

Original Article

APPL1 acts as a protective factor against podocytes injury in high glucose environment

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Abstract: APPL1, an intracellular adaptor protein, takes part in numerous metabolic reactions. Although APPL1 plays a key role in glucose metabolism via adiponectin pathway and has been proved associated with type 2 diabetes, little is known about its role in diabetic nephropathy. To explore the role of APPL1 in diabetic nephropathy, we upregulated the expression of APPL1 in cultured mouse podocytes by adenovirus infection and tested the effects of APPL1 overexpression in podocytes treated with high glucose. Here, a mouse podocyte cell line (generated from H-2Kb-tsA58 immortomouse) was cultured and divided into four groups: Group 1 (normal glucose, NG), Group 2 (high glucose, HG), Group 3 (HG and infected with control adenovirus) and Group 4 (HG and infected with Ad-APPL1). Cell vitality of Group 4 is significantly higher than Group 2, but notably lower than Group 1 ($P < 0.01$). The apoptosis rate of Group 4 was much lower ($P < 0.01$) than Group 2 and Group 3. A decrease in phase G0/G1 and an increase in phase S was observed in Group 4 compared with Group 2 ($P < 0.01$). These data suggested the protective role of APPL1 overexpression in high glucose condition. Moreover, the levels of Nephryn, AMPK and p-AMPK were decreased by high-glucose treatment, but increased by APPL1 overexpression. In conclusion, in the experimental high glucose condition, APPL1 acts as a protective factor against podocytes injury through regulating AMPK signaling, and may be a new therapy target for diabetic nephropathy.

Keywords: APPL1, podocytes, high glucose, AMPK

Introduction

APPL1 (Adaptor protein containing a PH domain, PTB domain and leucine zipper motif 1) is an intracellular transition protein that takes part in various metabolic reactions. The function of APPL1 in adiponectin pathway has been clearly clarified [1]. APPL1 directly interacts with the adiponectin receptor 1 (AdipoR1) via its PTB domain. Overexpression of APPL1 increases adiponectin signaling and adiponectin-mediated downstream events, such as the membrane translocation of glucose transport 4 (GLUT4) and glucose uptake. Overexpression of PTB-domain deleted APPL1 or silencing of APPL1 reduces adiponectin signaling and downstream events [1]. These data indicate that APPL1 plays a key role in glucose metabolism via adiponectin pathway.

Type 2 diabetes is a metabolic disorder that is characterized by hyperglycemia in the context

of insulin resistance and relative lack of insulin. The pathogenesis of type 2 diabetes has been explored extensively. Several mechanisms, including insulin resistance, cell dysfunction, inflammation, oxidative stress and endothelial damage, are revealed based on glucometabolic disorder [2]. As a key point in glucometabolic regulation system, APPL1 has been proved associated with type 2 diabetes. In APPL1 knockout mice model, deficiency of APPL1 was shown to impair glucose-stimulated insulin secretion [3]. In another study, APPL1 was observed counteracted obesity-induced insulin resistance and dysfunction in endothelial cells [4]. Moreover, in genetic level, the variability of APPL1 in single nucleotide polymorphisms increased the risk of coronary artery disease in type 2 diabetic patients [5].

Type 2 diabetes is a chronic disease associated with a ten-year-shorter life expectancy, which is partly due to a number of diabetic complica-

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tions [6]. Diabetic nephropathy is one of the most common diabetic complications. Recent studies suggest that podocyte injury is an important component of diabetic nephropathy. Podocytes, also known as glomerular visceral epithelial cell, is essential for the selective filtering function of the glomerular capillary wall [7]. During the course of diabetic nephropathy, morphological changes of injured podocytes firstly occur, accompanied with the decrease of structural components of the podocyte and the slit diaphragm, such as a3b1 integrin and Nephlin. Sustained damage of podocytes may lead to the detachment of podocytes from the glomerular basement membrane (GBM) and shedding into urine [8]. Previous study pointed out that administration of adiponectin in cultured adiponectin knockout podocytes increased the activity of AMPK and reduced the damage of podocytes [9]. APPL1 is regarded as a key link in adiponectin-signaling pathway between AdipoR1 and AMPK [1]. It is reasonable to suppose that APPL1 may involve in podocytes protection. In order to verify this hypothesis, in this study, we overexpressed APPL1 in cultured mouse podocytes, and observed the subsequent changes of proliferation, cell cycle, apoptosis and key protein expression of podocytes cultured in high glucose media. Our data suggested that APPL1 could protect podocytes from high-glucose induced injury and shed light on the therapy of diabetic nephropathy.

Materials and methods

Construction of adenoviral vectors expressing APPL1

Recombinant adenovirus expressing APPL1 were then generated using the AdEasy technology as previously described [10]. The mouse APPL1 gene (NCBI Reference Sequence: NM_145221.2) was amplified by reverse transcriptase polymerase chain reaction. The primer pairs were as follows: forward primer, 5'-ATAAGAAT GCGGCCGC ATGCCGGGGATCGA-CAAG-3'; reverse primer, 5'-ATAAGAAT GCGGCCGC TTATGCTCCGACTCTCTTTTTTTC-3'. Then the purified fragment was subcloned into pAdTrace-TO4 and subsequently used to generate adenoviral recombinants. Recombinant adenovirus (Ad-APPL1) was produced and amplified in packaging HEK293 cells as

described [10]. The Ad-APPL1 also co-express RFP. An analogous adenovirus expressing only RFP was used as a control (Ad-Ctrl). Viral supernatants were diluted in culture medium to give the desired concentration and added to logarithmic phase monolayer cell cultures. After 48 hours, the level of APPL1 expression was detected by western blotting.

Cell culture and grouping

The mouse podocyte cell line (generated from H-2K^b-tsA58 immortal mouse) was cultured as described previously [11]. Briefly, podocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10 U/ml mouse recombinant interferon at 33°C in 5% CO₂/95% air. To induce differentiation, podocytes were cultured in interferon-free medium at 37°C in 5% CO₂/95% air [7]. The cells were divided into four groups according to different experimental conditions: Group 1, cells were cultured with 1 g/L D-glucose (normal glucose); Group 2, cells were cultured with 4.5 g/L D-glucose (high glucose); Group 3, cells were infected with Ad-Ctrl and cultured with 4.5 g/L D-glucose; Group 4, cells were infected with Ad-APPL1 and cultured with 4.5 g/L D-glucose.

Cell proliferation assay

Podocytes were seeded in 96-well plates and cell proliferation was measured with CCK-8 Assay Kit (Dojindo Lab, Kumamoto, Japan) according to manufacturer's protocol. Briefly, at indicated time point, CCK8 solution (10 µl in 100 µl DMEM medium) was added to each well and incubated for 1 h. Optical density values (OD) at wavelength 450 nm was measured by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). All experiments were run in triplicates and repeated at least three times.

Cell cycle assay

The cell cycle was evaluated by flow cytometry using propidium iodide (PI, Sigma, St. Louis, MO, USA) staining on a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were plated in 6-well plates and treated as described above. After 48 h culture, the cells were harvested and fixed in 70% ethanol at -20°C overnight. Cells were then washed in PBS and stained with PI-RNase A solution as

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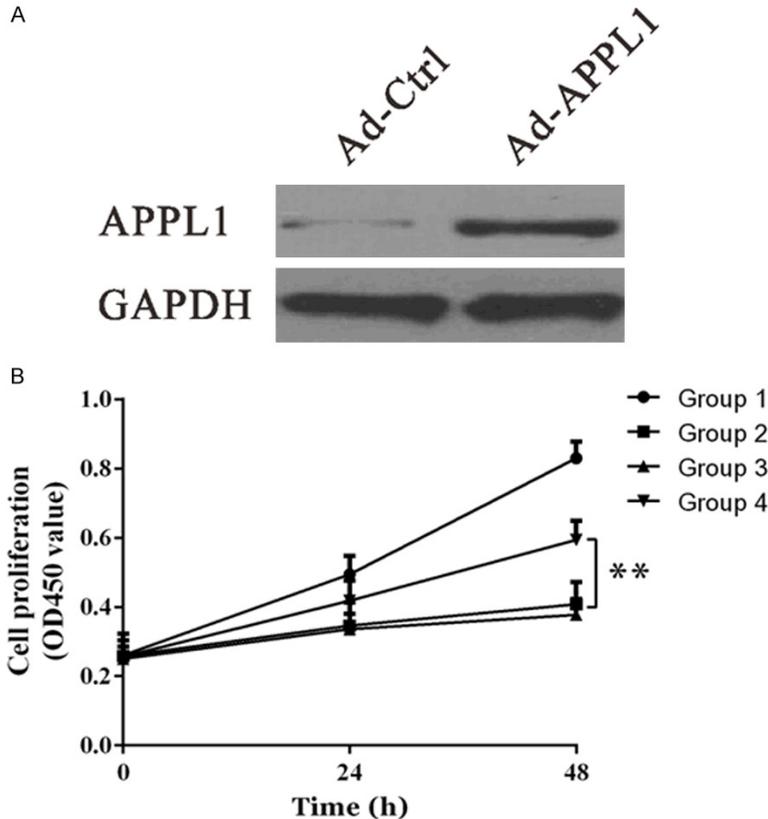


Figure 1. Effects of APPL1 overexpression on cell viability in high glucose media. A. APPL1 expression was detected by Western blotting. Lane 1, Ad-Ctrl, podocytes infected with control adenovirus; Lane 2, Ad-APPL1, podocytes infected with APPL1 recombinant adenovirus. B. The viability of podocytes was evaluated by the Cell Count Kit-8 (CCK-8). Data are presented as mean \pm SD (n=3). Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. ** $P < 0.01$ V.S. Group 3.

Table 1. Cell apoptosis rate (%)

	Group 1	Group 2	Group 3	Group 4
Early apoptosis	0.05 \pm 0.04	43.1 \pm 2.78	40.5 \pm 1.71	27.3 \pm 1.66**
Late apoptosis	0.03 \pm 0.04	0.03 \pm 0.04	0	0

Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. ** $P < 0.01$ V.S. Group 3.

described previously [12]. Experiments were performed in triplicate and 3×10^4 cells were analyzed per sample. G1, S, and G2/M fractions were quantified using CellQuest software (BD Biosciences) and manual gating.

Cell apoptosis assay

The percentage of cells actively undergoing apoptosis was determined by double stained with Annexin V-fluorescein isothiocyanate (FITC)

and PI. Podocytes were plated in 6-well plates and treated as described above for 48 hours. Cells were then harvested and then double-labeled with Annexin V-FITC and PI as described by the manufacturer (BD Biosciences). Cells were analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (BD Biosciences). At least 20,000 cells were acquired for each sample. The experiments were performed in triplicate.

Western blot

Cells were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, JRDUN Biotechnology, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and incubated on ice for 30 minutes. Cell lysis was centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant (20-30 μ g of protein) was run on SDS-PAGE gel and transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against APPL1, Nephrin, AMPK and p-AMPK were purchased from Abcam (Cambridge, MA, USA). Blots

were then incubated with goat anti-mouse secondary antibody (Beyotime, Cat#A0208) or goat anti-rabbit secondary antibody (Beyotime, Cat#A0216) and visualized by using the enhanced chemiluminescence (ECL) system (Millipore).

Statistical analyses

All data were expressed as the mean \pm standard deviation. ANOVA and LSD test were used

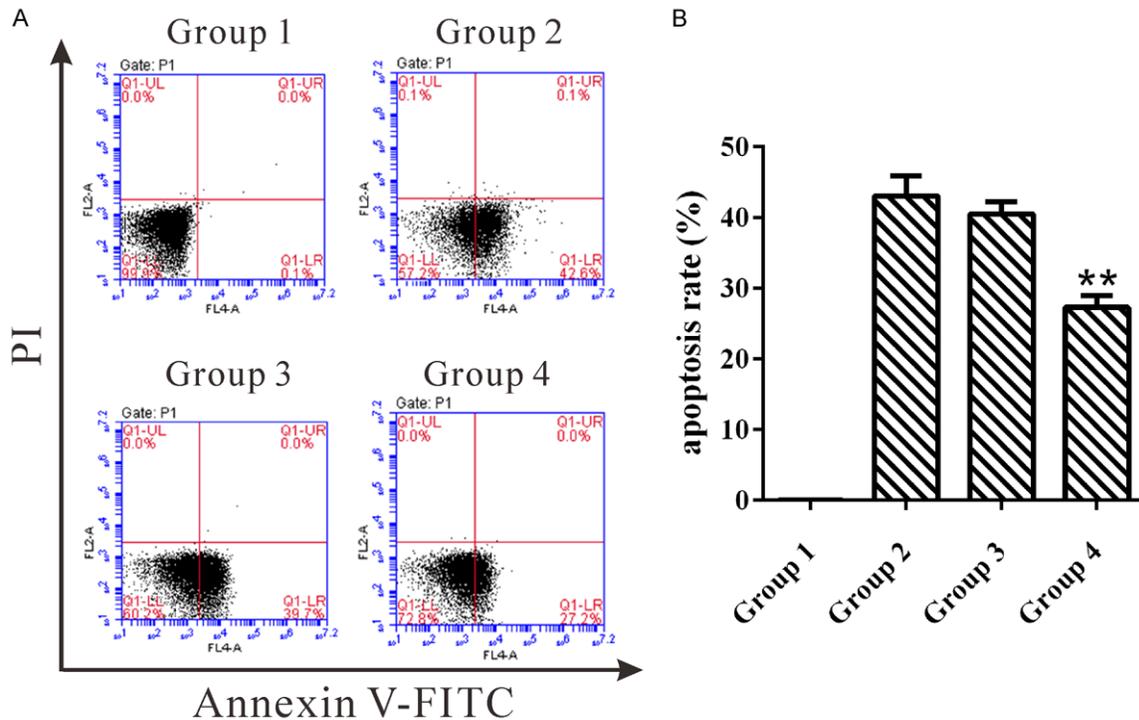


Figure 2. Effects of APPL1 overexpression on cell apoptosis in high glucose media. Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis. The lower right quadrant indicates early apoptotic cells (Annexin-V positive but PI negative). The upper right quadrant represents late apoptotic cells or necrotic cells (double positive). Data were based on at least 3 independent experiments, and shown as mean \pm SD. Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. ** $P < 0.01$ V.S. Group 3.

to compare groups of measurement data. All analyses were performed with SPSS 17.0 software. A P value < 0.05 was considered significant.

Results

Overexpression of APPL1 increased the proliferation of podocytes in high-glucose media

The podocytes were infected with recombinant adenovirus and the expression of APPL1 was detected by Western blotting (Figure 1A). The expression level of APPL1 was much higher in overexpression group (Ad-APPL1) than in control group (Ad-Ctrl).

Podocytes were divided into four groups according to different experimental conditions: Group 1, cells were cultured with 1 g/L D-glucose (normal glucose); Group 2, cells were cultured with 4.5 g/L D-glucose (high glucose); Group 3, cells were infected with Ad-Ctrl and cultured with 4.5 g/L D-glucose; Group 4, cells were infected with Ad-APPL1 and cultured with 4.5

g/L D-glucose. To investigate the effects of APPL1 on the podocytes proliferation, CCK-8 assay was performed at 24 and 48 h after viral infection. As shown in Figure 1B, high glucose treatment significantly reduced the vitality of podocytes (Group 2 V.S. Group 1). The cell vitality of podocytes infected with APPL1 overexpression recombinant virus (Group 4) is higher than Group 2 and Group 3 ($P < 0.01$), but lower than Group 1. No significant difference was observed between Group 2 and Group 3. These data indicated that APPL1 overexpression could partially protect podocytes from high-glucose-induced injury.

Overexpression of APPL1 inhibited high-glucose-induced podocytes apoptosis

The podocytes apoptosis was tested by Annexin V/PI staining and flow cytometry. The early apoptosis rates of four groups were shown in Table 1. A significant increase in apoptotic rate was observed in high glucose group when compared with normal glucose group. APPL1

APPL1 protected podocytes from high-glucose-induced injury

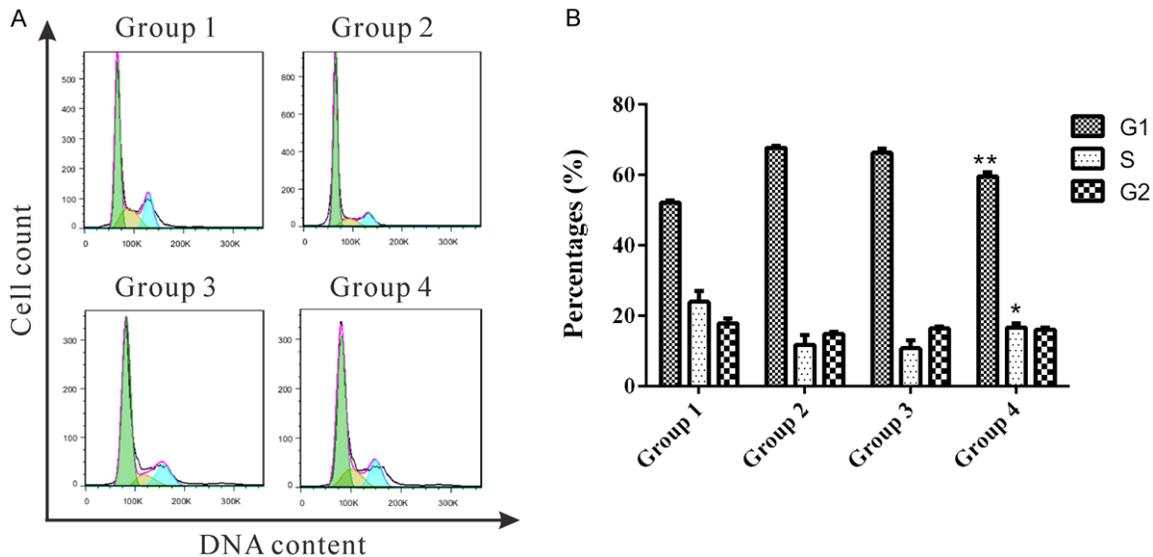


Figure 3. Effects of APPL1 on the cell cycle distribution of podocytes treated with high glucose. PI stain assay and flow cytometry were carried out to analyze the cell cycle. APPL1 overexpression induced G1-S transition in high glucose condition. Data are presented as mean \pm SD (n=3). Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. ** P <0.01 V.S. Group 3.

Table 2. Cell cycle distribution (%)

	Group 1	Group 2	Group 3	Group 4
G0/G1	52.19 \pm 0.58	67.76 \pm 0.52	66.34 \pm 1.12	59.56 \pm 1.29**
S	23.99 \pm 3.04	11.72 \pm 2.82	10.74 \pm 2.28	16.71 \pm 1.21*
G2/M	17.93 \pm 1.26	14.81 \pm 0.61	16.39 \pm 0.55	16.00 \pm 0.66

Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. * P <0.05, ** P <0.01 V.S. Group 3.

overexpression notably decreased the high glucose induced-apoptosis of podocytes (P <0.01, **Figure 2; Table 1**), which suggested a protective role of APPL1 in high-glucose-induced injury.

APPL1 overexpression reduced high-glucose-induced G0/G1 arrest in podocytes

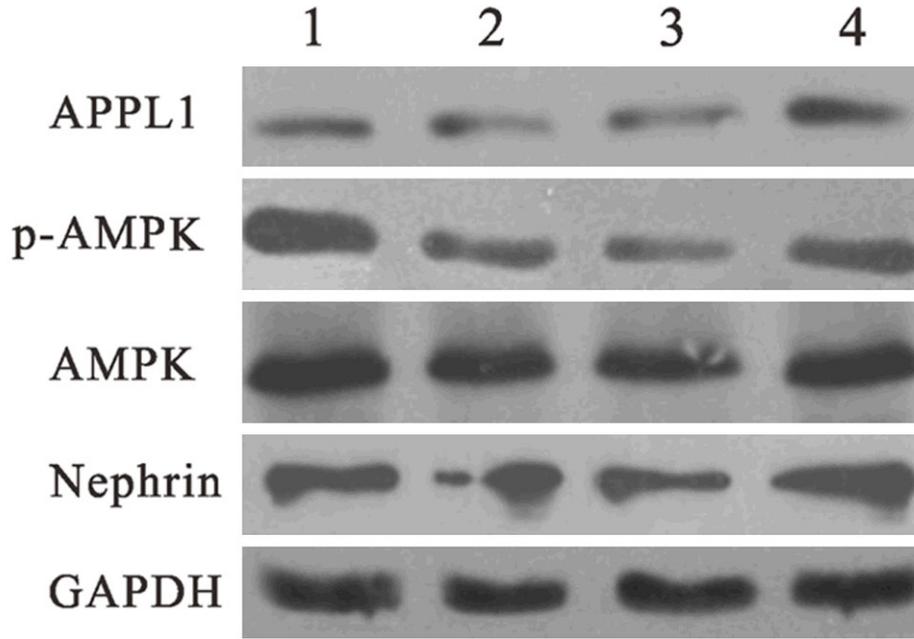
We then investigated the effect of APPL1 overexpression on the cell cycle progression. The distribution of G0/G1, S and G2/M phase was detected by PI staining and flow cytometry (**Figure 3**). High glucose treatment remarkably induced G0/G1 phase arrest (Group 2 V.S. Group 1), which may be the cause of the reduced proliferation rate. Compared with control adenovirus infection group (Group 3), APPL1 overexpression significantly decreased

the cell population in phase G0/G1, but increased the cell population in phase S when podocytes were exposed to high glucose (P <0.01, **Figure 3; Table 2**). These data implied that overexpression of APPL1 induced G1/S phase transition of podocytes treated with high glucose.

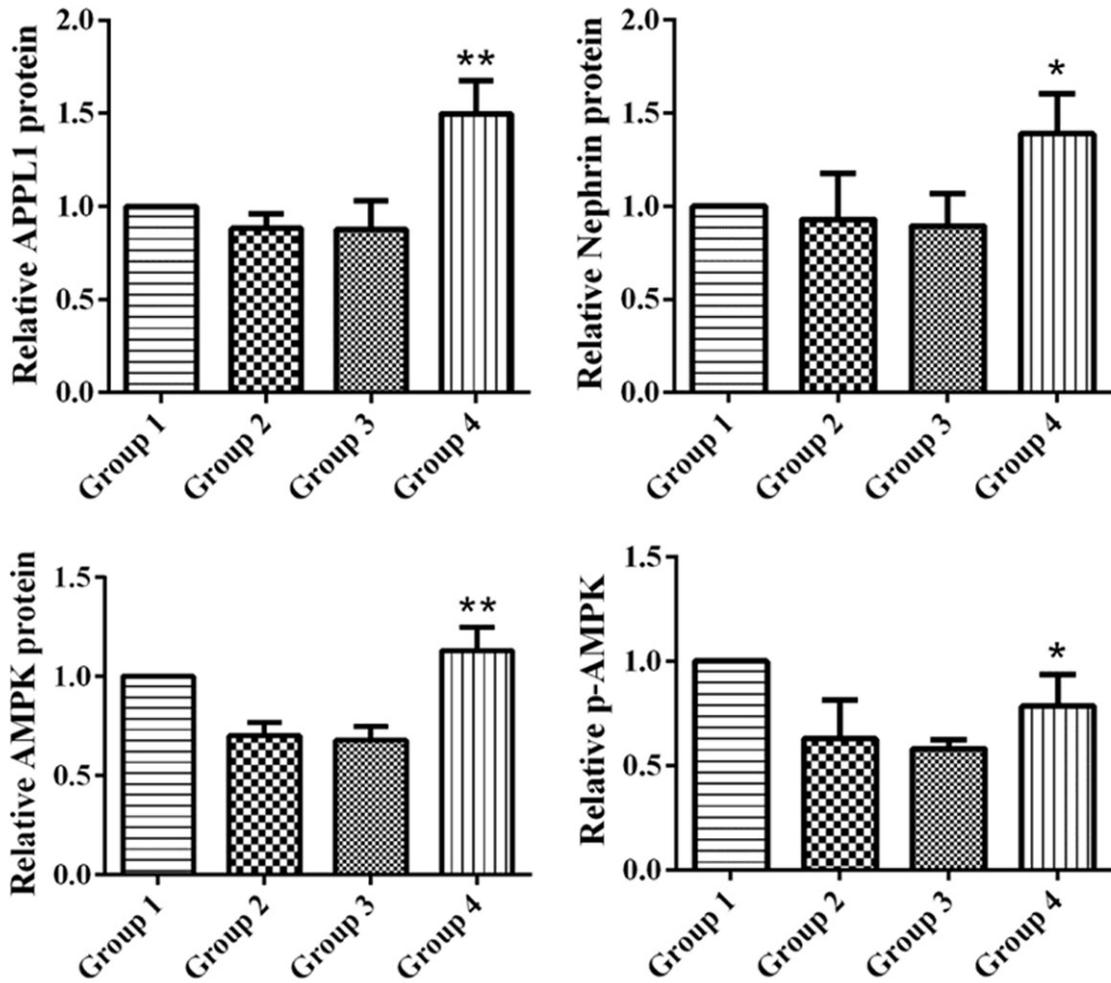
The effect of APPL1 overexpression on the expression of key proteins

The loss of Nephryn is an indicator of kidney and podocytes damage [16]. As shown in **Figure 4**, Nephryn expression was up-regulated by APPL1 overexpression (Group 4), which further indicated that high-glucose-induced podocyte injury was attenuated by APPL1 overexpression. AMPK is a key protein downstream of APPL1 pathway. In order to explore the possible mechanism of the protective role of APPL1 on high glucose induced-injury, AMPK, and p-AMPK was tested by Western blotting in the four groups. As shown in **Figure 4**, AMPK and p-AMPK was decreased in hyperglycemic condition. APPL1 overexpression significantly increased the levels of AMPK and p-AMPK, suggesting that APPL1 may exert its protective function on high-glucose induced injury through

A



B



APPL1 protected podocytes from high-glucose-induced injury

Figure 4. Effects of APPL1 overexpression on the expression of key proteins. A. Representative Western blot results showing the levels of Nephryn, AMPK and p-AMPK. GAPDH was used as an internal control. B. Quantification of Western blot results. All experiments were repeated three times. Data are presented as mean \pm SD. Group 1: Normal glucose; Group 2: High glucose; Group 3: High glucose and control adenovirus infection; Group 4: High glucose and Ad-APPL1 infection. $**P < 0.01$ V.S. Group 3.

regulating the expression and phosphorylation of AMPK.

Discussion

The human APPL1 gene was mapped to human chromosome 3p14.3-p21.1, it was originally cloned in two-hybrid screens as an interacting partner of Akt2, and was named APPL or DIP13 α [13]. The APPL1 protein consists of 709 amino acids and contains a PH (pleckstrin homology) domain, a COOH-terminal PTB (phosphotyrosine-binding) domain, and a Leucine zipper motif [13, 14]. Mao et al. firstly demonstrated that APPL1 interacts with N-terminal amino acids of AdipoR1 by its PTB domain and takes part in adiponectin signaling pathway. Overexpression of APPL1 in C2C12 myotubes could mimic the effect of adiponectin, such as increasing the phosphorylation level of AMPK and p38 MAPK, and enhancing the membrane translocation of glucose transporter 4 (GLUT4) to improve glucose uptake. Additionally, suppression of APPL1 by siRNA impaired adiponectin-stimulated phosphorylation of AMPK, p38MAPK and acetyl-CoA carboxylase (ACC), and a relationship between knock-down of APPL1 and the reduction of AMPK expression was observed [1]. In another study, APPL1 showed a clear effect in the cytoplasm localization of LKB1 by interacting with LKB1 using the BAR domain, thus inducing the phosphorylation of AMPK [15]. In the present study, we found that AMPK was decreased in mouse podocytes by high glucose treatment, but increased by APPL1 overexpression. These results suggested that APPL1 is a key link in adiponectin pathway between AdipoR1 and AMPK and plays an important role in glucometabolism.

High glucose condition leads to metabolic disorders, increasing of inflammatory cytokines, oxidative stress, and finally cell death. It will be a crucial challenge to reduce the cell death rate in metabolic syndromes. Several studies have indicated the potentiality role of APPL1 in this area. Chandrasekar et al. reported that IL-18-induced endothelial cell death was reversed, caspase-3 activity was decreased, and insulin

sensitivity was increased through the activation of AMPK mediated by APPL1 [16]. Here, we have investigated the role of APPL1 in podocytes damage in high glucose media. The present study firstly showed that the overexpression of APPL1 in mouse podocytes promoted cell proliferation (**Figure 1B**) and decreased cell apoptosis (**Figure 2**) in high glucose media. Additionally, APPL1 overexpression significantly decreased the cell number in phase G0/G1, but increased cell population in phase S (**Figure 3**), suggesting that a promoted role of APPL1 in cell cycle progression of podocytes in high-glucose media. Moreover, the increased AMPK and p-AMPK (**Figure 4**) also indicated that APPL1 may protect podocytes from high glucose condition by up-regulating the expression and increasing the activation of AMPK.

Although researches have revealed an association between APPL1 and type 2 diabetes [3-5], there was still no study concerned about the role of APPL1 in diabetic nephropathy. In diabetic nephropathy, podocytes as well as the structure proteins shed from GBM and discharged with urine [8]. The amount of urine podocytes indicates the severity of glomerular filtration function injury. Nephryn is the key protein maintaining glomeruli podocyte slit diaphragm (GPSD), and the loss of Nephryn was an indicator of kidney and podocytes damage [17]. Recently, Sharma et al. pointed out that AdipoR1 expressed on the surface of podocytes, which made podocytes be the direct target of adiponectin. Injecting adiponectin in cultured podocytes could activate AMPK, reduce podocytes damage, and improve the permeability and function of podocytes [9]. Here, we also observed an increasing of Nephryn expression after APPL1 overexpression, which suggested that APPL1 plays an important role to protect podocytes in diabetic nephropathy and alleviate proteinuria.

Taken together, this is an initiate research to discuss the role of APPL1 in podocytes injury induced by high glucose. In the experimental high glucose condition, APPL1 acts as a protective factor against podocytes injury through

upregulating the expression and activation of AMPK. The present data may provide a possible target for diabetic nephropathy treatment, although the detailed mechanism was needed to be explored.

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Disclosure of conflict of interest

None.

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