Role of mTOR signaling in the regulation of high glucose-induced podocyte injury

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Abstract. Podocyte injury, which promotes progressive nephropathy, is considered a key factor in the progression of diabetic nephropathy. The mammalian target of rapamycin (mTOR) signaling cascade controls cell growth, survival and metabolism. The present study investigated the role of mTOR signaling in regulating high glucose (HG)-induced podocyte injury. MTT assay and flow cytometry assay results indicated that HG significantly increased podocyte viability and apoptosis. HG effects on podocytes were suppressed by mTOR complex 1 (mTORC1) inhibitor, rapamycin, and further suppressed by dual mTORC1 and mTORC2 inhibitor, KU0063794, when compared with podocytes that received mannitol treatment. In addition, western blot analysis revealed that the expression levels of Thr-389-phosphorylated p70S6 kinase (p-p70S6K) and phosphorylated Akt (p-Akt) were significantly increased by HG when compared with podocytes that received mannitol treatment. Notably, rapamycin significantly inhibited HG-induced p-p70S6K expression, but did not significantly impact p-Akt expression. However, KU0063794 significantly inhibited the HG-induced p-p70S6K and p-Akt expression levels. Furthermore, the expression of ezrin was significantly reduced by HG when compared with podocytes that received mannitol treatment; however, α-smooth muscle actin (α-SMA) expression was significantly increased. Immunofluorescence analysis on ezrin and α-SMA supported the results of western blot analysis. KU0063794, but not rapamycin, suppressed the effect of HG on the expression levels of ezrin and α-SMA. Thus, it was suggested that the increased activation of mTOR signaling mediated HG-induced podocyte injury. In addition, the present findings suggest that the mTORC1 and mTORC2 signaling pathways may be responsible for the cell viability and apoptosis, and that the mTORC2 pathway could be primarily responsible for the regulation of cytoskeleton-associated proteins.

Introduction

Diabetic nephropathy (DN) is a common complication of diabetes that can promote the development of renal diseases (1). Patients with DN usually exhibit reduced filtration rates, albuminuria and ultimately renal failure (2). Multiple mechanisms have been implicated in the development and outcome of DN, including changes in hyperglycemia-induced metabolism, changes in hemodynamics and genetic predisposition (3). Patients with diabetes usually still develop massive and treatment-resistant proteinuria that can cause a rapid decline in renal function (4). Thus, further understanding of the pathogenesis in DN may help to improve renal and health outcomes in patients with diabetes.

Podocyte injury is a key event in the progression of DN that can induce the production of proteinuria and further cause the development of diabetic kidney disease (5). Podocytes have a limited ability to regenerate, thus the extent of podocyte injury is commonly regarded as an important prognostic determinant in DN (6). High glucose (HG) can result in glomerular injury, further induce chronic renal function loss and ultimately lead to the occurrence of end-stage renal disease (7-9). Previous studies have revealed that podocyte injury is an important early event leading to glomerular disease (10) in patients with DN (11,12). However, the underlying mechanisms involved in HG-induced podocyte injury remain unclear.

The mammalian target of rapamycin (mTOR), a serine/threonine kinase of the phosphoinositide 3-kinase-related kinase family, has been identified as the target of rapamycin (sirolimus) in mammals (13). mTOR is the core component of two distinct complexes complex 1 (mTORC1) and complex 2 (mTORC2) (14). As mTOR is specifically inhibited by rapamycin only when it is in mTORC1, mTORC1 has been initially defined as ‘rapamycin sensitive’, whereas mTORC2 has been defined as ‘rapamycin insensitive’ (15). The level of mTOR activity is associated with tubular cell proliferation (16), apoptosis (17-19) and autophagy (20). In has been revealed that the mTORC2/Akt/nuclear factor-κB signaling pathway can mediate the activation of transient receptor potential cation channel 6, which is involved in ADR-induced podocyte apoptosis (21). Inhibition of mTORC2-promotes the prevention of reactive oxygen species-induced apoptosis (22) and activation of mTORC1, which induces the expression of endoplasmic reticulum stress signaling and thus leads to apoptosis in HG-treated podocytes (23). In addition, dual targeting

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of mTORC1 and mTORC2 can promote the induction of autophagy in acute myeloid leukemia cells (24).

In the current study, it was hypothesized that the mTOR signaling pathway was involved in the regulation of HG-induced podocyte injury. Podocyte viability and apoptosis 24 h following HG treatment were assessed. In addition, the expression levels of mTOR signaling proteins and cytoskeleton-associated proteins were examined.

Materials and methods

Cell culture. Mouse podocytes (MPC5, provided by Professor Peter Mundel, Mount Sinai School of Medicine, New York, NY, USA) were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO₂. Cells were grown in RPMI 1640 medium containing 100 U/ml mouse interferon (IFN-γ; Peprotech EC Ltd., London, UK) at 33°C with 100% relative humidity and 5% CO₂, and were induced to differentiate at 37°C in a medium without IFN-γ for 10-14 days. Following differentiation for 10-14 days, the cells were subsequently divided into five groups: Normal glucose (NG; 5.6 mmol/l glucose), mannitol (M; 5.6 mmol/l glucose + 24.4 mmol/l HD), HG (30 mmol/l glucose), HG + rapamycin (30 mmol/l glucose +50 nmol/l rapamycin; MedChem Express, Monmouth Junction, NJ, USA) and HG + KU0063749 (30 mmol/l glucose +2 µmol/l KU0063794; MCE, NJ, USA) groups. The concentration of rapamycin or KU0063794 used was as described in previous studies (25,26). Cells were exposed to different conditions for 24 h and then harvested for further experiments.

Cell viability assessment. Podocytes were seeded on a 96-well plate overnight at 2x10⁴ cells/well and were subsequently incubated with NG (5.6 mmol/l glucose), M (5.6 mmol/l glucose + 24.4 mmol/l HD), HG (30 mmol/l glucose), HG + rapamycin (30 mmol/l glucose +50 nmol/l rapamycin) or HG + KU0063794 (30 mmol/l glucose +2 µmol/l KU0063794) for 24 h. Thereafter, cell viability was measured using the MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer's instructions. Briefly, a total of 10 µl MTT (5 mg/ml) was added into each well and then incubated for 4 h at 37°C. Next, 150 µl dimethyl sulfoxide was added to the 96-well plates. Finally, the absorbance was measured at 568 nm on a microplate reader. Results were calculated as percentages of the NG group (100%).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. Podocytes were harvested via trypsinization and washed twice with cold phosphate buffered saline (PBS). Cells were centrifuged at 750 x g and 4°C for 3 min and the supernatant was discarded. The cells were re-suspended in 1X PBS at a density of 5x10⁵-1x10⁶ cells/ml. A total of 500 µl of the sample solution was transferred to a 5-ml culture tube and incubated with 5 µl of FITC-conjugated Annexin V (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and 5 µl of PI (Nanjing KeyGen Biotech Co., Ltd.) for 15 min at room temperature in the dark. A total volume of 300 µl of 1X PBS was added to each sample tube, and the samples were analyzed with fluorescence activated cell sorting flow cytometers (Beckman Coulter, Inc., Brea, CA, USA) using CellQuest software (version 5.2.1; BD Biosciences, San Jose, CA, USA).

Western blot analysis. Cell proteins were extracted using lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and incubated on ice for protein extraction. Protein concentration was measured using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). The protein extract (40 µg), which was prepared from cells, was separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk (in PBS; pH 7.2; containing 0.1% Tween-20) for 2 h at room temperature and incubated at 4°C overnight with the primary antibodies, which was then followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:50,000; cat. no. BA1054; Wuhan Boster Biological Technology., Ltd., Wuhan, China) for 2 h at room temperature. The primary antibodies used were rabbit polyclonal anti-GAPDH (1:1,000; cat. no. AB-P-R 001; Hangzhou Xianzhi Biotechnology Co., Ltd., Hangzhou, China), rabbit polyclonal anti-phosphorylated p70S6 kinase (p-p70S6K; 1:600; cat. no. ab2571; Abcam, Cambridge, UK), rabbit polyclonal anti-p70S6K (1:1,200; cat. no. 14485-1-AP; ProteinTech Group, Inc., Wuhan, China), rabbit monoclonal anti-p-Akt (1:1,000; cat. no. 13038S, CST, MA, USA), rabbit monoclonal anti-Akt (1:1,000; cat. no. 4691S; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-εzrin (1:600; cat. no. 26056-1-AP; ProteinTech Group, Inc.) and rabbit polyclonal anti-α-smooth muscle actin (SMA;1:5,000; cat. no. 55135-1-AP; ProteinTech Group, Inc.). The immunoreactive proteins were visualized using ECL plus detection reagents (Thermo Fisher Scientific, Inc.). ImageJ software (version 1.48u; National Institutes of Health, Bethesda, MD, USA) was used for the densitometry analysis.

Immunofluorescence staining. Cells grown on glass cover slips were fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with PBS containing 0.5% Triton X-100 for 5 min. Following three washes with PBS, cells were incubated in a blocking buffer containing 3% bovine serum albumin (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) in PBS for 30 min at room temperature, followed by incubation with εzrin (ab75840) and α-SMA (ab5694) primary antibodies (1:100; Abcam) overnight at 4°C. Following this, the cells were stained with a secondary anti-body labeled with fluorescein (Alexa Fluor 488-conjugated AffiniPure anti-goat secondary antibody (BA1032); IgG-Cy3; 1:100; Wuhan Boster Biological Technology Co., Ltd.). Following counterstaining with 4,6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology) for 5 min at room temperature, cells were washed again with PBS and coverslips were transferred onto glass slides. Cells were observed and imaged were captured (magnification, x400) using fluorescent microscopy (BX53; Olympus Corporation, Tokyo, Japan). ImageJ software was used for fluorescence signal analysis. All experiments were performed a minimum of three times.
Inhibition of the mTOR signaling pathway suppresses HG-induced podocyte viability. As indicated in Fig. 1, HG significantly increased podocyte viability when compared with the M group. However, rapamycin treatment significantly suppressed podocyte viability when compared with the HG group. Notably, the dual mTORC1 and mTORC2 inhibitor, KU0063794, further suppressed HG-induced cell viability when compared with the HG + rapamycin group. These results indicate that the mTORC1 and mTORC2 signaling pathways may be involved in HG regulation of podocyte viability.

Inhibition of the mTOR signaling pathway suppresses HG-induced podocyte apoptosis. As indicated in Fig. 2, HG significantly increased podocyte apoptosis when compared with the M group. However, rapamycin treatment significantly suppressed the HG-induced effect when compared with the HG group. KU0063794 further suppressed the HG-induced effect on apoptosis when compared with the HG + rapamycin group. These results indicate that mTORC1 and mTORC2 signaling pathways may be involved in HG regulation of podocyte apoptosis.

Inhibition of mTORC2 signaling suppresses the effect of HG on the expression of ezrin and α-SMA. mTORC1 regulates various major processes, including protein and lipid synthesis, proliferation, apoptosis and autophagy (27). p70s6k is the downstream signaling protein of mTORC1. Generally, p70s6k has been regarded as the marker of the mTORC1 signaling pathway, and the change of p-p70s6k expression can indicate a change of mTORC1 activity (28). Notably, mTORC2 participates in the regulation of several members of the AGC subfamily of kinases, including protein kinase C-α, serum- and glucocorticoid-induced protein kinase 1 and Akt (27). In addition, Akt can regulate a series of cellular processes, including metabolism, survival, apoptosis, growth and proliferation, via the phosphorylation of several effectors (27). Akt is the downstream signaling protein of the mTORC2 signaling pathway (29). In the present study, a change of p-Akt expression was considered to indicate a change of mTORC2 activity. Thus, the activities of p70s6k and Akt were examined in podocytes. The present results indicated that the expression levels of p-p70s6k and p-Akt were significantly increased by HG when compared with the M group. Notably, rapamycin treatment significantly inhibited the HG-induced increase of p-p70s6k, but not p-Akt; however, KU0063794 significantly inhibited the HG-induced increase of p-p70s6k and p-Akt (Fig. 3). These results suggested that rapamycin significantly inhibited the mTORC1 signaling pathway and KU0063794 significantly inhibited the mTORC1 and mTORC1 signaling pathways. Ezrin and α-SMA are cytoskeleton-associated proteins that serve a key role in cell surface structure adhesion, migration and organization (30,31). In the present study, the expression of ezrin was significantly reduced when compared with the HG + rapamycin group. KU0063794, but not rapamycin, significantly suppressed the effect of HG on ezrin and α-SMA expression levels (Fig. 3). Furthermore, intense fluorescence demonstrated ezrin was predominantly localized on the cell membrane and in cytoplasm (Fig. 4A). A total of 24 h following HG treatment, the fluorescence density of ezrin was significantly decreased when compared with the M group. KU0063794, but not rapamycin, significantly suppressed the expression of α-SMA which was significantly increased, KU0063794, but not rapamycin, significantly suppressed the effect of HG on ezrin expression levels. Notably, weak fluorescence indicated α-SMA was also predominantly localized on the cell membrane and in the cytoplasm. A total of 24 following HG treatment, the fluorescence density of α-SMA was significantly increased. Similarly, KU0063794, but not rapamycin, significantly suppressed the effect of HG on the α-SMA expression levels (Fig. 4B). These results indicated that mTORC2 signaling, but not mTORC1 signaling, may primarily mediate the regulation of the cytoskeleton.

Discussion

Podocytes are highly specialized cells in the kidney, which can release growth factors for mesangial and endothelial cells, and can regulate glomerular filtration (32). Injury or loss to podocytes has a crucial role in the regulation of the pathological process of proteinuric kidney disease, including DN (33-35). Apoptosis and the functional loss of podocytes contribute to the development of DN (36). Previous studies have indicated that podocyte injury is associated with a reduced level of autophagy (37-39) and an increased level of inflammation (40,41) and apoptosis (23,42) during exposure to HG conditions. The present study revealed increased viability...
and apoptosis in podocytes 24 h following HG exposure. Furthermore, inhibition of mTORC1 by rapamycin significantly attenuated the HG-induced viability and apoptosis, and dual mTORC1 and mTORC2 inhibition by KU0063794 further attenuated the HG effect. In addition, KU0063794, but not rapamycin, was indicated to inhibit the effects of HG on the expression levels of ezrin and α-SMA.

The mTOR signaling pathway has important roles in regulating cell metabolism, growth, proliferation and apoptosis (19,43). Pharmacological inhibition of mTOR and selective targeting of mTORC1 or mTORC2 in podocytes has been suggested to elucidate their role in renal cell homeostasis (44). It has been reported that podocyte-specific embryonic knockout of mTORC1 in mice resulted in early albuminuria, later development of glomerulosclerosis, loss of weight and increase of mortality (45). Mice with podocyte-specific loss of mTORC2 did not demonstrate significant phenotypic differences when compared with the littermate controls, with the exception of transient albuminuria following protein overload in combination with the deletion of both mTOR complexes from podocytes.
Figure 3. Expression of mammalian target of rapamycin signaling-associated proteins and cytoskeleton-associated proteins in podocytes. (A) Representative image of western blot analysis. (B) Quantitative graph of the protein expression levels. Data were presented as the mean ± standard error of the mean and were representative of three independent experiments. Rapamycin significantly suppressed the HG-induced activation of p70s6k, but not Akt. KU0063794 significantly suppressed the HG-induced activation of p70s6k and Akt. HG significantly reduced the expression of ezrin and increased the expression of α-SMA. However, KU0063794, but not rapamycin, significantly inhibited the effect of HG on the expression of ezrin and α-SMA. **P<0.01 and ***P<0.001 as indicated. HG, high glucose; NG, normal glucose; M, mannitol; p-, phosphorylated; α-SMA, α-smooth muscle actin.

Figure 4. Immunofluorescence staining of cytoskeleton-associated proteins in podocytes. (A) Ezrin and α-SMA were primarily localized on the cell membrane and in the cytoplasm. Ezrin- and α-SMA-positive cells were stained with red fluorescence (magnification, x400). (B) Quantitative graph of the fluorescence density of ezrin- and α-SMA-positive cells. Data were presented as the mean ± standard error of the mean and were representative of three independent experiments. HG significantly reduced the fluorescence density of ezrin-positive cells and significantly increased the fluorescence density of α-SMA-positive cells. KU0063794, but not rapamycin, significantly inhibited the effect of HG on the fluorescence density of ezrin- and α-SMA-positive cells. ***P<0.001 as indicated. HG, high glucose; NG, normal glucose; M, mannitol; p-, phosphorylated; α-SMA, α-smooth muscle actin.
which caused an early (at 6 weeks old) fulminant proteinuric phenotype (44). These previous findings indicated that there may be some degree of interaction between mTORC1 and mTORC2 in regulating the development and homeostasis of podocytes. Over activation of mTORC1 can also lead to severe pathologic effects, including hallmarks of DN (46). A previous study revealed that inhibition of mTORC1 signaling activity prevented glomerulosclerosis and significantly ameliorates the progression of glomerular disease in DN (47), and inhibition of mTORC2 signaling activity reduced HG-induced podocyte apoptosis and attenuated albuminuria (48). Notably, previous findings have suggested that the activation of mTOR signaling is involved in HG-induced podocyte injury (23). It has been reported that the activated mTOR signaling can result in energy consumption, which in turn induces apoptosis in HG-treated podocytes (23). Furthermore, a previous study demonstrated that suppression of mTOR signaling protects against HG-induced apoptosis in β cells (49), indicating that mTOR signaling may serve crucial roles in the regulation of HG-induced apoptosis. In the present study, it was indicated that HG induced podocyte injury, which was reflected by the results on podocyte apoptosis, and altered the expression levels of cytoskeleton-associated proteins. However, the inhibition of mTORC1 activity significantly attenuated the HG effects, and dual mTORC1 and mTORC2 inhibition further attenuated the HG effects. These findings indicate that the mTORC1 and mTORC2 signaling pathways may be involved in HG-induced podocyte injury. As the expression levels of p-p70s6k and p-Akt can indicate the activation of mTORC1 and the mTORC2 signaling pathway, respectively (27), the expression levels of p-p70s6k and p-Akt were examined in podocytes in the present study. The following was indicated in the present study: HG significantly increased the p-p70s6k and p-Akt expression levels; rapamycin and KU0063794 significantly inhibited the increase in HG-induced p-p70s6k expression levels; rapamycin inhibited the increase in HG-induced p-Akt expression levels; and KU0063794 significantly inhibited the increase in the HG-induced p-Akt expression levels. These findings suggest that HG could significantly induce the activation of mTOR signaling pathway and that this may be suppressed by rapamycin or KU0063794.

Ezrin act as a linker between the cell membrane and the actin cytoskeleton. This linker function provides ezrin essential roles in various fundamental cellular processes, including cell adhesion, motility, cytokinesis, phagocytosis, determination of cell shape, polarity and surface structure, and integration of membrane transport with signaling pathways (50,51). It has been reported that abnormal expression of ezrin and actin initiates the morphological changes of arsenic-induced apoptosis in human esophageal epithelial cells (52). Ezrin is downregulated in glomeruli of patients with DN, and has been identified to serve a role in regulating the development of renal complications in diabetes through transport of glucose and organization of the actin cytoskeleton in podocytes (53). It has been reported that Ethanolic Ginkgo biloba leaf extract prevents renal fibrosis via mTOR signaling in DN (54), indicating that mTOR signaling may be involved in the regulation of cytoskeletal proteins. In the present study, it was revealed that HG significantly reduced the expression levels of ezrin, and KU0063794, but not rapamycin, suppressed the effect of HG on the ezrin expression levels. This finding suggested that the cytoskeleton-associated proteins may be primarily regulated by mTORC2 (and not the mTORC1) signaling pathway. Notably, α-SMA is the actin isoform that serves an important role in fibrogenesis (55). A previous study indicated that the α-SMA expression in the renal tubulointerstitium was highly upregulated in diabetic rats when compared with the non-diabetic rats (56). In the present study, it was revealed that HG significantly increased the expression of α-SMA, and KU0063794, but not rapamycin, significantly suppressed the effect of HG on α-SMA expression. This result further supported that the cytoskeleton-associated proteins may be primarily regulated by the mTORC2 (and not the mTORC1) signaling pathway. A previous study has reported that disruption of the podocyte cytoskeleton can induce podocyte apoptosis (57), indicating that cytoskeleton-associated proteins may serve important roles in regulating podocyte apoptosis. In the present study, it was demonstrated that HG induced podocyte apoptosis, which was accompanied by the disruption of cytoskeleton, including the decreased expression of ezrin and the increased expression of α-SMA. These results suggest that the altered expression of cytoskeleton-associated proteins in HG-treated podocytes may promote the development of apoptosis.

According to the present results, rapamycin significantly inhibited the increase of p-p70s6k, but not p-Akt, in HG-treated podocytes, indicating that rapamycin could significantly block mTORC1 activity. Rapamycin did not significantly suppress the effect of HG on the expression levels of ezrin and α-SMA, which suggested that the mTORC1 pathway may not be involved in the regulation of cytoskeletal proteins in podocytes. Figs. 1 and 2 demonstrate that mTORC1 and mTORC2 were involved in the regulation of cell viability and apoptosis in HG-treated podocytes. Previous studies have indicated that cytoskeletal proteins are associated with cell viability and apoptosis (52,58). The present results suggest that the role of the mTORC1 signaling pathway on cell viability and apoptosis may not be through the regulation of cytoskeletal proteins. During the development of DN, the formation of advanced glycation end products (AGEs) were significantly enhanced in the presence of HG levels (59). It has been reported that AGEs interact with the N-terminal domain of ezrin and inhibit its actions in proximal tubule cells (60). Notably, a previous study indicated that AGE treatment reduced podocyte adhesion to fibronectin and inhibited migration; however, ezrin overexpression completely reversed the AGE inhibition of podocyte adhesion to fibronectin and partially reversed AGE-induced inhibition of migration (61). This suggests that HG-induced podocyte injury may be mediated by an increased level of AGEs. Further in-depth study on the role of AGEs on podocytes is required.

In conclusion, the present study demonstrated that HG induced effects on podocytes, including increased podocyte apoptosis and changes in the expression levels of cytoskeleton-associated proteins 24 h following HG treatment. Notably, the cell viability was also increased following HG treatment. This suggests that with prolongation of the time of HG action, the cell viability may be reduced due to the increased level of apoptosis. These findings indicated that mTORC1 and mTORC2 signaling pathways may be involved in the regulation of podocyte viability and apoptosis, and that the mTORC2 signaling pathway may be the primary signaling pathway on podocyte injury.
pathway involved in the regulation of cytoskeleton-associated proteins.

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All data generated or analyzed during this study are included in this published article.

Authors’ contributions
QL, YZ and QJ performed the experiments. QL and JZ participated in data collection and drafted the manuscript. CW performed the statistical analysis and designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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