.44 mmol/L glucose and 20 μmol/L DMSO), HG+TGF-β1 inhibitor (24.44 mmol/L glucose and 10 μmol/L SB431542), HG+PI3K inhibitor (24.44 mmol/L glucose and 20 μmol/L LY294002), HG+NADPH oxidase inhibitor (24.44 mmol/L glucose and 10 μmol/L DPI), hydrogen peroxide (10 μmol/L H₂O₂), H₂O₂+TGF-β1 inhibitor (10 μmol/L H₂O₂ and 10 μmol/L SB431542), and H₂O₂+PI3K inhibitor (10 μmol/L H₂O₂ and 20 μmol/L LY294002) conditions, respectively. The data in the different experimental groups are expressed as the mean±SD. *n*=3. ^b*P*<0.05, ^c*P*<0.01, compared to the NS group. ^f*P*<0.01, compared to the HG group. ^h*P*<0.05, ⁱ*P*<0.01, compared to the H₂O₂ group.

treated with H₂O₂+SB431542 or H₂O₂+LY294002. These findings indicate that inhibiting the TGF-β1/PI3K/Akt pathway can suppress the proliferation of mesangial cells caused by hyperglycemia.

Figure 4. Bim protein (A) and mRNA (B) expression in the rat mesangial cells treated with different agents for 16 h. NS, MA, HG, HG+DMSO, HG+SB431542, HG+LY294002, HG+DPI, H₂O₂, H₂O₂+SB431542, and H₂O₂+LY294002 represent the normal glucose (5.56 mmol/L glucose), osmotic pressure (24.44 mmol/L mannitol), high glucose (24.44 mmol/L glucose), HG+solvent (24.44 mmol/L glucose and 20 μmol/L DMSO), HG+TGF-β1 inhibitor (24.44 mmol/L glucose and 10 μmol/L SB431542), HG+PI3K inhibitor (24.44 mmol/L glucose and 20 μmol/L LY294002), HG+NADPH oxidase inhibitor (24.44 mmol/L glucose and 10 μmol/L DPI), hydrogen peroxide (10 μmol/L H₂O₂), H₂O₂+TGF-β1 inhibitor (10 μmol/L H₂O₂ and 10 μmol/L SB431542) and H₂O₂+PI3K inhibitor (10 μmol/L H₂O₂ and 20 μmol/L LY294002) conditions, respectively. The data in the different experimental groups are expressed as the mean±SD. *n*=3. ^b*P*<0.05, ^c*P*<0.01, compared to the NS group. ^e*P*<0.05, ^f*P*<0.01, compared to the HG group. ⁱ*P*<0.01, compared to the H₂O₂ group.

Discussion

DN is characterized by the excessive accumulation of ECM with thickened glomerular and tubular basement membranes

Figure 5. The viability of rat mesangial cells treated with different agents for 16 h. NS, MA, HG, HG+DMSO, HG+SB431542, HG+LY294002, HG+DPI, H₂O₂, H₂O₂+SB431542, and H₂O₂+LY294002 represent the normal glucose (5.56 mmol/L glucose), osmotic pressure (24.44 mmol/L mannitol), high glucose (24.44 mmol/L glucose), HG+solvent (24.44 mmol/L glucose and 20 μmol/L DMSO), HG+TGF-β1 inhibitor (24.44 mmol/L glucose and 10 μmol/L SB431542), HG+PI3K inhibitor (24.44 mmol/L glucose and 20 μmol/L LY294002), HG+NADPH oxidase inhibitor (24.44 mmol/L glucose and 10 μmol/L DPI), hydrogen peroxide (10 μmol/L H₂O₂), H₂O₂+TGF-β1 inhibitor (10 μmol/L H₂O₂ and 10 μmol/L SB431542) and H₂O₂+PI3K inhibitor (10 μmol/L H₂O₂ and 20 μmol/L LY294002) conditions, respectively. The data in the different experimental groups are expressed as the mean±SD. *n*=3. ^c*P*<0.01, compared to the NS group. ^e*P*<0.05, ^f*P*<0.01, compared to the HG group. ^h*P*<0.05, compared to the H₂O₂ group.

and increased mesangial area^[1], and NO may play an important role in these changes^[13, 14]. However, the molecular mechanism for the effects of hyperglycemia on NO over-production in the kidneys remains unclear. In this study, we found that oxidative stress in response to hyperglycemia might promote NO over-production in rat MCs due to decreased Bim protein and mRNA expression and increased iNOS protein expression, and the iNOS over-production is at least partially a result of the TGF-β1-induced activation of the PI3K/Akt pathway.

The change in NO production is an important biochemical feature in DN. Several reports have shown that the NO level is significantly increased in the early stages of kidney disease in diabetic rats^[9, 17-19] and rat MCs under high glucose conditions^[10]. However, the molecular mechanism involved is not well understood. In the current study, NO release was significantly increased in the rat MCs treated with high glucose or H₂O₂ for 16 h and was then reduced by treatment with DPI (a NADPH oxidase inhibitor). These results indicate that excessive ROS generation stimulates NO over-production in MCs under high glucose conditions. Moreover, the NO level was substantially elevated in the MCs treated with TGF-β1, but was then reduced by SB431542 (a TGF-β1 receptor kinase inhibitor) or LY294002 (a PI3K inhibitor). The results suggest that the TGF-β1/PI3K/Akt pathway may mediate the effect of high glucose on NO release from MCs.

TGF-β1, a very important growth factor in DN pathogenesis, has been reported to be up-regulated in the kidney under diabetic conditions using *in vitro*^[20-23, 25] and *in vivo*^[6-8, 24] studies. In the present study, both high glucose and H₂O₂ led to

increased TGF- β 1 protein and mRNA expression in rat MCs, and these were then reduced by treatment with DPI, suggesting that ROS mediated high glucose-induced TGF- β 1 production. Our previous reports have demonstrated that TGF- β 1 protein and mRNA expression were increased in the renal cortices of diabetic rats^[6–8]. These findings indicate that TGF- β 1 plays an important role during hyperglycemia.

To investigate the mechanism associated with TGF- β 1-induced NO production in MCs, we examined the expression of iNOS, a major enzyme for NO biosynthesis after stress. High glucose dramatically increased iNOS protein expression in MCs, which was then reduced by treatment with SB431542 or LY294002. This result suggests that the TGF- β 1/PI3K pathway participates in iNOS generation in MCs under high glucose conditions. TGF- β 1 is required for NF- κ B-mediated modulation of iNOS activity when controlling the induction of the Epstein-Barr virus replication cycle^[40]. A report showed that TGF- β 1 stimulates NO production in astrocytes by recruiting distinct cell subpopulations^[41]. Therefore, the iNOS expression induced by the TGF- β 1/PI3K/Akt pathway may be a main reason for NO over-production in MCs cultured in high glucose (Figure 6). Our study is the first report on the mechanism of induced iNOS expression in MCs under high glucose conditions.

Figure 6. Possible mechanism for high glucose-induced nitric oxide overproduction in rat mesangial cells.

High glucose can stimulate the over-proliferation of MCs and ECM secretion in MCs, which can be reduced by hydrogen sulfide via decreased ROS and TGF- β 1 levels^[42]. In the current study, the rat MCs under high glucose conditions showed NO over-production, increased TGF- β 1 protein and mRNA expression, and increased cell proliferation (one of the most important pathological manifestations of diabetic kidney disease). Treatment with SB431542, LY294002, or DPI inhibited the mesangial cell proliferation caused by high glucose. These results indicate that excessive ROS generation causes MC proliferation under a high glucose condition, which may

be mediated by the TGF- β 1/PI3K/Akt pathway.

Reduced expression of the pro-apoptotic protein Bim can result in cell proliferation^[37]. The lack of Bim expression had a different impact on ECM production in renal endothelial and epithelial cell migration, adhesion, eNOS activity and NO bioavailability^[43]. In the current study, Bim protein expression was remarkably reduced in the MCs cultured in high glucose and was associated with increased cell proliferation and NO, which were both significantly inhibited by treatment with SB431542, LY294002, or DPI. Moreover, both high glucose and H₂O₂ showed similar effects on MCs. These results demonstrate that TGF- β 1/PI3K/Akt pathway activation by ROS contributes to reduced Bim protein expression in the MCs cultured in high glucose. We also found that the change in Bim mRNA expression in the rat MCs was consistent with Bim protein expression. Reports have indicated that the nuclear factor FoxO3a is controlled by the activation of the TGF- β 1/PI3K/Akt pathway, and Bim and MnSOD, which are associated with oxidative stress, represent two of the important downstream target genes of FoxO3a^[30–36]. Therefore, FoxO3a may regulate Bim mRNA in MCs cultured in high glucose. Moreover, our previous study showed that FoxO3a participate in the down-regulation of MnSOD at the protein and mRNA levels in MCs cultured in high glucose via activation of the TGF- β 1/PI3K/Akt pathway^[44]. Therefore, reduced Bim expression is one of the important factors for rat MC proliferation and subsequent NO over-production in diabetic kidneys (Figure 6). Additionally, a report has indicated that exposure of MCs for up to 24 h can increase the cell number; however, after that time, the cell number remained unchanged, and the cells underwent hypertrophy, reflecting the observations found in experimental and clinical studies *in vivo*^[45]. Therefore, the pathways discussed in the present study could be targeted during the early stage of DN.

In conclusion, TGF- β 1 up-regulation by oxidative stress in MCs under high glucose conditions leads to NO over-production, mediated by increased iNOS protein expression and decreased Bim expression, which are at least partially due to activation of the PI3K/Akt/FoxO3a pathway.

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Author contribution

Xiao-xing YIN designed the study and revised the paper; Yun-peng ZHAI performed most of the experiments; Qian LU performed some of the experiments and wrote this paper; Yao-wu LIU analyzed part of the data and made the figures; Qian CHENG and Cheng-lin LI performed some of the experiments; Ya-Qin WEI and Fang ZHANG purchased the reagents.

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