Astragaloside IV prevents damage to human mesangial cells through the inhibition of the NADPH oxidase/ROS/Akt/NF-κB pathway under high glucose conditions

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Abstract. Glomerular hypertrophy and hyperfiltration are the two major pathological characteristics of the early stages of diabetic nephropathy (DN), which are respectively related to mesangial cell (MC) proliferation and a decrease in calcium influx conducted by canonical transient receptor potential cation channel 6 (TRPC6). The marked increase in the production of reactive oxygen species (ROS) induced by hyperglycemia is the main sponsor of multiple pathological pathways in DN. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important source of ROS production in MCs. Astragaloside IV (AS-IV) is an active ingredient of Radix Astragali which has a potent antioxidative effect. In this study, we aimed to investigate whether high glucose (HG)-induced NADPH oxidase activation and ROS production contribute to MC proliferation and the downregulation of TRPC6 expression; we also wished to determine the effects of AS-IV on MCs under HG conditions. Using a human glomerular mesangial cell line, we found that treatment with AS-IV for 48 h markedly attenuated HG-induced proliferation and the hypertrophy of MCs in a dose-dependent manner. The intracellular ROS level was also markedly reduced following treatment with AS-IV. In addition, the enhanced activity of NADPH oxidase and the expression level of NADPH oxidase 4 (Nox4) protein were decreased. Treatment with AS-IV also inhibited the phosphorylation level of Akt and IκBα in the MCs. In addition, TRPC6 protein expression and the intracellular free calcium concentration were also markedly reduced following treatment with AS-IV under HG conditions. These results suggest that AS-IV inhibits HG-induced mesangial cell proliferation and glomerular contractile dysfunction through the NADPH oxidase/ROS/Akt/nuclear factor-κB (NF-κB) pathway, providing a new perspective for the clinical treatment of DN.

Introduction

Diabetic nephropathy (DN) is one of the main chronic complications of diabetes mellitus (DM) associated with capillaries, often leading to chronic renal failure and end-stage renal disease (1). Although the precise pathogenesis of DN has not been elucidated, a number of studies have demonstrated that glomerular hyperfiltration and mesangial expansion are the two characteristic pathophysiological changes occurring during the early stages of DN (2,3).

Mesangial cells (MCs), located within glomerular capillary loops, play an important role in the regulation of glomerular hemodynamics due to their contractile function (4). There is increasing evidence indicating that hyperglycemia directly stimulates MCs, which subsequently results in mesangial contractile dysfunction and glomerular hyperfiltration (5,6). The hypocontractility of MCs is closely related to the reduced calcium influx (7,8).

Canonical transient receptor potential cation channels (TRPCs), as members of the transient receptor potential (TRP) superfamily, are Ca^{2+} permeable cation channels widely expressed in a series of tissues and cells (9,10). The TRPC family includes seven related members, designated as TRPC1-7 (11). Among these, TRPC6 is closely associated with kidney disease (12,13). Möller et al (14) found that TRPC6 exists throughout the glomerulus in kidney tissues, particularly in MCs. Additionally, it has been dcemonstrated that hyperglycemia downregulates the expression of TRPC6 protein, which results in a decrease in intracellular calcium, leading to impaired MC contractile response and glomerular hyperfiltration (15). However, the underlying molecular mechanisms of TRPC6 protein downregulation in MCs have not yet been fully elucidated.

Hyperglycemia can also cause the abnormal proliferation of MCs in DN, leading to glomerular hypertrophy and fibrosis (16). Clinical and animal experiments have demonstrated an increase

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in the production of reactive oxygen species (ROS) in MCs, as the direct result of chronic exposure to high glucose (HG) (17,18). The overproduction of ROS can transduce and amplify glucose signaling, playing a key role in MC proliferation (19). ROS can also modulate Ca^{2+} channels by activating various signaling cascades (20).

In the kidneys, particularly in MCs, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the predominant source of ROS (21). The phagocyte NADPH oxidase consists of two membrane-associated subunits, $p22^{phox}$ and $gp91^{phox}$, and at least four cytoplasmic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTPase, Rac. NADPH oxidase in the resting state becomes activated upon interaction between the catalytic core (membrane-associated subunits) and the cytosolic regulatory subunits (22). There are six homologues of phagocytic $gp91^{phox}$ proteins expressed by distinct non-phagocytic cells (23). It has been reported that NADPH oxidase 4 (Nox4) is the key subunit of NADPH oxidase expressed in MCs, and Nox4-derived ROS is the major contributor to renal morphological changes and functional abnormalities in DN (24).

Akt, also known as protein kinase B (PKB), belonging to serine/threonine kinase family members, is one of the downstream effectors of phosphoinositide 3-kinase (PI3K) which participates in numerous signaling pathways involved in diverse physiological processes, including glucose metabolism, protein synthesis, cell proliferation, cell apoptosis (25,26). Kim *et al* reported that Akt was activated in renal damage in streptozotocin-induced diabetic mice (27). It has also been demonstrated that Akt is an important mediator of MC proliferation, and can be regulated by ROS (28).

Akt is not only the downstream signaling molecule of PI3K, but also the major upstream element in the activation of nuclear factor-κB (NF-κB). Akt promotes the transcriptional activity of NF-κB through a variety of mechanisms and the signaling cascade eventually leads to cell proliferation and migration (29). A number of studies have demonstrated that NF-κB is activated in MCs by hyperglycemia, and plays a crucial role in the progression of DN (30,31). In addition, a recent study found that ROS is the important messenger in the NF-κB signaling pathway (32). Of note, the promoter region of TRPC6 has NF-κB binding sites (33). Therefore, we hypothesized that the redox-sensitive NF-κB participates in the downregulation of TRPC6 in DN. Thus, we aimed to explore whether NADPH oxidase-derived ROS is involved in HG-induced cell proliferation and the downregulation of TRPC6 in MCs through the Akt/NF-κB pathway.

Astragaloside IV (AS-IV, 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosylcycloastragenol), a purified small molecular saponin, is one of the main active ingredients of Radix Astragali, which has been reported to possess comprehensive biological properties, including antioxidant, anti-inflammatory, immunoregulatory and anti-aging properties and to improve intellectual development (34). A recent studies indicated that AS-IV ameliorates proteinuria in rats with adriamycin nephropathy (35). It has also been reported that AS-IV significantly inhibits renal oxidative stress and apoptosis in STZ-induced diabetic rats (36). However, the protective effects and the precise mechanisms of action of AS-IV on oxidative stress-induced injury in MCs under HG conditions have not yet been fully elucidated.

The present study aimed to investigate the effects of AS-IV on HG-induced MC proliferation and the downregulation of TRPC6 through a mechanism associated with the inhibition of NADPH oxidase-mediated ROS production, Akt and NF-κB activation, in an attempt to provide a novel therapeutic approach for the treatment of DN.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Laboratories, Inc. (Logan, UT, USA). Penicillin/streptomycin solution, 0.05% Trypsin-EDTA, phosphate-buffered saline (PBS), and dimethyl sulfoxide (DMSO) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), α-mannitol (MA), diphenyleneiodonium (DPI), tempol, LY294002, sulfasalazine (Sul), Fluo3/AM ester, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma (St. Louis, MO, USA). The cell NADPH oxidase colorimetric assay kit was purchased from GenMed Scientifics Inc. (Boston, MA, USA). Antibodies were obtained from the following sources: anti-Nox4 polyclonal antibody (pAb), anti-phospho-Akt pAb and anti-Akt pAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-phospho-IκBα monoclonal antibody (mAb) and anti-IκBα mAb were from Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-TRPC6 mAb was from Abcam (Cambridge, UK); anti-β-actin mAb, horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-mouse IgG and anti-rabbit IgG were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

AS-IV was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China; purity >98%, HPLC). The chemical structure of AS-IV ($C_{41}H_{68}O_{14}$; molecular weight, 784) is depicted in Fig. 1. AS-IV was dissolved in DMSO to the concentration of 25 μmol/ml as a stock solution. The stock solution was diluted with DMEM into AS-IV solutions according to the respective group when used and the final DMSO concentration did not exceed 0.5% (v/v).

MC culture. The human mesangial cell line (HMC) was obtained from the Modern Analysis and Testing Center of Central South University (Changsha, China), and maintained in normal DMEM (5.6 mM glucose) supplemented with 10% FBS (v/v), 100 μ/ml penicillin and 100 μg/ml streptomycin at 37˚C in an atmosphere containing 5% CO_2. HG treatment was performed by culturing the cells in DMEM containing 25 mM glucose for 48 h.

Cell proliferation assay. Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates. When the cell confluence reached at 70-80%, the growth medium was replaced with DMEM containing 5.6 mM glucose and 0.5% FBS. After 24 h, the quiescent cells were treated with the indicated concentrations of glucose together with various concentrations of AS-IV (5, 10, 25, 50 and 100 μM) or 0.5% DMSO (vehicle control) for 48 h. The osmotic control medium was made by supplementing normal DMEM with 24.5 mM MA. Following incubation with the above-mentioned compounds, cell proliferation was determined by MTT assay (37). The absorbance was measured at 490 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).
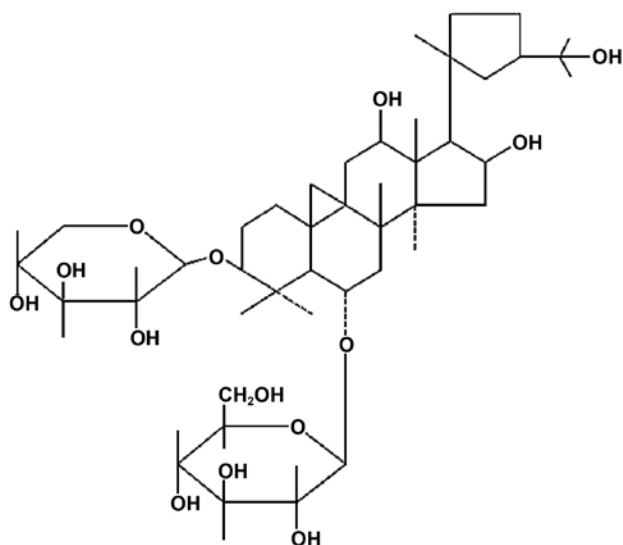
Figure 1. Chemical structure of astragaloside IV (AS-IV).

Measurement of total protein to cell count ratio. The ratio of the total protein content to the cell number is a well-established measure of cellular hypertrophy (38). The MCs were seeded into 6-well plates and were synchronized into quiescence in DMEM containing a normal glucose concentration and 0.5% FBS for 24 h. The cells were then stimulated with HG and treated with various concentrations of AS-IV (5, 10, 25, 50 and 100 μM) or 0.5% DMSO (vehicle control) for 48 h. Following incubation, the cells were washed twice with PBS and trypsinized. A small aliquot of the cells was used for cell counting by a hemocytometer. The remaining cells were lysed in RIPA buffer (Beyotime, Haimen, China), and the total protein content was measured by a protein quantitative reagent kit-BCA method (Beyotime). The total protein content to the cell count ratio was expressed as microgram protein per 10^4 cells.

Detection of intracellular ROS generation. The generation of ROS was detected using the membrane permeable indicator, DCFH-DA. The cells were seeded in 24-well plates at a density of $1x10^5$ cells/well. After being synchronized, the cells were cultured in DMEM containing 5.6 or 25 mM glucose with or without various concentrations of AS-IV (25, 50 and 100 μM) or tempol (100 μM) for 48 h, were then loaded with 10 μM DCFH-DA in serum-free DMEM containing 5.6 or 25 mM glucose at 37˚C for 30 min in the dark, and the cell culture plate was shaken every 5 min and washed three times with PBS in order to remove residual probes. Subsequently, intracellular ROS production were observed under a fluorescence microscope (Nikon, Tokyo, Japan; excitation at 488 nm, emission at 525 nm). The mean fluorescence intensity for each group of cells was determined using the Image-Pro Plus 6.0 analysis system (MediaCybernetics, Rockville, MD, USA).

NADPH oxidase activity assay. NADPH oxidase activity in the MCs was measured using the cell NADPH oxidase colorimetric assay kit (GenMed Scientifics Inc.) according to the manufacturer's instructions. Briefly, the cells grown in DMEM containing 5.6 or 25 mM glucose in the presence or absence of AS-IV (25, 50 and 100 μM) or DPI (10 μM) for 48 h were washed twice in PBS and scraped from the plate followed by centrifugation at 12,000 g, 4˚C, for 3 min, and suspended in PBS, followed by incubation with 250 μM NADPH. NADPH consumption was monitored by a decrease in absorbance at 340 nm for 5 min using a SpectraMax 190 Microplate Reader (Molecular Devices). NADPH oxidase activity was defined as picomoles per liter of substrate per minute per milligram of protein.

Western blot analysis. The cells were lysed with cold lysis buffer containing protease inhibitors. Equal amounts of protein extracts were fractionated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). After being blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST, pH 7.6) for 1 h at room temperature, the membranes were incubated with the indicated primary antibodies (Nox4, 1:200; Akt, 1:500; phospho-Akt, 1:500; IκBα, 1:1,000; phospho-IκBα, 1:200; TRPC6, 1:1,000 and β-actin, 1:1,000) overnight at 4˚C. The membranes membranes were rinsed three times with TBST and incubated with the respective secondary antibodies (1:10,000 dilutions of each antibody) for 1 h at room temperature. The protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and captured using a Bioshine ChemiQ 4600 Mini Chemiluminescence imaging system (Ouxiang, Shanghai, China). The optical density of each band was quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to the intensity of β-actin.

Fluorescence measurement of $[Ca^{2+}]_i$. Measurements of the intracellular free calcium concentration were performed using Fluo-3/AM fluorospectrophotometry. The MCs, grown on 24-well plates, were loaded with Fluo-3 by incubation for 40 min at 37˚C in the dark in HEPES buffer solution containing 5 μM Fluo-3/AM followed by washing three times with the same buffer. The cells were then incubated with Fluo-3-free HEPES buffer for an additional 20 min. Afer being trypsinized and collected, the fluorescence intensity (F) was monitored using an F-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan; excitation at 488 nm, emission at 530 nm). The maximal density (F_{max}) was measured using 0.1% Triton X-100, and the minimum (F_{min}) was measured using 20 mM EGTA. The concentration of calcium was calculated using the following formula: $[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F)$, where K_d represents the dissociation constant of Fluo-3 and calcium, and its value is 450 nM.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Each experiment was repeated at least three times independently. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical differences between two groups were analyzed by the unpaired Student's t-test and differences among multiple groups were analyzed by one-way ANOVA. In all cases, values of $P < 0.05$ were considered to indicate statistically significant differences.
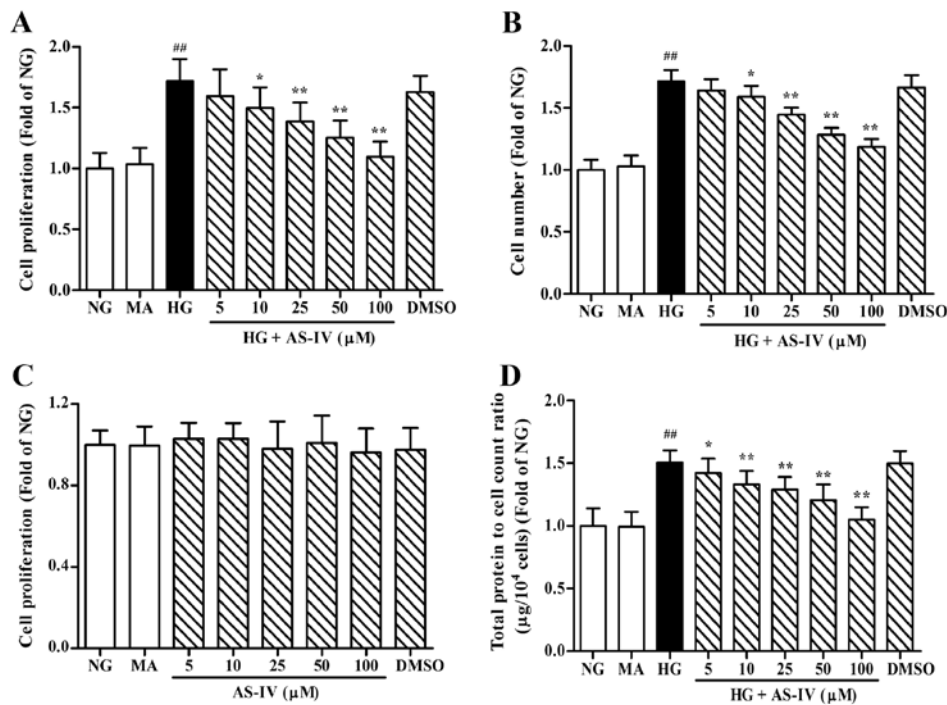
Figure 2. Astragaloside IV (AS-IV) suppresses mesangial cell (MC) proliferation and hypertrophy stimulated by high glucose (HG). (A and B) MCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.6 [normal glucose (NG)] or 25 mM HG in the presence or absence of AS-IV (5, 10, 25, 50 and 100 μM) or 25 mM α-mannitol (MA) or 0.5% dimethyl sulfoxide (DMSO) for 48 h. Following incubation, cell proliferation was examined by MTT assay and the cell number was calculated. (C) MCs were treated with NG in the presence or absence of AS-IV (5, 10, 25, 50 and 100 μM) or 0.5% DMSO for 48 h. (D) MCs were incubated under HG or NG conditions with or without AS-IV (5-100 μM) or 25 mM MA or 0.5% DMSO for 48 h. Cell hypertrophy was determined with the ratio of total protein content to the cell number which expressed as μg/10^4 cells. ##P<0.01 compared with cells cultured in NG; *P<0.05, **P<0.01 compared with cells cultured in HG. Data in the bar graphs represent the means ± standard deviation (SD) of three independent experiments.

Results

Effects of AS-IV on HG-induced MC proliferation and hypertrophy. To determine the effects of AS-IV on MC proliferation, the MTT assay and cell counting were employed. As shown in Fig. 2A and B, HG significantly stimulated the growth of the MCs in comparison to normal glucose (NG) conditions (P<0.01). The administration of AS-IV at the concentration range of 5-100 μM led to a significant inhibition of cell growth induced by HG. The vehicle control treated with DMEM containing HG and 0.5% DMSO also showed a significant increase in the proliferation of MCs. In addition, AS-IV at the examined concentrations had no effect on the viability of MCs under NG conditions, which suggested that the inhibitory effect of AS-IV upon cultured MCs was not due to its cytotoxicity (Fig. 2C). HG also markedly stimulated cell hypertrophy, defined as the protein content of MCs per unit cell number, which was abolished by the AS-IV administration in a dose dependent manner (Fig. 2D). Unlike HG, the addition of 24.5 mM MA to the medium did not exert an obvious effect on the proliferation and hypertrophy of MCs as compared with control, suggesting that the HG-triggered MC proliferation and hypertrophy were not the results of high osmolality within the medium.

Effect of AS-IV on HG-stimulated ROS generation in MCs. We examined the effects of AS-IV on HG-induced intracellular ROS generation by DCFH-DA fluorescent probe assay using fluorescence microscopy. As demonstrated in Fig. 3, the

MCs cultured under HG conditions for 48 h showed a significant increase in ROS generation compared to the cells cultured under NG conditions (P<0.01). To assess the antioxidative effects of AS-IV, we used tempol, a classic antioxidant, as a positive control. The effects of HG-induced ROS generation in the MCs were notably decreased by treatment with AS-IV (25, 50 and 100 μM) or tempol (100 μM), and AS-IV exerted its antioxidant effects in a dose-dependent manner.

Effect of AS-IV on HG-mediated NADPH oxidase activation in MCs. Since NADPH oxidase is the most important source of intracellular ROS, we used the NADPH oxidase inhibitor, DPI, as a positive control to assess the effects of AS-IV on HG-induced NADPH oxidase activity. As shown in Fig. 4A, HG resulted in a significant increase in NADPH activity that was markedly attenuated by AS-IV (25, 50 and 100 μM) or DPI (10 μM). As Nox4 is the key membrane subunit of NADPH oxidase expressed in MCs, we further examined whether AS-IV blocks the expression level of Nox4 protein. The protein level of the Nox4 subunit was notably upregulated under HG conditions compared to the control (P<0.01). Treatment with AS-IV (25, 50 and 100 μM) or DPI (10 μM) for 48 h markedly downregulated the protein expression level of Nox4 compared to the HG-treated group (Fig. 4B and C).

Inhibitory effects of AS-IV on HG-induced phosphorylation of Akt and IκBα in MCs. Given the role that Akt and NF-κB signaling plays in MC growth and proliferation, we examined the effects of AS-IV on Akt and NF-κB activation.
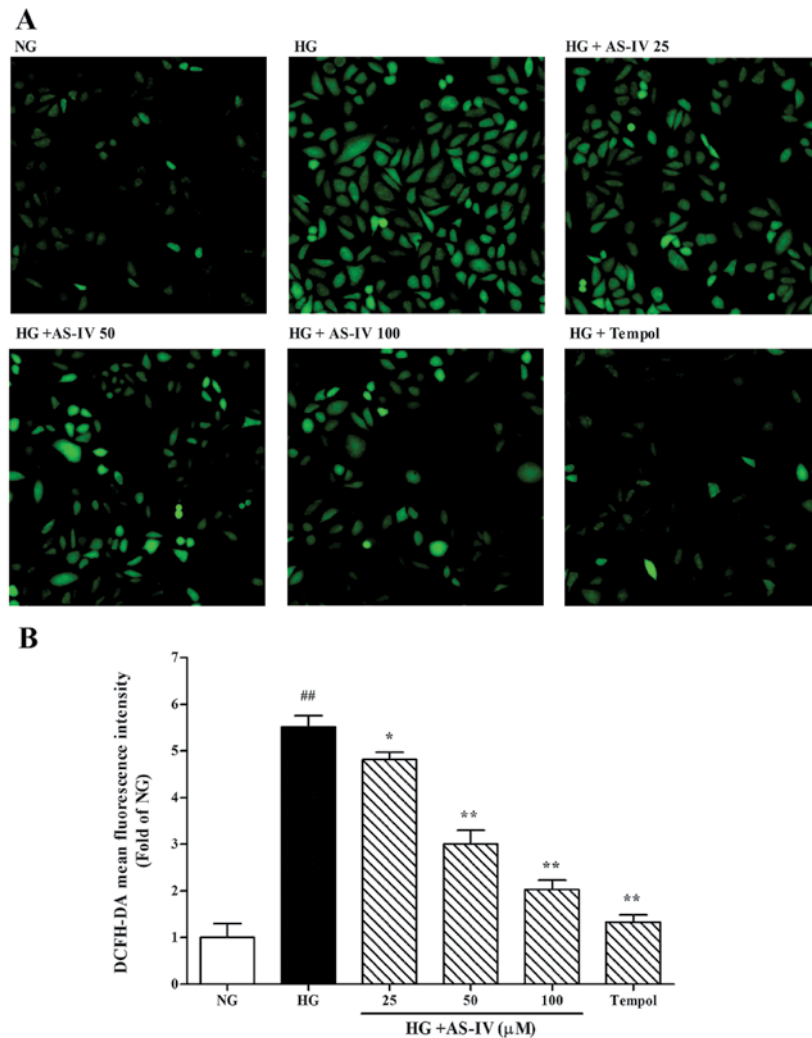
Figure 3. Astragaloside IV (AS-IV) inhibits high glucose (HG)-induced reactive oxygen species (ROS) generation in mesangial cells (MCs). (A) MCs were treated with normal glucose (NG) or HG in the presence or absence of AS-IV (25, 50 and 100 μM) or tempol (100μM) for 48 h. Cells were stained with the ROS-sensitive dye fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and observed under a fluorescence microscope. Magnification, x200. (B) The average fluorescence intensity of the NG group was calculated as 100%, and all data were normalized to the NG group. $^{\#\#}P<0.01$ compared with NG; $^{*}P<0.05$, $^{**}P<0.01$ compared with HG. Data in the bar graphs represent the means ± standard deviation (SD) of three independent experiments.
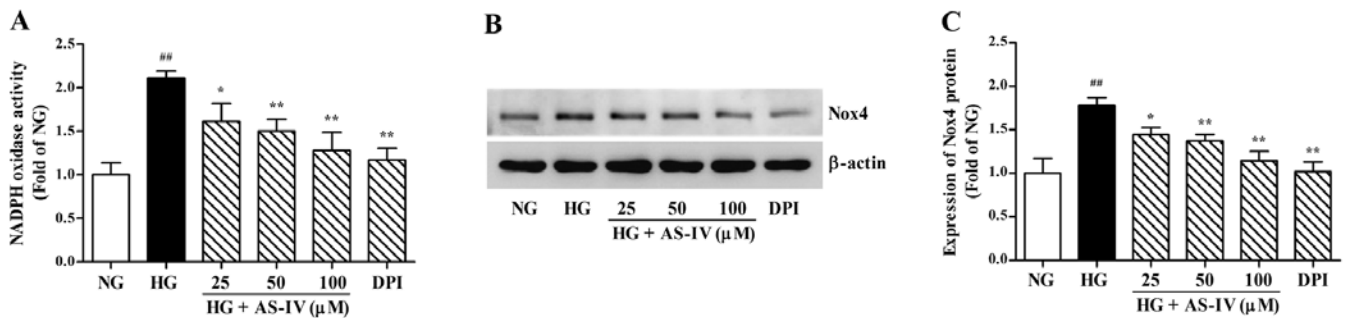

Figure 4. High glucose (HG)-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation was inhibited by astragaloside IV (AS-IV) in mesangial cells (MCs). (A) MCs were incubated with normal glucose (NG) or HG for 48 h and with or without AS-IV (25, 50 and 100 μM) or diphenylenei-odonium (DPI) (10 μM). The activity of NADPH oxidase was detected using the cell NADPH oxidase colorimetric assay kit. (B) The protein level of Nox4 after HG treatment for 48 h was determined by western blot analysis, normalized to β-actin. (C) Summary data from the experiments indicated in (B). The Nox4/β-actin ratios were expressed relative to the mean value of the control. $^{\#\#}P<0.01$ compared with NG; $^{*}P<0.05$, $^{**}P<0.01$ compared with HG. Values are the means ± standard deviation (SD) of three independent experiments.

The phosphorylation level of Akt and IκBα and the protein expression of total Akt and total IκBα were detected by western blot analysis. It was observed that HG induced Akt and NF-κB activation, as manifested by the fact that the
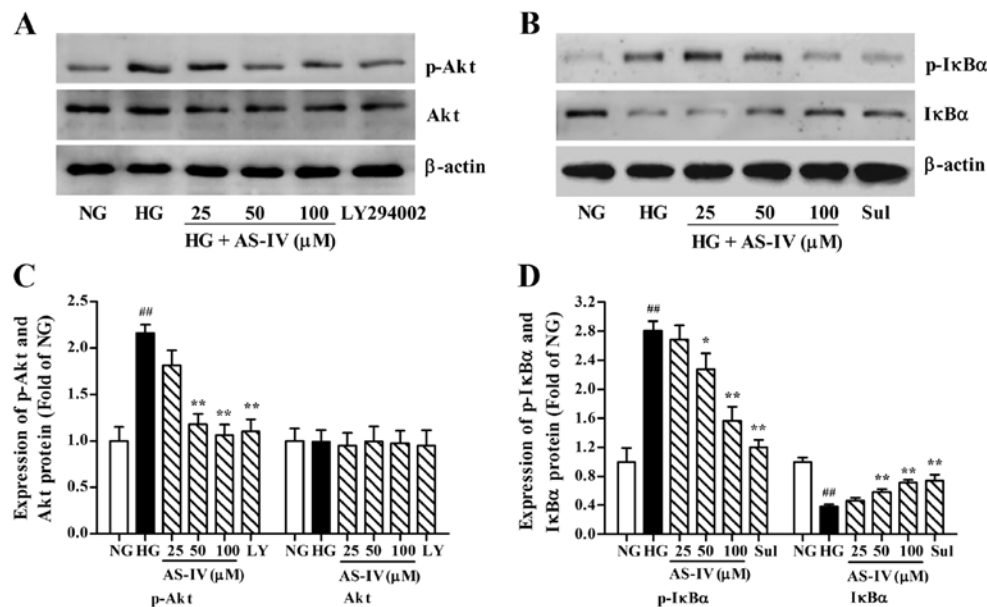
Figure 5. Astragaloside IV (AS-IV) suppresses high glucose (HG)-induced Akt and nuclear factor-κB (NF-κB) activation in mesangial cells (MCs). (A) MCs were cultured in normal glucose (NG) or HG with or without various concentrations of AS-IV (25, 50 and 100 μM) or LY294002 (10 μM) for 48 h. Akt phosphorylation and total Akt protein level was detected by western blot analysis. (B) MCs were cultured in NG or HG with or without various concentration of AS-IV (25, 50 and 100 μM) or the IκBα inhibitor, sulfasalazine (Sul, 0.5 mM), for 48 h, and the protein levels of p-IκBα and IκBα were detected by western blot analysis. β-actin was used as a loading control. (C and D) Summary data from the experiments indicated in (A) and (C), respectively. [##]P<0.01 compared with NG; [*]P<0.05, [**]P<0.01 compared with HG. Values are the means ± standard deviation (SD) of three independent experiments.

relative amount of phosphorylated Akt and IκBα and the degradation of IκBα were significantly higher compared to the control cells (Fig. 5). However, treatment with AS-IV or the PI3K inhibitor, LY294002 (10 μM), effectively abrogated the HG-induced Akt phosphorylation in the MCs, and the phosphorylation and degradation level of IκBα was also markedly inhibited by AS-IV or the IκBα inhibitor, Sul (0.5 mM). AS-IV did not affect the protein expression level of total Akt in the HG-cultured cells. These results suggest that AS-IV inhibits HG-induced Akt and NF-κB activation in MCs.

Regulatory effect of AS-IV on the NADPH oxidase/ROS/Akt/ NF-κB signaling pathway in MCs under HG conditions. To further investigate the signaling cascade involved in HG-induced NADPH oxidation, Akt and NF-κB activation, specific inhibitors of several signaling molecules were used. Treatment with NADPH oxidase inhibitor (DPI, 10 μM) or ROS inhibitor (tempol, 100 μM) blocked Akt and NF-κB activation in the MCs induced by HG (Fig. 6), indicating that NADPH oxidase activation induced by HG occurs upstream of Akt and NF-κB in MCs. The HG-induced Akt phosphorylation was abolished by treatment with PI3K inhibitor (LY294002, 10 μM) , whereas Sul (0.5 mM), an IκBα inhibitor, failed to inhibit Akt phosphorylation (Fig. 6A and C). IκBα phosphorylation and degradation were also inhibited in the presence of Sul, LY294002 and DPI or tempol (Fig. 6B and D). These data demonstrate that the PI3K/Akt pathway is upstream of NF-κB in MCs cultured under hyperglycemic conditions.

We also examined whether NADPH oxidase activation in MCs is dependent on Akt or NF-κB activation. The expression level of the Nox4 subunit was evaluated in the HG-exposed cells treated with or without LY294002, DPI, tempol or Sul. LY294002 and DPI effectively inhibited the HG-induced

increase in the expression of Nox4. However, no significant change in the expression level of Nox4 was observed in the MCs treated with tempol and Sul (Fig. 6E and F). Taken together, the above results suggest that NADPH oxidase activation and the PI3K/Akt pathway may function in parallel or may interplay with each other, which are upstream of NF-κB in HG-stimulated MCs.

Inhibitory effects of AS-IV on the HG-induced downregulation of TRPC6 and the reduction in calcium influx in MCs. TRPC6 is known as a Ca^{2+}-conductive cation channel and regulates the contractile function of MCs; it plays a pivotal role during the early stages of HG-induced damage to MCs (14,15). Thus, in this study, we examined the effects of AS-IV on the expression level of TRPC6 protein and the concentration of intracellular free calcium in MCs cultured under HG conditions. As illustrated in Fig. 7A and C, incubation of the MCs with HG for 48 h markedly decreased the expression level of TRPC6 protein compared with the cells cultured under NG conditions. The downregulation of TRPC6 induced by HG was markedly abrogated by treatment with AS-IV at a concentration of 25 to 100 μM and the TRPC6 agonist, a diacylglycerol analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG, 100 μM). Furthermore, in the presence of inhibitors of signaling molecules, such as LY294002, DPI, tempol and Sul, the HG-induced TRPC6 downregulation was markedly abolished (Fig. 7B and D). Ultimately, we detected the intracellular free calcium concentration in MCs using Fluo-3/AM fluorospectrophotometry. As shown in Fig. 7E, the HG-induced reduction in calcium influx in the MCs was also greatly ameliorated by AS-IV (25, 50 and 100 μM) or OAG (100 μM). These results suggest that AS-IV protects MCs against contractile dysfunction under HG conditions by upregulating the TRPC6 protein
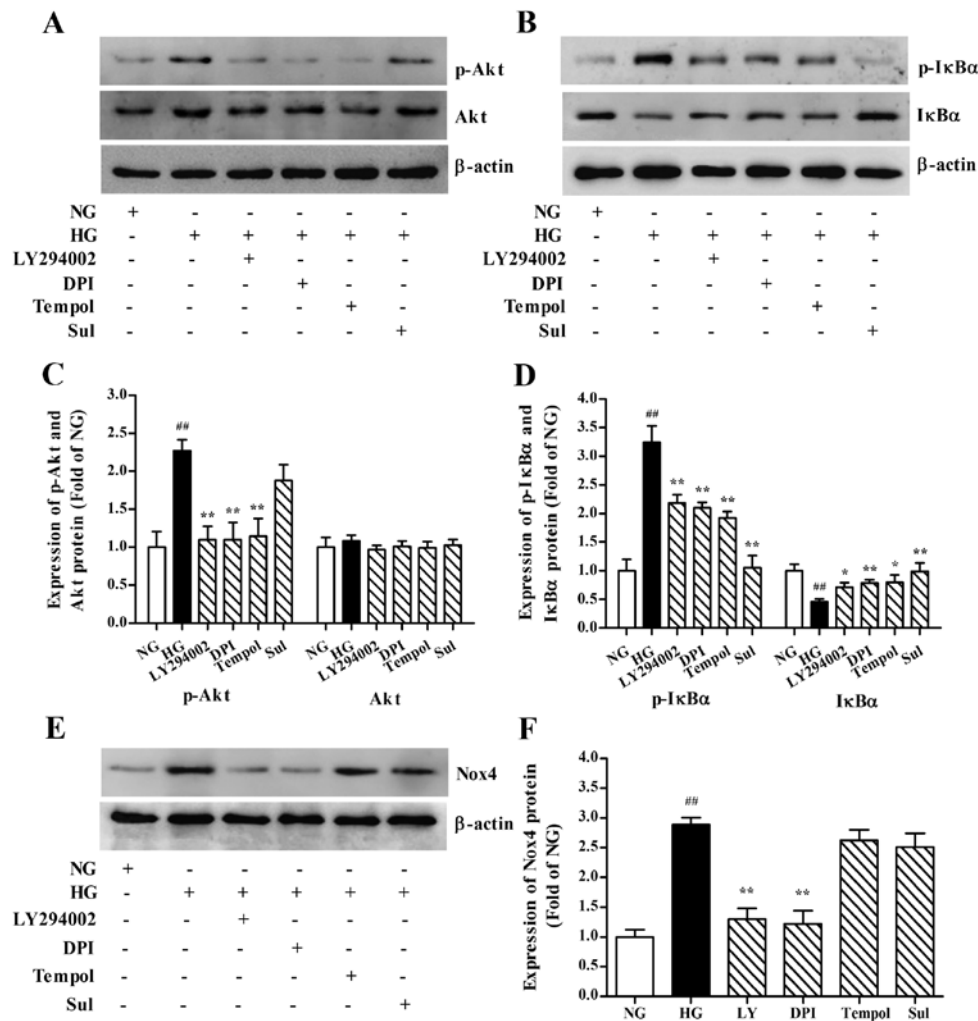
Figure 6. High glucose (HG)-potentiated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation is affected by the phosphoinositide 3-kinase (PI3K)/Akt pathway, but not nuclear factor-κB (NF-κB) in mesangial cells (MCs). (A, B and E) Cells were incubated with HG in presence or absence of various inhibitors [LY294002, 10 μM; diphenyleneiodonium (DPI), 10 μM; tempol, 100 μM; sulfasalazine (Sul), 0.5 mM] for 48 h. The phosphorylation level of Akt and IκBα, and the expression level of Nox4, total Akt and total IκBα protein were determined by western blot analysis. β-actin was used as a loading control. (C, D and F) Representative quantitative analysis for results of (C) Akt, (D) IκBα, and (F) Nox4 were expressed as fold change from the control. [##]P<0.01 compared with normal glucose (NG); [*]P<0.05, [**]P<0.01 compared with HG. Data in the bar graphs represent the means ± standard deviation (SD) of three independent experiments.

expression and increasing Ca²⁺ influx through the NADPH oxidase/ROS/Akt/NF-κB signaling pathway.

Discussion

Radix Astragali, the dried root of *Astragalus membranaceus* (Fisch.) *Bunge*, has long been used in traditional Chinese medicine for the treatment of cardiovascular diseases and diabetes (39). Recently, investigations into its active ingredients have attracted much attention due to the unique pharmacological properties of many of its constituents (34). AS-IV is a novel saponin extracted from Radix Astragali, and it has been reported to ameliorate podocyte apoptosis by attenuating ROS production and to prevent acute kidney injury by inhibiting oxidative stress (40,41). In a previous study of ours, we suggested that AS-IV significantly reduced H_2O_2-induced ROS overproduction in MCs (42). In order to further demonstrate that treatment with AS-IV can suppress oxidative stress-induced injury in DN, the present study was designed to

examine the protective effects of AS-IV on the morphological and functional abnormalities of MCs cultured under hyperglycemic conditions.

Hyperglycemia, a common condition occurring in diabetes, markedly increases the production of ROS in MCs (19). The redox imbalance between the production of ROS and the compensatory response from the endogenous antioxidant network results in oxidative stress. The interaction of the excessive ROS generation with biomolecules, such as lipids, proteins and DNA, can activate a series of cell signaling pathways, leading to severe kidney injury and dysfunction (43). The most prominent effect is MC proliferation, which often leads to glomerulosclerosis (GS), renal fibrosis or even end-stage renal failure (44). NADPH oxidase, a multicomponent enzyme, is the major source of ROS production in renal cells (23). NADPH oxidase was originally found in neutrophils (22). In many non-phagocytic cells, the Nox family is a homologue of gp91[phox], which is the catalytic subunit of NADPH oxidase, including several types,
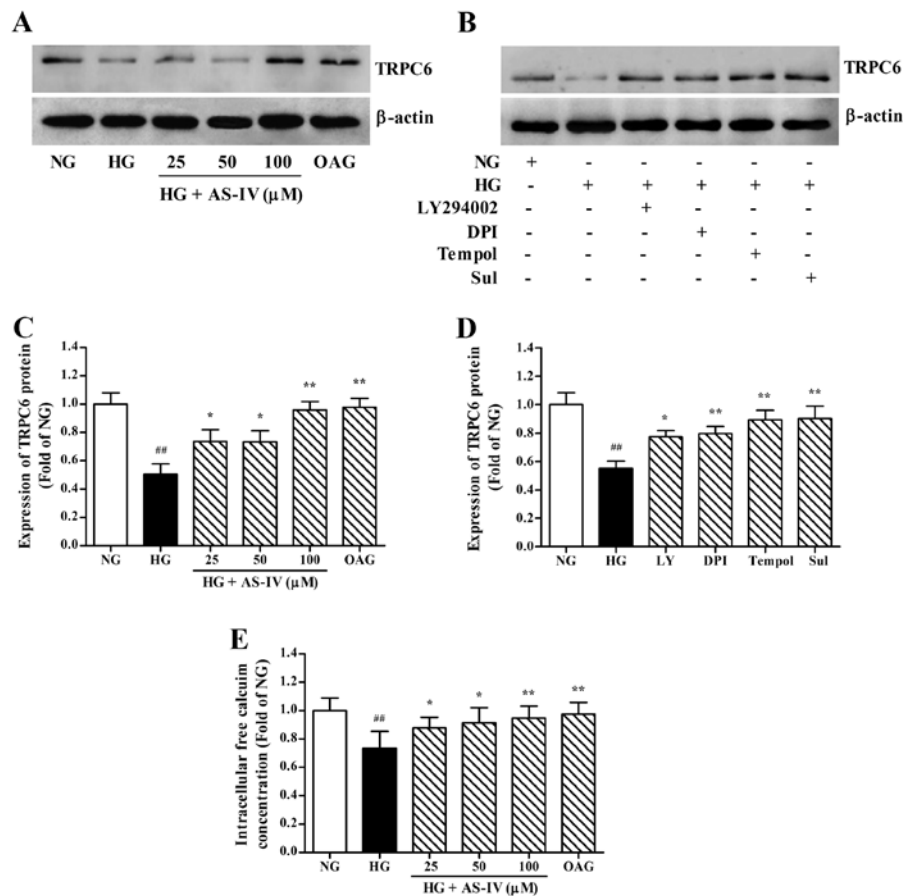
Figure 7. Treatment with astragaloside IV (AS-IV) inhibits the downregulation of canonical transient receptor potential cation channel 6 (TRPC6) protein in mesangial cells (MCs) and the reduction in calcium influx under high glucose (HG) conditions. (A) MCs were stimulated with HG for 48 h with or without AS-IV at various concentration (25, 50, and 100 μM) or 1-oleoyl-2-acetyl-sn-glycerol (OAG) (100 μM). (B) MCs were cultured in normal glucose (NG) or HG with or without different inhibitors of signaling molecules [LY294002, 10 μM; diphenyleneiodonium (DPI), 10 μM; tempol, 100 μM; sulfasalazine (Sul), 0.5 mM] for 48 h. The protein level of TRPC6 was detected by western blot analysis, and β-actin was used as a loading control. (C and D) Summary data from the experiments indicated in (A) and (B), respectively. (E) MCs were loaded with the fluoroprobe Fluo-3/AM, and the fluorescence intensity of intracellular calcium was detected by fluorospectrophotometry. The Ca^{2+} concentration was expressed as fold of NG. [##]$P<0.01$ compared with NG; [*]$P<0.05$, [**]$P<0.01$ compared with HG. Results represent the means $\pm$ standard deviation (SD) of three independent experiments.

such as Nox1, Nox2, Nox3, Nox4 and Nox5 (45). Of these, the Nox4 isoform is mainly found in MCs (46). The activity of NADPH oxidase and the expression level of Nox4 protein are both markedly increased in MCs under HG conditions. In this study, we found that treatment with AS-IV markedly supressed HG-induced intracellular ROS generation, as well as MC proliferation and hypertrophy. Consistently, our experiments demonstrated that AS-IV markedly attenuated the HG-stimulated NADPH oxidase activation and the overexpression of Nox4 in MCs. Our results indicated that there was no obvious direct cytotoxic effect of AS-IV on MCs. These results provide evidence that AS-IV may exert an inhibitory effect on HG-induced MC proliferation and hypertrophy by downregulating Nox4-derived ROS generation.

Since the activation of various cellular molecules, such as transcription factors, cytokines, hormones and protein kinases has been reported to contribute to the signal transduction cascades of DN (47,48), we investigated the mechanisms through which AS-IV prevents damage to human MCs induced by HG stimulation in detail in order to explore the underlying molecular mechanisms involved in the above-mentioned effects of AS-IV. Our results revealed that following HG stimulation,

the levels of several cellular phosphorylated molecules were decreased by treatment with AS-IV. Akt, one of the downstream effectors of PI3K, is involved in cell proliferation and hypertrophy (26). It has been demonstrated that Akt is activated in renal cells and is regulated by intracellular ROS (28). In the present study, we found that the phosphorylation level of Akt was increased by HG stimulation in MCs. Akt phosphorylation was also markedly reduced by treatment with AS-IV. Moreover, Akt plays a key role in promoting the transcriptional activity of NF-κB (29). NF-κB is one of the most important transcription factors, which can be activated by various stimuli in DN, such as hyperglycemia, advanced glycation end products (AGEs), angiogeninII (AngII), oxidative stress and the PI3K/Akt signaling pathway (49). Akt promotes the activation of NF-κB by activating the IκB kinase (IKK) to accelerate the phosphorylation and degradation of IκB, thereby promoting the translocation of NF-κB from the cytoplasm into the nucleus and subsequently binding to specific sequences in DNA, which in turn results in gene transcription. These signaling cascades eventually lead to MC proliferation (31). Our results revealed that HG enhanced the activation of NF-κB, and that the degradation and phosphorylation levels of IκBα were marekdly

decreased in the MCs treated with AS-IV. These data strongly suggest that the Akt/NF-κB signaling pathway is involved in the pathogenesis of DN.

In addition, we used specific inhibitors of signaling molecules as a control to compare the effectiveness of AS-IV in order to validate the upstream and downstream association among HG-induced NADPH oxidase, Akt and NF-κB activation. The phosphorylation levels of Akt and IκBα were markedly inhibited by the NADPH oxidase inhibitor, DPI, or the ROS inhibitor, tempol. Of note, the PI3K inhibitor, LY294002, also abolished the HG-stimulated Nox4 expression, as well as IκBα phosphorylation and degradation. However, the IκBα inhibitor, Sul, did not suppress Nox4 expression and Akt activation induced by HG. Studies have indicated that ROS, as an important stimulator of NF-κB activation, mediate the activation of Akt in MCs and other cultured cells (50). However, controversially, there are also data reporting that the intracellular ROS level is regulated by Akt (26). These discrepancies suggest the existence of a cross-talk between NADPH oxidase-derived ROS and Akt activation. Our results support this assumption and indicate that both the activation of NADPH oxidase and Akt may be required for HG-induced IκBα phosphorylation and degradation in MCs.

The early distinctive pathological characteristics of DN are not only MC proliferation, but also the hypocontractility of MCs, which is induced by the decreased Ca^{2+} influx. TRPC6 is Ca^{2+} permeable cation channel which plays a pivotal role in regulating Ca^{2+} signaling in MCs, proving a mechanism for impaired MC contraction in diabetes (10). It has previously been suggested that the abundance of TRPC6 protein in MCs is decreased by ROS and PKC in diabetes (51). Moreover, it has also been reported that NF-κB participates in the regulation of TRPC6 expression (33). The results of the present study revealed that exposure to HG resulted in the downregulation of TRPC6 protein and a reduction in free calcium concentration in the MCs, inducing the contractile dysfunction of MCs; the NADPH oxidase/ROS/Akt/NF-κB signaling pathway may also be involved in these effects, which were markedly supressed by treatment with AS-IV.

In conclusion, the present study indicates that hyperglycemia induces glomerular MC proliferation and the downregulation of TRPC6 protein by promoting Nox4 upregulation, ROS generation, Akt and NF-κB activation. Treatment with AS-IV inhibits HG-induced MC proliferation and contractile dysfunction through the NADPH oxidase/ROS/Akt/NF-κB signaling pathway. Therefore, we suggest that AS-IV may be a valuable candidate for the prevention and treatment of early DN. However, other relevant mechanisms underlying the effects of AS-IV require further investigation.

Acknowledgements

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