Transient receptor potential canonical 6 knockdown ameliorated diabetic kidney disease by inhibiting nuclear factor of activated T cells 2 expression in glomerular mesangial cells

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ABSTRACT

Purpose: Glomerular mesangial cell (GMC) dysfunction plays a vital role in the pathogenesis of diabetic kidney disease (DKD). Transient receptor potential canonical 6 (TRPC6) has been demonstrated to be involved in the development of DKD. However, the underlying mechanism remains unclear. The present study investigated the role of TRPC6 in GMC dysfunction and the related mechanism.

Methods: Diabetic rats and cultured GMCs were used in the experiment. The diabetic rat model was created by intraperitoneal injection of streptozotocin. Protein and mRNA levels were assessed by Western blotting and quantitative RT–PCR, respectively. Histological changes in the kidneys were observed by immunohistochemistry and hematoxylin and eosin. TRPC6 knockdown was achieved by adenovirus-mediated TRPC6 shRNA delivery in vivo and TRPC6 siRNA transfection in vitro.

Results: TRPC6 expression was increased in diabetic rat kidneys. Knockdown of TRPC6 attenuated diabetes-induced kidney functional deterioration. In addition, the increases in extracellular matrix components, including collagen IV, collagen I, and fibronectin production, as well as NFAT2 expression were also suppressed. In cultured GMCs, high glucose (25 mM, HG) treatment increased the expression of TRPC6. Knockdown of TRPC6 alleviated HG-induced increases in collagen IV, fibronectin, and NFAT2 expression. Knockdown of NFAT2 also inhibited the upregulation of proteins, including collagen IV and fibronectin, in HG-treated GMCs.

Conclusion: These results demonstrate that inhibition of TRPC6/NFAT2 signaling attenuates GMC dysfunction and the development of DKD and suggest that pharmacological targeting of TRPC6/NFAT2 in GMCs may provide beneficial effects for DKD.

INTRODUCTION

Diabetic kidney disease (DKD) is a complication of diabetes mellitus (DM) and is thought to be the main cause of end-stage kidney disease (ESKD) worldwide. Laboratory experiments and clinical studies have reported many therapeutic targets for DKD, including the renin-angiotensin system, sodium-glucose cotransporter 2, glucagon-like peptide-1, and dipeptidyl peptidase-4 [1,2]. Although therapeutic strategies have provided some beneficial effects on DKD-related kidney function deterioration, none of them are able to stop the progression of DKD [3]. Hence, it is imperative to reveal the underlying mechanisms of DKD and explore novel therapeutic interventions.

DKD is the final consequence of progressive damage in kidney resident cells subject to persistent hyperglycemic conditions. Glomerular basement membrane thickening and mesangial expansion are the typical early histological features in DKD that manifest as glomerular hypertrophy, and these features are followed by glomerulosclerosis in the late stage [4]. Glomerular mesangial cells (GMCs) play an essential role in regulating glomerular hemodynamics, maintaining extracellular matrix (ECM) homeostasis, and phagocytosing apoptotic cells [5]. Persistent hyperglycemia directly stimulates GMCs and leads to GMC dysfunction, such as cell overproliferation and migration; an increase in ECM generation capacity; the release of proinflammatory, profibrotic, and proapoptotic factors; and the activation of multiple signaling pathways. All these changes in GMCs are complementary to the pathological process of mesangial matrix expansion and glomerulosclerosis [6].

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Transient receptor potential (TRPC) 6, a Ca\(^{2+}\)-conductive cation channel, is one member of the seven-member family of TRPCs [7]. Transient receptor potential canonical 6 (TRPC6) is extensively expressed in kidneys, including GMCs, podocytes, and kidney tubular epithelial cells [8]. TRPC6 plays an important role in kidney function control, and TRPC6 dysregulation is associated with kidney pathophysiological changes. TRPC6 mutation results in hereditary focal segmental glomerulosclerosis [9]. The upregulation of TRPC6 is a common feature of patients with proteinuria, and overexpression of TRPC6 in mice causes proteinuria [10]. TRPC6 activation also plays a crucial role in podocyte injury by regulating actin cytoskeleton dynamics [11,12]. Inhibition of TRPC6 in podocytes and kidney tubular epithelial cells can attenuate DKD [13,14]. Previous studies have also shown that TRPC6 regulates the contractile function and proliferation of GMCs [15,16].

The downstream signaling events following TRPC6 activation include the activation of two Ca\(^{2+}\)-dependent transcription factors: nuclear factor of activated T cells (NFAT) and cAMP response element-binding protein [17,18]. The NFAT family has five members, and their activation is regulated by the Ca\(^{2+}\)-calcineurin signaling pathway [19]. In podocytes and cardiac cells, TRPC6-mediated Ca\(^{2+}\) influx activates calcineurin, which induces NFAT2 dephosphorylation and translocation into the nucleus and subsequently initiates downstream target gene transcription [20,21]. Recently, it was reported that TRPC6/NFAT2 activation mediated via CD-36 is implicated in palmitate-induced human GMC proliferation in type 2 DKD [22]. However, whether TRPC6/NFAT2 signaling is involved in hyperglycemia-induced GMC dysfunction in type 1 DKD remains unknown. Therefore, the purpose of our study was to observe the effect of TRPC6/NFAT2 signaling on GMC dysfunction in streptozotocin (STZ)-induced type 1 diabetic rats and in high glucose (HG)-cultured GMCs.

**Materials and methods**

**Animals and treatment**

Thirteen-week-old male SD rats were obtained from Nanjing Qinglongshan Animal Pasture. These rats were fed in a specific-pathogen-free environment at a temperature of 22°C with a 12-h light/dark cycle. Standard diet and water were provided. All rats were given free access to water and food. The experimental animal protocol of this study obtained consent from the Animal Welfare and Ethics Committee of Wannan Medical College (LLSC-2020-145). Intraperitoneal injection of STZ in citrate buffer (0.1 mol/L, pH 4.4) at 45 mg/kg body weight was used to induce diabetes in rats, and the equivalent amount of sodium citrate buffer was administered to the control group. STZ-injected rats with fasting blood glucose >16.7 mmol/L were designated as diabetic rats. Thirty diabetic rats were randomly divided into 2 groups (n = 15/group): the DM + Ad-null group (diabetic rats injected with negative control (NC) adenovirus) and the DM + Ad-shRNA-TRPC6 group (diabetic rats injected with TRPC6 shRNA adenovirus). Normal rats injected with NC adenovirus were designated the Ctrl + Ad-null group (n = 14). Adenovirus was injected into the left kidney via the kidney pelvis at a dose (HBAAV2/9-r-TRPC6 shRNA-null: 1.7 × 10\(^{12}\) vg/mL, HBAAV2/9-NULL NC: 1.8 × 10\(^{12}\) vg/mL). Fasting blood glucose and body weight were measured every two weeks. Rats were sacrificed 8 weeks after adenovirus injection.

**Renal pelvis injections**

Rats were intraperitoneally injected with mixed anesthetics (14 g urethane, 0.7 g α-chloralose, and 0.7 g borax per 100 mL normal saline, 0.6–0.7 mL/100 g). In addition, eye ointment was applied, and the hair of mice was shaved. The renal pelvis injection was always performed using a flank incision. If necessary, the kidney hilum was separated from the surrounding fat, and the kidney was gently pressed with closed forceps to visualize the renal pelvis, a small white area in the lower part of the kidney. Adenovirus liquid (Hanheng, China) was injected within 1–3 s using a 30-G 0.5-mL insulin syringe without a safety shield, and the needle was kept in place for approximately 5 s before being removal from the renal pelvis. The last steps involved placing the kidney back into the abdomen and suturing the rats with 5–0 silk.

**Kidney histology**

Freshly collected kidneys were fixed with 10% formalin, desiccated in a graded alcohol series, cleared with xylene, embedded in paraffin, and finally cut into slices of ~5 μm thickness. Using standard procedures, the treated kidney sections were stained with hematoxylin and eosin (HE) and Masson’s trichrome.

**Assessment of serum creatinine (SeCr) and urea nitrogen (BUN)**

Serum specimens were obtained from rats, and SeCr and BUN were assessed by strictly following the
instructions of the kit (JianCheng, China). SeCr and BUN were measured based on the light density of each sample at wavelengths of 546 nm and 640 nm, and these values are used to calculate amount of SeCr and BUN present in the sample. The sarcosine oxidase method is used to measure creatinine levels as noted in the Methods Section.

Immunohistochemistry

Standard immunohistochemical (IHC) staining was performed as previously described [23]. Kidney tissue sections were stained with COL-IV by IHC. The sections were developed with a DAB (3,3-diaminobenzidine) developing system (Vector Laboratories, Inc., Burlingame, CA, USA).

Cell culture and treatment

Rat GMCs (HBZY-1) were obtained from Yijishan Hospital affiliated with Wannan Medical College. GMCs were grown in DMEM (Sigma–Aldrich; Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (LONSERA, China) at 37 °C in 5% CO2 in a humid atmosphere. In addition, the cells were treated in medium containing 0.5% FBS for 24 h to ensure that the cells remained quiescent. Then, cells were treated with normal glucose (19.5 mM mannitol + 5.5 mM D-glucose, NG) or HG (25 mM D-glucose) for 48 h or 72 h.

RNA interference

Small interfering RNAs (siRNAs) used for the knockdown of rat TRPC6 or NFAT2 were purchased from RiboBio (Guangzhou, China). According to the manufacturer's instructions, GMCs were transfected with TRPC6 or NFAT2 siRNA and NC siRNA using Vigofect (Vigorous Biotechnology, China) transfection reagent at a final concentration of 50 nM. The sequences of the siRNAs were as follows: TRPC6 siRNA, 5'-CCTCTACTCCTACGAGAAGA-3'; TRPC6 reverse, 5'-CCCGTTTACGCAAGAAGAAGAGAGA-3'; GAPDH forward, 5'-GAACGGGAAGCTCACTGGA-3'; and GAPDH reverse, 5'-GGCTGCTTACACCACAGTT-3'. Using GAPDH as the internal control, the relative expression of TRPC6 was calculated by the 2-ΔΔCt method.

Western blotting

Total tissue and cell protein extracted from rat kidneys and GMCs were lysed with RIPA lysis buffer (Beyotime, China) and measured with a BCA Protein Assay Kit (Beyotime, China). Nuclear protein was extracted using the Nuclear and Cytoplasmic Protein Extraction kit (cat. no. P0028; Beyotime Institute of Biotechnology, Nanjing, China) following the manufacturer's instructions. The protein was separated by 10% SDS–PAGE and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk and incubated with the following primary antibodies overnight at 4 °C: TRPC6, collagen IV, collagen I, fibronectin, and NFAT2 from Abcam (Cambridge, MA, USA) as well as β-actin and histone 3 from Abclonal (Wuhan, China). On the next day, HRP-conjugated goat anti-rabbit or goat anti-mouse IgG, which served as the secondary antibody, was incubated with the membranes for 2 h at room temperature to detect immune response bands. ECL chemiluminescence was used for imaging.

Statistical analysis

Data are expressed as the mean ± standard deviation. The differences between the two groups were scrutinized using Student's unpaired t test and one-way ANOVA, and the differences among multiple groups were analyzed via the Newman–Keuls multiple comparisons test. p < 0.05 was considered statistically significant. GraphPad Prism 5.0 and ImageJ (National Institutes of Health) software were used to perform statistical analysis and density analysis of all western blots, respectively.

Results

Effects of TRPC6 knockdown on metabolic parameters in STZ-induced diabetic rats

After STZ injection, the changes in blood glucose and body weight were detected at 2, 4, 6, and 8 weeks. The body weight in the DM group and Ad-TRPC6-shRNA group was considerably lower than that noted in non-diabetic rats (Figure 1(A)). Compared with the blood
glucose level of nondiabetic rats, the blood glucose levels in the DM group and Ad-TRPC6-shRNA group were noticeably increased (Figure 1(B)). However, body weight and blood glucose did not obviously differ between the DM group and the Ad-TRPC6-shRNA group (Figure 1(A,B)). The SeCr level and the ratio of kidney weight to body weight in the DM group were markedly increased relative to the nondiabetic group, which could be substantially reduced by Ad-TRPC6-shRNA (Figure 1(C,D)). The BUN levels in the DM and Ad-TRPC6-shRNA groups were higher than those noted in nondiabetic rats, and no heterogeneity in the Ad-TRPC6-shRNA group or DM group was observed (Figure 1(E)).

Effects of TRPC6 knockdown on kidney morphological changes

Kidney sections were stained with HE, Masson's trichrome staining, and immunochemistry. The HE staining results showed that no obvious inflammatory cell infiltration was observed between different groups (Figure 2(A)). The Masson's trichrome staining results showed that compared with the control group, collagen accumulation occurred in the diabetic group, and compared with the diabetic group, Ad-TRPC6-shRNA treatment reduced collagen accumulation in the kidney tissue of diabetic rats (Figure 2(B)). The IHC results showed that collagen IV protein levels were higher in the diabetic group compared with the control group, and Ad-TRPC6-shRNA treatment reduced collagen formation in the kidney tissues of the diabetic rats compared to the diabetes group (Figure 2(C)).

Effects of TRPC6 knockdown on ECM production and NFAT2 expression

We observed that hyperglycemic conditions increased the level of Trp6 (Figure 3(A)). We next examined whether glomerulus and kidney tubular ECM accumulation could be prevented in response to Ad-TRPC6-shRNA. Western blotting results showed that compared with nondiabetic rats, fibronectin, collagen IV, and collagen I protein levels were obviously increased in the DM group, and this effect was reversed by Ad-TRPC6-shRNA delivery (Figure 3(B–D)). We also observed that hyperglycemic conditions increased NFAT2 protein expression, which was also inhibited by Ad-TRPC6-shRNA delivery (Figure 3(E)).

Effects of HG on TRPC6/NFAT2 signaling and ECM production in GMCs

Western blotting showed that the TRPC6 protein levels were considerably increased in GMCs subject to HG for
48 or 72 h (Figure 4(A)). The qRT–PCR results indicated that TRPC6 mRNA expression was increased in GMCs treated with HG for 48 h (Figure 4(B)). Collagen IV, fibronectin, and NFAT2 expression was significantly increased in GMCs treated with HG for 48 or 72 h compared with NG (Figure 5).

Effects of TRPC6 knockdown on ECM production in HG-treated GMCs

Compared with NC siRNA transfection, TRPC6 siRNA transfection for 48 h reduced TRPC6 protein levels by ~70% (Figure 6(A)). TRPC6 protein and mRNA levels in GMCs treated with HG were significantly increased. The increases in the TRPC6 protein and mRNA levels could be reversed by the TRPC6 siRNA (Figure 6(B,C)).

Western blotting results showed that fibronectin, collagen IV, and NFAT2 expression was considerably increased in GMCs treated with HG for 48 h. Additionally, the increase in fibronectin, collagen IV, and NFAT2 expression could be reversed by TRPC6 siRNA (Figure 7).

Effects of NFAT2 knockdown on ECM production in HG-treated GMCs

Western blotting indicated that nuclear NFAT2 protein levels were noticeably increased in GMCs treated with HG for 48 h, and this effect could be blocked by NFAT2 siRNA (Figure 8(A)). In GMCs treated with HG for 48 h, fibronectin and collagen IV significantly increased, and knockdown of NFAT2 by siRNA prevented the HG-induced increases in fibronectin and collagen IV (Figure 8(B,C)).

Discussion

The excessive accumulation of ECM in kidney glomeruli is increasingly regarded as an important contributor to the progression of DKD. In our study, we found that TRPC6 knockdown alleviated the overaccumulation of ECM induced by HG and the SeCr increase in type 1 diabetic rats. In vitro, HG-induced overexpression of NFAT2, TRPC6, collagen IV, and fibronectin in cultured GMCs was largely abolished by silencing TRPC6 with
siRNA. These results suggest that the TRPC6/NFAT2 signaling pathway is activated under hyperglycemic conditions and implicated in mesangial cell activation and subsequent ECM production.

Many studies have provided strong evidence for the beneficial effects of Trp6 knockout in proteinuria nephropathy. For example, Wang et al. and Kim et al. reported that knockout of Trp6 attenuates kidney dysfunction in puromycin amino-nucleoside nephrosis [24,25]. In addition, two recent studies reported that Trp6 knockout exhibited beneficial effects in rodent DKD models [13,26]. Notably, the beneficial effect of knockout of TRPC6 on inhibiting albuminuria in the type 1 diabetes Akita model was observed 12 and 16 weeks after diabetes, but this difference could not be observed at 20 weeks. A recent study from this group found that insulin resistance and glomerular damage could be aggravated by knocking out Trp6 in Akita mice [27]. In our study, TRPC6 expression in the kidney cortex of diabetic rats was significantly increased. To observe the role of TRPC6 in DKD, gene silencing of TRPC6 was performed by injecting adenovirus expressing TRPC6 specific shRNA (shRNA-TRPC6). However, gene delivery to kidney cells has always been a problem. The mammalian kidney has a strict filtering function, rejecting proteins larger than 50 kDa. In addition, podocytes in the glomerulus form a slit diaphragm with a diameter of only 10 nm. It is difficult to achieve sufficient expression levels in the kidney by traditional intravenous administration. Rubin et al. demonstrated the utility of direct kidney injections to circumvent the kidney size exclusion barrier [28]. Woodard et al. reported that renal pelvis injection allowed highly efficient gene transfer in multiple kidney cell types, including glomeruli, tubules, and collecting ducts [29]. Therefore, we also used renal pelvis injection

Figure 3. Effects of Ad-TRPC6-shRNA on the expression of TRPC6, fibronectin, collagen IV, and NFAT2 in STZ-induced diabetic rats. Representative Western blotting and quantification of the expression of TRPC6, fibronectin, collagen IV, collagen I, and NFAT2 in the kidney cortex of diabetic rats after Ad-TRPC6-shRNA treatment (A–F) (n = 5 in each group *p < 0.05, **p < 0.01).
to knock down the expression of Trp6. We confirmed that the TRPC6 knockdown exhibited renoprotective effects at an 8-week experimental period, which represents the early stage of DKD. The emergence of proteinuria is a manifestation of early DKD. In the 8-week DM group, the significant increase in proteinuria is a good indicator for kidney damage [30]. In this experiment, the lack of proteinuria data represents a limitation.

Regarding hyperglycemia, ECM accumulation in the glomerular and tubular compartments contributes to the progression of DKD into ESKD [31,32]. The mesangial matrix consists mainly of collagen IV and fibronectin. The tubular interstitial matrix is composed mainly of collagens I [33]. TRPC6 was found to be distributed in kidney tubules and glomeruli by immunofluorescence [34]. After knockdown of TRPC6, collagen I and collagen

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**Figure 4.** TRPC6 protein and mRNA levels were significantly increased in HG-treated GMCs. Representative western blotting and quantification of the expression of TRPC6 in HG-treated GMCs (A). qRT–PCR detection of TRPC6 mRNA in HG-treated GMCs (B) (n = 5 in each group, *p < 0.05, **p < 0.01).

**Figure 5.** Fibronectin, collagen IV, and NFAT2 were significantly upregulated in HG-treated GMCs. Representative western blotting and quantification of the protein expression of fibronectin (A), collagen IV (B), and NFAT2 (C) in HG-treated GMCs (n = 5 in each group, *p < 0.05, **p < 0.01, ***p < 0.001).
IV protein expression levels in kidney tissues of DM mice decreased, indicating that TRPC6 plays an important role in glomerular and kidney tubular injury caused by diabetes. Recent studies have shown that glomerulotubular balance and tubuloglomerular feedback affect the progression of DKD [35]. Therefore, we hypothesize that TRPC6 shRNA administration can alleviate ECM accumulation under hyperglycemia through direct glomerular infection or indirectly through tubuloglomerular feedback.

In our study, we focused on GMCs. GMCs in the interstitial cells in the kidney glomerulus are involved in the processes of DKD. In the priming of DKD, GMC activation leads to cell proliferation, overproduction of ECM proteins and thickening of the glomerular basement membrane [36,37]. Therefore, GMC activation is an indispensable factor in the initial pathophysiological changes of early DKD, but those changes will ultimately lead to glomerular sclerosis and kidney failure [38]. In our study, we observed that knockdown of TRPC6 by

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**Figure 6.** TRPC6 siRNA reversed HG-induced TRPC6 upregulation in GMCs. Representative Western blotting and quantification of endogenous Trp6 expression in GMCs transfected with NC siRNA or TRPC6 siRNA (A). GMCs were transfected with NC siRNA or TRPC6 siRNA for 24 h, followed by exposure to HG for 48 h (B,C). Representative western blotting and quantification of the expression of TRPC6 (B). qRT–PCR detection of TRPC6 mRNA (C) (n = 5 in each group, *p < 0.05, ***p < 0.001).

**Figure 7.** Effects of TRPC6 siRNA on ECM production in HG-treated GMCs. GMCs were transfected with NC siRNA or TRPC6 siRNA for 24 h followed by exposure to HG for 48 h. Representative western blotting and quantification of fibronectin (A), collagen IV (B), and NFAT2 (C) protein expression in HG-treated GMCs (n = 5 in each group, *p < 0.05, **p < 0.01).
siRNA reduced ECM overproduction induced by HG. In neonatal pig GMCs, hyperforin-induced activation of the TRPC6 channel inhibited GMC proliferation and triggered apoptotic pathways [39]. We hypothesize that the intracellular signal transduction mediated by the TRPC6 channel might vary depending on the cell types or different pathological stimuli.

NFAT is expressed in various tissues and can regulate the expression of various genes involved in the cell cycle and apoptosis, so it is considered to be essential for modulating cell survival, differentiation, and proliferation [40]. In cardiac cells, the increase in intracellular Ca\(^{2+}\) caused by TRPC6 channel opening is able to activate NFAT, and NFAT activation stimulates pathological cardiac hypertrophy [21]. In the kidney, TRPC6 channel opening-mediated activation of NFAT2 initiates downstream gene transcription and is associated with glomerulosclerosis [41,42]. TRPC6 activation is also observed to promote hyperforin-induced GMC apoptosis via the calcineurin/NFAT and FasL/Fas signaling pathways [41], and CD36-mediated TRPC6/NFAT2 signaling activation is involved in palmitate-induced human GMC fibrosis in type 2 DKD [22]. Consistent with these observations, our study showed that NFAT2 and TRPC6 expression levels were both increased in the kidneys of STZ-induced diabetic rats and in HG-treated GMCs, and the elevation in NFAT2 was largely abrogated by TRPC6 knockdown. In a follow-up study, we will further explore whether knockdown of NFAT2 can alleviate diabetic nephropathy.

In summary, our study indicates that TRPC6 is essential for the onset and progression of GMC dysfunction under HG conditions via NFAT2, and inhibition of TRPC6/NFAT2 signaling may provide new therapeutic strategies for the treatment of DKD.

**Conclusion**

In summary, our study shows that hyperglycemia induces GMC dysfunction through TRPC6/NFAT2 signal activation in type 1 DKD, suggesting that pharmacological targeting of TRPC6/NFAT2 signaling may represent an attractive therapeutic strategy for manipulating DKD, at least in the early stage.

**Consent for publication**

All authors agreed on the submission and the copyright policies of the journal.

**Ethical approval**

All animal studies (including the euthanasia procedure) were approved by the Animal Welfare and Ethics Committee of Wannan Medical College.

**Disclosure statement**

All authors declare there is no conflict of interest in this study.
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