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Increased TRPC6 expression is associated with tubular epithelial cell proliferation and inflammation in diabetic nephropathy

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ABSTRACT

Although TRPC6 expression is shown to be significantly elevated in a rat model diabetic nephropathy (DN), its expression and role in human DN are unclear. We thus explored the role of TRPC6 in the pathophysiology of tubular epithelial cell injury following DN. HK-2 cells were cultured in a high-glucose medium to induce a DN cell model. Ad-TRPC6 and TRP6 siRNA were transfected to overexpress and knock down TRPC6. We found that TRPC6 expression was significantly upregulated in DN tissues and cells. TRPC6 siRNA inhibited cell proliferation and promoted cell apoptosis in HK-2 cells treated with high glucose, whereas Ad-TRPC6 showed the opposite effect. Furthermore, Ad-TRPC6 significantly promoted release of IL-8 and IL-6. Subsequent experiments demonstrated that the signaling pathway of nuclear factor of activated T cells (NFAT) was activated by Ad-TRPC6 and deactivated by TRPC6 siRNA. The NFAT signaling inhibitor, FK-506, eliminated the effect of TRPC6 on HK-2 cells. These results suggest that TRPC6 was upregulated in DN and could promote cell proliferation and inflammation by inhibiting the NFAT signaling pathway in tubular epithelial cells.

1. Introduction

Diabetic nephropathy (DN) is a common complication of diabetes characterized by poor renal function and increased albuminuria, leading to end-stage renal disease and chronic renal failure (Cade, 2008; Shaheen and Al-Khader, 2005). About 40% of patients with diabetes worldwide have DN (Packham et al., 2012). Although it is well known that the initiating factor of kidney damage is glomerular injury in DN, (Okamura et al., 2011) recently found that tubular injury is correlated with DN progression and a key cause of chronic kidney injury. Moreover, apoptosis of tubular epithelial cells (TECs) is thought to be a crucial injurious event that triggers kidney injury in DN and contributes to tubular atrophy and interstitial fibrosis (Habib, 2013). However, little is known about the molecular mechanisms of tubular apoptosis in DN.

As a member of the transient receptor potential family of calcium channels, transient receptor potential channel 6 (TRPC6) is a receptor-activated, nonselective, cation channel that is homogeneously expressed throughout the central nervous system and peripheral tissues, including the kidneys (Riccio et al., 2002). It has been suggested that TRPC6 plays a significant role in diabetes (Graham et al., 2007; Graham et al., 2011) and DN (Ilatovskaya et al., 2015; Ma et al., 2015). Our previous bioinformatics study identified TRPC6 as an upregulated and differentially expressed gene in DN pathogenesis, suggesting that it may be a novel target for therapy (Ma et al., 2015). However, the role of TRPC6 expression in DN has not been investigated. The primary target of DN is renal TECs (Liu et al., 2015). TRPC6 expression has been reported in the cytoplasm of renal TECs (Liu and Ji, 2012).

Several signaling pathways, including RhoA and Ca^{2+} signaling, have been described as downstream effectors of TRPC6 channel activation (Singh et al., 2007; Tauseef et al., 2012). Nuclear factor of activated T cells (NFAT) is regarded as a Ca^{2+}-dependent transcription factor (Im and Rao, 2004), and recent studies indicated that NAFT signaling could be activated by overexpression of TRPC6 (Soni and Adebiyi, 2016). It has been well demonstrated that NFAT is crucial for controlling cellular energy homeostasis, and it can regulate cell survival, proliferation, apoptosis, migration, and differentiation in pulmonary arterial smooth muscle cells, cancer, and neural precursor and progenitor cells (Huang et al., 2011; Serrano-Perez et al., 2015). (Schlondorff et al., 2009) announced that TRPC6 might induce neonatal glomerular mesangial cell apoptosis by triggering ectopic activation of the NFAT pathway. However, the role of the NFAT signaling pathway in

Abbreviations: DN, diabetic nephropathy; NFAT, nuclear factor of activated T cells; qRT-PCR, quantitative real-time PCR; TECs, tubular epithelial cells; TRPC6, transient receptor potential channel 6

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ND is not fully understood.

Therefore, the present study aimed to assess expression of Trpc6 in patients with DN and in cells induced with DN by culturing in high glucose. The effects of Trpc6 ectopic expression on cell proliferation, apoptosis, and inflammatory cytokine release of TECs were estimated in vitro. Finally, involvement of the NFAT signaling pathway was investigated to explore the mechanisms by which Trpc6 modulates cell proliferation and apoptosis in TECs.

2. Materials and methods

2.1. Subjects

The kidney specimens (located > 5 cm away from the disease sites) were obtained from 35 patients with DN and 37 nondiabetic patients with kidney cancer who were consecutively recruited from the department of endocrinology at the Second Affiliated Hospital of Zhengzhou University. All patients with type 2 diabetes were between 35 and 75 years of age, and DN was diagnosed by pathological renal biopsy. The study was approved by the Human Ethical Review Committee, the Second Affiliated Hospital of Zhengzhou University, Henan, China, and performed according to the Declaration of Helsinki. Informed consent was provided by all involved patients.

2.2. Cell culture

Human renal TECs (HK-2 cells) from ATCC (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) under standard conditions. The cells were exposed to 100 μg/ml of nonglycated control bovine serum albumin (Co-BSA) or AGE-BSA (2221-10; BioVision, Mountain View, CA, USA) in an incubator with 5% CO_2 at 37 ° C for at least 2 weeks. High glucose was supplemented in the culture medium to induce a DN cell model. Mannitol was added to the culture medium as a control. After 24 h, differentiated TECs were divided into three groups: NG (normal glucose, 5.6 mmol/L), HG (high glucose, 30 mmol/L), and HM (NG + mannitol 25 mmol/L).

2.3. Cell transfection

To obtain suitable siRNA to knockdown TRPC6 expression, fragments of siRNA against human TRPC6 were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A non-specific siRNA served as a negative control (NC siRNA, Sense: 5′-UAAGGCUA UGAAGAGAUAC-3′, Anti-sense: 3′-AUUCCGAUACUUCUCUA UG-5′). The sequences for TRPC6 siRNAs were as follows: TRPC6 siRNA Sense: 5′-GGACCAGCAUACAUGUUUAdTdT-3′, Anti-sense: 3′-dTdTCCUGGUC GUAUGUACAAAU-5′. HK-2 cells were transfected with TRPC6 siRNA or NC siRNA using Lipofectamine 3000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Untreated cells with no transfection were used as the normal group.

A replication-defective adenoviral vector encoding wild-type TRPC6 (Ad-TRPC6) was constructed using the Qbiogene AdEasy Vector System (Qbiogene, Nottingham, UK), as previously described (Hamdollah Zadeh et al., 2008). Adenoviral vectors expressing green fluorescence protein (Ad-GFP) were used as controls for virus infection. After infection for 48 h, the NFAT signaling inhibitor FK-506 was purchased from Astellas Ireland Co. Ltd. (Killorglin, Ireland) and diluted in culture medium.

2.4. Reverse transcription and real-time PCR

Cells were harvested, and total RNA was extracted using Trizol reagent (Invitrogen). RNA samples were analyzed by electrophoresis and quantified. About 1 μg of RNA was used to synthesize cDNA using the M-MLV reverse transcriptase system (Takara, Japan). Subsequently, PCR amplifications were performed using SYBR® Premix Ex TaqTM (Takara) with MX3000P real-time PCR system (Stratagene). Amplification cycles were 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. The primer sequences used to detect TRPC6 were as follows: forward, 5′-TGGCAAGTCCAGCATACCTGTC-3′ and reverse, 5′-GTGTTTCTGCAGAGGTCCAGGAG-3′. NFATc2: forward, 5′-CTTCTCCAACACCAAAGTCC-3′ and reverse, 5′-CGTACCCGTGTGTT CTTCCT-3′. β-actin: forward, 5′-GACATCCGTAAAGACCTCTATGCC-3′ and reverse, 5′-ATAGAGCCACCAATC CACACAGAG-3′. Results were normalized to β-actin expression using the ΔΔC(t) method.

2.5. Western blot assay

Cells were harvested and then lysed in RIPA lysis buffer. The nuclear protein was extracted using a Nucleoprotein Extraction Kit (Sangon Biotech, China). Total protein concentrations were tested with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples (30–40 μg) were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. After blocking with nonfat dry milk, the sections were incubated with rabbit anti-TRPC6 polyantibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NFATc2 antibody (BD Pharmingen, San Diego, CA, USA), mouse anti-β-actin antibody or mouse anti-histone H3 antibody (Santa Cruz Biotechnology), followed by the respective horseradish peroxidase–conjugated secondary antibodies. Immunoreaction products were visualized with a diaminobenzidine kit (Zhongshan Bio-Tech Co., Beijing, China).

2.6. Cell counting Kit-8 assay

Cells in the logarithmic growth phase were placed in a 96-well plate, and cell proliferation was estimated by measuring the cell numbers at specific time points using a Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotechnology Co., Ltd.). In brief, the medium was replaced with 100 μL of fresh medium containing 10 μL of CCK-8 reagent and incubated for 2 h in a CO_2 incubator. Then, the OD value at 450 nm was determined by microplate reader (Bio-Rad Laboratories Inc.).

2.7. Apoptosis assay

To measure the influence of TRPC6 protein ectopic expression on apoptosis, apoptotic cells were detected using a cell death detection ELISA-Plus kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instruction. Absorbance was measured at 405 nm.

2.8. ELISA

Spontaneous IL-8 (Andreucci et al., 2010) and IL-6 (Gallo et al., 2014) released in supernatants of cells culture were measured using ELISA in accordance with the manufacturer's protocol.

2.9. Statistical analysis

Data are presented as means ± SEM. Statistical significance of differences between groups were assessed by *t* test and one-way ANOVA. *P* values < .05 were considered statistically significant. All experiments were performed independently at least three times.

3. Results

3.1. TRPC6 expression in DN patient tissues and HK-2 cells

To identify the role of TRPC6 in diabetic nephropathy, we analyzed TRPC6 expression in 35 patients with DN and 37 nondiabetic patients using qRT-PCR and Western blot analysis. The results showed that