TMEM16A exacerbates renal injury by activating P38/JNK signaling pathway to promote podocyte apoptosis in diabetic nephropathy mice

Running header: TMEM16A exacerbates renal injury by activating P38/JNK pathway in diabetic nephropathy mice

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Abstract

Diabetic nephropathy (DN) is one of the most common microvascular complication of diabetes mellitus (DM) as well as the main reason resulting in chronic renal failure. Transmembrane protein 16A (TMEM16A) plays an important role in multiple physiological actions. Here we found that it was up-regulated in high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) amplification, Western blot detection, Periodic Acid Schiff (PAS) staining and immunohistochemical analysis confirmed that TMEM16A deficiency alleviated renal injury in diabetic mice and TMEM16A knockout diabetic mice were protected from the HFD-induced reduction in Nephrin expression. To understand further the molecular mechanism of its function, podocytes treated with high glucose (HG, 30 mmol/L glucose) in *vitro* was chosen as a model to study its signal transduction pathway. Nephrin expression level in siRNA-TMEM16A group was significantly higher than that of the HG group (also called Model group). Flow cytometric analysis revealed that podocyte apoptosis in siRNA-TMEM16A group was significantly lower than that of the Model group. RT-PCR and Western blot exhibited that apoptosis-related genes including apoptosis-inducing factor (AIF) and cystinylaspartate specific protease-3/-9 (caspase-3/-9) were dramatically down regulated in siRNA-TMEM16A group, compared with Model group. Phosphorylation levels of P38 and JNK in siRNA-TMEM16A group were lower than that of the Model group. Thus, TMEM16A is one of the critical components of a signal transduction pathway that links renal

injury to podocyte apoptosis in DN.

Keywords: TMEM16A, diabetic nephropathy (DN), podocyte, apoptosis, P38/JNK

1. Introduction

DN is one of the most serious complications of DM, finally resulting in multiorgan failure and death for diabetic [1]. The major pathological changes of DN included glomerulus hypertrophy, glomerulus extracellular matrix accumulating, basement membrane thickening and nodular glomerulosclerosis [2-5]. The etiology and pathogenesis of DN are complex issues that involve glucose and lipid metabolism disorder, hemodynamic disorder, inflammatory mediator release, cell apoptosis and so on [6,7]. However, research of the pathogenesis of DM has been mainly focused on renal tubular epithelial cell transdifferentiation, renal interstitial fibrosis and mesangial cells function disorder [8,9]. With the establishment of podocyte cell line and the development of biological techniques, podocytes have been studied in depth. Research has suggested that podocyte lesion and podocyte apoptosis are closely to proteinuria, glomerulosclerosis and renal tubule-interstitial fibrosis [10,11]. Therefore, podocyte lesion may play important roles in the development of DN. Podocyte is a terminally differentiated epithelial cell, which involved in maintaining filtration barrier in the big molecule protein and glomerular [12-14]. We may come to the conclusion that podocyte apoptosis is a practical problem urgently to be solved.

TMEM16A silencing in vitro or vivo can inhibit the function of calcium-activated chloride channels. Studies have found TMEM16A is expression in lung, kidney,

pancreas and mediates many biologic actions such as exocytosis and nervous excitation [15-17]. In addition, high TMEM16A expression was prevalent in esophageal cancer, bladder cancer and breast cancer. These studies suggest that TMEM16A is closely related to cell division cycle and cell proliferation [15-18]. We suspect that TMEM16A may be related to apoptosis. In this study, we explore the possibility that TMEM16A may play a role in the development of DN TMEM16A knockout diabetic mice, and we assume that TMEM16A is related to podocyte apoptosis in DN.

2. Materials and methods

2.1 Animal studies

Eight-week-old wild type C57BL/6J mice were purchased from BRL⁺ Bio-Technique Co. Ltd (Shanghai, China). Eight-week-old TMEM16A^{-/-} mice were purchased from Cyagen Bio-Technique Co. Ltd (Suzhou, Jiangsu, China). Animal experiments were conducted accordance with the local ethics committee of lab animal center (Wuhan University, China), which follows the China Public Health Service's guide for care and use of animals. A total of 18 wild type (WT) male mice were randomly into 2 groups as follows: normal diet group (Norm-diet) and HFD/STZ group. A total of 18 TMEM16A^{-/-} male mice were randomly into 2 groups as follows: normal diet group (Norm-diet) and HFD/STZ group. In HFD/STZ group, WT mice and TMEM16A^{-/-} mice were place on HFD (19.7 kJ/g, 45% of energy as fat, Research Diets, Cyagen), and after 4 weeks of HFD feeding, the mice were injected intraperitoneally with low-dose STZ (100 mg/kg) to establish DN model [19]. All

mice had freely access to food/water and were maintained for 14 weeks of HFD feeding in accordance with the institutional animal care and use committee procedures of University. The normal diet-fed mice were used as nondiabetic controls. All mice were weighed weekly. By 10 weeks after STZ injection, urine samples over 24 h were collected. Then venous blood was collected via cutting off tail to detect blood glucose, triglyceride, free fatty acid and creatinine after 8 h of fasting subjects.

2.2 Cell culture and glucose treatment

Conditionally immortalized mouse podocytes were purchased from Yubo Bio-Technique Co. Ltd (Shanghai, China) and were cultured as described previously [20]. Murine podocytes were cultured in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibico, NY, Grand Island), 100 U/ml penicillin and 100 µg/ml streptomycin (Solarbio, Beijing, China). Cells were grown at 33 °C in a 5% CO₂ incubator (Heraeus, Japan) in the presence of 40 units/mL interferon-γ (IFN-γ, Sigma, St Louis, MO, USA). To induce differentiation, podocytes were maintained at 37 °C without interferon for 14 days. Podocytes were seeded in a 6-well plate at a density of 3×10^5 cells/mL with complete culture medium. After stable for 24 hours, podocytes were exposed to media containing 40 mM of D-glucose for 24 h [21], and then cells were harvested for further experimental study.

2.3 PAS staining

Formalin-fixed hemisected kidneys were embedded in paraffin and stained with PAS. Paraffin-embedded histological sections cut at 4 µm thickness were taken after

series of graded ethanol washings and deparaffinization. Then sections were placed in 1% periodic acid for 10 min followed by water wash. Placed under the conditions of protection from light accession Schiff's reagent (Sigma-Aldrich, USA) at room temperature reaction for 30 min, followed by washing with 0.5% potassium metabisulfite (Shifeng Bio-Technique Co. Ltd. Shanghai, China) for 2 min, washed thoroughly in water and mounted under coverslips in Glycergel. Glomerular volume and lipid accumulation were quantified by scanning 20 tangential glomeruli from each kidney section with Image J software and expression the oil red O positive area as a percentage of the total area.

2.4 Immunohistochemistry

Immunohistochemistry was performed according to the manufacturer's instructions (ZSGB Bio-Technique Co. Ltd, Beijing, China). Briefly, paraffin-embedded sections were deparaffinized with xylene and then rehydrated in a descending series of ethanol washes. Place the sections into the boil in 600 mL sodium citrate buffer for 20 min, and maintained at a room temperature to cool. The sections were treated with 3% H₂O₂ in methanol for 15 min and then were blocked with 10% normal goat serum for 10 min at 37 °C. The sections incubated with the primary antibodies to TMEM16A and Nephrin (Abcam) for overnight at 4 °C. Cell in see positive expression of brown-yellow granules.

2.5 RNA interference and cell transfection

Murine podocytes with D-glucose were seeded onto 12-well culture plates. The TMEM16A siRNA or control siRNA, both purchased from Genepharma (Shanghai,

China), were then transfected into murine podocytes with D-glucose at 60% confluency by using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The transfected cells were collected at 48 h after transduction and stored at -80 °C for the subsequent experiments.

2.6 Flow cytometry analysis

Podocytes were seeded onto 6-well plate at a density of 3×10^5 per well and were cultured for 24 h, podocytes were exposed to 40 mM of D-glucose (Model) and control siRNA (Model+mock) or 40 mM of D-glucose and TMEM16A siRNA (siRNA-TMEM16A). After 48 h, podocytes were collected and washed with PBS. Afterwards, podocytes were incubated with 5 μ l of Annexin V stock solution and 5 μ l of Propidium iodide (PI) (BD, San Jose, CA) for 30 min. Flow cytometric analysis the cell apoptosis (BD Bioscience).

2.7 RNA isolation and RT-PCR

Total RNA was extracted from the kidneys of mice and podocytes using TRIzol (Invitrogen), respectively. Then 2 μ g of RNA was used for complementary (cDNA) synthesis with a first strand cDNA kit (TransGen Biotech, Beijing, China), according to the manufacturer's protocol. PCR amplification was executed in ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA), using a SYBR Green PCR kit (Thermo). The PCR cycles were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, annealing/extension at 60 °C for 45 s. The primers were designed by Shanghai Sangon Company (Shanghai, China). The specific primer sequences for each gene were listed as the follows: 5' GAAGCGGAAGCAGCGCTATG 3' and 5'

AGTGGAGCCAGAGGGAAGGA 3' for TMEM16A (product: 132 bp); 5' GCCCACGGATGAGACAGTCA 3' and 5' GGCATCACTGAAGGCTTCGC 3' for Nephrin (product: 124 bp); 5' GCGGGTGCTTTCAAGCAGAA 3' and 5' TCCCAGAAGCACCTGCAGAC 3' for AIF (product: 145 bp); 5' CAGGGCACACAGGACTTGGGA 3' and 5' AGAGCCAGCAGTGAAGTCTCAGC 3' for caspase-3 (product: 139 bp); 5' GGCAGGCCCTTCCTCTCTTC 3' and 5' CAGGCACCAGGTGGTCTAGG 3' for caspase-9 (product: 135 bp) and 5' TGGCCTTCCGTGTTCTACC 3' and 5' TTCAGTGGGCCCTCAGATGC 3' for GAPDH (product: 121 bp). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.8 Western blot

Protein lysates were separated in 10% sodium dodecyl sulfate–polyacrylamide gels, electroblotted onto to a polyvinylidene fluoride membrane (Roche Diagnostics, Mannheim, Germany), then detected with TMEM16A, Nephrin, AIF, caspase-3, caspase-9, P38, phosphorylated (p-) P38, JNK, p-JNK and GAPDH proteins. Protein loading was estimated using mouse anti-GAPDH. Lab Works Image Acquisition and Analysis Software (UVP, Upland, CA, USA) were used to quantify band intensities. Antibodies were purchased from Cell Signaling Technology, Beverly, MA 01915, USA.

2.9 Statistical analysis

All experiment data are expressed as means $\pm$ SE. Any differences between two groups were evaluated by Student's t-test for unpaired variables and those among

three or four groups in vitro study were analyzed by one-way ANOVA and subsequent Tukey's test. Probability (P) values <0.05 was considered statistically significant.

3. Results

3.1 HFD/STZ-induced diabetic mice had been established successfully

As shown in Table 1, body weight was no significant difference between four groups. Compared with Norm-diet group in WT mice, kidney weight and kidney index were increased ($P<0.05$). Blood glucose, triglyceride and FFA of HFD/STZ groups in WT mice and TMEM16A^{-/-} mice were dramatically increased compared to that of Norm-diet group ($P<0.05$).

There was no significant difference in creatinine between four groups after HFD/STZ treatment. 24-h urinary albumin excretion (UAE) was significantly increased in HFD/STZ group of WT mice and TMEM16A^{-/-} mice. The blood glucose level higher than 16.7 mmol/L and positive urinary protein are considered DN mice [25]. According to our results, DN models are successfully established by HFD/STZ treatment can be used in further experimental study.

3.2 An intensive expression of TMEM16A was found in DN mice

In WT mice, the expression level of TMEM16A in HFD/STZ group was higher than that of Norm-diet group (Figure 1A-D). Positive expression of TMEM16A was observed in paraffin-embedded sections of kidney tissues in HFD/STZ group by immunohistochemical staining, and the average optical density was significantly decreased in HFD/STZ group, compared to Norm-diet group (Figure 1E and 1F). Upregulation of TMEM16A is observed in HFD/STZ group of WT mice.

3.3 Lowly expressed Nephrin was observed in HFD/STZ group of TMEM16A^{-/-} mice

As shown in Figure 2, Nephrin expressions in HFD/STZ group of WT mice and TMEM16A^{-/-} mice were lower than that of Norm-diet group, Nephrin expressions in HFD/STZ group of TMEM16A^{-/-} mice were higher than that of HFD/STZ group in WT mice ($P<0.05$; Figure 2A-C). Immunohistochemistry verified that there was significantly less of positive staining in HFD/STZ group of WT mice and TMEM16A^{-/-} mice than in the Norm-diet group (Figure 2D). PAS staining results showed that the area of glomerular mesangium matrix in HFD/STZ group was more than that of Norm-diet group. In addition, there was no significantly difference in mesangial matrix index between HFD/STZ group of WT mice and HFD/STZ group of TMEM16A^{-/-} mice (Figure 2E).

3.4 TMEM16A interference up-regulated Nephrin expression in podocytes with high-glucose

As is shown in Figure 3A-C, the expression levels of TMEM16A were significantly decreased in siRNA-TMEM16A group in comparison with Model group and Model+mock group. In addition, the expression levels of Nephrin in siRNA-TMEM16A group were lower than that of control group, but Nephrin expression in siRNA-TMEM16A group was significantly higher than Model group and Model+mock group.

3.5 TMEM16A interference reduced the apoptosis and decreased the expression levels of apoptosis related factors in podocytes with high-glucose.

Flow cytometric analysis revealed that the apoptosis rate of Model group and

Model+mock group were higher than that of control group, and the apoptosis rate of siRNA-TMEM16A group obviously lower than that of Model group and Model+mock group (Figure 3D and 3E). As shown in Figure 3FGH, the expression levels of apoptosis related factors including AIF and caspase-3/-9 were significantly increased in Model group and Model+mock group, comparison with control group. The expression levels of AIF and caspase-3/-9 in siRNA-TMEM16A group were dramatically lower than that of Model group and Model+mock group.

3.6 TMEM16A interference suppressed the activation of P38/JNK signaling pathway in podocytes with high-glucose.

As shown in Figure 4, podocytes of control group expressed low level of phosphorylated P38 and JNK proteins and podocytes of Model group and Model+mock group expressed high level of the phosphorylated P38 and JNK proteins. In addition, compared with Model group and Model+mock group, the phosphorylation protein levels of P38 and JNK in siRNA-TMEM16A group were significantly decreased.

Discussion

Studies have shown that macroalbuminuria in DN are associated with podocytes apoptosis, podocytes destroyed can cause macroalbuminuriab [11,27]. High TMEM16A expression is prevalent in various tumor tissues and is closely related to cell cycle and cell proliferation, and its roles in DN is to be elucidated [28,29]. Therefore, this study used HFD/STZ-induced animal models to explore the effects of TMEM16A on DN. Because STZ is hard to induce DN, HFD combined with STZ are

used for inducing obesity and insulin-resistance. Mice on a HFD have obviously proteinuria and glomerular injury. In our results, the blood glucose level of WT mice was significantly increased while urinary protein was positive after HFD/STZ treatment (Table 1), and then, it can aggravate kidney dysfunction while giving HFD and STZ stimulation [30]. The expression level of TMEM16A in WT mice of HFD/STZ group was detected by means of RT-PCR, Western blot and immunohistochemical staining, compared with WT mice of Norm-diet group, high expression of TMEM16A mRNA and protein was found in WT mice of HFD/STZ group. TMEM16A is highly induced in tubular epithelium in kidney (Figure 1). In HFD/STZ-induced DN model, kidney had injured, while the expression levels of TMEM16 were significantly increased. The high expressed TMEM16A may exacerbate kidney injury whereby damaging the tubular epithelium. This is an indication that TMEM16A may play a key role in the occurrence and development of DN. The occurrence of albuminuria is closely associated with impaired filtration barrier function of glomerulus. Nephrin plays a key role in maintaining the normal development and function of glomerulus which is specifically expressed in slit diaphragm of glomerulus [31], and the expression level of Nephrin is significantly decreased in DN patients. It shows that the expression level of Nephrin can embody glomerulus function of DN patients. The lower expression of Nephrin was found in kidney of WT mice with HFD/STZ than in kidney of WT mice with norm-diet. Compared with HFD/STZ group of WT mice, the expression level of Nephrin in HFD/STZ group of TMEM16A^{-/-} mice was significantly increased (Figure 2A-D).

The extension of mesangial matrix is one of the major characteristics of histopathologic change in DN. The mesangial matrix index of TMEM16A^{-/-} mice with HFD/STZ was lower than that of WT mice with HFD/STZ (Figure 2E). These results showed that TMEM16A deletion alleviates kidney injury and prompted that TMEM16A may be involved in the process of development in kidney injury of DN. The results showed that Nephritin expressions in Model group was significantly lower than that of control group while the expression level of Nephritin in siRNA-TMEM16A group was significantly higher than that of Model group (Figure 3A-3C). It showed that TMEM16A expression level can affect Nephritin expression level significantly. High expression of TMEM16A inhibits Nephritin expression level from another side proved that TMEM16A overexpression exacerbates glomerular injury. To prove it, we detected podocytes apoptosis by means of flow cytometry. The results showed that apoptosis degree of siRNA-TMEM16A group was significantly lower than that of Model group and Model+mock group (Figure 3D and 3E). The expression levels of apoptosis related proteins including AIF and caspase-3/-9 were detected. AIF is flavoprotein that located in mitochondria, having double function as performing oxidation reduction and promoting cell apoptosis. Caspase play a crucial role in the process of apoptosis. Our results showed that the expression levels of AIF and caspase-3/-9 in Model and Model+mock groups were significantly increased, and the expression levels of AIF and caspase-3/-9 in siRNA-TMEM16A group were dramatically decreased (Figure 3F-3H). Taken together, TMEM16 overexpression can aggravate kidney injury whereby promoting podocytes apoptosis. P38/JNK signaling

pathway is an important part of mitogen-activated protein kinase family and is one of apoptotic signaling transduction pathways. In our results, the p-P38 and p-JNK proteins in Model and Model+mock groups were significantly higher than that of control group, the levels of p-P38 and p-JNK proteins in siRNA-TMEM16A group were significantly lower than that of Model and Model+mock groups (Figure 4). It showed that high expression of TMEM16A can activate the P38/JNK signal pathway.

In conclusion, TMEM16A is one of the critical components of a signal transduction pathway and can exacerbate renal injury caused by podocyte apoptosis by activating P38/JNK signaling pathway in DN mice.

Disclosure

The authors report no conflicts of interest in this work

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Table 1 Physical and biochemical parameters of experimental animals

	Wild-type mice		TMEM16A ^{-/-} mice	
	Norm-diet	HFD/STZ	Norm-diet	HFD/STZ
Body weight (g)	24.22±1.44	28.32±2.12	26.4±1.71	29.51±2.26
Kidney weight (g)	0.13±0.01	0.28±0.02 [*]	0.18±0.01	0.33±0.02 ^{**##}
Kidney index (%)	0.58±0.02	0.98±0.22 [*]	0.61±0.13	1.01±0.4 ^{**#}
Blood glucose (mmol/L)	5.9±0.95	22.79±1.34 [*]	5.2±0.42	17.1±1.68 ^{*#}
Triglyceride (mg/dL)	136.7±10.15	298.77±17.33 ^{**}	140.7±13.2	230.7±20.37 ^{**##}
FFA (mmol/L)	1.63±0.15	3.67±0.66 [*]	1.56±0.34	2.98±0.54 ^{*#}
Creatinine (μmol/L)	63.27±4.14	70.05±5.22	64.9±3.57	72.63±4.38
UAE (μg/24h)	38.92±3.27	261.03±10.11 ^{**}	37.07±2.84	173.27±14.72 ^{**##}

Mice were fed a HFD for 4 weeks and then injected with a single low dose of STZ

(intraperitoneal at 100mg/kg) followed by continued HFD feeding for an additional

10 weeks. Values are means ± SD for 9 mice in each group.

* $P < 0.05$ and ** $P < 0.01$ vs. Norm-diet groups of wild-type mice and TMEM16A^{-/-}

mice; # $P < 0.05$ and ## $P < 0.01$ vs. HFD/STZ group of wild-type mice.

Figure 1

An intensive expression of TMEM16A was found in HFD/STZ-induced diabetic wild-type mice. (A and B) RT–PCR analysis of TMEM16A mRNA levels in kidney tissues of HFD/STZ-induced diabetic wild-type mice; (C and D) Western blot analysis of TMEM16A protein levels in kidney tissues of HFD/STZ-induced diabetic wild-type mice; (E and F) Immunohistochemical analysis of TMEM16A positive expression in kidney tissues of HFD/STZ-induced diabetic wild-type mice. GAPDH was also detected as the control of sample loading. Data were presented as means $\pm$ SD, n=3, ** P <0.01 vs. Norm-diet groups of wild-type mice. magnification 20 $\times$.

Figure 2

Loss of TMEM16A expression reversed kidney injury of HFD/STZ-induced diabetic mice. (A) RT–PCR analysis of Nephlin mRNA levels in kidney tissues of Norm-diet and HFD/STZ groups of wild-type mice and TMEM16A^{-/-} mice; (B and C) Western blot analysis of Nephlin protein levels in kidney tissues of Norm-diet and HFD/STZ groups of wild-type mice and TMEM16A^{-/-} mice; (D) Immunohistochemical analysis of Nephlin positive expression in kidney tissues of Norm-diet and HFD/STZ groups of wild-type mice and TMEM16A^{-/-} mice; (E) PAS staining analysis of glomerular injury in kidney tissues of Norm-diet and HFD/STZ groups of wild-type mice and TMEM16A^{-/-} mice. GAPDH was also detected as the control of sample loading. Data were presented as means $\pm$ SD, n=3, ** P <0.01 vs. Norm-diet groups of wild-type

mice and TMEM16A^{-/-} mice; ^{##}*P*<0.01 vs. HFD/STZ group of wild-type mice. magnification 40×.

Figure 3

TMEM16A interference up-regulated Nephrin expression levels and inhibited podocyte apoptosis. (A) RT–PCR analysis of Nephrin and TMEM16A mRNA levels in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte; (B and C) Western blot analysis of Nephrin and TMEM16A protein levels in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte; (D and E) Flow cytometry analysis of apoptosis rate in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte; (F) RT–PCR analysis of AIF and caspase-3/-9 mRNA levels in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte; (G and H) Western blot analysis of AIF and caspase-3/-9 protein levels in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte. GAPDH was also detected as the control of sample loading. Data were presented as means ± SD, *n*=3, **P*<0.05 and ***P*<0.01 vs. Control group; ^{##}*P*<0.01 vs. Model group and Model+mock group.

Figures 4

TMEM16A interference suppressed the activation of P38/JNK signaling pathway in podocyte. (A and B) Phosphorylation levels of P38 in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte were assessed by Western blot using anti-phospho-P38 antibody and antibody to P38; (C and D) Phosphorylation levels of

JNK in control, Model, Model+mock and siRNA-TMEM16A groups of podocyte were assessed by Western blot using anti-phospho-JNK antibody and antibody to JNK. Data were presented as means $\pm$ SD, n=3, * P <0.05 and ** P <0.01 vs. Control group; ## P <0.01 vs. Model group and Model+mock group.

Figure 1

Figure 2

Figure 3

Figure 4